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Hypoxic exposure to increase exercise performance: beneficial aspects and potential drawbacks

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Stefan **Leuven, April 2019**

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LIST OF ABBREVIATIONS

1RM	1-repetition-maximum
2,3-DPG	2,3-di-phosphoglycerate
2OG	2-oxoglutarate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMS	Acute mountain sickness
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
a-vO_2 diff	Arteriovenous oxygen difference
B2M	Beta-2-microglobulin
BNIP3	Bcl-2/adenovirus EIB 19kD-interacting protein 3
CA3/9	Carbonic anhydrase 3/9
C_aO_2	Arterial oxygen content (unit: mL O_2 per dL blood)
CI	Confidence interval
CO	Carbon monoxide
CON	Control group (cfr. paper 2 and 3)
COX4	Cytochrome c oxydase subunit 4
CS	Cytrate synthase
CSA	Cross-sectional area
CycloA	Cyclophilin A
dm	Dry muscle
EPO	Erythropoietin
FENO	Fraction of nitic oxide in the exhaled air
FIH-1/2/3	Factor inhibiting hypoxia-inducible factor-1/2/3
F_iO_2	Fraction of inspired oxygen
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1/3/4	Glucose transporter 1/3/4
H	Group training in hypoxia and receiving placebo (cfr. paper 1)
HACE	High-altitude cerebral edema
HAPE	High-altitude pulmonary edema
Hb	Hemoglobin
Hbmass	Total hemoglobin mass
HF	High Frequency power
HHb	Deoxyhemoglobin
HIF	Hypoxia-inducible factor
HIIT	High-intensity interval training
HK	Hexokinase
HN	Group training in hypoxia and receiving nitrate supplements (cfr. Paper 1)
HR	Heart rate
HRE	Hypoxic responsive elements
HRV	Heart rate variability
HSP27/70	Heat shock protein 27/70
HVR	Hypoxic ventilatory response
IHT	Intermittent hypoxic training
LDHA	Lactate dehydrogenase-A
LF	Low frequency power
LF/HF	Sympathovagal balance
LLS	Self-reported Lake Louise consensus scoring system
Ln	Natural logarithm
MAPK	Mitogen-activated protein kinase
MCT1/4	Monocarboxylate transporter 1/4
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MVC	Maximal voluntary contraction
N	Group training in normoxia and receiving placebo (cfr. paper 1)
NBC	Na^+/HCO_3^- cotransporter
NES	Nuclear export signal
NFAT	Nuclear factor of activated T-cells
NHE1	Sodium-proton exchanger 1
NIRS	Near-infrared spectroscopy
NN50	The number of pairs of adjacent normal R–R intervals that differ by more than 50 ms
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthases
O_2^-	Superoxide
O_2Hb	Oxyhemoglobin

P_aCO_2	Arterial partial pressure of carbon dioxide
P_AO_2	Alveolar partial pressure of oxygen
P_aO_2	Arterial partial pressure of oxygen
PCr	Phosphocreatine
PDK4	Pyruvate dehydrogenase kinase 4
PFK	Phosphofructokinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator-1α
PHD1/2/3	Prolyl hydroxylase domain protein 1/2/3
PI3K	Phosphatidylinositol 3-kinase
P_iO_2	Intracellular partial pressure of oxygen
pNN50	Percentage of adjacent RR intervals that differ from each other more than 50 ms
PO_2	Partial pressure of oxygen
pVHL	von Hippel-Lindau tumor suppressor protein
P_vO_2	Venous partial pressure of oxygen
Qmax	Maximal cardiac output
qPCR	Quantitative polymerase chain reaction
REDD1	Regulated in development and DNA damage responses 1
RMSSD	The square root of the mean squared differences between adjacent normal R–R intervals
ROS	Reactive oxygen species
RPL19	Ribosomal protein L19
RSA	Repeated-sprint ability
RSH	Repeated-sprint training in hypoxia
RSN	Repeated-sprint training in normoxia
SaO_2	Arterial oxygen saturation, measured in arterial blood
SDNN	SD of all normal RR intervals
sEPO	Serum erythropoietin
sFer	Serum ferritin
SIT	Sprint interval training
SOD1	Superoxide dismutase 1
SpO_2	Arterial oxygen saturation, estimated by pulse oximetry
SV	Stroke volume
Tf	Transferrin
Tfam	Mitochondrial transcription factor A
Tfr	Transferrin receptor
tHb	Total hemoglobin (cfr. near-infrared spectroscopy)
TOI	Tissue oxygen index
TR	Group participating in 'live high-train low' program (cfr. paper 2 and 3)
TR_{HYP}	Leg trained in hypoxia (cfr. paper 2 and 3)
TR_{NOR}	Leg trained in normoxia (cfr. paper 2 and 3)
TT_{30min}	30-min simulated time-trial (cfr. paper 1)
$\dot{V}_E$	Minute ventilation
VEGF	Vascular endothelial growth factor
VO_2max	Maximal oxygen uptake
$\dot{V}_T$	Tidal volume
W_{30s}	30-s modified Wingate test (cfr. paper 1)
βhm	Buffering capacity of homogenized muscle
f	Breathing frequency
Δ	Delta (changes in …)
η^2_p	Partial eta squared

ENGLISH SUMMARY

Throughout history, mankind has always tried to push the boundaries in physical performance and capabilities. In the quest to reach worlds' highest summits (i.e. mountaineers) and greatest heights (i.e. balloonists), high-altitude pioneers soon experienced the detrimental effects of progressively decreasing oxygen availability at altitude. Acute altitude exposure impairs exercise performance and may induce mild to life-threatening forms of high-altitude illness, of which acute mountain sickness (AMS) is the most common form. It is characterized by headache in conjunction with non-specific symptoms such as gastro-intestinal distress, weakness, dizziness, and insomnia. Rate of ascend and the altitude reached are the primary predictors of its incidence and severity. Although some individuals are less or more prone to AMS development than others, no adequate physiological testing procedures are currently available to assess individual susceptibility. In fact, prior acclimatization to hypoxia protects against future AMS development, individual susceptibility may not be a fixed trait. Furthermore, altitude acclimatization may also enhance sea-level exercise performance. It is thus no surprise that elite athletes commonly implement altitude training in their preparation for important competition. Altitude training comprehends both living and training at altitude, the latter either in a setting of 'living high' or 'living low'. 'Living high' stimulates erythropoiesis and ventilatory acclimatization, whilst hypoxic training has been postulated to stimulate muscular adaptations. Nowadays, commercialization of hypoxicators has enabled athletes to simulate altitude-related oxygen deprivation (hypobaric hypoxia) within the normal lowland habitat (normobaric hypoxia). Nonetheless, despite decades of research, the effectiveness and underlying mechanisms of altitude training to increase muscular exercise performance are not clear and remain topic of considerable debate. Therefore, the primary aims of the present thesis were to (i) investigate muscular and performance adaptations to supramaximal hypoxic *versus* normoxic training in a setting of either 'living low' (**Study 1**) or 'living high' (**Study 2-3**), and (ii) to identify and describe physiological responses to hypoxia associated with susceptibility and development of AMS (**Study 4**).

Initial hypoxic training studies typically compared high-intensity endurance training at similar absolute training workloads in hypoxic *versus* normoxic conditions. Training in hypoxia induced unique muscular adaptations involved in capillarization and oxidative energy turnover. Nonetheless, superior performance benefits were not consistently reported. Furthermore, training at similar relative intensities (i.e., percentage of maximal oxygen uptake (VO_2max) in either normoxia or hypoxia) does not seem to stimulate either muscular or performance adaptations. In fact, ambient hypoxia impairs the neuromechanical training load during high-intensity endurance exercise, *i.e.* the speed or power output at which training can be performed. Concomitant reductions in muscle oxygen fluxes and neuromechanical loading likely leads to inferior training adaptations. Hence the 'live high – train low' strategy has long been held best practice in altitude training.

In the last few years, focus has shifted from endurance to supramaximal-intensity (above VO_2max) interval training in hypoxia. Particularly sprint training in hypoxia has received more scientific attention. This training strategy allows similar neuro-mechanical loading of the muscle whilst concurrently superimposing additional hypoxic stress. This hypoxic stress has been postulated to stimulate activation of a major transcription factor called hypoxia-inducible factor (HIF). HIF is a heterodimer composed of an oxygen-regulated HIF-α subunit and a constitutively expressed HIF-β subunit. HIF induces expression of genes involved in muscle pH-handling, glycolytic metabolism, and capillarization. In hypoxia, myocellular metabolism shifts away from oxidative energy turnover towards the glycolytic metabolism. It is thus reasonable to postulate that supramaximal-intensity training in hypoxia may stimulate myocellular pH-handling capacity, glycolytic metabolism, and eventually glycolytic exercise performance.

In a first study (**Study 1**), we hypothesized sprint interval training in hypoxia might induce superior muscular and performance adaptations compared to similar training in normoxia. Additionally, we postulated dietary nitrate supplementation during training in hypoxia to increase the absolute training workload and hence training adaptations. Twenty-seven healthy recreationally-active men were randomly allocated to perform 5 weeks of sprint interval training in either normoxia (F_iO_2: 20.9%, n=10) or normobaric hypoxia (F_iO_2: 15.0%, ~2750 m). Subject training in hypoxia received either placebo (n=8) or dietary nitrate (6.45 mmol NaNO3, n=9) supplements. Training was scheduled 3 times a week and involved four to six 30-s all-out cycling sprints interspersed by 4.5 min of active recovery. Biopsies from the *vastus lateralis* muscle were sampled and VO_2max, 30-min time-trial performance, and 30-s sprint performance

were assessed before and after the study intervention. VO_2max (~11-16%), 30-min time-trial performance (~4-8%), and 30-s sprint performance (~6-12%) increased in all groups, irrespective of ambient hypoxia or nitrate supplementation. Accordingly, citrate synthase maximal activity, a marker of muscle oxidative capacity, increased without differences between the groups. *In-vitro* measured muscle buffering capacity remained constant from the pretest to the posttest. Type IIx muscle fibers decreased in all groups. However, only in subjects training in hypoxia with dietary nitrate supplementation, a significant increase in type IIa muscle fibers was observed. From this study we conclude that sprint interval training in hypoxia did not increase muscular or performance adaptations more than similar training in normoxia. In addition, this study also indicates that dietary nitrate supplementation during training in hypoxia may affect exercise-induced shifting in fiber type composition.

Training adaptations are subject to high inter-individual variance. In parallel study designs (*i.e.* **Study 1**), such variance induces 'noise' which may mask small but potentially relevant benefits of training in hypoxia. Therefore, in a second study (**Study 2**), normoxic and hypoxic training was performed within a single individual. During 5 weeks, 3 times per week, ten healthy recreationally-active men trained one leg in normoxia (F_iO_2: 20.9%, n=10) and the other leg in normobaric hypoxia (F_iO_2: 12.3%, ~4300 m). Training consisted of high-intensity knee-extension exercise on a leg extension apparatus. Two sets of 4-6 consecutive 90-s series of each 30 muscle contractions (1 s concentric, 1 s eccentric, 1 s rest) were performed at 20-25% of the subjects' 1-repetition maximum. Training was performed in the setting of intermittent 'living high'. Indeed, during this 5-week training period, 5 consecutive days per week, participants 'lived high' for ~15.5 h per day in progressively increasing normobaric hypoxia (F_iO_2: 16.4-14.0%, ~2000-3250 m). The remaining time was spent in normoxia. Iron supplementation (105 mg elemental iron per day) was administered to subjects with serum ferritin concentrations below 100 $\mu g \cdot L^{-1}$. V*astus lateralis* muscle samples were taken before and after the study intervention. Two-legged VO_2max and single-legged 3-min time-trial performance and repeated-sprint ability were assessed in the pretest and the posttest. Serum erythropoietin (EPO) during 'living high' was measured in normoxia and subsequently after each first night in hypoxia. Total hemoglobin mass expansion from the pretest to the posttest was measured with the CO-rebreathing procedure. We hypothesized hypoxic *versus* normoxic high-intensity knee-extension training to induce superior adaptations in muscular pH-handling capacity and hence glycolytic exercise performance. We also postulated intermittent 'living high' to continuously increase serum EPO and hence total hemoglobin mass and VO_2max. Against our hypothesis, training failed to increase in-vitro measured muscle buffering capacity, irrespective of the ambient training conditions. Accordingly, abundancy of myocellular proton-extruding proteins remained constant from the pretest to the posttest. No changes in fiber type composition were observed, whilst muscle capillarization similarly increased (~15%) in both groups. Three-minute time-trial performance (~10-15%) and repeated-sprint ability (~8%) increased similarly following training in normoxia and hypoxia. Changes in muscle blood volume (~circulation) during repeated sprints, however, increased only following training in hypoxia. A two-fold increase in serum EPO was measured throughout the 5 weeks of 'living high' and adequately predicted the increase in total hemoglobin mass (r=0.78). Iron supplementation did not affect changes in total hemoglobin mass, however, the analysis was performed on a small sample size (n=5 in each group) and thus interpretation should be performed with caution. Nonetheless, it did prevent a ~30% drop in serum ferritin concentration. Total hemoglobin mass significantly increased by 2.6%. Regardless hematological adaptations, VO_2max remained stable from the start to the end of the study intervention. Taken together, hypoxic *versus* normoxic high-intensity knee-extension training did not stimulate muscular or performance adaptations. However, muscle blood circulation during repeated sprints increased only following training in hypoxia. Intermittent 'living high' continuously increased serum EPO and correlated with the increase in total hemoglobin mass.

Besides muscle samples at rest (**Study 2**), we also sampled *vastus lateralis* muscle tissue at the end of a 10-min arterial occlusion of the leg. Indeed, in **Study 3**, we examined the effects of normoxic *versus* hypoxic training in the setting of 'living high' on the HIF-pathway response to brief muscular ischemia. More specifically, HIF-1α stabilization, nuclear co-localization, and target gene expression was assessed in the pretest and the posttest, before and during brief ischemia-induced hypoxia of the leg. Also changes from the pretest to the posttest in protein abundancy of prolyl hydroxylase domain protein 2 (PHD2) at rest were assessed. In the presence of oxygen, PHD2 hydroxylates HIF-1α and hence initiates HIF-1α degradation. PHD-2 becomes inactive in hypoxia, causing HIF-1α to accumulate and translocate to the nucleus to heterodimerizes with HIF-1β. Note thar PHD-2 has been identified as a HIF-1 target gene and that muscle contractions induce myocellular hypoxia, particularly during exercise in ambient hypoxia. Repetitive

exercise-induced HIF-1 pathway activation may thus potentially increase PHD2 expression and thereby reduce HIF-1α accumulation at a given myocellular oxygen pressure. In support for this hypothesis, short-term endurance training has previously been shown to increase PHD2 protein content and attenuate acute exercise-induced HIF-1 pathway activation. However, as HIF-1α also acts as a metabolic and hormonal sensor, attenuation of HIF-pathway activation to a given exercise stimulus following short-term training may also result from reduced oxidative stress and inflammation. Compared to acute exercise, these confounding factors are less prominent during 10 minutes of muscle ischemia. Therefore, we hypothesized ischemia-induced HIF-pathway activation to be less pronounced following 5 weeks of high-intensity knee-extension training in normoxia, and even more so in hypoxia. Near-infrared spectroscopy analyses showed 10 min of arterial occlusion to prominently reduce muscle oxygenation, to levels well below those observed during normoxic or hypoxic knee-extension exercise. Immunohistochemical analyses showed ~54% of myonuclei to yield a positive staining for HIF-1α at baseline conditions, both before and after the 5-week study intervention. In the pretest, ten minutes of arterial occlusion increased the fraction of HIF-1α-positive nuclei to ~64%. Occlusion did not alter HIF-1α protein content or its target gene expression, this conceivably required a longer hypoxic episode. Against our hypothesis, 5 weeks training in normoxia nor hypoxia affected ischemia-induced HIF-1α translocation to the nucleus. In keeping with this finding, Western blot-derived HIF-1α, HIF-2α, and PHD2 protein content at rest remained stable throughout the 5-week study intervention. We speculate that training intensity determines changes in the HIF-pathway sensitivity to hypoxia or metabolic stress. Indeed, previous research showed short-term repeated-sprint training not to alter PHD2 expression. Furthermore, HIF-1α protein content increased following 12 weeks of resistance training in men. In genetically modified rodents, overexpression and knock-out of muscle HIF-1α was associated with the glycolytic and endurance phenotype, respectively. In addition, six weeks high-intensity interval training in mice increased HIF-1α protein in the gastrocnemius mice. From the present study, we conclude that muscle cells at rest abundantly express nuclear HIF-1α protein, and even more so during brief ischemia-induced hypoxia. Short-term high-intensity knee-extension training in normoxia nor hypoxia affected the HIF-pathway response to brief hypoxia.

Finally, in **Study 4**, we aimed to (i) identify pertinent markers of AMS susceptibility and (ii) describe physiological responses to hypoxia in subjects afflicted either less or more by AMS. Sixteen healthy male volunteers were included in this study. VO_2max was assessed in normoxia and acute hypoxia (F_iO_2: 12.7%). Subsequently, subjects were exposed to 24 h of normobaric normoxia and hypoxia equivalent to ~4000 m altitude (F_iO_2: 12.7%) during which five 30-min submaximal exercise bouts (1.5 $W \cdot kg^{-1}$) simulated the workload associated with normal ascend rates to alpine huts and summits. Physiological parameters believed to be involved in the development of AMS were assessed at rest and during exercise. Nine subjects developed mild AMS (AMS_{LOW}) whilst the remaining 7 subjects developed severe AMS (AMS_{HIGH}). The preliminary VO_2max tests in normoxia nor hypoxia adequately predicted AMS development. However, at ~2 h into the 24-h hypoxic trial, minute ventilation and blood oxygenation were lower in AMS_{HIGH} than AMS_{LOW}. Concurrently, vagal heart rate variability decreased in AMS_{HIGH} but not AMS_{LOW}. In fact, vagal modulation of the heart progressively decreased throughout the 24-h hypoxic trial in AMS_{HIGH}, whereas only a mild and transient withdrawal was observed in AMS_{LOW}. From early on in the 24-h hypoxic trial, exercise elicited greater blood lactate concentrations and arterial oxygen desaturation in AMS_{HIGH}. Subjects in AMS_{LOW} successfully completed the 5 submaximal cycling bouts in hypoxia. In contrast, 5 out of 7 subjects in AMS_{HIGH} could not maintain the prescribed workload. Multiple regression analyses identified arterial oxygen saturation (S_pO_2) in hypoxia at rest and during submaximal exercise as significant predictors of the overall sum of AMS symptoms (r=32) or the peak severity of AMS symptoms (r=53) throughout the hypoxic trial, respectively. This study suggest that individuals with low S_pO_2 at rest and during submaximal exercise within the first hours in hypoxia should be closely monitored for subsequent AMS development. Furthermore, subjects struggling to maintain the groups' hiking pace are conceivably more likely to be afflicted by AMS. The phenotype of AMS is characterised by early and progressive withdrawal of vagal cardiac regulation.

NEDERLANDSE SAMENVATTING

Het is eigen aan de mens om de grenzen van zijn fysieke kunnen af te tasten en te verleggen. Pioniers in de ballonvaart en alpinisme ondervonden al snel de nadelige effecten van afnemende zuurstofbeschikbaarheid met toenemende hoogte. Acute blootstelling aan grote hoogte tast het inspanningsvermogen aan. Afhankelijk van de hoogte en de snelheid waarmee deze bereikt werd kan en zal hoogteziekte zich ontwikkelen. De meest voorkomende vorm van hoogteziekte betreft acute hoogteziekte (acute mountain sickness, AMS). AMS wordt getypeerd door hoofdpijn in combinatie met aspecifieke symptomen zoals gastro-intestinale klachten, zwakte en vermoeidheid, duizeligheid, en slapeloosheid. Sommige personen zijn meer vatbaar voor de ontwikkeling van AMS dan anderen. Toch is de voorbeschiktheid voor AMS een op zijn minst gedeeltelijk plastisch gegeven. Pre-acclimatisatie aan hoogte verlaagt immers de kans op latere AMS-ontwikkeling. Het acclimatisatieproces kan ook het prestatievermogen op zowel zeeniveau als hoogte verbeteren. Reeds jaren maken atleten gebruik van hoogtetraining ter voorbereiding van belangrijke competitie. De term hoogtetraining omvat zowel level als trainen op hoogte. Leven op hoogte stimuleert de aanmaak van rode bloedcellen en verhoogt zo de zuurstoftransportcapaciteit vereist voor uithoudingsprestaties. Trainen op hoogte wordt geacht om aanpassingen ter hoogte van de spier te stimuleren. Hoogtetraining kan plaatsvinden op natuurlijke hoogte (hypobare hypoxie) of op gesimuleerde hoogte (normobare hypoxie). Om de afgenomen beschikbaarheid van zuurstof op hoogte te simuleren op zeeniveau wordt zuurstof uit de omgeving onttrokken of verdund door toevoeging van stikstof. Of dat training in hypoxie inderdaad de trainingsrespons verhoogt is nog steeds een onderwerp van debat. De primaire doelstellingen van deze doctoraatsthesis zijn dan ook de volgende: (i) onderzoeken of dat training aan supramaximale intensiteit op gesimuleerde hoogte een toegevoegde trainingsrespons van het musculaire systeem en de fysieke prestatie induceert (**Studie 1-3**), en (ii) respectievelijk beschrijven en identificeren van de fysiologische respons op acute normobare hypoxie en parameters die duiden op predispositie voor AMS (**Studie 4**).

De eerste hypoxische trainingsstudies uit de jaren ’90 vergeleken veelal hoog-intensieve uithoudingstraining aan eenzelfde absolute trainingsintensiteit in normoxische en hypoxische trainingscondities. Daaruit bleek training in hypoxie unieke adaptaties te induceren, waaronder een verhoogde toename in capillarisatie en enzymen betrokken in oxidatieve energieproductie in de spier. Echter werden grotere toenames in prestatievermogen niet consistent geraporteerd. Meer nog, hypoxische training aan eenzelfde relatieve intensiteit bleek potentieel nefast voor de toename in prestatie. Acute blootstelling aan hypoxie verlaagt namelijk de aerobe inspanningscapaciteit. Gedurende hoog-intensieve uithoudingstraining boet de neuromechanische belasting en zuurstofflux ter hoogte van de spier in. De ‘leef hoog – train laag’ strategie werd daarom jarenlang als de optimale hoogtetrainingsstrategie beschouwd.

De voorbije jaren heeft de focus zich verlegd van hoog-intensieve uithoudingstraining naar supramaximale interval training (intensiteit boven de maximale zuurstofopname), doorgaans onder de vorm van sprinttraining. Deze aanpak laat toe een additionele hypoxische prikkel te bieden aan de spier, zonder te moeten inboeten in de neuromechanische belasting. Sprintprestaties worden namelijk niet beïnvloed door acute blootstelling aan normobare hypoxie. De additionele hypoxische stress wordt verondersteld een belangrijke transcriptiefactor ‘hypoxie-induceerbare factor’ (HIF) te activeren. HIF bestaat uit een zuurstofgereguleerde HIF-1α subeenheid en een constitutief tot expressie gebrachte HIF-1β subeenheid. HIF induceert onder meer de expressie van genen verantwoordelijk voor de pH-homeostase in de spiercel, het glycolytische metabolisme, en de capillarisatie. Inderdaad, onder andere via HIF-activatie shift het metabolisme in hypoxie van oxidatieve naar het anaerobe glycolytische energieproductie. Daaruit vloeit de gangbare hypothese dat training aan supramaximale intensiteit in hypoxie grotere adaptaties teweegbrengt in musculaire buffercapaciteit, glycolytische metabolisme, en lactische prestatievermogen.

In een eerste studie (**Studie 1**) werd onderzocht of dat sprint interval training (SIT) in hypoxie superieure musculaire en prestatiegerelateerde adaptaties induceert vergeleken met identieke training in normoxie. Daarenboven werd getest of dat nitraatsupplementatie tijdens training in hypoxie via een verhoogde trainingskwaliteit de trainingsadaptaties verder verhogen kan. Zevenentwintig gezonde mannen werden op gerandomiseerde wijze toegewezen aan SIT in normoxie (F_iO_2: 20.9%, n=10), SIT in hypoxie + placebo (F_iO_2: 15.0%, ~2750 m, n n=8), of SIT in hypoxie + nitraatsupplementatie (F_iO_2: 15.0%, 6.45 mmol NaNO3, n=9). Gedurende 5 weken, driemaal per week, voerden de deelnemers 4 à 6 maximale sprints van 30 seconden uit per sessie. Tussen de sprints werd gedurende 4,5 min actief gerecupereerd. Voor en na de trainingsinterventie werden spierbiopten genomen uit de *vastus lateralis*.

Maximale zuurstofopname (VO_2max), power output gedurende een gesimuleerde tijdrit van 30 minuten, en de power output gegenereerd over een sprint van 30 seconden werden gemeten in de pretest en de posttest. VO_2max (~11-16%) nam toe in elke groep, ongeacht de trainingscondities. Training verhoogde ook de power output gemeten tijdens de tijdrit (~4-8%) en de sprint (~6-12%), zonder verschillen tussen de groepen. De maximale enzymatische activiteit van citraatsynthase, een merker voor de oxidatieve capaciteit van de spier, nam toe in de drie groepen, opnieuw zonder groepsverschillen. Training verhoogde de *in-vitro* buffercapaciteit van de spier in geen enkele groep. Het relatieve aandeel type IIx spiervezels nam af in elke groep, maar alleen na hypoxische training in combinatie met nitraatsupplementatie werd een toename in type IIa spiervezels geobserveerd. Uit deze studie concluderen we dat sprint interval training in hypoxie geen meerwaarde biedt ten opzichte van indentieke training in normoxie. Verder lijkt nitraatsupplementatie betrokken te zijn in de shift in vezeltypering gedurende training in hypoxie.

Er bestaan grote interindividuele verschillen in de respons op kracht- en uithoudingstraining. Dergelijke interindividuele variatie genereert 'ruis' op de studiedata waardoor kleine maar potentieel belangrijke verschillen tussen parallelle interventiegroepen gemaskeerd kan worden. Daarom werd in een tweede studie (**Studie 2**) training in normoxie *versus* hypoxie vergeleken binnen eenzelfde subject. Tien gezonde recreatief-actieve mannen trainden één been in normoxie (F_iO_2: 20.9%) en het andere been in hypoxie (F_iO_2: 12.3%, ~4300 m). Training bestond uit twee sets van 4 à 6 series. Elke serie bestond uit 30 spiercontracties (1 s concentrisch, 1 s excentrisch, 1 s rust) gedurende 90 seconden. De intensiteit werd ingesteld aan 20 à 25% van de subjecten hun 1-repetitie maximum. Gedurende deze trainingsperiode verbleven de subjecten op intermittenterende basis op gesimuleerde hoogte. Gedurende 5 weken, 5 opeenvolgende dagen per week, verbleven de deelnemers 15,5 uur per dag in progressief toenemende normobare hypoxie (F_iO_2: 16.4-14.0%, ~2000-3250 m). De rest van de tijd brachten ze door in normoxie. Subjecten met ferritineconcentraties onder 100 $\mu g \cdot L^{-1}$ kregen op dagelijkse basis een ijzersupplement (105 mg ijzer) toegediend. Spierstalen van de *vastus lateralis* werden genomen voor en na de studie-interventie. Van elk been apart werd de power output gemeten gedurende een korte tijdrit van 3 minuten en gedurende een herhaalde sprinttest (20 sprints van ~15 seconden met 15 seconden recuperatie). VO_2max werd gemeten voor, en één en twee weken na de studie-interventie. Serum erytropoëtine (EPO) werd gemeten bij het ontwaken na iedere eerste nacht in hypoxie. Expansie in totale hemoglobinemassa werd beoordeeld op basis van de 'CO-rebreathing'-procedure. De volgende twee hypotheses werden getest: (i) hoog-intensieve training van de kniestrekkers in hypoxie stimuleert adaptaties in de spier en het prestatievermogen, en (ii) intermittent verblijf op gesimuleerde hoogte induceert een toename in EPO, totale hemoglobinemassa, en VO_2max. Training in normoxie noch hypoxie verhoogde de *in-vitro* musculaire buffercapaciteit. Ook de concentraties van proteïnen verantwoordelijk voor protontransport over het myocellulaire membraan bleven ongewijzigd. Veranderingen in spiervezeltypering werden niet geobserveerd. Wel nam de capillarisatie toe met ~15%, ongeacht de trainingscondities. Prestatie tijdens de 3-minuten tijdrit en de herhaalde sprinttest nam in beide benen toe, zonder verschillen tussen de trainingscondities. Hypoxische maar niet normoxische training stimuleerde de lokale bloedcirculatie tijdens de herhaalde sprinttest. Serum EPO verdubbelde doorheen de volledige studie-interventie en voorspelde de toename in totale hemoglobinemassa (+2.6%, r=0.78). Ijzersupplementatie stimuleerde de toename in hemoglobinemassa niet, maar verhinderde wel een afname in serum ferritineconcentraties. VO_2max werd niet significant verhoogd door de studie-interventie. Uit deze studie concluderen we dan ook dat hoog-intensieve training van de kniestrekkers in hypoxie de trainingsadaptaties in de spier en het prestatievermogen niet verhoogt vergeleken met identieke training in normoxie. Training in hypoxie leek wel de lokale bloedcirculatie van de kniestrekkers tijdens herhaalde sprints te stimuleren. Intermittent hoogteverblijf in gradueel toenemende normobare hypoxie verhoogt de serum-EPO-concentraties op consistente wijze doorheen de volledige studie-interventie en voorspelt de toename in totale hemoglobinemassa.

In het experiment van **Studie 2** werden naast de spierbiopten in rust ook spierstalen genomen op het einde van een 10 minuten arteriële occlusie van het been. Inderdaad, in **Studie 3** werd het effect onderzocht van leven en trainen in normoxie *versus* hypoxie op ischemie-geïnduceerde activatie van de HIF-pathway. Meer specifiek werd HIF-1α stabilisatie, nucleaire translocatie, en targetgenexpressie onderzocht voor en tijdens 10 minuten arteriële occlusie. Ook werd de proteïneconcentratie van prolyl hydroxylase domain protein 2 (PHD2) gekwantificeerd in de pretest en de posttest. In normoxie wordt HIF-1α gehydroxyleerd door PHD2. Dit initieert het degradatieproces van HIF-1α. In cellulaire hypoxie accumuleert HIF-1α en transloceert ze naar de nucleus om te binden met HIF-1β. PHD2 werd in voorgaande studies geïdentificeerd als een HIF-1 targetgen. Aangezien spiercontracties de myocellulaire zuurstofdruk

verlagen is het realistisch te veronderstellen dat training in normoxie PHD2 expressie stimuleert. Meer nog, gezien blootstelling aan gesimuleerde hoogte het verval in intracellulaire zuurstofdruk tijdens spiercontracties accentueert, lijkt het een logische hypothese dat hypoxische *versus* normoxische training de expressie van PHD2 nog meer zou stimuleren. Indien deze hypothese klopt, dan zou een bepaalde afname in myocellulaire zuurstofdruk na een kortstondige trainingsperiode een lagere HIF-pathway activatie induceren. Inderdaad, uithoudingstraining verlaagt PHD2 proteïneconcentraties en vermindert de HIF-pathway respons op acute spiercontracties. HIF doet echter ook dienst als metabole en hormonale sensor. De afgenomen activatie van de HIF-pathway kan dus ook wijzen op een trainings-geïnduceerde afname in de inflammatoire en oxidatieve stress respons op eenzelfde acute inspanning. Vergeleken met acute inspanning spelen deze variabelen een veel kleinere rol tijdens kortstondige ischemie. In **Studie 3** werd getest of dat 5 weken hoog-intensieve training van de kniestrekkers (i) de ischemie-geïnduceerde HIF-pathway activatie vermindert, en (ii) of dat deze afname meer uitgesproken is na training in hypoxie. Nabij-infrarood spectroscopie applicatie op de *vastus lateralis* toonde aan dat arteriële occlusie een hoge graad van hypoxie in de spier sterk induceerde. Immunohistochemische analyses toonde een hoge aanwezigheid van HIF-1α aan in de myonuclei in rust (~54%), zowel voor als na de studie-interventie. In de pretest verhoogde ischemie-geïnduceerde hypoxie de myconucleaire co-lokalisatie van HIF-1α tot ~64%. De occlusie induceerde echter geen meetbare toename in HIF-1α proteïneconcentraties of targetgen transcriptie. In tegenstelling tot onze hypothese beïnvloedde training in normoxie noch hypoxie de occlusie-geïnduceerde nucleaire translocatie van HIF-1α. Ook PHD2 proteïneconcentraties bleven constant van de pretest tot de posttest. We speculeren dat trainingsintensiteit een bepalende factor speelt in de sensitiviteit van de HIF-1-pathway. Immers verhoogde sprinttraining in een voorgaande studie de expressie (mRNA) van PHD2 niet. Wel verhoogde krachttraining HIF-1α in rust. Daarnaast toonden knaagdierstudies ook reeds aan dat overexpressie en knock-out van HIF-1α in de spier leidt tot een respectievelijk glycolytisch en uithoudingsgetraind fenotype. We concluderen uit **Studie 3** dat tijdens kortstondige hypoxie HIF-1α naar de nucleus transloceert, maar dat training aan hoge intensiteit in normoxie of hypoxie deze translocatie niet beïnvloedt.

In een laatste studie (**Studie 4**) werd getracht om (i) pertinente merkers van AMS-predispositie te identificeren, en (ii) de hypoxische respons te beschrijven in subjecten met *versus* zonder ernstige symptomen van AMS. Zestien gezonde mannen werden onderworpen aan een stapsgewijze VO_2max-test in normoxie en normobare hypoxie (F_iO_2: 12.7%). Twee weken later volgden twee 24-uur sessies, eenmaal in normoxie en andermaal normobare hypoxie (F_iO_2: 12.7%, ~4000 m), gedurende dewelke vijf submaximale inspanningen van 30 minuten werden uitgevoerd aan een intensiteit (1.5 $W \cdot kg^{-1}$) die een beklimming naar Alpiene hutten en toppen simuleert. Een reeks van fysiologische parameters die belangrijk geacht worden in de ontwikkeling van AMS werd gemeten in rust en tijdens inspanning. Negen subjecten ontwikkelde milde symptomen van AMS (AMS_{LOW}), de resterende 7 subjecten ontwikkelde ernstige AMS (AMS_{HIGH}). De preliminaire VO_2max-test in normoxie noch hypoxie voorspelde de gradatie of incidentie van latere AMS-ontwikkeling. Daarentegen waren arteriële zuurstofsaturatie (S_pO_2) en minuutventilatie na 2 uur in hypoxie lager in AMS_{HIGH} dan AMS_{LOW}. Vergeleken met normoxie daalde vagale hartritmevariabiliteit in hypoxie in AMS_{HIGH} maar niet AMS_{LOW}. Meer nog, AMS_{HIGH} vertoonde een progressieve afname in vagale modulatie van het hart doorheen de 24 uur in hypoxie. Daarentegen was deze afname mild en van voorbijgaande aard in AMS_{LOW}. In hypoxie leidde submaximale inspanning tot een grotere toename in bloedlactaat en lagere S_pO_2 in AMS_{HIGH} dan AMS_{LOW}. Alle subjecten in AMS_{LOW} volbrachten de 5 submaximale inspanningen in hypoxie, terwijl 5 van de 7 subjecten in AMS_{HIGH} niet in staat waren om de inspanning aan de opgelegde intensiteit te volbrengen. Multipele regressieanalyse weerhield alleen S_pO_2 in rust als voorspellende factor (r=32) voor de totale som van AMS symptomen doorheen de hypoxische sessie. S_pO_2 tijdens submaximale inspanning voorspelde 53% van de maximale intensiteit van AMS ervaren op gesimuleerde hoogte. Uit deze studie besluiten we dat subjecten met lage S_pO_2 in rust en tijdens submaximale inspanning nauw gevolgd moeten voor latere AMS-ontwikkeling. Ook subjecten die het moeilijk hebben om het wandeltempo op hoogte aan te houden zijn meer waarschijnlijk om symptomen van AMS te vertonen. AMS gaat bovendien gepaard met een progressieve afname in vagale modulatie van het hart.

PART I

INTRODUCTION AND OUTLINE

Introduction and outline

1. Background

1.1. Brief history of high altitude medicine and physiology

The colorful history of high altitude medicine and physiology dates back many centuries. In the 'Book of Han', covering the classical Chinese history as written in 101 AD, a Chinese official warns for the dangers of 'great headache mountains' which, as he states, may cause one to become feverish, losing color and develop gastro-intestinal distress and headache. However, only in the 17th century scientific breakthroughs started to unravel the mechanisms by which altitude exposure mediates the physiological effects on the human body. Indeed, not long after Torricelli E. developed the mercury barometer in 1643, Pascal B. demonstrated barometric pressure to drop with increasing altitude. Hundred years later, well into the 18th century, scientific experiments yielded significant advances in the understanding of the composition of air and the function of its different respiratory gases. First, in 1774, Priestley J. isolated oxygen from air by heating mercuric oxide in an inverted glass container. The gas emitted was found to intensify the flame of a burning candle, as well as to keep a mouse alive four times longer compared to its control sealed in a container with normal air. Three years later, French chemist Lavoisier A. was the first to accurately describe the three main respiratory gasses:

'Eminently respirable air (oxygen) that enters the lung, leaves it in the form of chalky aeriform acid (carbon dioxide) … in almost equal volume … Respiration acts only on the portion of pure air that is eminently respirable … the excess, that is its mephitic portion (nitrogen), is a purely passive medium which enters and leaves the lung … without change or alteration. The respirable portion of air (oxygen) has the property to combine with blood and its combination results in its red colour.'

Around the same period, the Montgolfier brothers developed the hot air balloon capable of carrying men high into the sky. Adventurous balloonists reaching continuously greater altitudes reported about deleterious effects of high-altitude exposure on human functioning, with the most tragic testimony coming from the 'Zenith' aeronauts in 1875. Three French aeronauts attempted to break men's altitude record whilst also studying the meteorological phenomena of the upper atmosphere. Environmental physiologist Bert P., now often referred to as the 'father of altitude physiology', had previously conducted experiments on the Zenith aeronauts in his self-constructed hypobaric chambers. As his work had shown lowered partial pressure of oxygen to be responsible for the deleterious effects of high altitude, he advised the aeronauts to carry voluminous leather bags with oxygen-enriched air during their flight. Nonetheless, despite their precautions and presumably due to valve malfunctions and too little oxygen, all three aeronauts lost consciousness during their ascent to an altitude of approximately 7500 m. According to a self-registering barometer, the hot air balloon further ascended to an altitude of approximately 8600 m before descending back to sea level. Only one of the three aeronauts survived the ordeal, his companions had deceased due to severe hypoxic exposure during their ascent.

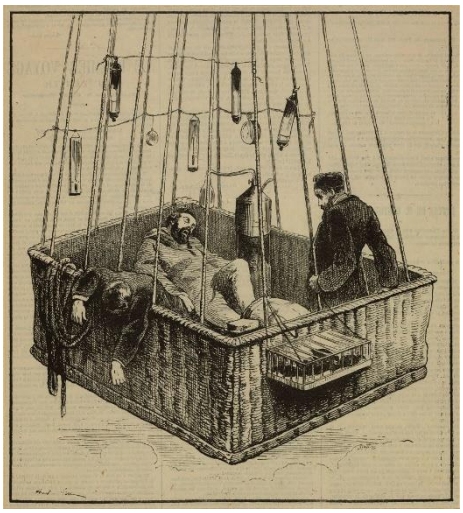


Figure 1. Zenith balloonist losing consciousness due to the lack of oxygen during their ascend to high altitude. Paris, April, 1875. (Tissandier Collection/Library of Congress, with permission).

With its summit around 8848 m, Mt Everest peaks above the maximal elevation the Zenith hot air balloon had reached during its tragic ascent. Unsurprisingly, it has long been held scientifically impossible to summit world's highest mountain, let alone without the aid of supplemental oxygen. Since the first expedition to Everest summit in 1922, many mountaineers had likewise failed to reach the top due to the harsh environmental conditions. It was only in 1953 that Norgay T. and Hillary E. summited Mt Everest, albeit with the aid of supplemental oxygen. It took another 25 years before Habeler P. and Messner R. summited and returned from earths' highest mountain without the use of oxygen supplementation. Their astonishing achievement attested the remarkable capability of the human body to adapt to its hypoxic environment. Indeed, in contrast to the early balloonists greatly suffering from the acute exposure to hypobaric hypoxia, Habeler and Messner had allowed their bodies to slowly acclimatize to the low partial pressure of oxygen (PO_2) at altitude, enabling them to better endure the hypoxic stress they faced during their ascent.

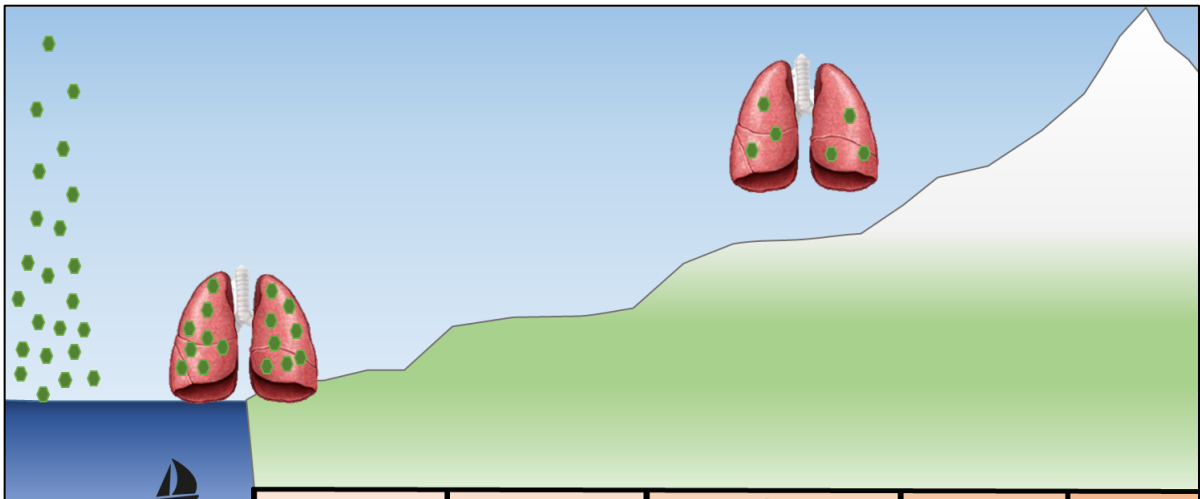
For athletes and coaches always seeking to enhance exercise performance, they reasoned that hypoxic adaptations might benefit exercise performance both at altitude and sea level. This perceptive was reinforced following the controversial 1968 Olympic Games, which were held in Mexico City at an altitude of ~2300 m. For the first time in history, an international sport event of this proportion was organized at an altitude uncommon for the majority of the athletes. Hence the many objections raised by coaches, athletes and scientist warning for potential risks, fearing athletes might faint, lose consciousness or even die by maximally exerting themselves in oxygen-deprived air. Nevertheless, the Games went on as planned, and fortunately no major adverse events attributed to elevation in altitude occurred. Of specific interest were the performances of East African athletes, common to living and training at moderate altitude. Indeed, these athletes outperformed the competition in almost all long-distance running events, as they still do nowadays. Their excellence in endurance performance was believed to result from acclimatization to altitude. This stimulated also Western lowlanders to routinely implement various kinds of altitude training in their preparation for major competition. Albeit its popularity, the success rate of this strategy is not consistent and currently still topic of considerable debate. Clear and uniform guidelines concerning optimal altitude training strategies are still lacking. Currently, the 'live high-train low' strategy is considered golden standard, for this strategy is commonly accepted to induce erythropoiesis by accumulating hypoxic exposure time without compromising high oxygen fluxes and power output during high-intensity endurance or interval training. Training 'high' whilst living 'low' remains highly controversial, for its beneficial effects on exercise performance have been found lacking or modest at best. The last two decades, however, increasing data point towards the

pivoting role of training intensity whilst training 'high', indicating potential benefits of hypoxic *versus* normoxic training regarding muscular and performance adaptations following supramaximal training in the form of repeated sprints. Clearly, the underlying mechanisms for altitude training to increase endurance exercise performance remain largely unclear and warrant further in-depth investigations.

1.2. Hypoxia

1.2.1. Environmental altitude and hypoxia

Barometric pressure is primarily defined by the pressure induced by the atmospheric mass above the point of measurement. As such, barometric pressure and likewise ambient partial pressure of oxygen (PO_2) decrease with increasing elevation, an environmental condition referred to as hypobaric hypoxia. Ambient PO_2 is the product of barometric pressure and the fraction of inspired oxygen (F_iO_2), the latter remaining constant at around 20.9% throughout earths' atmosphere. At sea level, environmental PO_2 can be lowered via hypobaric chambers reducing the environmental pressure in the chamber, or via reduction in F_iO_2 by oxygen extraction or nitrogen dilution, inducing so-called normobaric hypoxia. In the present thesis we differentiate between five levels of (simulated) altitude according to the definition by Bärtsch and Saltin [1] as follows: near sea level (0 – 500 m), low altitude (500 – 2000 m), moderate altitude (2000 – 3000 m), high altitude (3000 – 5500 m) and extreme altitude (> 5500 m) (**Figure 2**).



	Near sea level	Low altitude	Moderate altitude	High altitude	Extreme altitude
Altitude (m)	0 - 500	500 – 2000	2000 – 3000	3000 – 5500	> 5500
Barometric pressure (mmHg)	760 - 716	716 - 596	596 - 526	526 - 379	< 379
Oxygen partial pressure (mmHg)	159 - 150	150 - 125	125 - 110	110 - 79	< 79
Simulated altitude (% F_iO_2)	20.9 – 19.7	19.7 – 16.4	16.4 – 14.5	14.5 – 10.4	< 10.4

Figure 2. Nominal classification of altitude zones.

Classification of altitude zones according to the definition by Bärtsch and Saltin, 2008 [1]. Corresponding barometric pressure, oxygen partial pressure, and ambient oxygen percentage at matching simulated altitude in normobaric hypoxia are given.

1.2.2. The oxygen cascade from the atmosphere to the cell

A continuous flow of oxygen from ambient air to the cell is required to maintain cellular oxidative energy turnover, metabolism and survival. Both at altitude and sea level, the ambient PO_2 represents ~20.9% of the barometric pressure. Hence this corresponds to ~160 mmHg (20.9% of 760 mmHg) at sea level. During inhalation, water vapor is added to the air whilst passing through the nose, mouth, larynx and trachea. As the inhaled air is humidified and warmed to body temperature while it makes its way to the alveoli, water vapor pressure increases to about 47 mmHg and so decreases PO_2 of the inspired air by ~10 mmHg ([20.9% of (760 mmHg minus 47 mmHg)] equals 149 mmHg) [2].

In the alveoli, PO_2 (P_AO_2) depends on the rate of oxygen diffusion to the arteries and the refreshment rate of alveolar air (**Figure 3**). During acute and even more so during extended exposure to hypoxia, the drop in P_AO_2 is attenuated by hypoxic stimulation of peripheral chemoreceptors increasing the drive to breathe [3,4]. At rest, the transit time of red blood cells through the pulmonary capillaries is about 0.75 – 1.00 s and thus largely sufficient for oxygen to reach equilibration across the alveolar-capillary membrane (~0.25s) [5,6,7]. The small drop in PO_2 from the alveoli to the arteries (<10 mmHg) largely results from a minor mismatch between pulmonary ventilation and perfusion. During whole body exercise at high intensity, it is not uncommon for elite endurance athletes to experience a small drop in arterial PO_2 (P_aO_2, pressure exerted by uncombined O_2 molecules dissolved in the plasma) and arterial oxygen saturation (S_aO_2) consequent to relative hypoventilation, ventilation-perfusion mismatch, and diffusion limitations due to the decreased pulmonary capillary transit time or transient pulmonary edema [8]. It is believed that large volumes of aerobic training desensitize peripheral chemoreceptors and thus induce relative hypoventilation [9-11], potentially making athletic populations more susceptible for performance impairment at altitude [12]. At altitude, decreased P_AO_2 reduces the driving pressure for oxygen to diffuse from the alveoli to the pulmonary capillaries. However, mixed venous blood enters the lung capillaries with lowered PO_2 (P_vO_2) attenuating the gradient reduction towards the pulmonary capillaries. Still, the potential to increase P_aO_2 at altitude remains limited, as per definition P_aO_2 cannot exceed P_AO_2. Therefore, oxygen loading to hemoglobin takes place at the steep part of the oxygen-hemoglobin dissociation curve where the affinity of hemoglobin for oxygen is lower, potentially further inhibiting the diffusion rate.

Furthermore, it is important to note that in healthy subjects ventilation-perfusion inequality is limited, both at sea level and altitude. In contrast, prominent ventilation-perfusion inequality and thus alveolar-arterial PO_2 differences can be observed in patients with pulmonary pathologies such as chronic obstructive pulmonary disease or high altitude pulmonary edema (HAPE) [13-15].

Further down the oxygen cascade, in the capillaries surrounding the metabolically active tissues, oxygen is extracted across the capillary membrane, the interstitium and the cytoplasmic membrane into the cytoplasm, where myoglobin facilitates intracellular oxygen transport to the mitochondria for oxidative phosphorylation. Using proton nuclear magnetic resonance spectroscopy, cytosolic oxygen pressure in the muscle cell (P_iO_2) at rest has been estimated to be around 34 and 23 mmHg in normoxia and in hypoxia (10% F_iO_2), respectively [16-17]. During maximal knee extensor exercise, however, cytosolic PO_2 has been reported to decrease to about 3.1 mmHg in normoxia and 2.3 mmHg in hypoxia (12% F_iO_2) [17]. Here, at the final destination of oxygen (cytoplasm and mitochondria),

a plethora of cellular events will be initiated during cellular hypoxia to withstand and adapt to ongoing and future episodes of hypoxic stress (elaborated in detail below).

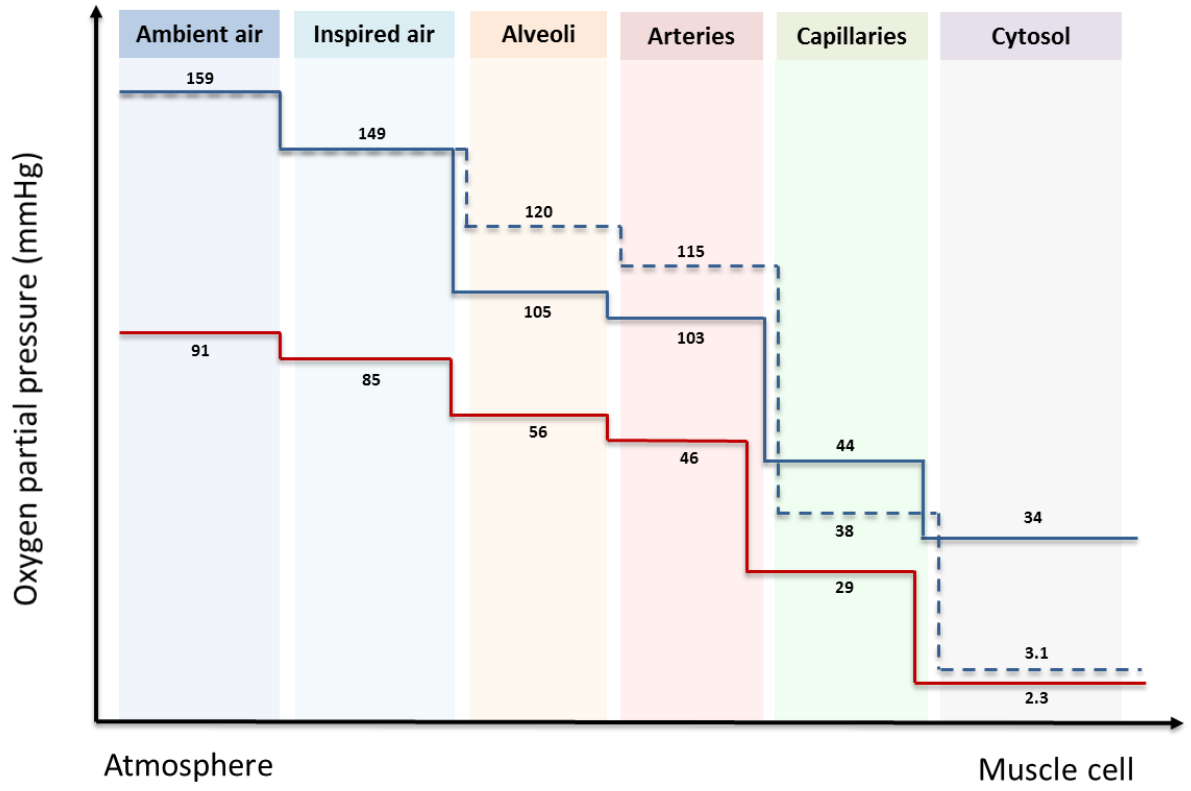


Figure 3. Oxygen transport from ambient air to the cytosol of muscle cells.
Oxygen transport throughout the body at rest in normoxia (solid blue line) and during maximal knee extensor exercise in normoxia (dashed blue line) and hypoxia (12% F_iO_2; solid red line). Figure drawn using data from Richardson *et al., 1995 and 2006* [16,17].

1.2.3. Intracellular hypoxia and oxygen sensing via the HIF pathway

Intracellular hypoxia may be caused by either systemic or local hypoxia. In the former case, a fall in arterial oxygen content (C_aO_2, expressed as mL O_2 per dL blood) impairs oxygen delivery to the tissues throughout the whole body. Amongst others, a reduction in C_aO_2 may be induced by low ambient PO_2, diffusion limitations across the alveolar-capillary membrane, hypoventilation, ventilation-perfusion mismatch, anemia, or heart failure. On the other hand, local blockage or narrowing of an artery to the heart, skeletal muscle, or any other organ may create a local hypoxic environment for tissues located distally to the blockage, without directly interfering with oxygen delivery to other parts of the body. Along the same line, mitochondrial oxygen utilization during muscle contractions can induce local hypoxia in muscle cells. Whatever the cause of intracellular hypoxia, defining it as '*a deviation from the normal amount of oxygen*' may not fully compass its severity and consequences. Indeed, it may be more appropriate to define intracellular hypoxia conform its consequences on metabolic energy delivery, or the magnitude by which intracellular hypoxia activates hypoxia inducible factors (HIF) and therefore cellular reprogramming.

HIFs are heterodimeric complexes composed of an α- (HIF-1α, HIF-2α and HIF-3α) and a β-subunit. The stability and activity of the α-subunit are controlled by hydroxylation reactions at its proline and asparagine residues, respectively (**Figure 4**). The hydroxylation reaction is performed by Fe^{2+} and 2-oxoglutarate (2OG) dependent dioxygenases which require O_2 for enzymatic activity. In normoxia, prolyl hydroxylase domain proteins (PHD) 1-3 hydroxylate the proline residues of the HIF-α subunit, thereby recruiting the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is part of an E3-ubiquitin ligase complex responsible for ubiquitination and targeting of HIF-α for proteosomal destruction [18]. In hypoxia, however, HIF-α escapes proteolysis and translocates to the nucleus. Translocation of HIF-α from the cytoplasm to the nucleus is a regulated process. Indeed, two separate nuclear localization signals have been identified in HIF-1α, both of which capable of interacting with specific nuclear import receptors [19,20]. As a shuttling protein, HIF-1α also is capable of migrating back from the nucleus to the cytoplasm. Nuclear export is controlled by p42/p44 mitogen-activated protein kinases (MAPK), which phosphorylates one or two HIF-1α serine residues in close proximity of a nuclear export signal (NES) [21]. Upon phosphorylation, NES is masked for CRM1-dependent nuclear export, hence promoting accumulation of HIF-1α in the nucleus. Here, HIF-1α interacts with its β-subunit to form a fully functional transcription factor HIF-1. By recognition and interaction with hypoxic responsive elements (HRE) in the promoter or enhancer region of its target genes, this major transcription factor regulates the expression of more than hundreds of genes [22]. Importantly, also HIF transcriptional activity is subject to regulation. Similar to its stabilization mechanism, hydroxylation reactions are controlling this process. Through oxygen-dependent hydroxylation of HIF-α asparagine residues by factor inhibiting HIF-1 (FIH-1), FIH-1 suppresses the interaction of HIF and its transcriptional co-activator CBP/p300. Ingeniously, as FIH-1 remains active at lower oxygen tensions than PHD2, FIH-1 further regulates the activity of the early stabilized HIF-1α that escaped proteosomal degradation under mild hypoxia [23-25].

Noteworthy, also multiple interweaved oxygen-independent mechanisms, involving reactive oxygen species (ROS), growth factors and inflammatory markers, are known to induce activation of HIF-1α [28,29]. Exercise induces both cellular hypoxia and ROS formation. In mitochondria, leakage of unpaired electrons from the electron transport chain to O_2 constitutes the primary source of superoxide (O_2^-) formation, the precursor of most other ROS [30]. ROS has been shown to induce HIF-1α stabilization in normoxia via multiple pathways [29]. For instance, O_2^- can oxidize ferrous iron (Fe^{2+}) to its ferric state (Fe^{3+}), making it useless as a cofactor of the PHD enzyme. Also via activation of the phosphatidylinositol 3-kinase (PI3K) and ERK mitogen-activated protein kinase (MAPK) pathways, ROS-induced growth-factor signaling is believed to stimulate the translation of HIF-1α mRNA into protein [28,31]. Furthermore, ROS also increases the synthesis of inflammatory mediators, which on their turn are known to stimulate transcription and synthesis of HIF-1α [30]. Current literature taken together, HIF-α stabilization in the face of environmental or local hypoxia indicates a biological read out of hypoxia-induced cellular reprograming activity, but it should always be interpreted against the background of HIF as a sensor of both metabolic and hormonal disturbances.

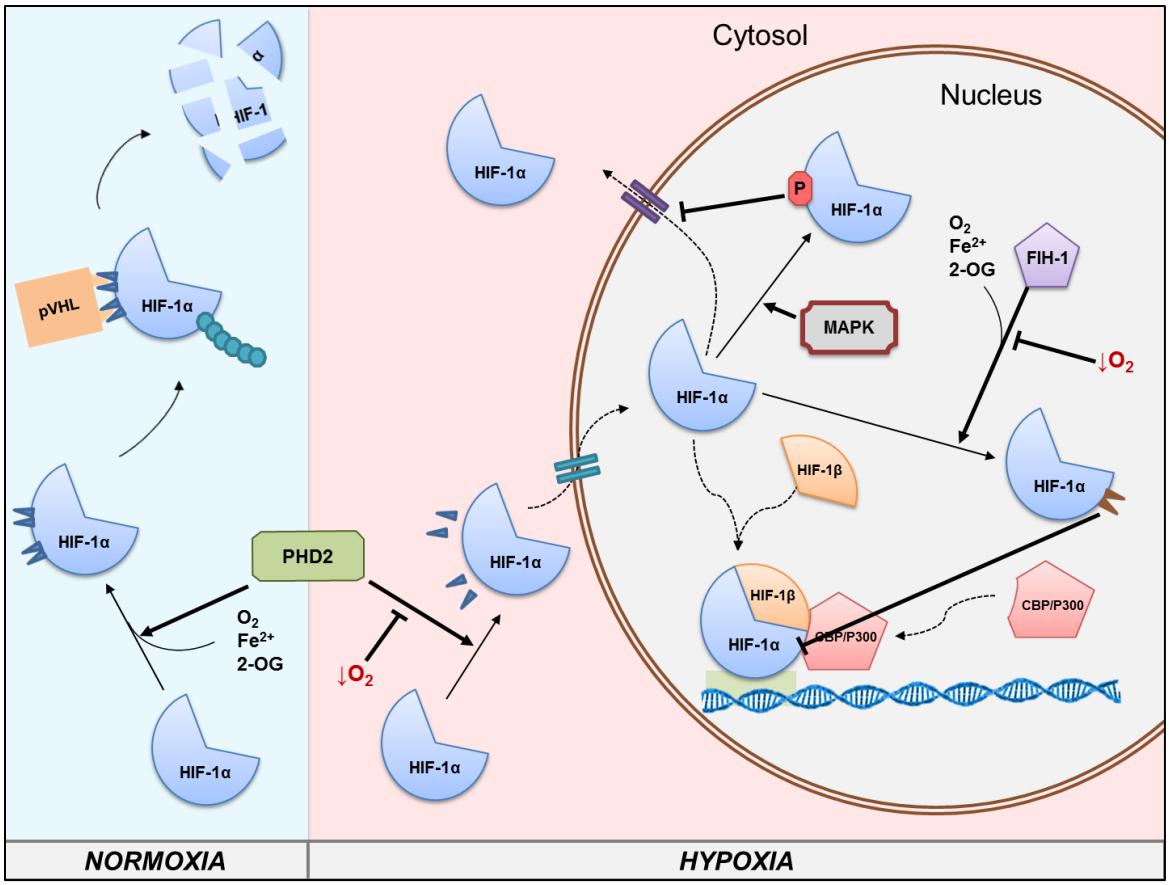


Figure 4. Oxygen-dependent regulation of hypoxia-inducible factor-1α.
In the presence of oxygen (O_2), ferrous iron (Fe^{2+}) and 2-oxoglutarate (2-OG), prolyl hydroxylase domain containing enzyme 2 (PHD2) hydroxylates the proline residues of hypoxia-inducible factor-1α (HIF-1α), thereby creating a binding site for the von Hippel-Lindau protein (pVHL). pVHL is part of a ubiquitin ligase complex, ubiquitinating and thus targeting HIF-1α for proteosomal degradation. In hypoxia PHD2 activity is inhibited, resulting in HIF-1α accumulation and translocation to the nucleus via nuclear importers. HIF-1α is a shuttling protein, capable of both entering and leaving the nucleus. Nuclear export is mediated via nuclear exporters, a regulated mechanism controlled by mitogen-activated protein kinase (MAPK). Once HIF-1α heterodimerizes with HIF-1β, it forms a functional transcription factor (HIF-1) initiating gene transcription after interaction with hypoxic response element in the promoter of its target genes. Transcriptional activity, however, also requires recruitment of co-activator CBP/300. In normoxic conditions, factor inhibiting HIF-1 (FIH-1) hydroxylates the asparagine residues of HIF-1α, inhibiting CBP/300 recruitment. Noteworthy, FIH-1 remains active at lower oxygen tension than PHD2, by this means working in tandem regulating HIF-1α activity over the physiological range of myocellular oxygen concentrations.

1.3. Acute and chronic adaptations to hypoxia

In order to preserve cellular oxygen homeostasis and hence tissue integrity in hypoxia, a machinery of physiological responses is initiated in an oxygen-dependent way. In general, hypoxia-induced adaptations aim to increase oxygen delivery on the one hand, whilst suppressing oxygen-consuming processes on the other hand. Two distinct oxygen-sensing mechanisms control these regulatory adaptations: the chemoreceptors and the HIF pathway. The latter controls cellular metabolic and structural reprogramming and hormone production by altering the transcription rate of HIF target genes. As such, HIF acts as a master regulator of erythropoiesis, angiogenesis, vascular tone and energetic substrate oxidation. Chemoreceptors on the other hand act through activation of oxygen-sensing glomus cells in the carotid and aortic bodies. Both carotid and aortic bodies are sensitive to

blood oxygenation, P_aCO_2 and pH. However, chemoreceptors in the carotid body are particularly sensitive for P_aO_2 and primarily stimulate ventilation, whereas aortic chemoreceptors are mainly responsive to C_aO_2 and primarily stimulate heart rate [31,32]. Upon activation chemoreceptors fire action potentials to the cardiovascular and respiratory control centers in the central nervous system. These respiratory and adrenergic centers in turn respond by stimulating minute ventilation and cardiac output so to compensate for the lowered C_aO_2. Hypoxic exposure thus affects human physiology in a tissue-specific manner, as will be discussed in greater detail below.

1.3.1. Ventilatory response

As P_aO_2 decreases upon acute exposure to hypoxia, peripheral chemoreceptors in the carotid body respond by firing action potentials to the cardiorespiratory centers of the central nervous system. In an effort to restore blood oxygenation, the latter responds by increasing minute ventilation in a biphasic manner [4]. Indeed, during the initial ~5 min of hypoxic exposure, minute ventilation rapidly increases to slowly decrease again in the subsequent 15 min to reach a new steady state around two-thirds of the initial increase. The first phase is defined as the acute hypoxic ventilatory response, whereas the second phase is known as the hypoxic ventilatory decline. Following the acute response, during the next hours, days and weeks, ventilation steadily rises again as a result of increasing sensitivity of the carotid body and sensitization of the central nervous system for its input signals [3]. This process is defined as 'ventilatory acclimatization to hypoxia' and allows partial restoration of P_AO_2 towards normoxic values (**Figure 5**). This acclimatization process is believed to occur at least partially via expansion of type 1 glomus cells, as well as by central nervous system sensitization for afferent input [31]. As previously described, such adaptation enables better blood oxygenation and thus PO_2 in tissues downstream the oxygen cascade (**Figure 3**).

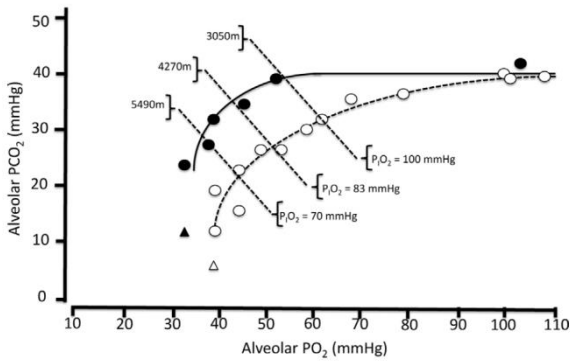


Figure 5. Ventilatory acclimatization to hypoxia partially restores alveolar oxygen pressure.
Filled and open symbols represent values of unacclimatized and acclimatized subjects, respectively. Reproduced from Ainslie *et al.*, 2013 [4], with permission.

1.3.2. Hematological response

Alveolar hyperventilation in ambient hypoxia decreases arterial partial pressure of CO_2 (P_aCO_2) and so increases blood pH, a state called respiratory alkalosis (**Fout! Verwijzingsbron niet gevonden.**). For blood pH to restore to its normal acid-base balance, the kidneys need to excrete bicarbonate during the first days of 'living high'. Still, complete restoration of blood pH will not be reached, causing the oxyhemoglobin dissociation curve to shift leftwards (**Figure 7)** [34]. As this shift facilitates oxygen loading from the alveoli to hemoglobin in the pulmonary capillaries, and animals living at high altitude seemingly having adapted to altitude by increasing their hemoglobin-oxygen affinity [35], this is believed to be a strategy to cope with low ambient PO_2 at altitude.

Altitude	Equivalent	Pb (mm Hg)	Estimated Pao_2 (mm Hg)	Estimated Sao_2 (%)	$Paco_2$ (mm Hg)
Sea level	—	760	90–100	97–99	38–42
5280 ft (1610 m)	Denver	623	65–80	93–97	32–42
8000 ft (2440 m)	Machu Pichu	564	45–70	88–95	31–36
12,000 ft (3660 m)	La Paz, Bolivia	483	42–53	80–89	24–34
17,500 ft (5330 m)	Everest Basecamp	388	38–50	65–81	22–30
29,000 ft (8840 m)	Everest Summit	253	28–32	54–62	10–14

Figure 6. Typical arterial partial pressures of oxygen and carbon dioxide with increasing altitude. Pb, barometric pressure; P_aO_2, arterial partial pressure of oxygen; P_aCO_2, arterial partial pressure of carbon dioxide; S_aO_2, arterial oxygen saturation. Figure reproduced from Davis & Hackett, 2017 [36], with permission.

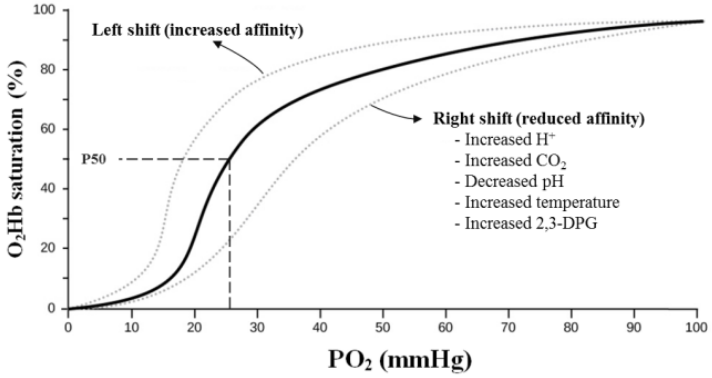


Figure 7. Oxyhemoglobin dissociation curve.
The normal oxyhemoglobin dissociation curve in normoxia is represented by the solid line. P50 is the partial pressure of oxygen (PO_2) at which 50% of Hb is saturated with oxygen. H^+, CO_2, pH, temperature and 2,3-di-phosphoglycerate (2,3-DPG) influence the structure of Hb and hence its affinity for oxygen. In humans, normal P5O fluctuates around 28 mmHg, though considerable variation has been reported [35]. Despite increased levels of 2,3-DPG in acclimatizing lowlanders [37], respiratory alkalosis overrules its influence and causes P50 to decrease via a leftwards shift of the oxygen dissociation curve [34].

Upon the first 24 h of 'living high' at 2500 m, plasma volume rapidly decreases by ~6%, concomitantly increasing hemoglobin concentrations and hematocrit values by 0.5 $g \cdot dL^{-1}$ and 1.5 points, respectively [38]. Throughout the following days, plasma volume progressively decreases, reaching a new steady state within about one week. Although these changes become more prominent at increasing altitude [38], as is the production of erythropoietin (EPO) [39], the observed elevations in hemoglobin concentrations are almost entirely attributable to loss of plasma volume. Indeed, increments in total hemoglobin mass (Hbmass) are not expected within this time frame, for a minimum altitude exposure of approximately two weeks is required to elicit significant increments in Hbmass (**Figure 8**) [40]. In general, Hbmass increases by ~1.1% per 100 h of altitude exposure above 2100 m [41]. However, responses are largely variable within and between individuals [42-44]. Unfortunately, pertinent markers of individual altitude sensitivity are lacking, and predicting the hematological response on an individual basis remains difficult if not impossible at this stage.

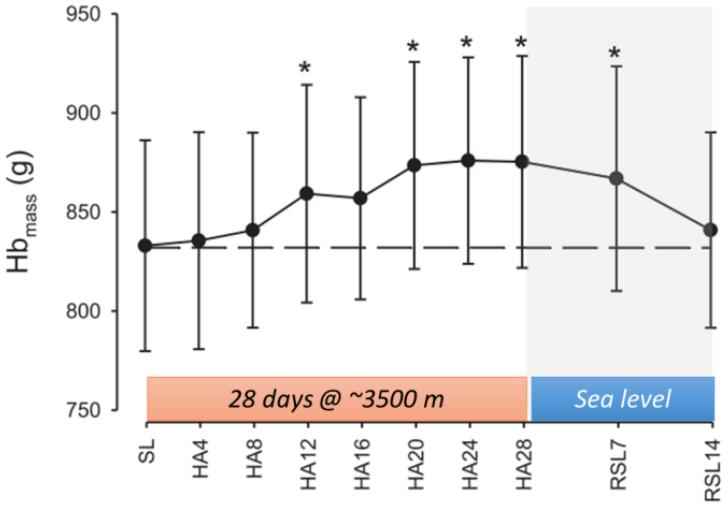


Figure 8. Expansion and decay of total hemoglobin mass during 28 days 'living high' at 3454 m followed by 14 days of 'living low'. Data represent mean ± SEM hemoglobin mass (Hbmass) in 9 subjects at sea level (SL) and every 4th day of living at high altitude (HA). Decay in Hbmass was measured on day 7 and 14 after return to sea level (RSL).
*, $P < 0.05$ *versus* SL. Figure adapted from Siebenmann *et al.*, 2015 [40], with permission.

Hypoxia-induced stimulation of renal EPO production is primary regulated by the HIF-2α pathway (elaborately reviewed in [45-47]). Serum EPO (sEPO) levels increase in a dose-dependent manner in response to reductions in blood oxygenation (or magnitude of altitude exposure) [37,39,48]. HIF-2 controls both the number and expression levels of renal EPO-producing cells [45,49]. The sEPO response to altitude is biphasic; reaching its peak within the first 48 h and gradually declining back towards sea-level values over the following days and weeks (**Figure 9**) [43,48,50]. Although not yet fully understood, this biphasic response suggests one or several negative feedback loops to control the production of EPO. Firstly, PHD2 and PHD3 genes are known HIF targets, increasing in content with decreasing HIF-α protein abundance during long-term hypoxic exposure [51,53]. Indeed, oxygen-sensitive alterations in PHD expression allow cells to reset their normoxic setpoint to their new environmental PO_2 [52,53]. Secondly, hypoxia-induced increases in C_aO_2 over time in conjunction with facilitated oxygen extraction by renal EPO-producing cells by virtue of elevated 2,3-DPG concentrations in acclimatizing lowlanders presumably also contribute to this phenomena [37,54]. Finally, one should keep in mind that sEPO is the product of EPO production, degradation and excretion [55]. However, whether altitude exposure affects the rate of EPO degradation is not yet known.

EPO acts as an anti-apoptotic agent on colony-forming units-erythroids. These are erythrocytic progenitor cells located in the bone barrow, which in the presence of EPO proliferate and successively differentiate to proerythroblasts, normoblasts, reticulocytes and eventually erythrocytes. After an acute increase in EPO, it takes about 3 to 4 days for the first reticulocytes to appear in the blood, and about 7 days for the colony-forming units-erythroids to transform into fully differentiated erythrocytes [56]. Administration of recombinant human EPO aggressively increases Hbmass, i.e. by ~18% following 2 weeks of 3-weekly injections of 250 IU/kg in recreational athletes [57]. This response is dose-dependent [58-61]. It was initially postulated that in lowlanders the hematological response to 'living high' could be predicted by the magnitude of rise in endogenous sEPO levels during the initial phase of altitude training [42]. However, this was contradicted by follow-up studies showing divergent sEPO and Hbmass responses despite high inter- and intra-individual variability in both these parameters [40,43,62,63]. Still, the underlying mechanism for the lacking correlation remains unclear.

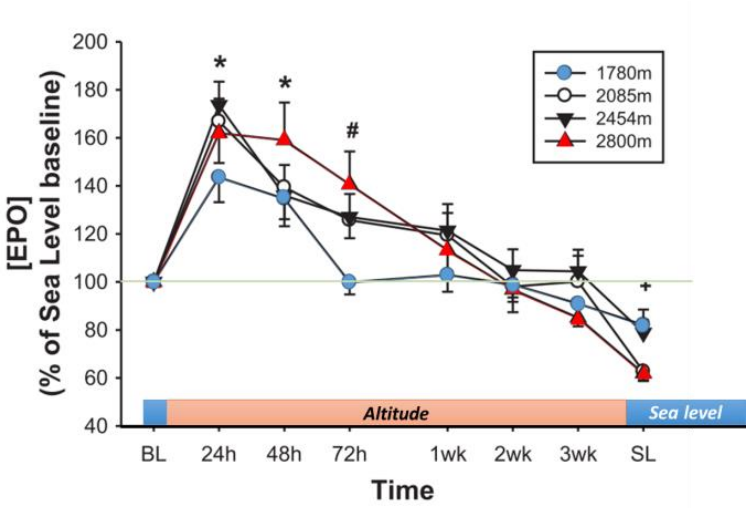


Figure 9. Erythropoietin concentrations throughout 4 weeks of 'living high' at a given altitude.

Data represent mean ± SEM erythropoietin concentrations ([EPO]) expressed as percentage of pre-altitude baseline (BL) throughout 4 weeks of 'living high' and upon the second day of return to sea level (SL). *, $P < 0.05$ vs. BL in all four altitude groups; #, $P < 0.05$ vs. BL in the 2085-m, 2454-m, and 2800-m groups only; +, $P < 0.05$ vs. BL in the 2085-m and 2800-m groups only. Figure adapted from Chapman *et al.*, 2014 [48]. with permission.

1.3.3. Cardiac response

Already more than a century ago, Douglas *et al.* [64] reported about the tachycardic response upon ascent to altitude. Heart rate results from sympathetic and parasympathetic modulation of the intrinsic heart rate. The autonomic regulation of the heart is, amongst others, influenced by the input from the peripheral chemoreceptors [65]. Indeed, consequent to increased sympathetic activation in conjunction with vagal withdrawal [66], heart rate at rest and during submaximal exercise increases proportionately to the magnitude of hypoxia [67]. At least during acute hypoxic exposure at rest, the increase in cardiac output is solely attributed to the chronotropic response, for stroke volume remains unaltered in these conditions [68]. Following several days of acclimatization, however, cardiac output returns to sea-level values, despite persistent elevation in heart rate and thus due to decreasing stroke volume [69]. As infusion-induced normalization of plasma volume restores stroke volumes in lowlanders acclimatizing to altitude, loss of plasma volume is believed to be at least partially responsible for the lowered stroke volume at altitude. Though some studies have reported heart rate to normalize following acclimatization [70,71], most compelling evidence indicates that sympathetic hyperactivity is a persistent phenomenon not allowing full normalization to sea-level values [72,73]. In contrast to heart rate at rest and during submaximal exercise, maximal heart rate is decreased during both acute and chronic exposure to hypoxia [74]. In hypobaric hypoxia corresponding to 300-2800 m, maximal heart rate on average decreases by ~2 beats per 1000 m altitude added [75]. A greater decline of ~8 beats per min per 1000 m has been reported during altitude exposures corresponding to 3300 to 6300 m [76]. Reduction in maximal heart rate at altitude is believed to be a mechanism to preserve functional integrity of the heart. Indeed, increasing heart rate requires increasing coronary blood flow. However, from a given combination of exercise intensity and hypoxemia at altitude, the coronary blood flow requirement might surpass coronary perfusion capacity [74]. In addition, further increasing cardiac output and therefore pulmonary capillary transit time against a background of diffusion limitation would only further aggravate arterial oxygen desaturation.

1.3.4.Muscular response

1.3.4.1. Cellular and myocellular metabolic and transcriptional response

The cellular response to hypoxia has been studied extensively in tumor cell lines, and to a lesser extent in vivo in rodents and humans. Extrapolation of *in vitro*-obtained mechanistic data to *in vivo* situations in humans should thus be performed with caution. Nonetheless, continuously expanding literature allows for increasingly greater insights in the HIF pathway. Clearly, activation of HIF target genes endeavors maintenance of oxygen homeostasis by increasing oxygen delivery on the one hand, whilst decreasing oxygen consumption on the other hand [77-80] (**Figure 10**). As previously highlighted, high rates of mitochondrial oxygen utilization may cause local cellular hypoxia. Hypoxic induction of pyruvate dehydrogenase kinase (PDK) inhibits the conversion of pyruvate to acetyl-CoA. HIF-induced PDK activity thus inhibits the oxidation of pyruvate, whilst stimulating the anaerobic conversion to lactate [79-82]. In addition, HIF has also been found to induce mitochondrial autophagy via the expression of Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNIP3) [77]. Clearly, under hypoxic conditions oxidative energy provision is downregulated and must be compensated for by increased glycolysis. The use of glucose as an energy substrate is more suitable than fatty acids under conditions of limited O_2 availability, given that 'anaerobic' glucose catabolism (glucose → pyruvate → lactic acid) allows energy provision without the need for O_2. The latter is, however, at the expense of pH homeostasis and therefore only allows high rates of anaerobic energy provision for a limited amount of time. Nonetheless, in hypoxia, even 'aerobic' conversion of glucose (glucose → pyruvate → acetyl-CoA → oxidative phosphorylation) is preferred over fatty acid catabolism because the ATP yield per O_2 molecule (glucose: 6 ATP per O_2 molecule) is ~7% higher than that for fatty acids (palmitate: 5.6 ATP per O_2 molecule). It is thus not surprising that several glucose transporters and nearly all glycolytic enzymes probably are under the control of HIF [79]. Activation of lactate dehydrogenase-A (LDHA) stimulates the conversion of pyruvate to $lactate^- + H^+$. For the cell to be able to better cope with such increase in proton production, HIF also activates target genes encoding for pH-regulating proteins such as monocarboxylate transporter 4 (MCT4), sodium-proton exchanger 1 (NHE1) and carbonic anhydrase 9 and 12 (CA9,12) [79-83]. That being said, at maximum aerobic exercise capacity, high-altitude natives and acclimatized lowlanders have been reported to generate lower than expected peak post-exercise blood lactate concentrations, a phenomenon referred to as the lactate paradox [84]. It was suggested that in chronic hypoxia, an improved metabolic matching between pyruvate production through glycolysis and pyruvate oxidation by the mitochondria following its conversion to acertyl-CoA would result in less pyruvate excess and hence lactate production. However, the lactate paradox and the aforementioned underlying mechanism have been challenged by more recent experiments and remain a matter of debate [85].

Finally, as stated previously in paragraph 1.3.2., HIF also stimulates erythropoiesis via transcription of EPO [47] and genes involved in iron uptake and metabolism [86]. More importantly at the muscular level, induction of vascular endothelial growth factor (VEGF) stimulates angiogenesis to facilitate more homogeneous muscle perfusion and O_2 extraction [87].

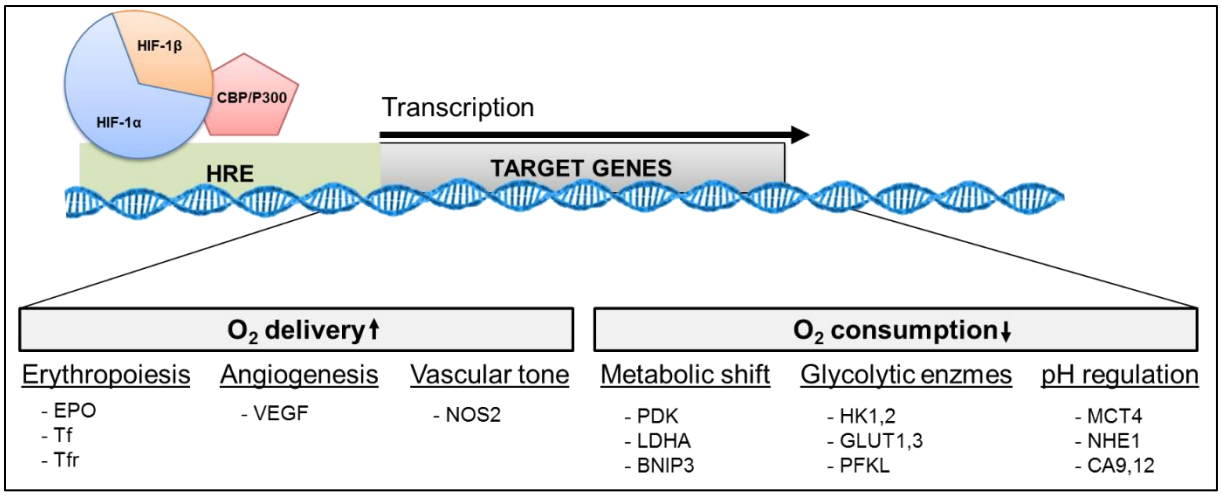


Figure 10. Major HIF-1 target genes controlling oxygen delivery and consumption.
Hypoxia-inducible factors (HIF-1, HIF-2) are major transcription factors controlling the expression of more than hundreds of genes that enable the cell and organism to adapt and survive local and ambient hypoxic conditions. HIF enhances the delivery of oxygen via interaction with hypoxic responsive elements (HRE) and hence activation of genes encoding for erythropoietin (EPO), transferrin (Tf), transferrin receptor (Tfr), vascular endothelial growth factor (VEGF) and nitric oxide synthase 2 (NOS2). HIF activation shifts the substrate metabolism away from oxidative phosphorylation in the mitochondria via induction of pyruvate dehydrogenase kinase (PDK), lactate dehydrogenase-A (LDHA) and Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNIP3). Glycolysis and the anaerobic metabolism are stimulated to compensate the reduction in oxidative energy-turnover via induction of hexokinase 1 and 2 (HK1,2), glucose transporter 1 and 3 (GLUT1,3) and phosphofructokinase L (PFKL). Such a metabolic shift is associated with increased proton production, partially covered by HIF-induced induction of pH-regulating genes monocarboxylate transporter 4 (MCT4), sodium-proton exchanger 1 (NHE1) and carbonic anhydrase 9 and 12 (CA9,12). Validation of HIF target genes via [77-83].

1.3.4.2. HIF-1α stabilization in skeletal muscle during 'living high'

Muscle tissue frequently undergoes episodes of hypoxic stress, either because of mitochondrial oxygen consumption or due to impaired oxygen flux at some point throughout the oxygen cascade. Whether or not environmental hypoxia is sufficient to stabilize HIF-1α in resting skeletal muscle has been a recent topic of debate [88]. However, recent observations from our laboratory have shown HIF-1α to stabilize within 6 hour of passive exposure to gradually increasing normobaric hypoxia from sea level to ~5300 m (10.7% FiO_2) [80]. Still, the minimal degree of hypoxia needed to elicit HIF-1α stabilization in human skeletal muscle is still unknown. Also the time course of HIF-1α stabilization in human during prolonged exposures to hypoxia remains to be elucidated. Nevertheless, cell culture and in vivo data in mice have already provided evidence for the operation of a functional feedback loop limiting the hypoxic response over time. Indeed, during sustained hypoxic exposure, HIF-1α acts in a biphasic response, albeit tissue specific, resetting its 'normoxic' setpoint downward by stimulating the expression of PHD1-3 [52,53]. Hence HIF itself, rather than altered oxygenation status, is the mediator of this negative feedback loop. This might also explain why muscle HIF-1α protein content is not increased in sea-level natives living at Everest base camp (~5300 m) for 19 or 66 days [89].

The cellular response to hypoxia primarily aims to shut down oxygen-consuming processes. Hence it is not surprising that protein synthesis, estimated to account for about one third of cellular energy expenditure in differentiating mammalian cells [90], is almost completely inhibited in anoxic conditions [91]. Energy starvation associated with severe micro-environmental hypoxia leads to a drop in ATP/ADP ratio, thereby stimulating AMP-activated protein kinase (AMPK). This kinase, together with activation of HIF, suppresses the mammalian target of rapamycin (mTOR) signaling pathway and thereby protein synthesis [92,93]. In vivo studies investigating muscle wasting during high altitude sojourns indicated a threshold for muscle atrophy to occur from about ~5000 m and beyond [94]. It is tempting to speculate that this 'threshold' corresponds to the HIF-1α stabilization threshold. However, one must interpret these results with caution because changes in physical activity level, food intake, sleep quality, as well as cold exposure conceivably may act as confounding factors [95,96]. Muscle cross-sectional area in subjects sojourning 8-10 weeks at Everest base camp (~5300 m) has been reported to decrease by about 10% [97]. This was associated with increased capillary density, but not capillary-to-fiber ratio, indicating that no neovascularization had taken place. Muscle mitochondrial volume density was decreased by about 20%, which corroborates decreased oxidative enzymes activity [98]. 'Operation Everest II', a project in which subjects sojourned in a hypobaric chamber for 40 days to simulate an ascent to Mt Everest, confirms the aforementioned field observations with regard to muscle wasting, absence of neovascularization, and unaltered or decreased mitochondrial content and oxidative enzymes [99,100]. In a more recent project, the Caudwell Xtreme Everest expedition, muscle samples were taken from lowlanders at sea level and at Everest base camp (~5300 m), either 19 days after initiating their ascent to basecamp or after 66 days of altitude with ascents beyond 6400 m [89]. Muscle oxidative capacity, as measured via mitochondrial density and protein content of citrate synthase and complex I and IV of the electron transport chain decreased in participants living 66 days at extreme altitude only. Mean muscle fiber area or capillarization changed in neither groups.

Importantly, in the context of athletes engaging in altitude training for several weeks, sojourning at moderate altitudes (2000-3000 m) affects neither skeletal muscle mass [94], nor mitochondrial content or function [101], nor pH-regulating proteins and buffering capacity [102].

1.3.4.3. HIF-1α stabilization in skeletal muscle during 'training low' *versus* 'training high'

Muscle contractions are distinctly more potent than low inspired PO_2 to decrease P_iO_2 (see paragraph 1.2.2.). Superimposing ambient hypoxia to muscle contractions ('train high') only marginally exacerbates contraction-induced drop in P_iO_2 [17,103]. Hence exercise in either hypoxia or normoxia similarly increases muscle HIF-1α mRNA [104], protein content [105], nuclearization [105] and DNA binding activity [105]. Thus any exercise-induced drop in P_iO_2 probably is ample to fully activate HIF-1, irrespective of arterial O_2-saturation. Nonetheless, a recent study showed submaximal endurance exercise in severe hypoxia (F_iO_2: 10.7) but not in normoxia to increase HIF-1α protein content [80]. Along the same line, high-intensity endurance training in hypoxia but not in normoxia, has been found to induce some unique muscular adaptations [106] (see paragraph 1.6.3. for in-depth discussion).

1.4. Maladaptive responses to hypoxia

Exposure to ambient hypoxia alters blood flow in a tissue-specific manner. Systemic, muscular and cerebral arterioles vasodilate in attempt to compensate for decreased CaO_2 by enhancing tissue blood flow and concomitantly O_2 delivery. Lung vasculature on the other hand undergoes

vasoconstriction in hypoxia to minimize pulmonary shunting. Although these adaptive mechanisms may aid maintenance of oxygen homeostasis, they are also believed to play a role in the underlying mechanisms of high-altitude sickness. Indeed, excessive cerebral blood flow has been associated with cerebral edema and increased intracranial pressure [107]. Traditionally, this has been considered the primary mechanism underlying acute mountain sickness (AMS) and high-altitude cerebral edema (HACE), in which AMS was considered the early manifestation HACE. Whereas the former is characterized by rather benign symptoms of headache and general malaise, the latter is associated with severe life-threatening symptoms secondly to central nervous system impairment [108]. Though related, newly emerging paradigms also show differences in the complex pathophysiology of AMS and HACE [109].

Likewise, the hypoxic pulmonary response may be maladaptive in its excessive effectuation. In ambient hypoxia, all alveoli experience lower O_2 tension, resulting in a widespread though sometimes rather inhomogeneous pulmonary vasoconstriction. Increased cardiac output and inhomogeneous pulmonary vasoconstriction lead to localized hyperperfusion of the pulmonary capillary network. These effects are associated with high hydrostatic pressures, in turn causing leakage of fluids into the interstitium and the alveoli. When plasma leakage exceeds the drainage capacity of the lymphatics, high-altitude pulmonary edema (HAPE) develops and requires urgent treatment [108].

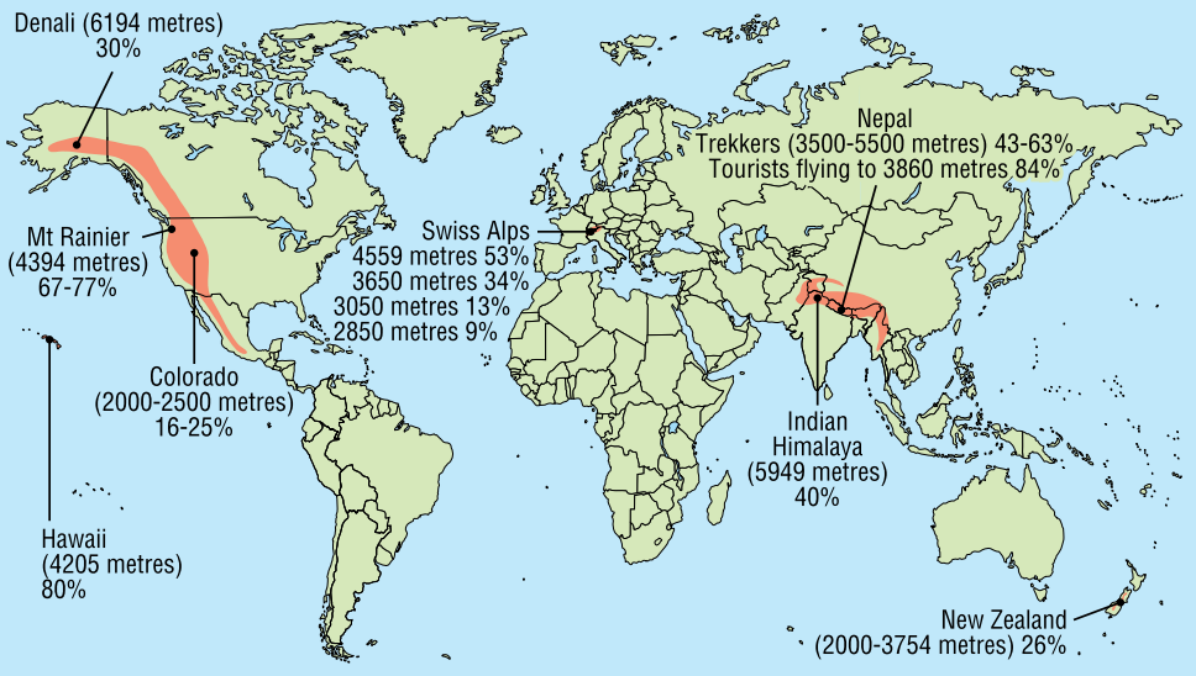


Figure 11. Global incidence of acute mountain sickness in the Himalaya, European Alps, Hawaii's volcanos, and the mountainous regions in North America and New Zealand.
Reproduced from Barry and Pollard, 2003 [110], with perimission.

1.4.1.Acute mountain sickness

The occurrence of headache in conjunction with fatigue, dizziness, sleep disturbance, and/or gastro-intestinal discomfort shortly after a rise in altitude is known as a syndrome called AMS [111]. AMS is the most common form of high-altitude illness, affecting approximately 25% of visitors traveling to ~2000 - 3000 m [112], and more than half the population traveling above ~4000 m [108,113,114] (**Figure 11**). In general, symptoms develop 6 to 12 h after arrival to new altitude, though earlier or later symptom

development should also be considered to reflect AMS until proven otherwise. In mild AMS, symptoms generally spontaneously dissolve within 24-48 h when subjects remain at the same altitude and are provided adequate rest, hydration, and possibly pharmacological treatment in the form of general pain killers and acetazolamide. If symptoms do not resolve or further aggravate, descend to lower altitude and/or O_2 supplementation is the primary treatment strategy [36]. Ignoring symptoms of AMS and further ascending to greater altitude aggravates the altitude illness therefore potentially progressing into HACE. This life-threatening disorder needs to be treated urgently, as central nervous system impairment may progress within hours and result in loss of consciousness and eventually death.

1.4.2. Pathophysiology of acute mountain sickness

The incidence of AMS increases along the severity of environmental hypoxia. Accordingly, arterial oxygen saturation (SpO_2) in hypoxia has been shown to correlate with AMS development [115]. Cerebral hypoxemia triggers an increase of cerebral blood flow [116,117]. In subjects with AMS, the capacity of the cerebral arteries to anticipate rapid surges in pressure is impaired [118,119]. Impaired autoregulation is postulated to induce local surges in cerebral blood flow and capillary pressure, thereby causing disruption of the blood-brain barrier and vasogenic edema. As such, AMS was long believed a sequel of high intracranial pressure due to brain swelling. However, in clear contrast to HACE [120,121], no hard evidence has been found for disruption of the blood-brain barrier in AMS [119,122,123]. Hence only mild vasogenic edema is observed in AMS positive subjects. Importantly, this mild edema is of similar extent compared to healthy subjects exposed to similar levels of hypoxia [124,125]. Indeed, there appears to be no evident correlation between elevated cerebral arterial inflow and AMS symptom severity [126,127]. Thus, against the early 'tight-fit' hypothesis wherein AMS susceptible individuals are believed to have a 'tight' *versus* 'compliant' brain, the latter hypothetically allowing greater asymptomatic increases in cerebral blood volume or edema, clear evidence for AMS to be caused by high intracranial pressure remains poor [122,128]. Furthermore, the mild volume increases typically observed in the AMS brain (5-10 mL, <1% brain volume) are far below the threshold (~50-120 mL) above which symptoms are triggered via pain sensitive fibers in the meninges or via activation of the trigeminovascular system [122,124,129]. There is, however, evidence showing AMS-specific redistribution of the cerebral fluid from the extracellular to the intracellular space [124,125]. The underlying mechanism of such intracellular (cytotoxic) astrocytic swelling remains to be elucidated, but may be consequent to impairment of Na^+/K^+-ATPase activity.

Also cerebral oxidative and nitrosative stress and local neurogenic inflammation may be involved in the pathophysiology of AMS. Indeed, these brain-borne stressors have been documented to correlate with AMS severity [123]. Systemic oxidative stress recently has been documented to correlate with SpO_2 and AMS severity [130]. It is postulated that free radicals and local neurogenic inflammation may stimulate the activation of the trigeminovascular system and so induce the AMS syndrome [109]. Attenuated AMS development has been reported following antioxidant supplementation in one study [131]. Another study showed subjects with high dietary antioxidant intake to be somewhat protected to AMS [130]. However, contradicting findings regarding the prophylactic effects of antioxidants have also been reported [132]. Clearly, the pathophysiology of AMS remains unclear and warrants further studies to allow precautionary strategies in AMS susceptible subjects.

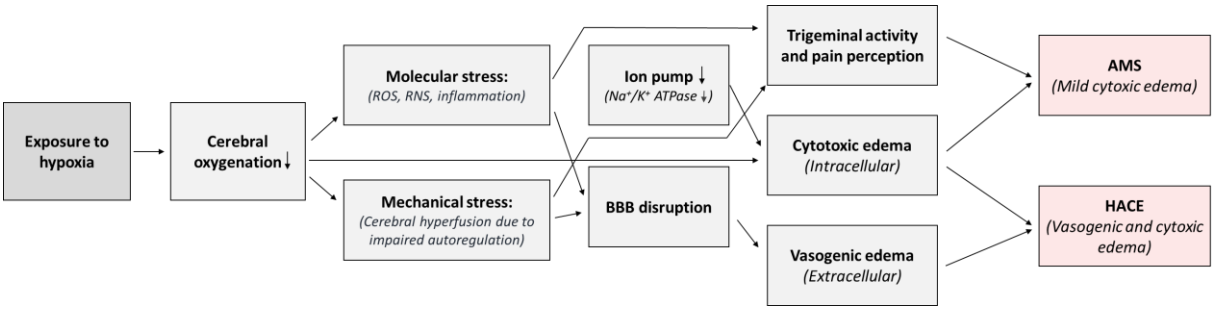


Figure 12. Pathophysiology of acute mountain sickness and high altitude cerebral edema.
Whereas molecular activation of the trigeminovascular system is currently considered the primary mechanism mediating acute mountain sickness (AMS), vasogenic edema is considered the principal mechanism leading to high-altitude cerebral edema (HACE) [133]. ROS: reactive oxygen species, RNS: reactive nitrogen species.

1.4.3. Predictors of acute mountain sickness

Effective altitude, rate of ascent, altitude pre-exposure, and individual susceptibility have been identified as the primary determinants of AMS (**Figure 13**) [108,113,134]. Despite the fact that previous history of AMS is known to be a strong independent risk factor [112,134], adequate testing procedures to assess individual susceptibility for AMS are still lacking. There is evidence for genetic predisposition [135,136], though genetic variants strongly associated with AMS are lacking. Awareness of one's susceptibility prior to ascend could allow timely implementation of preventive strategies, such as pharmacologic prophylaxis (acetazolamide), hypoxic preconditioning, and alterations in the ascend profile. Nonetheless, pertinent screening protocols enabling to accurately predict AMS are lacking to date.

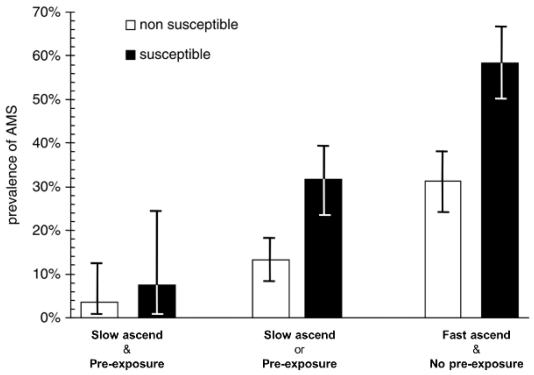


Figure 13. Prevalence of acute mountain sickness in susceptible and non-susceptible mountaineers on the morning after arrival at 4559 m.
Subjects reporting to never or seldom experience headache and other symptoms of acute mountain sickness (AMS) were considered not susceptible to AMS. Gradual ascend to altitude over a period of more than 3 days was defined as slow ascend. Subjects who had spent more than 4 days at an altitude above 3000 m two months prior to their ascend to 4559 m were classified as pre-exposed to altitude. Reproduced from Schneider *et al.*, 2002 [134], with permission.

Hypoxemia is the primary upstream factor triggering AMS. High ventilation at altitude protects against blood deoxygenation by virtue of more frequent refreshment of the alveolar air. Indeed, a lower rate of ventilatory acclimatization during the first days at altitude has been reported in subjects with AMS [137]. Early reports documented high pre-ascent hypoxic ventilatory response (HVR) to protect against AMS [138,139]. However, the majority of follow-up studies could not confirm these findings [137,140-143]. The HVR is biphasic, characterized by an acute increase in minute ventilation, which subsequently

decreases to slowly increase again in the course of time. Traditional HVR measurements represent the acute hypoxic ventilatory response, but not minute ventilation following the ventilatory decline. Given that AMS often develops within 6 hours of hypoxic exposure, this second phase in the biphasic HVR might be of greater importance for prediction of individual susceptibility to AMS. Nonetheless, conflicting results have also been reported concerning SpO_2 as a predictor of AMS. In a retrospective study, 63 subjects who previously experienced AMS on average showed 5% lower SpO_2 levels following 20-30 min of hypoxic exposure as compared to 87 non-susceptible subjects [144]. However, there was considerable overlap in SpO_2 drop between the groups. In the meantime, multiple follow-up studies have shown SpO_2 not to be of clinical relevance in the context of AMS prediction [145-148].

Exposure to hypoxia is associated with reduced parasympathetic regulation of the heart [149,150] and increased sympathetic nerve output to skeletal muscle [151]. The increments of plasma noradrenaline and adrenaline with increasing absolute exercise intensity are greater in hypoxic compared to normoxic conditions [152,153]. During acute hypoxic exposure at rest, either increased [154-158], decreased [135,153,159,160], or unaltered [150,161-163] plasma or urinary noradrenaline concentrations/exretions have been reported. However, more consistent findings of elevated plasma noradrenaline levels are found following the first few days at altitude [151]. Interestingly, hypoxia-induced elevations in sympathetic activity may be associated with AMS development. In a large group of 50 healthy male lowlanders both acute and extended exposure to hypobaric hypoxia (3658 m) markedly increased 24-h urinary catecholamine output, but only in symptomatic subjects [164]. Accordingly, higher venous and arterial noradrenaline and adrenaline levels have been observed in subjects developing AMS [156,165]. Indeed, symptoms of AMS positively correlate with arterial/venous noradrenaline/adrenaline concentrations in hypoxia [161,165]. In line with these findings, pharmacological beta-adrenergic blockade seems to attenuate AMS symptom development [166]. As sympathetic activity increases oxygen consumption and edema formation by sodium retention, sympathetic hyperactivity may indeed be involved in hypoxic maladaptation. However, one should keep in mind that the above findings do not show a cause-effect relationship *per se*, as sympathetic hyperactivity may also result from the experienced AMS symptoms and hypoxic stress. Furthermore, contrasting findings recently reported 8 hours of normobaric hypoxia to decrease urinary noradrenaline excretion, and even more so in subjects afflicted by AMS [135]. More research is warranted to clarify the exact role of parasympathetic and sympathetic regulation in hypoxic (mal-) adaptation and its possible role in AMS development.

Spontaneous changes in heart rate can easily be assessed by analysis of beat to beat RR intervals. Heart rate variability (HRV) reflects, amongst other factors, a qualitative measure of the activity of sympathetic and parasympathetic cardiac efferent nerve traffic [167]. The analysis of HRV in both the time and frequency domain might be useful to evaluate differences in the cardiac autonomic response to hypoxic exposure between subjects. **Table 1** summarizes typical HRV parameters, their corresponding abbreviations, as well as the interpretation in the context of autonomous nerve activity. Hypoxic exposure is known to attenuate cardiac autonomic regulation [159,168-174]. Interestingly, Karinen *et al.* [175] reported pronounced parasympathetic withdrawal at 2400 m, as reflected by decreased HF and RMSSD, to predict AMS development on further ascent to 3000 – 4300 m (RMSSD < 30 ms sensitivity and specificity of 92% and 58%, respectively). Along the same line, retrospective analysis showed low HFnu (< 20%) and high LF/HF ratio (> 1.3) at low altitude (1317 m) to be associated (odds ratio 7.0) with AMS development at 3440 m [176]. In the same study, subjects with AMS presented with lower TP, HF and HFnu in conjunction with a higher LF/HF ratio at 3440 m. Accordingly, in subjects transported to 3150 m, RMSSD and PNN50 increased after the first night at altitude only in subjects without AMS [170]. Conversely, these parameters tended to decrease in those displaying AMS symptoms. Surprisingly, low pre-ascend (normoxic) LF/HF (cutpoint $\leq$ 2.28 au) was recently reported to have moderate

diagnostic value (sensitivity: 85%; specificity: 88%) [177]. Notwithstanding the aforementioned findings, the potential to use HRV as a predictor of AMS is still a matter of debate. Both elaborate laboratory [178] and mountaineering [174] studies have failed to find a pertinent association between parameters of HRV and AMS. Further research exploring these relations is warranted.

Table 1 | Conventional heart rate variability measures

Variable	Unit	Definition	Attributed meaning
Time domain measures (time domain analysis)			
SDNN	ms	SD of all normal RR intervals	Total variance
RMSSD	ms	The square root of the mean squared differences between adjacent normal R–R intervals	Vagal activity
NN50		The number of pairs of adjacent normal R–R intervals that differ by more than 50 ms	Vagal activity
pNN50	%	Percentage of adjacent RR intervals that differ from each other more than 50 ms	Vagal activity
Frequency domain measures			
Total power	ms^2	Area under the entire power spectral curve (usually ≤0.40), variance of all normal RR intervals	Total variance
LF	ms^2	Low frequency power (0.04–0.15Hz)	Vagal activity
HF	ms^2	High Frequency power (0.15–0.40 Hz)	Combination of vagal (25%) and sympathetic (75%) activity + baroreceptor activity
LFnu	nu	Normalized low frequency power (LF/(LF+HF))	Vagal modulation
HFnu	nu	Normalized high frequency power (HF/(LF+HF))	Sympathetic modulation
LF/HF		Ratio of the low-to high frequency power	Index of sympathovagal balance

Nu: normalized units. Table adapted from [167,179], with permission.

1.5. Exercise training and performance

1.5.1. Limiting factors and trainability of exercise performance in normoxia

1.5.1.1. Endurance performance in normoxia

1.5.1.1.1. Whole body endurance performance

Maximal endurance performance is primarily determined by the maximal oxygen consumption that can be maintained for a given time, and the efficiency with which cellular oxygen utilization is translated into power output or speed [180]. The maximal oxygen consumption ($L \cdot min^{-1}$) that can be maintained for a given time is per definition set by VO_2max. The percentage of VO_2max at which endurance performance can be maintained correlates well with the second lactate threshold, which on its turn is at least partially determined by the oxidative capacity of the muscle [180]. Both VO_2max and the relative percentage at which the lactate threshold occurs can be increased by training (**Figure 14)**.

In the traditional view, the circulatory capacity to deliver oxygen to contracting muscles rather than the muscular capacity to extract and utilize the oxygen delivered, is considered the rate limiting factor of VO_2max [181]. Indeed, at whole-body VO_2max, *in-vitro* data from permeabilized muscle fibers suggest mitochondria to be activated to only ~70% of their maximal oxidative potential [182,183]. In addition, oxygen supplementation during whole body exercise has been shown to increase pulmonary [184] and leg [185] VO_2max, again advocating for oxygen delivery limitations. Accordingly, cross-sectional data show VO_2max to correlate well with maximal cardiac output (r = 0.97) [186] and Hbmass (r = 0.97) [187], both of which determine blood oxygen transport capacity. Furthermore, training-induced increases in maximal cardiac output (Qmax) linearly correlate with the concomitant increase in VO_2max [188]. Enhancement in Qmax is attributable to changes in maximal stroke volume. Maximal stroke volume is enhanced via cardiac eccentric hypertrophy, increased ventricular compliance, increased ventricular preload (~end-diastolic volume) due to higher blood volume, and increased ventricular contractility. Whether normoxic training increases blood volume and thereby end-diastolic volume solely via well-reported expansion in plasma volume [189], or in conjunction with higher erythrocyte volume remains a matter of debate. In recreational athletes, nine months of endurance training increased Hbmass and plasma volume by 6.4% and 11.6%, respectively [190]. In athletes, however, Hbmass seems to remain rather stable, independent of training volume and training phase [191-193]. The erythropoietic effects of normoxic training thus seem to be specific to one's training status, if any effects occur at all.

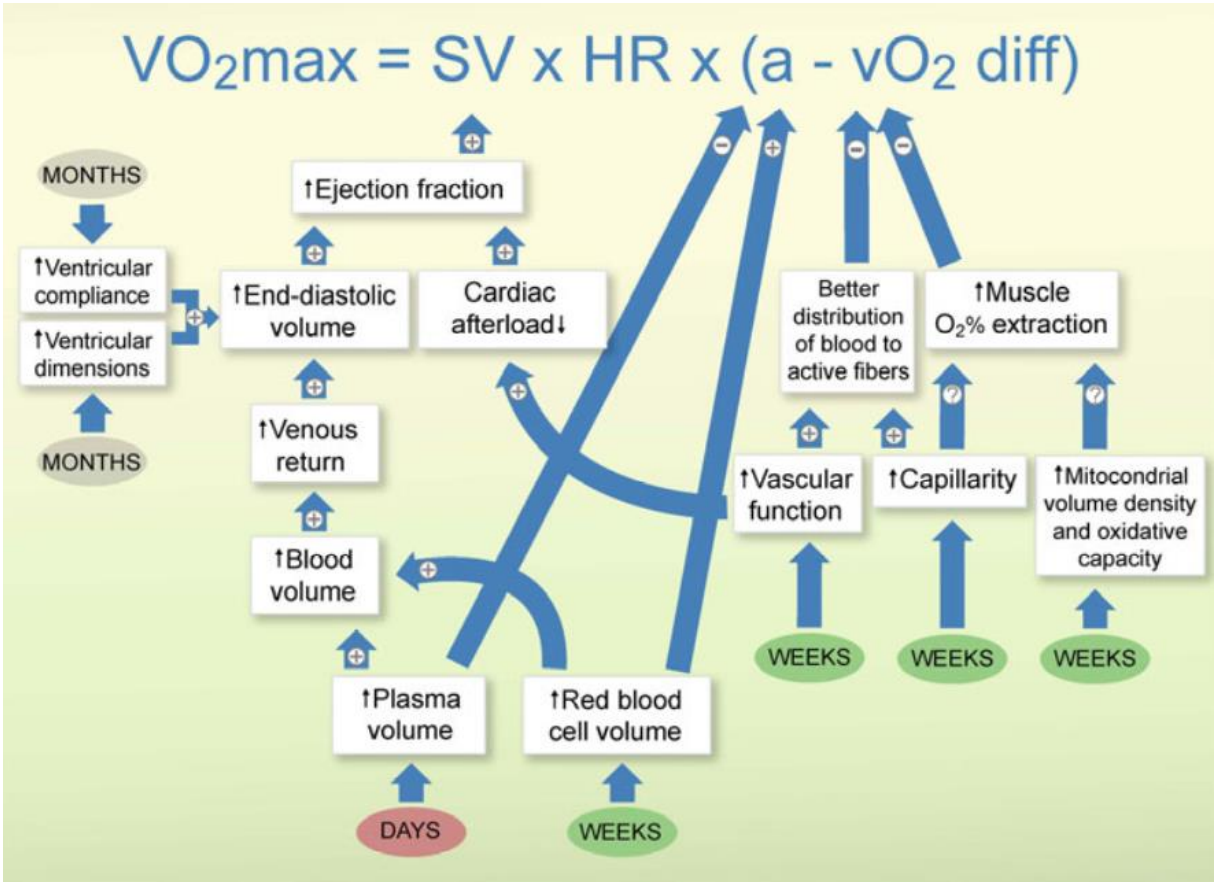


Figure 14. Enhancement in maximal oxygen uptake by exercise training.
Maximal oxygen uptake (VO_2max) is determined by the product of maximal cardiac output and arteriovenous oxygen difference (a-vO_2 diff). Cardiac output is the product of stroke volume (SV) and heart rate (HR). Reproduced from Lundby *et al.*, 2016 [186], with perimission.

1.5.1.1.2. Small muscle mass endurance performance

Skeletal muscle can accommodate a much larger blood flow than the heart can supply during whole body exerice [194]. Indeed, Qmax is inadequate to maintain blood pressure during vasodilation off all major arteries supplying blood to the active muscles during whole body exercise. Furthermore, muscle mitochondrial respiratory capacity surpasses the maximal oxygen delivery rate when a large muscle mass (half or more of the body's muscle mass) is engaged [182]. In contrast, maximal exercise using only a small muscle mass is not limited by Qmax, for a greater fraction of cardiac output can be directed to a small isolated muscle region. Indeed, muscle blood flow [194] and mass specific VO_2max (VO_2max divided by the activated muscle mass) [195] during one-legged knee extension exercise far exceed values observed during maximal two-legged cycling. Maximal arteriovenous oxygen differences (a-vO_2 diff) also are lower during one-legged exercise because the extremely short mean capillary transit times are insufficient for diffusional gas equilibration. Of note, it is believed that high capillary density and tortuosity in trained muscles enhance oxygen extraction capacity by virtue of longer capillary transit times and thus exchange of gasses, metabolites and substrates.

Against this background, it is not surprising that a recent meta-analysis reports that higher maximal a-vO_2 difference does not correlate with elevation in whole body VO_2max [188]. Indeed, seven weeks of one-legged endurance training increased one-legged but not two-legged cycling pulmonary VO_2max [196]. Maintenance of blood pressure during whole body exercise obviously receives priority to maximizing muscle blood flow, consequently limiting the capability to match blood flow to muscular respiratory capacity whenever a large fraction of muscle mass is recruited. The above arguments strongly advocate oxygen delivery to be the rate limiting factor of VO_2max, yet a recent study reports two weeks of high-intensity interval training to improve VO_2max (+6%) solely by expansion of muscle mitochondrial volume (+20%) in the absence of increased Qmax or blood volume [197]. The higher oxygen extraction capacity during maximal two-legged cycling in the latter study was believed to result from a greater oxygen gradient between the capillaries and mitochondria, for the expansion of mitochondria might have created a larger oxygen sink during exercise whilst diffusive capacity might have increased due to training-induced angiogenesis. Furthermore, a recent study shows enzyme activity of succinate dehydrogenase and estimated mitochondrial oxidative capacity to strongly correlate (r^2 = 0.81 and 0.89, respectively) with whole body VO_2max across heart failure patients, sedentary controls and highly trained cyclists with a mitochondrial overcapacity at whole body VO_2max of only 11% [198]. In conclusion, VO_2max is predominantly limited by oxygen supply, but oxygen extraction capacity should also be taken into account.

1.5.1.2. Single and repeated-sprint performance in normoxia

1.5.1.2.1. Energy systems

Single all-out sprint performance is determined by the maximal amount of energy that can be produced to generate power (product of force and velocity) over a short period of time (≤ 30 s). Therefore, sprint performance as measured during a 30-s all-out Wingate test (absolute power output) correlates well with lower extremity lean mass [199]. Maximal power output for a given mass of recruited muscle is constrained by the maximal capacity of energy-delivering processes on the one hand, and the capacity to handle accumulation of energy catabolites on the other hand. The relative energy supply from phosphocreatine (PCr), anaerobic glycolysis, and aerobic glycolysis during a single 30-s sprint has been estimated around 16, 55, and 29%, respectively [200]. During consecutive sprints, however, anaerobic glycolysis is inhibited and energy supply gradually shifts towards aerobic glycolysis. Thus, the relative energetic contribution of the aforementioned energetic processes shifts to respectively 16, 21 and 63%

during the final of three 30-s sprints interspersed by 4-min recovery episodes. A similar pattern of energetic shifting away from the anaerobic glycolytic metabolism also occurs during shorter 6-s sprints interspersed by brief 30-s recovery periods [201]. With the exception of the initial sprints in the series, repeated-sprint exercise should therefore be considered predominantly aerobic in nature.

The energetic contribution of PCr to successive sprints depends on the recovery time, and the capacity to resynthesize PCr prior to the next sprint. For example, PCr decreases to respectively 55 and 27% of its initial concentration after respectively one and five consecutive 6-s sprints interspersed by 30 s of recovery [202]. Following a recovery period of 30 s, PCr restores to respectively 69 and 45% of its initial value, whilst a 3-min recovery period enables resynthesis to respectively 90 and 84% of its baseline content [202]. Accordingly, after a 30-s sprint PCr is depleted to 16-20% of its resting value and is partially restored to 65, 78, and 85% after respectively 1.5, 4.0 and 6.0 min of recovery [203,204]. The rate of PCr resynthesis is an important determinant in repeated-sprint performance [203]. PCr resynthesis rate depends on oxidative metabolism pathways [205,206], hence subjects with high *versus* low VO_2max and mitochondrial respiratory capacity exhibit faster recovery rates and enhanced fatigue resistance during repeated-sprint exercise [207].

1.5.1.2.2. pH homeostasis

Most prominent adaptations in maximal glycolytic enzyme activity and anaerobic capacity (*i.e.*, as measured by maximal accumulated oxygen deficit during brief (1-2 min) all-out performance) typically occur following exercise training involving short (<30-s) all-out efforts interspersed by relatively long (~ 10min) recovery intervals [207]. Such training sessions induce high levels of lactate and H^+ accumulation, metabolites known to inhibit key glycolytic enzymes [208-210] and impair excitation-contraction coupling and force production [211-213]. Therefore, it is postulated that the capacity to maintain intracellular pH homeostasis via both H^+ buffering and extrusion plays a pivotal role in supramaximal performance [214-217]. A primary defense mechanism against perturbations in intracellular pH is provided by the neutralizing reactions of small peptides (i.e. carnosine), proteins, bicarbonate and inorganic phosphates(*i.e.*, HPO_4^{2-}). The *in vitro* intracellular buffering capacity (βhm) is traditionally measured on muscle homogenates via acid titration and expressed as the required amount of H^+ to alter intracellular pH with a single unit. As the H^+ accumulation quickly surpasses βhm, cellular H^+ export is required for maintenance of proper cell function. Because of its electric charge, H^+ cannot passively diffuse through the cellular membrane. Therefore proton efflux is mediated via protein-linked transport (reviewed in [218]) (**Figure 15**). At rest, pH homeostasis is primarily regulated by the Na^+/H^+ exchange (NHE) system, more specifically by the NHE1 isoform in human skeletal muscle. In addition, also myocellular bases such as bicarbonate are believed to contribute to pH homeostasis at rest. These bases need to be imported via Na^+/HCO_3^- cotransporters (NBC) and perhaps as well by $Cl^-/HCO3^-$ exchangers. As both NHE1 and NBC depend on a high Na^+ gradient across the membrane, extrusion of Na^+ via ATP consuming Na+/K+-pumps must also be taken into account. Unlike in resting conditions, removal of H^+ during exercise is primarily mediated via proton-linked transport of lactate by monocarboxylate transporters (MCTs). Whereas MCT1 mainly regulates cellular import of $lactate^-$ and H^+, export is mediated via the MCT4 isoform. Accordingly, higher concentrations of MCT1 are found in oxidative fibers [219-221]. In these fibers, lactate is converted back to pyruvate and Acetyl-Coenzyme A so to be oxidized as an energetic substrate. Conversely, high concentrations of MCT4 and NHE1 are typically found in glycolytic muscle fibers [221,222].

1.5.1.2.3. Trainability of muscle pH-handling capacity

MCT1 has been reported to increase following both low- [223,224] and high-intensity training [225-228]. However, high-intensity muscle contractions seem needed to elevate muscle membrane MCT4 content [225-227]. The magnitude of enhancement in MCT4 content generally is less pronounced compared

to its MCT1 isoform. High-intensity training has also been reported not to increase MCT1 and/or MCT4 [229-234]. Accordingly, discordant results have been reported regarding training-induced changes in NHE1, with some studies observing training-induced elevations in muscle NHE1 content [233,235-237], and others who do not [229,231,232]. Preliminary evidence suggest that the NBCe1 isoform can also be enhanced by training [238]. Finally, discordant data have been reported regarding training-induced changes in βhm, with most [226,230,233,238-243] but not all high-intensity interval training (HIIT) studies [244-247] failing to increase βhm.

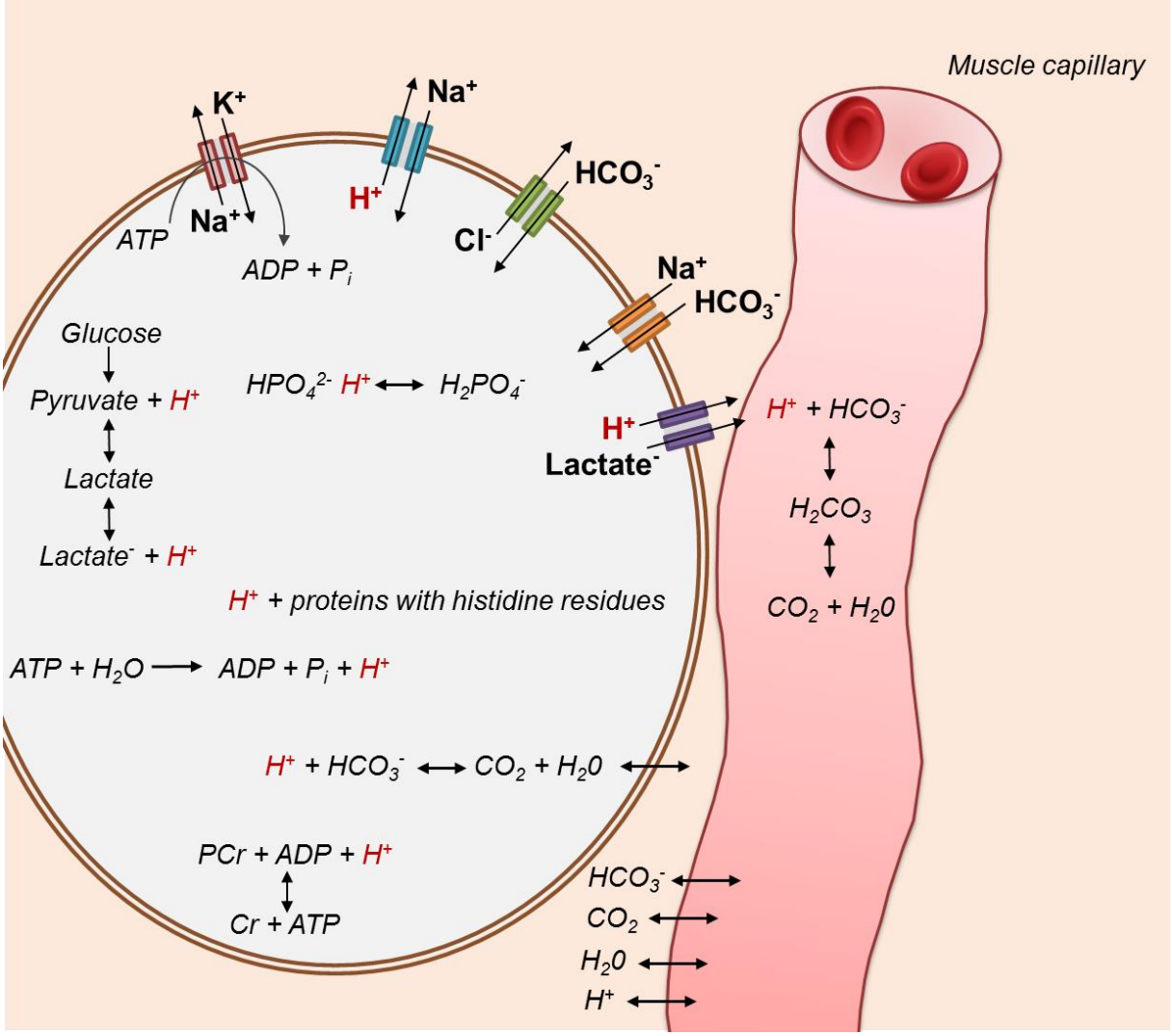


Figure 15. Schematic representation of the major regulators of pH homeostasis in the muscle cell. Physico-chemical buffering (inorganic phosphates, bicarbonate, and proteins with histidine residues (i.e., carnosine and anserine)), metabolic buffering (phosphocreatine), and transmembrane fluxes of protons regulate pH homeostasis in the muscle cell.

1.5.2.Exercise performance in hypoxia

The effects of acute altitude exposure on exercise performance are obvious from the comparison of athletic performances during the Mexico Olympic Games in 1968, held at an altitude of ~2300 m, with the world records existing at that time (**Figure 16)**. Hypoxia negatively affects all endurance performances (*i.e.*, track and field events longer than 800 m) [248-250]. Conversely, sprint

performances (*i.e.*, 100 – 400 m in track and field events) improve consequent to lowered air resistance [248-250]. As described above, endurance performance is primarily determined by the capacity to deliver oxygen to the working muscles. Every alteration in one of the different steps of the O_2-cascade impacts on muscular oxygen delivery. Decreased inspired PO_2 at altitude lowers the capacity to deliver O_2 to the working muscles. By this means, VO_2max linearly decreases with falling ambient PO_2, at a rate of 6-8% per 1000 m altitude [251]. Indeed, the decline in VO_2max is due to alterations in oxygen delivery, as the decrease in SpO_2 accounts for about 70 to 86% of the decrease in VO_2max [75,252]. In some high-velocity endurance sports, however, the positive effects of 'thin air' on air resistance outweighs the negative effects on VO_2max. In fact, models integrating both aforementioned parameters predict 1-h cycling performance (*i.e.*, the world hour record) to benefit from altitude exposure up to 3650 – 3750 m above sea level, above which distance covered is predicted to decrease again [253].

In line with the above example, sprint performances by definition are enhanced at moderate altitude. Whilst these short all-out disciplines likewise benefit from decreased air resistance, the potential negative effects of decreased O_2 availability are negligible. Indeed, in contrast to endurance exercise, sprint performance depends less on aerobic energy provision and is therefore barely, if at all affected by ambient hypoxia [254]. Moreover, in endurance-trained cyclists, impaired aerobic energy provision during a 30-s all-out cycling sprint in normobaric hypoxia (10.4% F_iO_2, ~5300 m) is compensated by increased anaerobic energy provision. Indeed, cyclist were capable of maintaining power output in hypoxia by increasing relative anaerobic energy provision from 71 to 77% [255]. However, as sequential sprint efforts are performed, hypoxia likewise impairs repeated-sprint performance [256-258]. This amongst others translates into impaired performance in team sport competitions such as soccer at altitude [259].

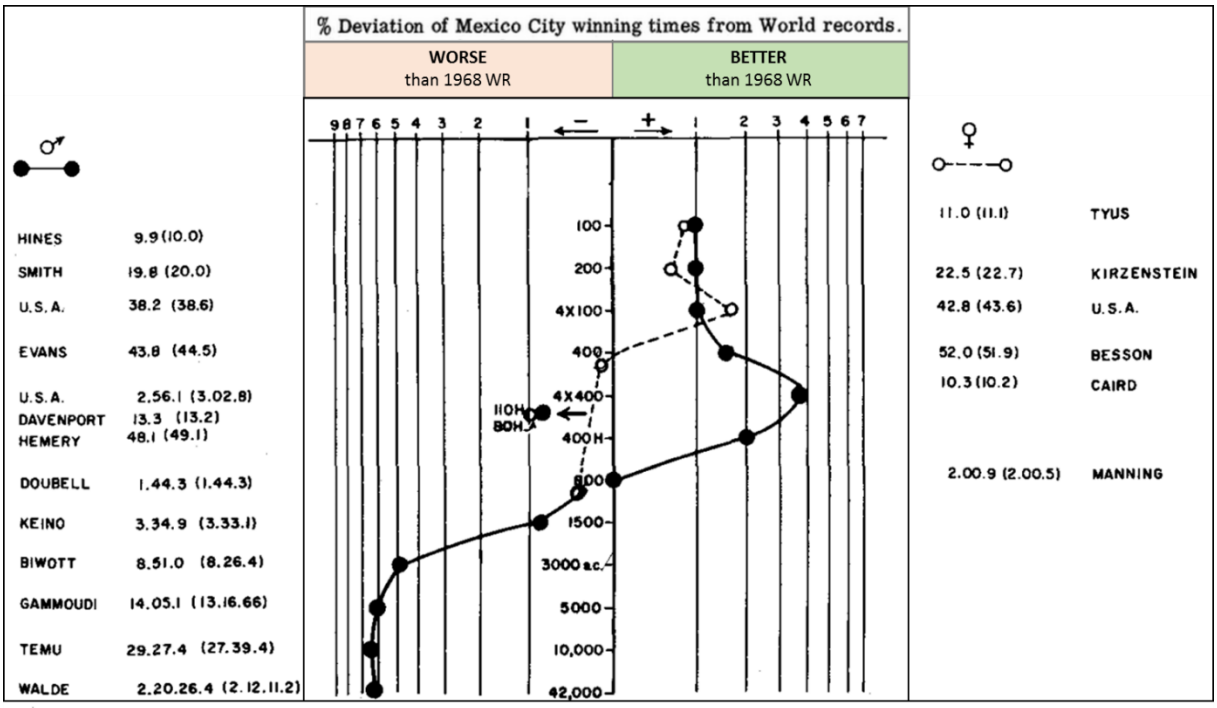


Figure 16. Percentage deviation of winning times of 1968 Olympic Games from the 1968 World records. Figure adapted from Jokl *et al.*, 1969 [248], with permission.

1.5.3.Altitude training to increase sea-level exercise performance

1.5.3.1. Living high

Depending on the strategy applied, altitude training aims to increase exercise performance via either hematological or muscular adaptations. 'Living high' typically involves a two to four-week sojourn at moderate altitude (2000 – 3500 m) so as to increase Hbmass (*paragraph 1.3.2*). Every 100 hours of hypoxic exposure above 2100 m in either hypobaric or normobaric hypoxia, on average increases Hbmass by 1.08% [41]. However, this requires a minimum daily hypoxic exposure time of 12 to 13 hours. Below such dose changes in Hbmass remain largely absent [260,261]. Noteworthy, great inter-individual variability in hematological responses exists [42,43]. Therefore, athletes are often classified as 'responders' *versus* 'non-responders'. However, recent evidence rejects such classification, given that the hematological response to sequential hypobaric altitude exposures is inconsistent within individuals [44]. Besides, the hematological response is negatively affected by a high initial Hbmass [262,263], caloric deficit [44], and illnesses/injuries during 'living high' [44,263,264]. Also normal pre-altitude iron stores seem requisite for a good hematological response [265]. Accordingly, iron supplementation at altitude has been postulated to stimulate the increase in Hbmass, though further research remains warranted [266].

Every 1% increase in Hbmass on average increases VO_2max by 0.6-0.7% [267]. This has been considered the primary mechanism generating the ergogenic effects of 'living high', though involvement of non-hematological mechanisms should not be excluded (see point:counter point series in 'Journal of Applied Physiology' 2005, volume 99). In support for non-hematological adaptations, testimonials by athletes and coaches point peak performance to occur two weeks after a two to four-week period of 'living high' [268]. By that time, Hbmass already decreased to pre-altitude levels [40]. However, delayed peak performance might be attributed to the taper period, which is habitually implemented briefly after an intensive training period at altitude. Nonetheless, also adaptations in muscle pH-handling capacity, mitochondrial efficiency and increased exercise efficiency have been postulated to contribute to acclimatization-induced performance enhancements [269]. Indeed, in several older studies, 'living high' was found to increase muscle buffering capacity (βhm) [270-272]. However, these studies have been contested by more comprehensive follow-up investigations [102,273].

It is indeed unlikely for moderate altitude exposure at rest to trigger hypoxia-induced adaptations of skeletal muscle, for P_iO_2 in resting muscle is only marginally reduced by ambient hypoxia as compared to the P_iO_2-dropping effect of muscle contractions. Whilst severe ambient hypoxia reduces P_iO_2 from ~34 to ~23 mmHg [16], even normoxic exercise reduces P_iO_2 to levels as low as ~3 mmHg (see *paragraph 1.2.2*). Myocellular HIF-1α has been postulated to stabilize around 8 mmHg P_iO_2 [274], a threshold well below the ongoing P_iO_2 in muscle cells during hypoxia at rest. On the other hand, mitochondrial volume loss has consistently been reported following extreme-altitude mountaineering expeditions (>5500 m, reviewed by Murray & Horscroft [275]). However, this effect may not be specific due to concomitant drop of energy intake, changing nutritional factors, and loss of muscle mass [276].

Lastly, athletes aiming to improve exercise performance by sojourning at altitude typically reside at moderate altitude, for living at moderate altitude is, in contrast to high altitude, not associated with muscle mass loss [276]. Neither does moderate altitude exposure negatively affect sleep quality and recovery as does high or extreme altitude [277]. In contrast to mountaineering studies, a recent investigation found 4 weeks of passive exposure to 3454 m to increase intermyofibrillar mitochondrial density against the face of unaltered body mass, though without any other peripheral adaptations such as muscle oxidative enzyme activity, mitochondrial efficiency or muscle capillarity [278]. Noteworthy, Jacobs *et al.* previously reported attenuated skeletal muscle respiratory capacity but improved mitochondrial efficiency in one study [279], whilst no changes in mitochondrial efficiency were found in another study

[101] following a similar altitude intervention. Clearly, the effects of 'living high' at moderate to high altitude on mitochondrial modulations are not yet well understood, but all data taken together indicate that mitochondrial adaptations are absent or small at best.

1.5.3.2. Training high

Exercise training requires cardiovascular stress, elevated O_2-fluxes, neuromechanical loading, and/or hormonal changes for training adaptations to occur. Hypoxia increases the cardiovascular strain, but may impair the O_2-flux and neuromechanical loading. A seminal paper by Levine & Stray-Gudnersen (1997) showed high-intensity endurance performance (5000 m run, ~16-18 min) to improve following 'living high – training low', but not after 'living high – training high' [280]. Since then, the 'live high – train low' concept is considered to the preferred altitude training strategy. However, emerging concepts suggest training during 'living high' should preferentially be performed either near sea level or at altitude, depending on its content and goals. Training sessions resistant against hypoxia-induced attenuation in absolute training load or intensity may allow high mechanical workload whilst simultaneously stimulating unique muscular gene expression via HIF-1α activation and/or increased metabolic stress. Therefore, supramaximal high-intensity interval training (HIIT) in hypoxia has recently gained more attention in both the research and practical setting.

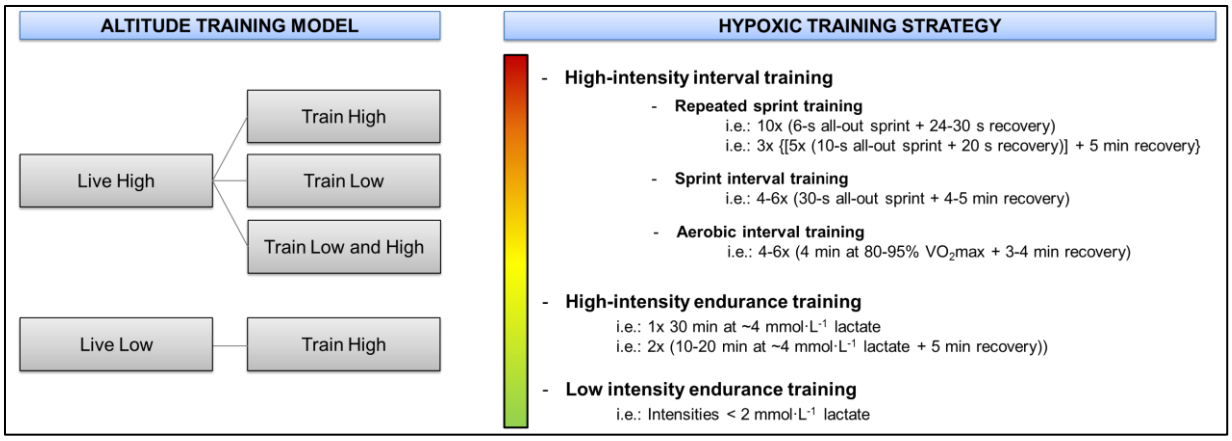


Figure 17. Altitude training models and researched hypoxic training strategies.
Resistance training was omitted as a potential training mode, given that molecular signaling and training strategies promoting muscle protein synthesis and hypertrophy fall beyond the primary scope of the present thesis.

1.5.3.2.1. Endurance training in hypoxia

In the nineties, a series of studies using one-legged endurance training reported muscular oxidative enzymes to increase more following training in hypoxic *versus* normoxic conditions [281-283]. These findings seemed consistent in experiments comparing normoxic and hypoxic training at similar absolute workloads. However, hypoxic training at similar relative workloads (%VO_2max in hypoxia *versus* %VO_2max in normoxia) has been reported to inhibit adaptions in muscle oxidative function [284]. Still, two-legged high-intensity endurance cycling at training workloads matched for relative intensity increased volume density of subsarcollemal mitochondria following hypoxic but normoxic training [285-287]. Nonetheless, no specific effects on the respiratory capacity of skeletal muscle was reported in another recent study [288]. Although enhanced mitochondrial adaptations occurred in some of the aforementioned

experiments, these studies showed training in hypoxic conditions to stimulate increases in VO_2max or aerobic power in hypoxic but not normoxic testing conditions [285-287].

Short-term endurance training in hypoxia elevated muscle HIF-1α mRNA levels more than similar training in normoxia [287,289]. In fact, gene expression related to oxygen transport (myoglobin, VEGF), oxidative metabolism (NADH6, SDH, COX1, COX4), mitochondrial biogenesis (TFAM, PGC-1α), glycolysis (PFK, GLUT-4), and pH regulation (CA3, MCT1) was stimulated by training in hypoxia [287,289]. Additionally, a systematic literature review showed muscle capillarity to increase more following hypoxic endurance training, albeit with the proviso that capillary length density and not capillary-to-fiber ratio was assessed [290].

Hoppeler *et al.* elegantly summarized the relevant literature as follows: "*Muscle structural, biochemical and molecular findings point to a specific role of hypoxia in endurance training ... hypoxia as a supplement to training is not consistently found to be of advantage for performance at sea level. There is some evidence mainly from studies on untrained subjects for an advantage of hypoxic training for performance at altitude"* [106].

1.5.3.2.2. Sprint training in hypoxia

Because maximal power [255] and anaerobic capacity [291] are well-maintained in hypoxia, attention has recently shifted from hypoxic endurance training towards hypoxic HIIT in the form sprint training. This strategy may allow for more explicit systemic and muscular adaptations due to elevated hypoxic and oxidative stress in conjunction with pertinent neuromuscular and neuromechanical loading. For instance, six weeks of hypoxic but not normoxic sprint interval training (SIT, **Figure 17**) has previously been shown to increase maximal activity of phosphofructokinase (PFK) [234], a key enzyme in the glycolytic metabolism. This feature was postulated to be induced by increased glycolytic ATP turnover during the hypoxic training workouts [254,255]. Nonetheless, no superior changes in performance were noted in this study [234]. More consistent performance enhancements were found following repeated-sprint training in hypoxia (RSH, **Figure 17**). Indeed, Faiss *et al.* showed increased capacity to perform repeated sprints to exhaustion in normoxia following both cycling and cross-country RSH, but not following similar training in normoxia (RSN) [292,293]. Remarkably, RSH enhanced oscillations in muscle blood volume (~perfusion) during repeated sprints more than RSN. Whilst the latter finding was recently confirmed by another research group [294], the associated performance enhancements were not. However, emerging evidence from two recent studies shows RSH induced by voluntary hypoventilation to decrease arterial oxygenation (SpO_2 ~90%) and enhance the number of sprints during a RSA test to exhaustion in highly trained swimmers and rugby players [295,296].

Taken together, hypoxic supplementation to sprint training might stimulate training adaptations by increased glycolytic activity on the one hand, and muscle perfusion on the other hand. A third potential mechanism involves the hypoxic induction of a unique gene transcript primarily mediated via stimulation of the HIF-1 pathway. Indeed, both short-term endurance [287,289] and sprint training [293,297] in hypoxia have been shown to elevate muscle HIF-1α mRNA levels more than similar training in normoxia. HIF-1α induces transcription of genes involved in glycolysis and pH regulation (*paragraph 1.3.4*). Therefore hypoxic training might stimulate high-intensity exercise performance by stimulating glycolytic capacity and the ability to extrude and neutralize intracellular H^+. Indeed, evidence supporting this hypothesis comes from Faiss *et al.* [293] showing RSH but not RSN to increase gene transcription of muscle MCT4 and carbonic anhydrase 3 (CA3) (**Figure 18**). Additionally, high-intensity endurance training in hypoxia but not normoxia has been shown to stimulate muscle GLUT-4 and PFK transcription in another study [289] (**Figure 18**). Conversely, Green *et al.* reported Na^+-K^+-pump

expression to decrease with single-leg endurance training in hypoxia, *versus* an increase following similar training in normoxia [283].

Elevated levels of steady-state mRNA following such short-term training periods are considered to potentially reflect long-term training adaptations [298]. However, they are by no means a substitute for protein quantification. Therefore they should be interpreted as supportive data rather than a primary outcome for evidence of muscular adaptations [296]. Adaptations in muscle protein concentrations following hypoxic training are understudied. Three weeks of combined endurance and interval training in hypoxia (~3000 m) did not stimulate adaptations in MCT-1 or MCT-4 content compared to similar training in normoxia [300]. Neither did two weeks of RSH (~3000 m) *versus* RSN in the setting of 'living high' [297]. Nor did six weeks of SIT in hypoxia (~3000 m) *versus* normoxia [234].

In conclusion, hypoxic supplementation to sprint training alters muscle gene transcription, but evidence for enhanced pH-regulating proteins content is still lacking. Importantly, growing data indicates hypoxic training to increase muscle blood perfusion capacity. More research is warranted to evaluate whether or not certain hypoxic training strategies could enhance exercise performance in normoxia and, if so, by which underlying mechanisms.

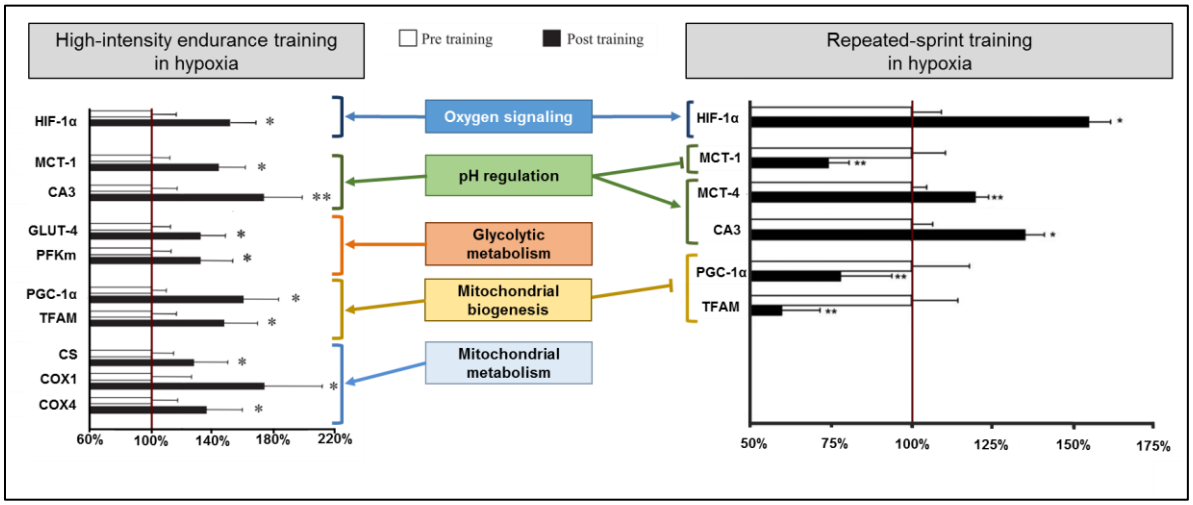


Figure 18. Hypoxia-specific upregulation in gene transcription (mRNA) .
Relative mRNA concentrations of selected gene transcripts in skeletal muscle before and after 6 weeks of high-intensity endurance training in hypoxia (**Left panel**, adapted from [289]) or before and after 4 weeks of repeated-sprint training in hypoxia (**Right panel**, adapted from [293]). Similar high-intensity endurance training in normoxia did not induce any significant changes in relative mRNA concentrations of the selected gene transcripts. With exception for increased MCT1, similar repeated-sprint training in normoxia did not induce any significant changes in relative mRNA concentrations of the selected gene transcripts. **HIF-1α**, hypoxia inducible factor-1α; **MCT1**, monocarboxylate transporter-1; **MCT4**, monocarboxylate transporter-4; **CA3**, carbonic anhydrase III; **GLUT-4**, glucose transporter 4; **PFKm**, 6-phosphofructokinase, muscle type; **PGC-1α**, peroxisome proliferator-activated receptor gamma coactivator 1α; **TFAM**, mitochondrial transcription factor A; **CS**, citrate synthase; **COX1**, cytochrome oxidase 1**; COX4**, cytochrome oxidase 4. *, $P < 0.05$ *versus* before training.

1.6. Normobaric vs. hypobaric hypoxia

Terrestrial altitude induces environmental hypoxia consequent to decreasing barometric pressure (hypobaric hypoxia). Normobaric hypoxia simulates the lowered ambient oxygen availability at altitude by reducing the percentage of oxygen in the air. For long, the mechanism by which ambient PO_2 is reduced has been considered inconsequential for the physiological response to hypoxia. Nowadays, it is widely accepted that minor differences between normobaric and hypobaric hypoxia exist. However, whether or not these differences should be considered to be of any clinical consequence remains a debated matter (point:counter point series in Journal of Applied Physiology Volume 12, [301]).

Notwithstanding, a growing body of evidence shows ambient pressure to affect physiological responses to hypoxia. Compared to normobaric hypoxia, hypobaric hypoxia has been associated with lower minute ventilation, lower tidal volume, higher breathing frequency and lower P_aCO_2 levels [302]. Hence hypobaric hypoxia induces greater alveolar dead space ventilation associated with ventilatory alkalosis and hypocapnia. Hypobaria also alters Starling forces stimulating fluid extrusion from the capillaries to the alveoli, thereby potentially inhibiting gas exchange efficiency [303]. Most studies have found SpO_2 and S_aO_2 to be lower in hypobaric compared to normobaric hypoxia [304-307]. However, when the hypoxic exposure time is prolonged these differences tend to disappear [308-312]. Nonetheless, higher incidence and severity of AMS symptoms have been observed in hypobaric hypoxia [309,312]. Along the same line, a recent experiment showed greater reduction in SpO_2 and inferior cycling time trial performance in hypobaric compared to normobaric hypoxia [313]. In contrast, similar erythropoietic adaptions have been reported following 'living high', irrespective of the hypoxic modality used [314]. Available literature data taken together, hypobaric hypoxia may be considered a more severe hypoxic stimulus for a given PO_2 compared to normobaric hypoxia, and thus in general inducing slightly more pronounced physiological responses. Experiments in the current PhD project have been performed in normobaric hypoxia. Extrapolations from these experiments to hypobaric hypoxia should therefore be performed with caution.

1.7. Nitrate supplementation

Nitric oxide (NO) is a gaseous signaling molecule involved in the regulation of vasodilation, muscle contractility and mitochondrial function [314,315]. Nitric oxide synthases (NOS) catalyze oxidation of L-arginine, a conditionally essential amino acid, to produce NO (**Figure 19**) [317]. In hypoxic conditions, an alternative O_2-independent pathway compensates the compromised oxidation of L-arginine via stepwise reduction of nitrate (NO_3^-) to nitrite (NO_2^-) and eventually NO [318]. NO plays a critical role in hypoxic vasodilation, attempting to restore to the balance between O_2 demand *versus* delivery [319] . Increasing the bio-availability of NO via nitrate supplementation, either via dietary (i.e., nitrate-rich beetroot juice or lettuce) or pharmacological (i.e., $NaNO_3$ capsules) supplementation, reduces the O_2 cost of exercise via enhanced mitochondrial efficiency [320,321] and reduced O_2 cost of muscle contractions [322]. The former was postulated to occur via attenuation of proton leakage across the mitochondrial membrane due to downregulation of uncoupling protein 3 and ADP/ATP translocase, whereas the latter was hypothesized to occur via the effects of NO on actin-myosin ATPase and sarcoplasmic reticulum calcium ATPase. Nitrate supplementation in rodents has also shown to increase blood flow towards hindlimb muscles during submaximal treadmill running, with these effects occurring preferentially in muscles abundant in glycolytic fibers [323]. Likewise contractility and calcium handling proteins in fast-twitch muscle fibers increased in nitrate-fed rodents compared to placebo-fed controls [324] . Interestingly, nitrate supplementation increased muscle oxygenation and exercise tolerance at 115 but not 35 rates per minute cycling exercise [325], reinforcing the hypothesis that nitrate supplementation particularly enhances glycolytic muscle fiber function.

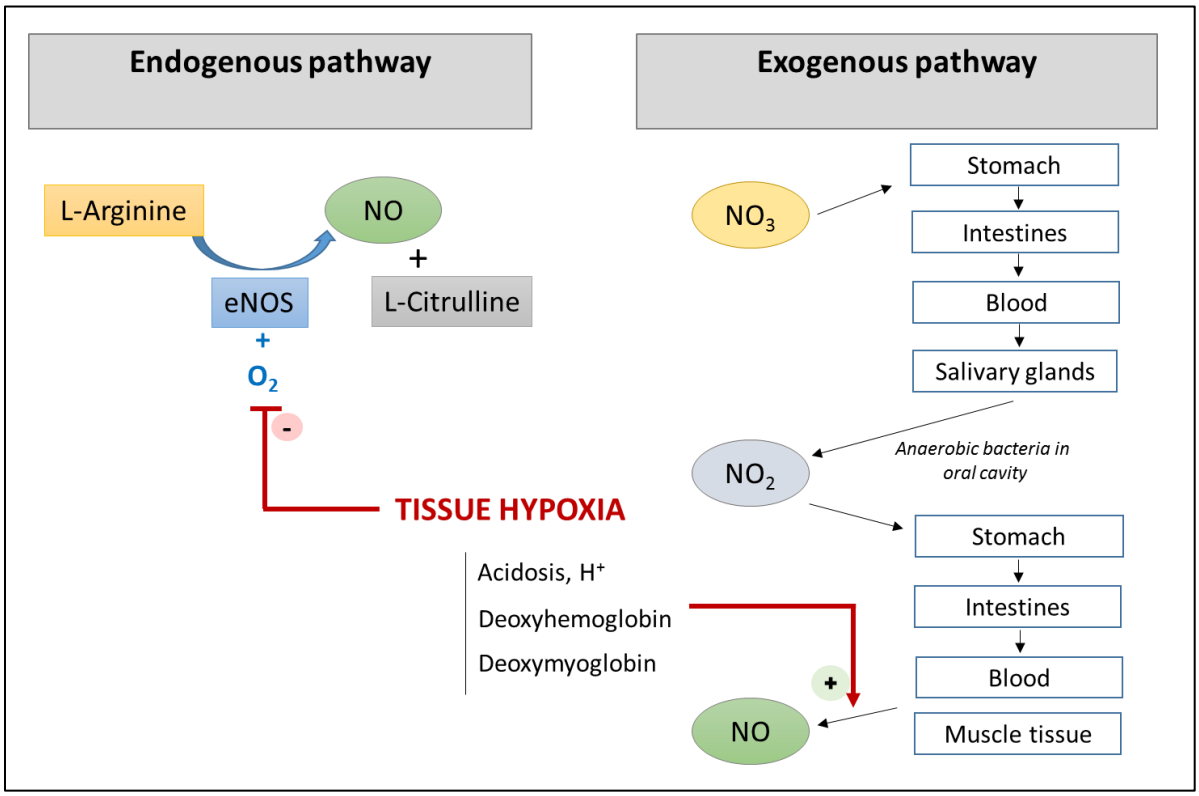


Figure 19. Schematic overview of the endogenous and exogenous nitric oxide production. eNOS, endothelial nitric oxide synthase; NO, nitic oxide; NO_3, nitrate; NO_2, nitrite.

In the last few years, a substantial amount of research has focused on the ergogenic effects of nitrate supplementation. Recently, a meta-analysis was conducted on 76 eligible trials, which showed strong evidence for nitrate supplementation to improve endurance exercise capacity. as measured by time to exhaustion tests, but not for time trial performances [326]. The performance enhancing effects seem specifically applicable for maximal exercise of 5 to 30 min of duration [315]. However, growing evidence also shows nitrate supplementation to increase intermittent high-intensity exercise performance [327-330] and repeated supramaximal exercise/sprint performance [327,331,332].

Because the L-arginine pathway is inhibited in hypoxia, it has been postulated that enhanced NO production via exogenous nitrate reduction might exert greater effects in hypoxic *versus* normoxic environments. Accordingly, one study showed nitrate supplementation to improve exercise tolerance in hypoxia (F_iO_2: 13.1%) but not in normoxia [333]. Other studies have also reported higher arterial and muscle oxygenation [334], time to exhaustion [333,335,336] and time trial performance [337,338] in nitrate *versus* control-fed subjects in hypoxic conditions. Therefore, it has been postulated that nitrate supplementation during training might increase training quality (*i.e.*, power output) and therefore training adaptations. However, Puype *et al.* found no superior training adaptations following 6 weeks of high-intensity endurance training in hypoxia (F_iO_2: 12.5%) with or without nitrate supplementation [339]. In contrast, two normoxic training studies in which nitrate supplements were administered before each sprint interval training session reported beneficial effects on the maximal work rate during incremental exercise testing [340,341].

2. Experimental aims and hypotheses

For decades athletes have implemented altitude training aiming to improve sea-level exercise performance. Since the seminal study by Levine and Stray-Gunderson as published in 1997 [280], 'living high' whilst 'training low' has become the preferred mode of altitude training. This strategy allows for high neuro-mechanical loading and O_2-fluxes during training near sea level whilst stimulating erythropoiesis during daily living at altitude. Still, considerable inter and intra-individual responses have been reported [42-44,342], yet pertinent markers for individual altitude sensitivity are lacking.

With the development of commercial devices capable of mimicking altitude at sea level, 'training high' whilst 'living low' has received increasingly more interest. Initial studies evaluating high-intensity endurance training in hypoxia were promising, demonstrating unique muscular adaptations not observed with similar training at sea level [106]. However, superior performance benefits were not consistently reported. Impaired mechanical training load in hypoxia has been postulated as the primary impeding factor for hypoxic training to induce greater training effects. Therefore, research has recently shifted focus from hypoxic high-intensity endurance training to hypoxic supramaximal high-intensity interval training [343,344]. This training strategy may allow similar neuro-mechanical training loads whilst superimposing an additional hypoxic stressor. Likewise, nitrate supplementation during training, especially when performed in hypoxia, might increase training quality (see *paragraph 1.7*).

Therefore, in a **first study**, we hypothesized sprint interval training in hypoxia to induce superior muscular adaptations and performance enhancements than similar training in normoxia. In addition, we evaluated whether nitrate supplementation prior to each hypoxic training session would stimulate training quality and therefore training adaptations as compared to a placebo-supplemented control group.

Because high inter-individual variance in training adaptions has the potential to mask small but potentially relevant benefits of training in hypoxic conditions [345], in a **second study** we performed normoxic *versus* hypoxic HIIT within a single individual. In this study, subjects performed single-legged HIIT, with one leg in hypoxia and the other leg in normoxia. Again, we hypothesized hypoxic *versus* normoxic training to induce superior muscular and performance adaptations. Training was performed in the setting of 'living high'. Five consecutive days per week, 15.5 h per day, participants 'lived high' in progressively increasing normobaric hypoxia. We assessed the hematological adaptations to this discontinuous 'living high' protocol and aimed to identify pertinent markers predicting individuals' response.

In a **third study**, we examined the role and regulation of HIF-1α and its target genes in response to brief hypoxic stress. Therefore, muscle biopsies were taken from the quadriceps *vastus lateralis* from subjects participating in 'study 2', before and after 10 min of femoral arterial occlusion, before and after 5 weeks of either normoxic or hypoxic HIIT. We postulated HIIT, especially when performed in hypoxic conditions, to attenuate the myocellular response to severe hypoxic stress due to hypoxic preconditioning. Specifically, we examined the translocation, stabilization and transcriptional activity of HIF-1α.

Finally, in a **fourth study**, we aimed to identify pertinent markers of hypoxia sensitivity. Therefore, subjects were exposed to 24 h of normobaric hypoxia at a simulated altitude expected to induce AMS in half the study population. Several parameters believed to be important in the development of AMS were monitored throughout the experiment, potentially allowing further understanding of this pathology aiding the development of adequate screening procedures to identify subjects at risk.

Study 1. Effects of hypoxia and dietary nitrate supplementation on adaptations to sprint interval training

- ***Hypothesis 1:*** Sprint interval training in hypoxia stimulates improvements in endurance and sprint exercise performance via hypoxia-specific adaptations in muscular pH-handling capacity, fiber type composition and oxidative capacity.
- ***Hypothesis 2:*** Dietary nitrate supplementation during hypoxic sprint interval training stimulates training adaptations, at least in part by increasing power output during training.

Study 2. Muscular, haematological, and physiological adaptations to training 'low' vs. 'high' whilst intermittent 'living high'

- ***Hypothesis 1:*** Hypoxic high-intensity interval training in the setting of 'living high' stimulates muscle fiber hypertrophy, muscular capillarization, and pH-handling capacity more than similar training in normoxia, leading to superior performance enhancements.
- ***Hypothesis 2:*** Five weeks of intermittent 'living high' at progressively increasing altitude induces high levels of serum EPO and efficiently increases hemoglobin mass and maximal aerobic capacity.

Study 3. Effects of high-intensity interval training in normoxia vs. hypoxia on muscular ischemic responses

- ***Hypothesis 1:*** High-intensity interval training in the setting of 'living high' attenuates the muscular response to a brief episode of ischemia-induced hypoxic stress. This effect is more pronounced following training in hypoxia compared to normoxia.

Study 4. Identification of markers associated with susceptibility to AMS

- ***Hypothesis 1:*** AMS-susceptible subjects display unique physiological markers both during normoxic and hypoxic exposure enabling early identification of AMS-susceptibility.

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PART II

RESEARCH ARTICLES

Paper 1

Nitrate intake promotes shift in muscle fiber type composition during sprint interval training in hypoxia

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Abstract

Purpose: We investigated the effect of sprint interval training (SIT) in normoxia, vs. SIT in hypoxia alone or in conjunction with oral nitrate intake, on buffering capacity of homogenized muscle (βhm) and fiber type distribution, as well as on sprint and endurance performance.

Methods: Twenty-seven moderately-trained participants were allocated to one of three experimental groups: SIT in normoxia (20.9% F_iO_2) + placebo (N), SIT in hypoxia (15% F_iO_2) placebo (H), or SIT in hypoxia + nitrate supplementation (HN). All participated in 5 weeks of SIT on a cycle ergometer (30-s sprints interspersed by 4.5 min recovery-intervals, 3 weekly sessions, 4–6 sprints per session). Nitrate (6.45 mmol $NaNO_3$) or placebo capsules were administered 3 h before each session. Before and after SIT participants performed an incremental VO_2max-test, a 30-min simulated cycling time-trial, as well as a 30-s cycling sprint test. Muscle biopsies were taken from m. vastus lateralis.

Results: SIT decreased the proportion of type IIx muscle fibers in all groups ($P < 0.05$). The relative number of type IIa fibers increased ($P < 0.05$) in HN ($P < 0.05$ vs. H), but not in the other groups. SIT had no significant effect on βhm. Compared with H, SIT tended to enhance 30-s sprint performance more in HN than in H ($P = 0.085$). VO_2max and 30-min time-trial performance increased in all groups to a similar extent.

Conclusion: SIT in hypoxia combined with nitrate supplementation increases the proportion of type IIa fibers in muscle, which may be associated with enhanced performance in short maximal exercise. Compared with normoxic training, hypoxic SIT does not alter βhm or endurance and sprinting exercise performance.

Keywords: sprint interval training, hypoxia, intermittent hypoxic training, nitrate, muscle fiber type composition, muscle buffering capacity, carnosine, citrate synthase

Introduction

Interest in intermittent hypoxic training (IHT) to boost endurance and high-intensity exercise performance in athletes is growing [1,2]. This might partly be explained by the commercialization of user-friendly, normobaric hypoxicators to simulate altitude within the normal lowland habitat. Well-controlled studies have provided evidence that high-intensity hypoxic endurance training can enhance muscle mitochondrial and capillary density [3-6], as well as stimulate other markers of mitochondrial metabolism and biogenesis [7-10]. IHT in the form of both endurance training [4,10] and sprint training [11,12] elevated muscle phosphofructokinase (PFK) mRNA and/or activity, as well as other markers of glycolytic metabolism and pH regulation. Nonetheless, research into the effects of IHT on sea-level exercise performance is equivocal, with the current literature suggesting higher training intensities involving anaerobic energy input to be more favorable than predominantly aerobic workouts [1,2,13]. This might be explained by impaired workload in endurance training due to inhibition of oxidative energy provision, resulting in higher glycolytic energy contribution and premature fatigue development [14-16].

Attention has recently shifted toward IHT in the form of sprint training [11,12,17], because maximal power [15] and anaerobic capacity [18] are well maintained in hypoxia. This may allow for more explicit systemic and muscular adaptations due to elevated hypoxic and oxidative stress in conjunction with pertinent neuromuscular and neuromechanical loading [19,20]. Support for such a contention comes from recent studies showing that repeated-sprint training in hypoxia (RSH), characterized by several short (<30 s) sprints interspersed with incomplete recovery (exercise-to-rest ratio <1:4), increased hypoxia-inducible factor-1α (HIF-1α) mRNA [11], and repeated-sprint ability (RSA) performance [11,17,21] more than identical repeated-sprint training in normoxia. However, the beneficial effect of RSH on exercise performance [21] has been debated [22] and needs to be confirmed. HIF-1α is implicated in the regulation of the genes controlling the expression of proteins involved in glycolysis and pH regulation [23]. In line with this, RSH has been shown to increase gene transcription of monocarboxylate transporter 4 (MCT-4) and carbonic anhydrase III (CA3) in muscle [11]. We have shown that 6 weeks of sprint interval training (SIT), characterized by 30-s sprints interspersed with long recovery periods of 4–5 min, increases muscle MCT-1, but not MCT-4 protein content, irrespective of whether the training was performed in normoxia or in hypoxia [12]. SIT in hypoxia but not in normoxia also elevated PFK activity [12], presumably due to increased glycolytic ATP turnover to compensate for impaired aerobic energy production during the hypoxic training workouts [14,15]. Given that post-exercise phosphocreatine (PCr) resynthesis is impaired under hypoxic conditions [24,26], long recovery time between sprints is required to allow substantial recovery of PCr prior to each sprint [27,28] and may assist maintenance of high power output throughout the training session.

As the contribution of glycolysis to energy provision increases, buffering capacity becomes a pivotal determinant of the capacity to maintain high muscle power outputs. Counter-intuitively, the extent of H^+ accumulation in muscle fibers during training does not seem to be the primary stimulus for the development of higher buffering capacity of homogenized muscle (βhm). Indeed, rat [29] and human [30] studies have shown that bicarbonate-induced myocellular alkalosis during work-matched interval training did not inhibit adaptations in βhm. Furthermore, a large acidic load (pH < 6.8) during training has been reported to reduce βhm, possibly due to cumulative transient decreases in βhm during consecutive training sessions [31]. In line with this rationale, performing SIT in hypoxic conditions might even impair βhm compared with similar training in normoxia. Alternatively, however, both ‘live high–train high’ [32,33] and ‘live high–train low’ experiments [34] have shown that ‘live high’ compared to ‘live low’ enhances βhm. However, these findings have also been recently debated [35,36], and the specific effect of IHT on βhm during ‘live low’ conditions remains unclear.

Oral nitrate supplementation can enhance endurance exercise performance in hypoxia [37-39], presumably by enhancing mitochondrial efficiency [40] and/or by reducing the energy cost of muscle contraction [41]. It is well-documented that the fraction of aerobic energy provision gradually increases during intermittent sprints due to impaired re-activation of glycolysis [28]. Recent data indicate that nitrate intake increases blood flow and contractility to a greater extent in fast-glycolytic than in slow-oxidative whole muscle and muscle fibers [42,43]. This may explain the more explicit effects of nitrate supplementation on performance during high-intensity exercise requiring greater input of type II fibers for production of high power outputs at high contraction velocities [37,44-46]. Higher muscle blood flow during recovery [47] could conceivably facilitate the clearance of waste metabolites during intermittent maximal exercise bouts and could, amongst the other aforementioned mechanisms, contribute to increased total work output during resistance training [48]. Furthermore, nitrate supplementation in hypoxia was shown to stimulate the rate of post-exercise muscle PCr resynthesis [26,37]. Taken together, these results suggest that oral nitrate supplementation could enhance performance during SIT in hypoxia and by this means potentiate training adaptations.

We, therefore, aimed to investigate whether SIT performed in hypoxic conditions elicited greater muscular and performance adaptations compared to similar training performed in normoxic conditions. Secondly, we aimed to investigate whether oral nitrate supplementation during training enhanced the effects of SIT in hypoxia.

Materials and methods

Participants

Thirty healthy men were recruited from the student population at the KU Leuven by word of mouth and via announcements on social media. To avoid confounding effects due to prior altitude acclimatization, participants who were exposed to altitudes higher than 1500 m during the 6 months prior to the study were excluded from participation. From the initial sample of 30 eligible participants, one did not complete the study due to SIT intolerance, and two withdrew from the study for reasons unrelated to the study protocol. Twenty-seven participants completed the full study protocol and were included in the final data analyses (for general characteristics see **Table 1**). Participants were recreationally active [2.7 ± 1.6 h (SD) exercise participation per week; i.e., soccer, basketball, cycling, running, swimming, strength training], but had not engaged in a consistent training program or any sport at a competitive level. Participants were non-smokers and did not use medication or dietary supplements in the 3 months prior to the study or during the period of the study. They were instructed to maintain their habitual physical activity level and normal diet throughout the study. Participants received a summary table of nitrate-rich foods and were instructed to avoid these foods throughout the study period. The study was approved by the KU Leuven Biomedical Ethics Committee (B322201316517) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent after clearing medical screening and being fully informed about the content of the experiments and the risks involved.

Study protocol

The study involved a test before (pretest) and after (posttest) a 5-week controlled SIT program. The pretest and posttest consisted of two experimental sessions separated by a 2-day interval. Between 3 and 2 weeks prior to the start of the study, participants completed two sessions of familiarization with the experimental procedures. In the first familiarization session, participants performed a maximal

incremental VO_2max-test on a cycle ergometer (Avantronic Cyclus II, Leipzig, Germany). The initial workload was set at 70 W and was increased by 30 W per min until volitional exhaustion. Respiratory gas exchange was measured continuously during the test (Cortex MetaLyzer II, Leipzig, Germany), and the highest oxygen uptake measured over a 30-s period was defined as the maximal oxygen uptake rate (VO_2max). Participants then cycled for 15 min at 50 W to recover, after which a 30-s modified Wingate test (W_{30s}) was performed. To avoid limitations of power output by co-ordination problems due to excessive cadence increase (cadence > 120 rev·min^{-1}), cadence during W_{30s} was fixed at 100 rev·min^{-1} by using the isokinetic mode setting of the cycle ergometer. In the second familiarization session, participants completed a 30-min simulated time-trial (TT_{30min}). They were instructed to keep their cadence between 80 and 100 rev·min^{-1}, and adjust the resistance to develop the highest possible mean power output (W). Following familiarization, participants were matched into triplets by VO_2max, mean power output during TT_{30min}, mean power output duringW_{30s}, as well as body mass and height. Thereafter, the triplets were randomly assigned to one of three experimental groups. One group performed the SIT program in normoxia (F_iO_2 = 20.9%, n = 10) and received a placebo supplement (N). All other participants trained in hypoxia (F_iO_2 = 15.0%, ~2750 m), with eight participants receiving a placebo (H) and nine participants receiving a nitrate (HN, n = 9) supplement. Participants were not blinded for the normoxic vs. hypoxic training conditions. Supplements were ingested 3 h prior to each training session so as to produce high plasma nitrite levels during the training in HN [49]. Nitrate was administered in the form of capsules containing 6.45 mmol $NaNO_3$ (~400mg molecular NO_3^-). Placebo capsules contained an equivalent amount of sodium (6.45 mmol) in the form of NaCl. All supplements were identical in appearance, and were administered single-blinded in N and double-blinded in H and HN.

TABLE 1 | Participant characteristics

	N	H	HN
Age (y)	23 ± 3	24 ± 2	25 ± 2
Height (cm)	180 ± 8	180 ± 6	182 ± 6
Body mass (kg)	74.0 ± 10.2	79.5 ± 12.1	78.5 ± 11.7

Data are mean ± SD and represent baseline characteristics of the participants training in normoxia (F_iO_2 = 20.9%) while receiving placebo supplementation (N, n =10), and participants training in hypoxia (F_iO_2 = 15.0%) while receiving either placebo (H, n = 8) or nitrate supplements (HN, n = 9).

SIT training program

All SIT sessions were performed in the same normobaric hypoxic facility (SportingEdge, Sherfield on London, UK) set at either 20.9% F_iO_2 (N) or 15.0% F_iO_2 (~2750 m; H and HN). The ambient O_2 fraction was checked before the start of each training session ($MaxO_2^+$ A Scuba, Maxtec, Utah). Participants cycled on cycle ergometers (Avantronic Cyclus II, Leipzig, Germany) that were calibrated prior to the start of the study. Participants completed three training sessions per week, with each separated by 48-h of recovery. Each session consisted of intermittent 30-s maximal sprints, interspersed by 4.5 min active recovery intervals at 50W. Cadence during the sprints was fixed at~100 rev·min^{-1} by using the isokinetic operation mode of the ergometers. The number of sprints was increased

from four in weeks 1–2, to five in weeks 3–4, and six in the final week. Including 5-min warm-up and cool-down @50 Watt, the training sessions lasted 30 min in week 1, increasing to 40 min in week 5. During each session participants were given verbal encouragement to perform maximally during each sprint. To evaluate the effect of SIT on arterial oxygenation, arterial oxygen saturation (SpO_2) was monitored in the final week of the training period by pulse oximetry (Nellcor OxiMax N-600x, Mallinckrodt, St. Louis, MO) with a sensor placed 2 cm above the eyebrow.

Pretest and posttest

All exercise testing was performed in normoxia. Participants were instructed to refrain from any strenuous physical activity for at least 48 h prior to the pretest. In order to minimize potential diet-induced variations in muscle metabolism, participants received a standardized carbohydrate-rich dinner (~1500 kcal; 65% carbohydrate, 15% fat, 20% protein) on the evening before each experimental day. For the first session they reported to the laboratory between 12:00 a.m. and 4:00 p.m. All participants received a standardized breakfast (~750 kcal, 70% carbohydrate, 10%fat, 20%protein) between 7:00 and 10:30 a.m. Participants completing sessions beyond 1:30 p.m. also received a standardized lunch (~650 kcal, 70% carbohydrate, 10% fat, 20% protein), with the last meal consistently being served between 3 and 2.5 h prior to the start of the experiments. Following a 1-h rest in a comfortable chair, a percutaneous needle biopsy (100–200 mg) was taken from the middle portion of the belly of the right m. *vastus lateralis* using a 5-mm Bergström-type needle under suction. Muscle samples were dissected in two parts. One part was rapidly frozen in liquid N_2 and stored at −80°C until subsequent biochemical analyses. The other part was frozen in isopentane on liquid N_2 and stored at −80°C for later histochemical analyses. Following the biopsy, participants warmed up for 20 min at incremental workloads corresponding to 70% (10 min) and then 90% (10 min) of their average power output recorded during the TT_{30min} familiarization session. During TT_{30min} heart rate was monitored continuously (Polar, Kempele, Finland) and blood lactate concentration was measured (Lactate Pro1, Arkray, Japan) at 10-min intervals from an earlobe capillary blood sample. Participants were allowed to drink water ad libitum and received on-line feedback about the time remaining to completion. No verbal encouragement was given. At the end of the experimental session, participants were instructed to refrain from any strenuous physical activity, before returning to the laboratory for the second session 2 days later. For this second session they arrived between 6 a.m. and 11 a.m. after an overnight fast. Following a 20-min rest period, they performed a maximal incremental VO_2max-test on the cycle ergometer (Avantronic Cyclus II, Leipzig, Germany). Initial workload was set at 70 W and was increased by 30 W every 3 min until volitional exhaustion. Thereafter participants cycled for 15 min at 50 W to recover, whereupon the W_{30s} commenced. The cycle ergometer was set in the isokinetic mode with cadence fixed at 100 rev·min^{-1}. During both tests heart rate was monitored continuously (Polar, Kempele, Finland). Standardized verbal encouragement was given only during W_{30s}. Respiratory gas exchange was continuously measured (Cortex MetaLyzer II, Leipzig, Germany) during the incremental test, VO_2max was determined as the highest oxygen uptake rate measured over a 30-s period. Maximal power output (MPO) was calculated by summing the workload during the last full stage, plus 30 W multiplied by the fraction of the final stage completed. Capillary blood samples for lactate determination (Lactate Pro1, Arkray, Japan) were taken from the earlobe at the end of each workload during the incremental test, and power outputs corresponding to 2 and 4 mmol·L^{-1} blood lactate levels were extrapolated on the lactate-power curve. Blood lactate was also determined 1, 2, and 3 min after theW_{30s}. Room temperature (18–20°C), oxygen content (20.9%), air humidity (40%) as well as air ventilation were standardized. Pretests and the posttests were performed on the same days of the week and time of the day within each participant. The posttest commenced 3 or 4 days following the last training session to eliminate acute physiological effects due to the prior training session.

Analysis of muscle samples

Citrate synthase activity - Enzymatic activity of citrate synthase (CS) was measured by standard colorimetric method. Briefly, 5 mg of wet muscle tissue was dissolved in 400 µL of ice-cold homogenization buffer (5 mM Hepes, 1 mM EGTA, 0.1% Triton X-100, 1 mM Dithiothreitol, pH 8.7). Protein concentration was determined with a DCprotein assay (Bio-Rad). After dilution to 0.5 $\mu g \cdot \mu L^{-1}$, samples and standards were loaded on a 96 well plate to perform the assay in triplicate. CS catalyzes the reaction between acetyl coenzyme A and oxaloacetic acid resulting in citric acid and CoA with a thiol group (CoA-SH). Measurement of its activity is based on the binding of CoA-SH to 5,5′-dithio-bis- (2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoate (TNB).The spectrophotometric absorbance intensity of TNB was measured at 412 nm and CS activity was calculated and expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$. The average coefficient of variation (CV) as determined from the triplicate measures was 4.4%.

Buffering Capacity of Homogenized Muscle (βhm) – Details of the titration method for analysis of βhm have been described elsewhere [50]. Briefly, freeze-dried muscle samples (1.7–2.5 mg dm) were dissected from blood and connective tissue and homogenized on ice in a sodium fluoride containing buffer (33.3 µl 10 mM NaF per mg dm). The homogenates were warmed in a hot water bath at 37.4°C for 5 min. Basal pH measurement was performed with a glass microelectrode (MI-410, Microelectrodes, Bedford, NH, USA) connected to a pH meter (Lab 850, Schott Instruments GmbH, Mainz, Germany). The homogenates were first adjusted to pH ~7.2 with sodium hydroxide (0.02 M NaOH). Then a serial addition of 2 µL hydrochloric acid (0.01MHCl) was titrated until a pH of ~6.1 was reached. After each titration, the homogenates were briefly vortexed to ensure a homogeneously mixed solution. The number of moles of H^+ per kg dry muscle required to change pH from7.1 to 6.5was interpolated from the fitted titration trend line and expressed as mmol H^+ per kg dm per unit pH as a unit for βhm. Each sample was measured in duplicate from which the mean was taken. The average CV as determined from the duplicate measures was 5.1%.

Muscle Carnosine Concentration – Details of muscle carnosine concentration determination by high performance liquid chromatography (HPLC) have been described elsewhere [51]. Briefly, about 5 mg of dry muscle was dissected from blood and connective tissue and extracted in a buffer containing perchloric acid (0.5MPCA) and 1 mMEDTA. After centrifuging the samples for 4 min at 13000 $rev \cdot min^{-1}$ at 4°C, the supernatant was collected and neutralized with a potassium bicarbonate containing buffer (2.1 M KHCO). Samples were placed on ice for 5min to allow CO_2 to escape, after which they were centrifuged at 5000 $rev \cdot min^{-1}$ for 4min at 4°C. The supernatant was then filtered through a 0.22 µm membrane filter where after 20 µL of supernatant was injected into a Perkin-Elmer HPLC system with an Atlantis HILIC Silica column (4.6 × 150 mm, 3 µm). Mobile phase A contained 0.65 mM ammonium acetate in ultrapure water/acetonitrile (25:75 ratio) at a pH of 5.5. Mobile phase B contained 4.55 mM ammonium acetate in ultrapure water/acetonitrile (70:30 ratio) at a pH of 5.5. A linear gradient from 100% phase A to 100% phase B in 13 min at a flow rate of 1.4 $mL \cdot min^{-1}$ was used for separation. Separation was monitored at a wavelength of 214 nm with a UV detector. The average CV as calculated from 12 duplicate injections in the HPLC system was 1.5%.

Muscle Fiber Type Composition – Serial 7-µm-thick cryosections were cut with a cryostat at -20°C. Cryosections were blocked for 60 min in phosphate buffered saline (PBS) containing 1% BSA. Hereafter they were incubated in primary antibodies for myosin heavy chain I (MHCI) (BA-F8, Developmental Studies Hybridoma Bank) and MHCIIa (SC-71, Developmental Studies Hybridoma Bank) dissolved in PBS with 0.5% BSA for 120 min. Dilutions of primary antibodies for MHCI and MHCII were 1:50 and 1:100, respectively. After washing in PBS, cryosections were incubated in

appropriate conjugated secondary antibodies (type I: Alexa 647 goat anti- mouse IgG2b, Invitrogen, diluted 1:300 in PBS with 0.5% BSA; type IIa: Alexa 350 goat anti-mouse IgG1, Invitrogen, diluted 1:300 in PBS with 0.5% BSA) for 60 min. Additionally, together with the secondary antibodies, membranes were stained using wheat germ agglutinin (WGA) Texas Red (Life Technologies). Slides were visualized by fluorescence microscopy (Nikon E1000, Nikon, Boerhavedorp, Germany). The epifluorescence signal was recorded using Cy5, DAPI, and Texas Red excitation filters for visualization of type I fibers, type IIa fibers, and cell membranes, respectively. Muscle fibers were classified as type I, type IIa, or type IIx (unstained fibers). Photos of the slides were analyzed with ImageJ software (version 1.41, National Institutes of Health, USA). Only fibers with adequate cross-sections showing no signs of distortion or folding were counted. 225 ± 27 (SD) fibers were analyzed per biopsy.

Statistical analysis

Differences in baseline values between N and H and between H and HN were tested using a Student's t-test. Main and interaction effects were evaluated by two-way (group × time) repeated measures ANOVA (SigmaStat and SigmaPlot software, Chicago, IL, USA).We performed two separate ANOVA's to test the two a priori hypotheses: N was compared with H to evaluate whether SIT yielded different effects in hypoxia vs. normoxia; H was compared with HN to evaluate whether nitrate administration was able to potentiate the effects of training in hypoxia. Tukey's honestly significant difference post hoc-test was run whenever appropriate to identify specific effects. A probability level $P < 0.05$ was considered statistically significant. All data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated.

Results

Arterial O_2-saturation during training (Figure 1)

Arterial O_2-saturation (SpO_2) was continuously measured during the SIT sessions in week 5. In N, resting SpO_2-values were 98.7 ± 0.4 and 97.6 ± 0.5% at the start and at the end of the sessions (n.s.). Corresponding values in H were lower, both at the start (91.0 ± 0.8%) and at the end of the sessions (89.3 ± 1.4%; $P < 0.05$). Compared with sprint 1, post-exercise SpO_2-values were lower in the latter sprints of the session in both groups. Each sprint also reduced SpO_2 more in H (–6.0 ± 0.6%) than in N (−1.9±0.2%, $P < 0.05$). SpO_2-values were not significantly different between H and HN: average SpO_2 during SIT was 85.0± 0.6% in H vs. 86.7 ± 0.3% in HN ($P = 0.20$).

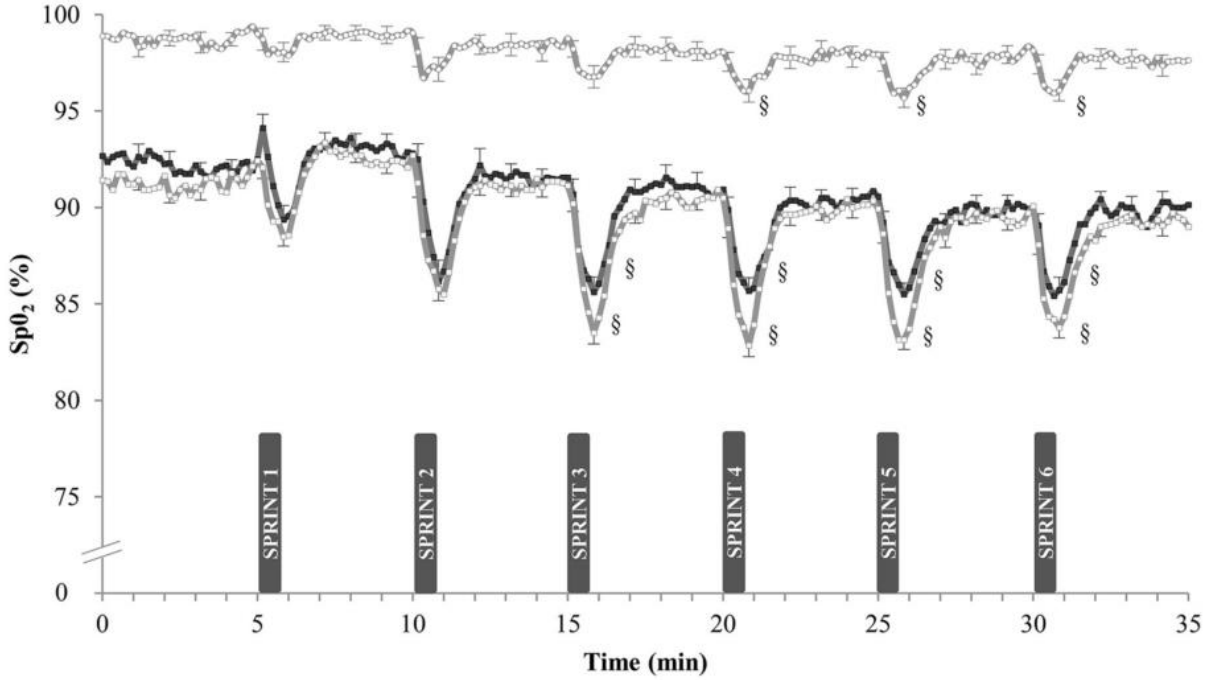

Figure 1 | Effect of SIT on arterial oxygen saturation
Data are mean ± SEM and represent arterial oxygen saturation (SpO_2) during the final week of SIT. Participants performed six 30-s all-out sprints interspersed by 4.5 min of active recovery on a cycle ergometer. One group trained in normoxia (F_iO_2 = 20.9%) while receiving placebo (N, ○). The other groups trained in hypoxia (F_iO_2 = 15.0%) while receiving either placebo (H, □) or nitrate supplements (HN, ■). §, $P < 0.05$ compared to SPRINT 1.

Training performance (Figure 2)

The number of sprints per session was increased from four in weeks 1–2, to five in weeks 3–4, and to six in week 5. Irrespective of the experimental group, mean power output in sprint 1 on average was 667 ± 31 W, decreasing to 561 ± 25 W in the final sprint of the session. There were no significant differences in power output between experimental groups at any time during training. Average total work done (kJ) per training session per week was similar between the groups from the start to the end of the training period (**Figure 2**). Accordingly, total work output over the 5-week training period was 1340 ± 39 kJ in N, 1311 ± 52 kJ in H, and 1261 ± 51 kJ in HN ($P > 0.50$). Blood lactate concentrations at the end of the training sessions peaked at ~14–15 mmol·L^{-1} on average in all groups.

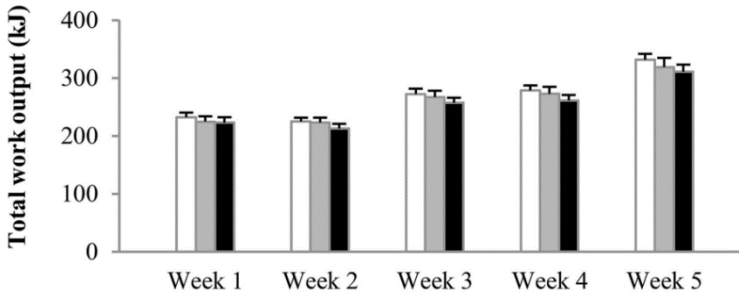

Figure 2 | Effect of hypoxia and nitrate intake on training workload. Data are mean ± SEM and represent average total work output (kJ) per training session per week. The number of sprints per session was increased from four in weeks 1–2, to five in weeks 3–4, and six in the final week. One group trained in normoxia (F_iO_2 = 20.9%) while receiving placebo (N, open bars). The other groups

trained in hypoxia (F_iO_2 = 15.0%) while receiving either placebo (H, gray bars) or nitrate supplements (HN, black bars).

Exercise performance (Table 2)

Baseline values of the incremental exercise test, TT_{30min} and W_{30s} were not significantly different between N and H (P > 0.05) or H and HN (P > 0.05). Compared with the pretest, VO_2max in the posttest was increased (P < 0.05) by ~16% in N vs. ~11% in both H and HN (P < 0.05), but there were no significant differences in VO_2max or change in VO_2max between N and H (P = 0.26) or between H and HN. Similarly, SIT increased (P < 0.05) time to exhaustion (10–11%), peak power output (8–10%), and power output corresponding to 4 mmol·L^{-1} blood lactate concentrations (5–11%) in the three experimental groups without significant differences between N and H or H and HN. Mean power output during the $TT3_{0min}$ in the pretest was, on average, ~200 W. Training tended to increase mean power output during the TT_{30min} in N (+4%, P = 0.062) and significantly increased power output during the TT_{30min} in H and HN (+8%, P < 0.05), but there were no significant differences between N and H or H and HN. Blood lactate concentrations during the TT_{30min} were, on average, ~6 mmol·L^{-1} at a heart rate of ~174 b·min^{-1} in each group in both the pretest and the posttest (data not shown). Mean power output during W_{30s} in the pretest also was similar between the groups (660–670 W). SIT increased power output by ~6% in N and H (P < 0.05), vs. +12% in HN (P < 0.05), yet differences between Hand HN were not significant (HN, P = 0.085). Accordingly, W_{30s} produced similar peak blood lactate concentrations (~10–12 mmol·L^{-1}) in the three experimental groups in both the pretest and the posttest.

TABLE 2 | Effects of training and nitrate supplementation on physiological parameters and exercise performances.

	N		H		HN	
	Pretest	Posttest	Pretest	Posttest	Pretest	Posttest
INCREMENTAL VO_{2max} TEST						
VO_{2max} (mL·min^{-1}·kg^{-1})	53.5 ± 2.6	62.3 ± 3.4*	54.3 ± 4.9	60.2 ± 3.5*	51.2 ± 2.2	56.9 ± 2.3*
Time to exhaustion (min)	23.1 ± 1.1	25.5 ± 1.0*	23.8 ± 1.8	26.4 ± 1.7*	22.4 ± 1.1	25.0 ± 1.2*
Peak power (W)	275 ± 12	295 ± 11*	278 ± 18	304 ± 17*	264 ± 11	290 ± 12*
Peak heart rate (beats·min^{-1})	188 ± 3	186 ± 3	188 ± 2	188 ± 3	190 ± 4	191 ± 3
Peak blood lactate (mmol·L^{-1})	11.2 ± 0.7	11.4 ± 0.4	11.0 ± 1.1	11.8 ± 1.0	12.1 ± 0.9	12.5 ± 0.6
Power output at 2 mmol·L^{-1} (W)	175 ± 10	189 ± 13#	161 ± 20	179 ± 14*	148 ± 14.1	172 ± 11*
Power output at 4 mmol·L^{-1} (W)	216 ± 10	227 ± 13*	213 ± 18	230 ± 14*	196 ± 12.0	217 ± 11*
TT_{30min}						
Mean power output (W)	203 ± 10	211 ± 12#	205 ± 16	221 ± 15*	193 ± 13	209 ± 12*
W_{30s}						
Mean power output (W)	662 ± 23	699 ± 21*	677 ± 25	719 ± 34*	663 ± 45	746 ± 41*
Peak blood lactate (mmol·L^{-1})	9.2 ± 0.7	11.1 ± 0.9*	11.5 ± 0.7	12.4 ± 0.5	9.8 ± 0.7	12.4 ± 0.4*

Data are mean ± SEM for the maximal incremental VO2max-test, the 30-min simulated time-trial (TT_{30min}), and the 30-s Wingate test (W_{30s}) in the pretest and in the posttest. One group trained in normoxia (F_iO_2 = 20.9%) while receiving placebo (N, n = 10). The two other groups trained in hypoxia (F_iO_2 = 15.0%) and received either placebo (H, n = 8) or nitrate supplements (HN, n = 9).
**P < 0.05 compared to the pretest. #P < 0.10 compared to the pretest*

Muscle fiber type composition (Table 3)

The relative number of type I (~45–50%), type IIa (~40–45%), and type IIx fibers (~10%) in m. *vastus lateralis* in the pretest was similar between N and H ($P > 0.05$ for all fiber types) as well as between H and HN ($P > 0.05$ for all fiber types). SIT reduced the proportion of type IIx fibers in all the groups ($P < 0.05$). In the posttest the proportion of type IIa fibers was higher in HN than in H ($P < 0.05$), with training significantly increasing the proportion of IIa fibers from 45 to 56% in HN ($P < 0.05$), but not in N (main effect of time $P = 0.07$) or H ($P = 0.40$). Similar changes occurred for fiber-specific CSAs, which also were similar between N and H or H and HN in the pretest ($P > 0.05$ for all fiber types). SIT reduced the relative CSA of type IIx fibers in all the groups ($P < 0.05$). Conversely, type IIa relative CSA increased in HN (+11%, $P < 0.05$) only and was significantly greater in HN compared to H in the posttest ($P < 0.05$). SIT did not alter mean fiber CSA (μm^2) of the different fiber types, except for type IIx mean fiber CSA in H which increased from the pretest to the posttest ($P < 0.05$). However, type IIx fibers were observed in only half of the participants in H ($n = 4$), in which only few type IIx fibers were observed in the pretest [44 ± 33(SD)] and in the posttest [30 ± 28 (SD)]. Hence interpretation should be performed with caution. Mean fiber-specific CSAs were not significantly different between N and H or H and HN at any time.

TABLE 3 | Effects of training and nitrate intake on muscle fiber composition.

	N		H		HN	
	Pretest	Posttest	Pretest	Posttest	Pretest	Posttest
RELATIVE FIBER NUMBER (%)						
Type I	49 ± 3	49 ± 3	51 ± 5	53 ± 5	45 ± 4	39 ± 2
Type IIa	42 ± 3	49 ± 3	42 ± 4	44 ± 4	45 ± 2	56 ± 2*,†
Type IIx	9 ± 2	2 ± 1*	7 ± 2	3 ± 2*	10 ± 2	5 ± 1*
RELATIVE FIBER CSA (%)						
Type I	48 ± 3	51 ± 2	48 ± 5	50 ± 5	44 ± 4	38 ± 2
Type IIa	44 ± 3	47 ± 2	45 ± 5	47 ± 4	47 ± 2	58 ± 2*
Type IIx	8 ± 2	2 ± 1*	7 ± 2	3 ± 1*	8 ± 2	4 ± 1*
FIBER CSA (μm^2)						
Type I	4769 ± 604	5214 ± 501	5269 ± 204	5843 ± 462	4998 ± 402	5106 ± 434
Type IIa	4952 ± 429	4769 ± 411	5978 ± 281	6554 ± 348	5365 ± 534	5497 ± 573
Type IIx	4507 ± 444	4302 ± 428	4929 ± 664	6078 ± 474*	3976 ± 405	4438 ± 537

Data are mean ± SEM for muscle fiber cross-sectional area (CSA) and fiber number in m. vastus lateralis in the pretest and in the posttest. One group trained in normoxia (F_iO_2 = 20.9%) while receiving placebo (N, n = 10). The other groups trained in hypoxia (F_iO_2 = 15.0%) and received either placebo (H, n = 8) or nitrate supplements (HN, n = 9).

**P < 0.05 compared to pretest. †P< 0.05 group × time interaction.*

Muscle biochemistry (Table 4)

Baseline values for muscular buffering capacity, carnosine content, and maximal citrate synthase activity measured in homogenized muscle tissue were not significantly different between N and H or H and HN ($P > 0.05$). Buffering capacity of homogenized muscle was unaffected by SIT irrespective of the experimental condition. SIT on average increased muscle carnosine content by ~13% in H and HN (main effect of time $P < 0.05$, no significant post hoc time effects within H ($P = 0.11$) or HN ($P = 0.13$)), but not in N (+6%, $P = 0.072$). Nonetheless, the increase in carnosine content was not significantly different between N and H ($P = 0.48$), and there were no significant differences in muscle carnosine between the groups in either the pretest or the posttest. Maximal citrate synthase activity

increased by 54% in N ($P < 0.05$) and by just under half that in H (22%) and HN (25%) ($P < 0.05$), yet changes were not significantly different between N and H ($P = 0.10$).

TABLE 4 | Effects of training and nitrate intake on biochemical measurements in muscle.

	N		H		HN	
	Pretest	Posttest	Pretest	Posttest	Pretest	Posttest
βhm (mmol H^+·kg dm^{-1}·pH^{-1})	136 ± 6	136 ± 6	138 ± 5	137 ± 6	125 ± 6	128 ± 6
Carnosine content (mmol·kg dm^{-1})	33.3 ± 2.3	35.3 ± 2.2	33.8 ± 2.9	38.2 ± 2.9	30.2 ± 2.2	34.0 ± 2.6
Citrate synthase activity (μmol·min^{-1}·g^{-1})	190 ± 19	292 ± 24*	231 ± 10	282 ± 16*	216 ± 17	269 ± 8.2*

Data are mean ± SEM for buffering capacity of homogenized muscle (βhm), carnosine content, and citrate synthase activity in the pretest and the posttest. One group trained in normoxia (F_iO_2 = 20.9%) while receiving placebo (N, n = 10). The other groups trained in hypoxia (F_iO_2 = 15.0%) and received either placebo (H, n = 8) or nitrate supplements (HN, n = 9).
**P < 0.05 compared to pretest.*

Discussion

The most striking results from the current study are that (a) 5 weeks of SIT, performed in hypoxic conditions, significantly increased the fraction of type IIa muscle fibers, but only when completed with concomitant dietary nitrate supplementation and (b) SIT performed in hypoxic conditions does not ameliorate physiological adaptations yielding enhanced aerobic or anaerobic endurance exercise performance.

We administered 400 mg of molecular nitrate ~3 h before each SIT session in HN, which has previously been shown to significantly increase the plasma nitrite concentration [52] and improve oxygen-efficiency of the skeletal muscle during exercise [40,41]. We postulated that this might delay fatigue development and thereby attenuate the reduced training intensity often shown during hypoxic sprint training [53,54]. Training workloads were, however, similar between all groups, regardless of whether the training was performed in normoxia (N) or in hypoxia, either with (HN) or without (H) nitrate supplementation. Thus, the degree of neuromechanical activation during training was similar between all experimental conditions.

Even in the absence of fiber hypertrophy, fiber type transition from IIx to IIa might be expected during short-term SIT involving 30-s sprints in healthy volunteers (for review, see [55]). In keeping with the published findings [56], 5 weeks of SIT did not induce muscle fiber hypertrophy in the current study. Consistent with this, SIT in normoxia reduced the relative type IIx fiber number, whilst the proportion of type IIa fibers tended to increase. SIT in hypoxia (H) also reduced type IIx fiber number, but did not alter the fraction of type IIa fibers. Interestingly, when nitrate supplementation was provided during SIT in hypoxic conditions (HN), relative type IIa fiber number increased from 45 to 56%.

Previous evidence indicates that nitric oxide (NO) plays a pivotal role in MHC-based muscle fiber type transition [57-59]. NO is suggested to increase inhibitory phosphorylation of glycogen synthase kinase (GSK)-3β in rat fast-twitch muscle, promoting nuclear factor of activated T-cell c1 (NFATc1) dephosphorylation and nuclear accumulation, resulting in a fast-to-slow fiber type transition [58]. Furthermore, pharmacological inhibition of NO-synthase (NOS) activity by NG-nitro-L-arginine-methyl-ester (L-NAME) negated overload-induced type II to I fiber type transition [57]. Results from both

rodent [42,43] and human [45,46] exercise studies indicated that oral nitrate intake exerts its actions primarily in type II muscle fibers by increasing blood flow [43], by elevating sarcoplasmic reticulum calcium stores and the expression of calcium handling proteins such as calsequestrin 1 and the dihydropyridine receptor [42], as well as by reducing muscle metabolic perturbation [37]. Nitrate supplementation also stimulated rate of force development [42] and power output [45,46] during high-intensity and high-velocity muscle contractions. Thus, while endogenous NO production during acute exposure of lowlanders to hypoxia is reduced, due to inhibition of the L-arginine–NOS pathway [60], exogenous nitrate provides an alternative pathway to stimulate NO production via nitrate to nitrite to NO conversion [60]. Given that NO probably plays an important role in exercise-induced MHC-based adult fiber type transitions, it is plausible that oral nitrate intake during SIT in hypoxia served as an adequate back-up mechanism for NO-induced muscle fiber transformation to compensate for impaired NOS activity. However, in contrast to what could be expected from the current literature, i.e., stimulation of type IIx to IIa and IIa to type I fiber type transition [57-59], nitrate supplementation during hypoxic SIT did not stimulate the transition of type IIa to type I muscle fibers. Follow-up studies are needed to elucidate the cellular mechanisms by which oral nitrate intake can stimulate the conversion to type IIa muscle fibers during SIT in hypoxia.

Despite a decrease in the fraction of IIx muscle fibers in all conditions and an increase in the fraction of IIa muscle fibers in HN, there was no change in βhm with training in any of the three groups. We postulated that SIT in hypoxia, due to an enhanced contribution of glycolysis to ATP production, might impair βhm compared with identical training in normoxia. Contrary to our hypothesis, however, SIT did not alter βhm, regardless of whether the training was completed in normoxia or hypoxia. Our data shows that SIT is not an adequate strategy to improve βhm, at least in healthy recreationally active volunteers during short-term training. Findings from the present and earlier studies support the opinion that high-intensity interval training (HIT) at workloads corresponding to 120–170% of the lactate threshold [30,50] is probably more effective to raise βhm than explicit 'glycolytic training' via SIT [61,63]. Adaptations of βhm during long-term SIT in elite sprinters may be different, given their substantially higher proportion of type II fibers and higher glycolytic capacity.

The physicochemical buffer capacity of muscle (~βhm) is composed of proteins, inorganic phosphate, bicarbonate, and the histidine-containing dipeptide carnosine. The histidine containing dipeptide content of muscle (carnosine in human muscle) contributes ~7–8% to total βhm [64-66], and elevations through training or dietary supplementation remain a plausible mechanism for increasing βhm. Accordingly, we determined the effect of SIT on muscle carnosine content and observed no significant effect in normoxic conditions. However, a main effect of time (pretest vs. posttest), though without significant post hoc effects, was found for the increase in muscle carnosine content following training in hypoxic conditions (H and HN), yet the training-induced changes were not significantly different between N and H. Earlier experiments in our laboratory [12] have shown that SIT in hypoxia did not increase muscle carnosine content (unpublished observations), suggesting some equivocality in the findings across our own studies. This would seem in line with the existing literature, given that SIT has previously been shown to substantially increase muscle carnosine content in one study [67], although no such training-effect has been shown in any other longitudinal intervention study using SIT [63] or resistance training involving either short (~10 s) [68] or longer (~20–45 s) series of maximal muscle contractions in normoxia [69]. Future studies performing single-fiber determination of muscle carnosine are warranted for clear interpretation of training-induced changes in fiber-specific myocellular carnosine content.

Buffering capacity of homogenized muscle, as measured in the present study, is different to muscle buffering capacity in vivo and reflects the altered state and chemistry of muscle after homogenization. Assuming that there would be no change in any other source of myocellular buffering,

it could be estimated that a 13% increase in muscle carnosine content (i.e., 4mmol·kg dm^{-1}) would increase homogenized muscle buffering capacity over the titration pH range of 7.1 to 6.5 by 2.21 $mmolH^{+}\cdot kg\ dm^{-1}\cdot pH^{-1}$, given that at pH 7.1 and pH 6.5 34.9 and 68.1% of the additional carnosine content is already in the protonated form, respectively [64]. This represents an increase of ~1.7% of the initial βhm in the present study and falls below the detection limit of the titration assay used. However, as we don't know what muscle buffering capacity in vivo is over this range, we cannot calculate the importance of this increase.

We also evaluated the effect of SIT on muscle oxidative capacity by measuring citrate synthase (CS) maximal activity. SIT substantially elevated CS activity (+25–50%), independent of whether the training was performed in normoxia or in hypoxia. These findings are in contrast with reports from single-leg studies reporting greater increases in CS activity following endurance training (30 min at 65–75% maximal work capacity) in hypoxic conditions compared to training at similar absolute workloads in normoxic conditions [7-9]. However, if the training workloads are matched for relative intensity, endurance training in hypoxic conditions abolishes rather than augments training-induced changes in muscle oxidative function [70]. Training workloads during SIT in the present study were similar between normoxic and hypoxic conditions in both absolute and relative terms. In line with previous reports, SIT in normoxic conditions increased CS activity (for review see [71]), but sprint training in hypoxic conditions did not augment adaptations in muscle oxidative capacity [12,13].

The hypothesis driving the current study was underpinned by the notion that performing SIT in hypoxic conditions, alone or in combination with oral nitrate intake, might yield specific physiological adaptations to boost exercise performance. Anaerobic glycolysis accounts for about 50% of the total ATP production during a 30-s maximal exercise bout [72]. It is therefore reasonable to postulate that a higher proportion of type IIa muscle fibers, providing a higher capacity for glycolytic ATP production, should be ergogenic during a 30-s all-out exercise. As discussed above, hypoxic SIT in conjunction with oral nitrate supplementation increased relative type IIa muscle area compared with hypoxic SIT alone. Interestingly, this also tended to translate into a greater gain in power output during W_{30s} (+12% in HN vs.+6% in H, P = 0.08), which indicates that short-term oral nitrate supplementation in conjunction with SIT may be a valid strategy to enhance performance in 'glycolytic' exercise events such as a 400-meter dash, by contributing to a beneficial fiber type shift. Future studies should seek to confirm this possibility. It is also important to emphasize that we did not study the effect of nitrate intake during SIT in normoxia, neither did we study exercise performance in hypoxia. Thus, we cannot extrapolate our findings to these conditions. Contrary to 30-s sprint performance, determinants of aerobic exercise capacity were similar between the groups. Five weeks of SIT enhanced cycling power output corresponding to 2 and 4 $mmol\cdot L^{-1}$ blood lactate levels, VO_2max, and 30-min cycling time-trial performance, independent of whether the training was performed in normoxia, or in hypoxia with or without oral nitrate supplementation. These findings indicate that short- term SIT in hypoxic compared to normoxic conditions is not an advantageous strategy for enhancing normoxic endurance exercise performance in recreationally active individuals.

In conclusion, the current experiment demonstrated that oral nitrate supplementation during short-term sprint-interval training increased the proportion of type IIa muscle fibers in muscle, which may contribute to enhanced performance in short maximal exercise events requiring a very high glycolytic rate. Compared with SIT in normoxia, SIT in hypoxia did not generate beneficial physiological adaptations yielding enhanced aerobic or anaerobic endurance exercise performance.

Author contributions

Conception and design of the study: PH and SD. All authors contributed to the collection and interpretation of the data, and reviewed and approved the final manuscript written by PH and SD.

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Conflict of interest statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper 2

Physiological adaptations to hypoxic versus normoxic training during intermittent living high

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Abstract

Purpose: In the setting of 'living high', it is unclear whether high-intensity interval training (HIIT) should be performed 'low' or 'high' to stimulate muscular and performance adaptations.

Methods: Therefore, 10 physically active males participated in a 5-week 'live high-train low or high' program (TR), whilst eight subjects were not engaged in any altitude or training intervention (CON). Five days per week (~15.5 h per day), TR was exposed to normobaric hypoxia simulating progressively increasing altitude of ~2000–3250 m. Three times per week, TR performed HIIT, administered as unilateral knee-extension training, with one leg in normobaric hypoxia (~4300 m; TR_{HYP}) and with the other leg in normoxia (TR_{NOR}).

Results: 'Living high' elicited a consistent elevation in serum erythropoietin concentrations which adequately predicted the increase in hemoglobin mass ($r = 0.78$, $P < 0.05$; TR: +2.6%, $P < 0.05$; CON: −0.7%, $P > 0.05$). Muscle oxygenation during training was lower in TR_{HYP} vs. TR_{NOR} ($P < 0.05$). Muscle homogenate buffering capacity and pH-regulating protein abundance were similar between pretest and posttest. Oscillations in muscle blood volume during repeated sprints, as estimated by oscillations in NIRS-derived tHb, increased from pretest to posttest in TR_{HYP} (~80%, $P < 0.01$) but not in TR_{NOR} (~50%, $P = 0.08$). Muscle capillarity (~15%) as well as repeated-sprint ability (~8%) and 3-min maximal performance (~10–15%) increased similarly in both legs ($P < 0.05$). Maximal isometric strength increased in TR_{HYP} (~8%, $P < 0.05$) but not in TR_{NOR} (~4%, $P > 0.05$).

Conclusion: In conclusion, muscular and performance adaptations were largely similar following normoxic vs. hypoxic HIIT. However, hypoxic HIIT stimulated adaptations in isometric strength and muscle perfusion during intermittent sprinting.

Keywords: altitude training, high-intensity interval training, muscle hemodynamics, pH regulation, EPO, hemoglobin mass

Introduction

Since the 1968 Olympic Games in Mexico City (2300 m) athletes have used altitude training to prepare for endurance exercise performances at sea-level. Extensive research has shown 'living high' while 'training low' to be the better approach in altitude training, because this strategy enables athletes to spend sufficient time in hypoxia to stimulate erythropoiesis whilst maintaining high mechanical work output and oxygen fluxes during training [1,2]. Today, normobaric hypoxic installations are easily available. Thus athletes can continue living in their normal home environment, while 'living high' in normobaric hypoxia, yet training at sea level. Indeed, hypobaric and normobaric hypoxic exposure recently were reported to be equally efficient in increasing total hemoglobin mass (Hbmass) [3]. The potential of 'living high' to increase Hbmass [4,5] and thereby raise maximal oxygen uptake rate (VO_2max) [6,7] is now widely recognized. Nonetheless, considerable inter-individual variability exists in the responses to hypoxia [8,9]. Moreover, even within a given individual, Hbmass does not respond consistently to sequential altitude training exposures [10]. Unfortunately, pertinent markers for individual altitude sensitivity are still lacking.

Hypoxia-induced stimulation of serum erythropoietin (sEPO) production drives the increase in Hbmass at altitude [11-13]. The sEPO response to altitude is biphasic. sEPO as a rule peaks within 48 h, where after it gradually declines towards sea-level values over the following days and weeks [8,14-16]. This is probably at least partly due to increasing arterial oxygen content [17-19], together with facilitated O_2 extraction by EPO-producing cells in kidney and liver by virtue of elevated erythrocyte 2,3-diphosphoglycerate concentration [19,20]. In addition, cells chronically exposed to hypoxia have been shown to lower their normoxic setpoint, hence blunting hypoxia signaling for the ongoing oxygen tension whilst allowing hypoxia-sensitive mechanisms to respond to new deviations in cellular oxygen availability [21,22]. It has been postulated that the magnitude of sEPO increment during the initial phase of altitude training can predict the eventual increment of Hbmass and even VO_2max [8]. However, this opinion was contradicted by later studies showing divergent sEPO and Hbmass responses in conjunction with high inter-individual variability [9,15,23,24]. A strategy to maintain high sEPO levels during prolonged exposure to hypoxia might be to either gradually increase the degree of hypoxia, or discontinuously administer the hypoxic episodes to avoid desensitization.

Over the last two decades, interest in the beneficial effects of intermittent hypoxic training (IHT) has grown [25,26]. Indeed, a series of seminal studies reported greater upregulation of oxidative enzymes [27-29] and mitochondrial biogenesis [30-33] in muscle following endurance training in hypoxia compared to similar training in normoxia. Nonetheless, published data with regard to the effects of IHT on sea-level exercise performance are equivocal [26]. Hypoxic training may upregulate myocellular oxidative potential, indeed, yet workload in endurance training sessions is reduced due to impaired oxidative energy provision [34]. Therefore, attention has recently shifted to IHT in the form of sprint training, which allows for similar or even higher training intensities to be performed at altitude [35,36]. Furthermore, growing evidence indicates that hypoxic resistance training may stimulate muscle anabolism [37,38]. Accordingly, heavy resistance training in hypoxia has recently been reported to induce greater strength gains than similar training in normoxia [39].

The precise physiological mechanism underlying specific muscular adaptations to IHT remains to be elucidated. Training in hypoxia stimulates hypoxia-inducible factor-1α (HIF-1α) transcription [30, 40-42]. In addition, acute hypoxia (10.7% F_iO_2), compared to normoxia, was recently found to stabilize HIF-1α protein in muscle both at rest and during submaximal exercise [43]. Repeated-sprint training in hypoxia (RSH) has also been found to stimulate the transcription of monocarboxylate transporter 4 (MCT4) and carbonic anhydrase III (CA3) genes more than similar training in normoxia [44]. Furthermore, a recent meta-analysis showed RSH, compared to similar training in normoxia, to

induce greater improvements in sea-level repeated-sprint performance [45]. Yet sprint interval training in both normoxic and hypoxic conditions failed to increase muscular buffering capacity [46]. Recent observations also indicate that RSH may facilitate muscular perfusion during intermittent contractions [40,47,48]. Furthermore, there is evidence to indicate that training in hypoxia may stimulate angiogenesis in muscle tissue [49].

The primary aim of the present study was to test the hypothesis that 'living high' in conjunction with high-intensity interval training (HIIT) in hypoxia, compared to identical training in normoxia, enhances high-intensity exercise performance at sea level by stimulating beneficial muscular adaptations. In the current study, HIIT was administered in the form of unilateral knee-extensions in order to be able to compare hypoxic and normoxic training within the same individual during a single period of 'living high'. Compared with whole-body exercise, oxygen delivery to muscles during unilateral knee-extension exercise is markedly increased due to elevated capillary perfusion [50]. Nonetheless, preliminary experiments in our laboratory showed that muscle oxygenation is significantly reduced by environmental hypoxia during intermittent knee-extension exercise, which makes the exercise model suitable for the purpose of this study. The secondary aim of this study was to assess the hematological and physiological responses to a discontinuous 'living high' protocol during which altitude simulated by normobaric hypoxia was gradually increased.

Materials and methods

Participants

Following a medical screening, including a blood sample to assess iron status, 18 young healthy men (mean ± SD; 23.9 ± 3.0 y, 1.80 ± 0.07 m, 70.1 ± 6.6 kg) were recruited to participate in this study. Exclusion criteria upon admission were: exposure to altitude above 1,500 m in the 6 months prior to the study; intake of any medication or nutritional supplements during the 3 months prior the study; smoking; any kind of cardiopulmonary or musculoskeletal disorder. Subjects were recreational sports participants (4.8 ± 2.8 h per week, P = 0.76 between the experimental groups), yet none were engaged in training or sport at high competitive level. Subjects were instructed to maintain their habitual physical activities during the study period. Starting 1 week prior to the intervention, all subjects with serum ferritin concentrations (sFer) lower than 100 µg·L^{-1} received iron supplements delivering 105 mg elemental iron plus 500 mg Vitamin C per day (Ferograd©, Abbott S.p.A., Campoverde, Italy). The study was approved by the KU Leuven Biomedical Ethics Committee (B322201525619) and conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent after being fully informed about the content of the study and the risks associated with participation.

Study design

The study involved two experimental groups, of which one was enrolled in a 5-week controlled 'live high-train low or high' intervention using normobaric hypoxia (TR, n=10), whilst the other group served as an untrained control group living low at sea level (CON, n=8) (**Figure 1**). During this period, every Tuesday, Thursday and Saturday, in TR but not in CON one leg was trained by knee-extension exercise at 12.3% F_iO_2 (~4300 m; TR_{HYP}), whilst the other leg was trained at sea level (TR_{NOR}). Before (pretest) and at the end (posttest) of the intervention period a series of measurements was performed to assess physiological and exercise performance adaptations to 'live high-train low or high'. Furthermore, muscle biopsies were taken from both the legs to evaluate muscular adaptations to TR_{HYP} *versus* TR_{NOR}. Between 1 and 3 weeks prior to the pretest, the subjects participated in 3 familiarization sessions with an interval of at least 2 days. These sessions served to habituate the subjects to the exercise tests to be performed in the pretest and the posttest. In the first familiarization session, subjects first performed a

maximal voluntary contraction test and repeated-sprint ability test with the right leg. Following a 15-min rest period, similar testing was conducted with the left leg. In the second familiarization session, subjects first performed a 3-min maximal performance test with the right leg and subsequently with the left leg. The third familiarization session was similar to the second familiarization session, consisting of 3-min maximal performance testing of both the legs. Due to the nature of the intervention, subjects enrolled in TR were susceptible to experience an 'expectancy-effect' which might lead to improved exercise performance in the posttest due to psychological factors [51]. In order to also induce an expectancy-effect in CON, all subjects received placebo supplements containing branched-chain amino acids (400 mg·day^{-1}) and dry chicory powder (200 mg·day^{-1}). The subjects were told that this novel 'plant extract' was believed to have the potential to mimic altitude training effects on red blood cell mass.

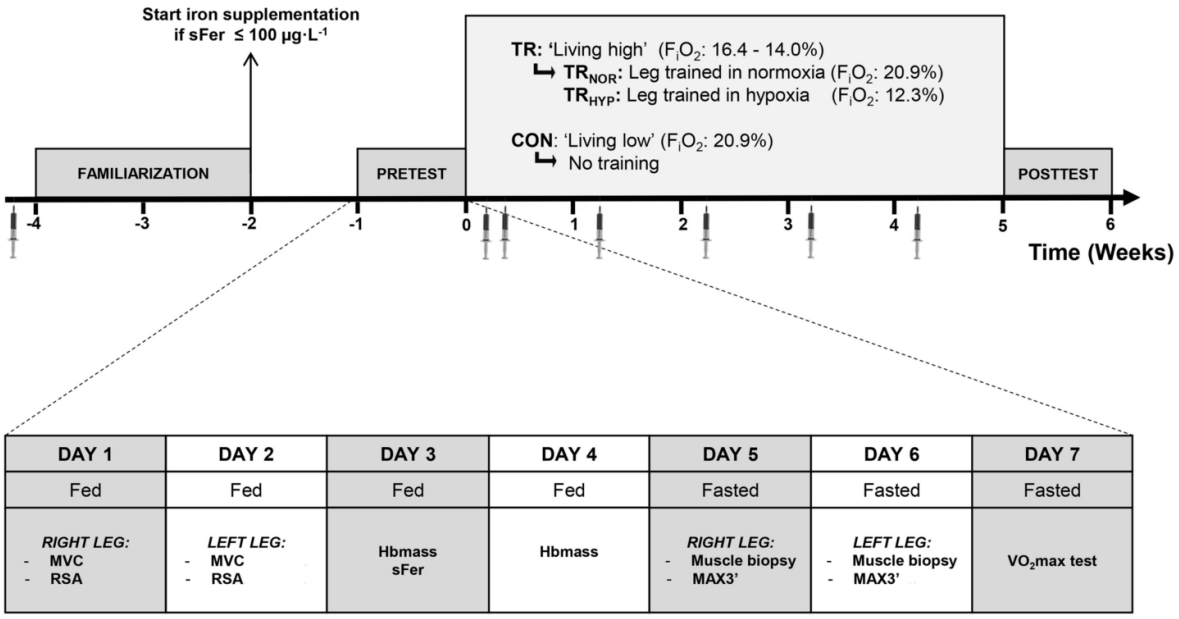


Figure 1 | Schematic presentation of the study protocol.
The study involved two experimental groups, of which one group was enrolled in a 'live high' intervention using normobaric hypoxia (TR, n = 10), whilst the other group lived at sea level (CON, n = 8). In TR one leg was trained in 12.3% F_iO_2 (TR_{HYP}, ~4,300m), whilst the other leg was trained in 20.9% F_iO_2 (TR_{NOR}). The week before (pretest) and the week after (posttest) the intervention period a series of measurements were performed. During the intervention period venous blood samples were taken on a weekly basis. MVC, maximal voluntary contraction; RSA, repeated-sprint ability; Hbmass, total blood hemoglobin mass; sFer, serum ferritin; MAX3′, 3-min maximal performance test; VO_2max, maximal oxygen uptake rate.

Normobaric hypoxia exposure – 'living high'

The subjects in TR lived in a major hypoxic facility (232 m^2; b-CAT, Tiel, the Netherlands), from Monday evening to Saturday morning. The facility (Bakala Academy, KU Leuven, Belgium) includes 5 large double bedrooms (~26 m^2) and a major living/dining/recreational room (~70 m^2) to spend non-sleeping time. On Saturdays and Sundays they lived in their normal home environment at sea level. On weekdays they spent on average ~15.5 h per day (range: 14.3 – 16.6 h) in the hypoxic facility,

accumulating ~390 h in total (range: 378 – 395 h) over the 5-week intervention period, which in the conditions of the current study is equivalent to a hypoxic dose of 1016 km·h [5]. The remaining time was spent outside the hypoxic facility at sea level. From day 1 to day 30 of the intervention period, F_iO_2 in the hypoxic facility was gradually decreased from 16.4% (~2000 m altitude) to 14.0% (~3250 m altitude) (see **Figure 2**). Every morning upon wake-up the subjects measured their arterial oxygen saturation (%SpO_2) using a portable pulsoximeter (Nonin Medical, Onyx 9590, Plymouth MN, USA), filled in the Lake Louise Questionnaire [52] and registered the hours of effective sleep in a diary.

Hypoxic vs. normoxic HIIT training

All training sessions were supervised by the investigators and consisted of unilateral knee extensions on a leg-extension apparatus (GLCE365, Body-Solid, Illinois, US). The leg-extension apparatus was adapted to limit knee-extension amplitude during training sessions from 95° to 175° knee angle. During each training session the subjects performed two sets of knee extensions with a 5-min passive recovery interval in between. Each set consisted of 4-6 series of 30 contractions at 20-25% of the 1-repetition-maximum (1RM), with 30 s of rest in between the series. The number of contraction series increased from four in sessions 1-5, to five in sessions 6-10, and eventually six in sessions 11-14. The relative load increased from 20% of 1RM in sessions 1-7 to 25% of 1RM in sessions 8-14. During each series the rate of muscle contraction and relaxation was paced by both auditory and visual feedback to obtain a 3-s duty cycle with 1s extension:1s relaxation:1s rest. 1RM was re-evaluated at the start of each next week to adjust training workloads. The subjects were randomly assigned to a group training with the dominant leg in hypoxia, whilst the contralateral leg was trained in normoxia, or vice versa. In each session the subjects thus trained one leg in the normobaric hypoxic facility (b-CAT, Tiel, The Netherlands) at 12.3% F_iO_2 (~4300 m), whilst the contralateral leg was trained in an adjacent normoxic room at 20.9% F_iO_2. The subjects performed 3 training sessions per week, and the order of left vs. right leg training was alternated between the sessions. The oxygenation status of m. *vastus lateralis* was assessed by near-infrared spectroscopy (NIRS) during all training sessions in week 1 and week 5, as previously described [53]. Immediately following each training session the subjects ingested 25 g of a standard whey protein concentrate mixture (Whey Shake, Sports 2 Health, Hofstade, Belgium) to stimulate muscular repair.

Pretest and posttest protocol

The protocol for the pretest and the posttest was identical, and consisted of 7 experimental sessions (**Figure 1**). The experiments involved multiple unilateral knee-extension tests, an incremental VO_2max test, biopsy sampling of m. vastus lateralis, and measurement of total hemoglobin mass. The order of the sessions was scheduled to avoid interference between the different measurements. From 48 h prior to the pretest and posttest the subjects were instructed to refrain from any strenuous activity other than the exercises prescribed by the study protocol. The last training session was scheduled 3 days prior to the posttest. All subjects consumed a standardized carbohydrate-rich dinner (~1,200 kCal; 65%carbohydrates, 15% fat, 20% protein) on the evening prior to each experimental day, and received a standardized breakfast (~960 kCal; 70% CHO, 10% Prot, 20% Fat) and lunch (~980 kCal; 64% CHO, 16% Prot, 20% Fat) on the next day. However, experimental sessions on day 5–7 were performed after an overnight fast. Furthermore, on the 7th day of the pretest, subjects spent the night in the hypoxic facility set at 20.9% F_iO_2. In the morning between 7 and 8 a. m. arterial oxygen saturation was measured upon wake-up (%SpO_2, Nonin Medical, Onyx 9590, Plymouth MN, USA) and a blood sample was taken from an arm vein for assay of serum erythropoietin (sEPO) concentration (see below). These measurements were taken as the normoxic baseline. During the subsequent 5-week 'living high' intervention %SpO_2 was measured every morning upon wake-up, and blood samples for follow-up of sEPO changes were collected each Tuesday between 7 and 8 a.m. in identical conditions as in the pretest.

Exercise testing procedures

Maximal voluntary contraction (MVC) – All knee-extension tests were performed separately for the left and the right leg. Maximal isometric contraction force was assessed on a self-constructed, motor-driven dynamometer as previously described [54]. MVC was measured at a knee-angle of 135° extension (180° being fully extended). Subjects first warmed up by 3 sets of 30 submaximal dynamic contractions with 30-s rest intervals in between. Subsequently the subjects performed five 5-s maximal isometric knee-extensions, with a 1-min passive recovery interval in between. The highest mean torque (Nm) over a 2-s time frame was selected. The best performance out of five attempts was taken as MVC. The typical error for the MVC measurements was 6.2%, which is within the normal reliability range for MVC testing [55].

Repeated-sprint ability (RSA) test – The RSA test was performed on the same dynamometer following 10 min of passive rest after the MVC testing. Unilateral isokinetic knee-extensions from 95° to 175° knee angle were performed at a rate of $90°\cdot s^{-1}$. Immediately after extension the leg was passively returned to the starting position ($180°\cdot s^{-1}$). The subjects were verbally encouraged to produce maximal force in each contraction. The RSA test consisted of 20 sets of 10 contractions. Each set was interspersed by a 15-s rest period (~1:1 work-to-rest ratio). The dynamic torque (Nm) productions were continuously digitized (250 Hz) by an on-line computer and both mean and peak (highest value out of 10 contractions) torque per set were automatically calculated. The typical errors for the overall mean and peak torque measurements were 3.5% and 3.7%, respectively.

3-min maximal performance test (MAX3') – By analogy with the assessment of MVC and RSA, MAX3' also was performed on the isokinetic dynamometer. The subjects first warmed up by 3 sets of 30 submaximal dynamic knee extensions (30% of MAX3' in the familiarization sessions) with 30-s rest-intervals in between. Thereafter, subjects performed 140 maximal unilateral isokinetic contractions at a rate of 1.3 Hz (active extension and passive flexion movements at a rate of $90°\cdot s^{-1}$ and $180°\cdot s^{-1}$, respectively). Subjects were instructed to produce the highest possible total work output over the 3-min exercise bout. Initial target torque was taken from the familiarization sessions and real-time torque output was shown on a screen to assist the subjects in setting the optimal exercise intensity during the initial 30 contractions. Beyond 30 contractions the on-line feedback was stopped and only remaining time to finish was shown for the next 150 s. The typical error for the measurement of total work output in MAX3' was 3.5%.

Incremental VO_2max test – A maximal incremental exercise test was performed on a bicycle ergometer (Avantronic Cyclus II, Leipzig, Germany). Initial workload was set at 100 Watt and was increased by $20\ W\cdot min^{-1}$. Respiratory gas exchange was measured using a breath-by-breath open circuit spirometry system (Cortex Metalyzer IIIb, Leipzig, Germany). The highest average oxygen uptake over a 30-s time period was taken as VO_2max. Heart rate was continuously monitored (Polar RS800CX, Kempele, Finland). One week after the posttest the subjects in TR performed an additional VO_2max test to re-evaluate aerobic capacity following a 7-day return to sea level (~normoxia). The typical error for VO_2max measurement was 3.4%, which corresponds with, or is favorable to literature data [56-59].

Total hemoglobin mass assay

Hbmass was measured with a slightly modified version of the optimized CO-rebreathing procedure [60], as previously described by Steiner and Wehrlin [61]. Briefly, a bolus CO of 1.0 mL per kg body mass was administered and rebreathed for 2 min. The percentage of carboxyhemoglobin was measured (ABL90 Flex, Radiometer, Copenhagen, Denmark) before (5 samples), and 6 and 8 min after administration of the CO bolus, by analysis of capillary blood samples (70 µL) taken from a preheated earlobe. The increase in carboxyhemoglobin was used to calculate the absolute Hbmass (g). A duplicate

measurement of Hbmass was performed the next day. The typical error for the Hbmass measurements, calculated from the duplicate measures, was 1.8% which is in line with other work in the field [62].

Blood sampling and analyses

During the medical screening and in the pretest and the posttest, blood (12 mL) was sampled from an arm vein into EDTA and serum separator tubes (Vacuette, Greiner Bio-One, Vilvoorde, Belgium) prepared for whole blood and serum analyses. Furthermore, additional blood samples were taken during the 'living high' period (see above) for follow-up of sEPO. Samples were allowed to clot at room temperature before serum was separated by centrifugation and either immediately analyzed for serum ferritin concentrations (sFer) via immunoturbidimetric assay (Roche Modular P Analyser, Roche Diagnostics, Switzerland) or stored at -80°C for later analysis of sEPO. White blood cell count (DxH 800, Beckman-Coulter, Namen, Belgium) was used for control of possible infection-induced alterations in sFer. However, white blood cell count test results were consistently normal throughout the study in all subjects. sEPO concentration was assessed in duplicate using a commercially available sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA). The intra-assay typical error was 4.6% which is within the margins reported by the manufacturer.

Near-infrared spectroscopy

A Niro-200 NIRS instrument (Hamamatsu, Japan) was used to measure tissue oxygen index (TOI) and changes in oxyhemoglobin (ΔO_2Hb), deoxyhemoglobin (ΔHHb) and total hemoglobin (ΔtHb) content in the vastus lateralis muscle during RSA testing in the pretest and the posttest, as well as during training sessions in the first and the last week of the intervention period. A NIRS probe was placed centrally on the belly of the vastus lateralis muscle after local shaving of the skin. An elastic non-compressive bandage was wrapped around the probe to prevent interference with external light and to avoid movement of the probe during testing. A surgical pen marked the margins of the probe on the skin in order to allow for identical repositioning of the probe in later testing. The NIRS signal was recorded at 2 Hz. Before analysis, all data points were processed with a Butterworth filter (4^{th} order, cutoff frequency of 0.05 Hz) in a mathematical software program (Matlab R2011a, The Mathworks, Natick, MA, USA). This filter was part of a custom made Matlab script in which data frames were selected manually. The minimum value of every negative peak (i.e., at the end of the knee-extensions set for tHb) and the maximum value of every positive peak (i.e., at end of recovery intervals for tHb) during each of the 20 sets of the RSA test were selected to evaluate the magnitude of changes (Δ) in NIRS parameters. Typical error of the average ΔtHb throughout the RSA test during muscle contractions and recovery was 23.0 and 21.8%, respectively. The corresponding typical errors for ΔTOI were 19.7 and 20.6%, respectively. The corresponding typical errors for ΔHHb were 17.2 and 17.2%, respectively, and 17.0 and 17.1% for ΔO_2Hb. During the training sessions in week 1 and week 5 mean values of TOI and tHb were calculated for each 5-s interval during the sets and for each 10-s interval during the recovery interval between the two sets. Due to the experimental design of the present study, typical errors of NIRS-derived parameters during training sessions could not be calculated.

Muscle biopsies

Subjects reported to the laboratory between 6 and 11 a.m. after an overnight fast. Following a 30-min rest period in the supine position, a biopsy was taken from *vastus lateralis* muscle using a Bergström-type needle through a single 5-mm incision in the skin (2% xylocaine without epinephrine, 1 mL subcutaneously) [63]. Muscle biopsies were taken by a medical doctor who accumulated extensive experience with the muscle biopsy procedure over years. Immediately following the biopsy, local pressure was applied until bleeding had completely stopped. The incision was then carefully sealed with adhesive strips (Steri-Strip, 3M Health Care, St. Paul, MN) and covered with a sterile plastic gauze

(OpSite, Smith & Nephew, London, UK). Muscle samples were divided into two parts. One part was rapidly frozen in liquid nitrogen and stored at -80°C for biochemical analyses. The other part was frozen in isopentane cooled in liquid N_2 and stored at -80°C for histochemical analyses.

Analysis of muscle samples

Buffering capacity of homogenized muscle (βhm) – βhm was evaluated by the titration method as previously described [64]. Briefly, 2-3 mg dry muscle (dm) was dissected from connective tissue and homogenized in a sodium fluoride containing buffer (33.3 µL 10 mM NaF·mg dm^{-1}). The homogenates were warmed to 37.0°C in a warm water bath. Basal pH was measured with a microelectrode (MI-410, Microelectrodes, Bedford, NH, USA) connected to a pH meter (Lab 850, Schott Instruments GmbH, Mainz, Germany) and subsequently adjusted to pH > 7.1 with sodium hydroxide (0.02 M NaOH). Then, via titration of 2 µL hydrochloric acid (0.01 M HCl) pH was stepwise adjusted until pH reached values below 6.1. βhm was expressed as mmol H^+·kg dm^{-1} required to decrease pH with a given unit. Twenty-three samples were analyzed in duplicate. In accordance with previous reports [46], the typical error of the βhm measurement was 5.4%.

Muscle fiber type composition and capillarization – Serial 7-µm-thick cryosections were cut with a cryostat at -20°C. Cryosections were blocked for 1 h in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Slides were then incubated for 2 h in primary antibody against CD31 (DakoCytomation, Heverlee, Belgium) in a 1:500 dilution in 0.5% BSA in PBS. The cryosections were washed and incubated in a biotinylated rabbit anti-mouse IgG (H&L) antibody (DakoCytomation) (1:500 in 0.5% BSA in PBS) for 1 h at room temperature. Following a quick wash, slides were incubated overnight in primary antibodies against myosin heavy chain I (BA-F8, Developmental Studies Hybridoma Bank) and myosin heavy chain IIa (SC-71, Developmental Studies Hybridoma Bank) dissolved in PBS with 0.5% BSA (diluted 1:50 and 1:600, respectively). After washing, cryosections were incubated in appropriate conjugated secondary antibodies 488 goat anti-mouse IgG2 (Invitrogen) and Alexa Fluor 350 goat anti-mouse IgG1 (Invitrogen) in a 1:1000 dilution in 0.5% BSA in PBS. The slides were washed again and incubated in streptavidin (1:100 dilution in PBS) for 30 min, after which they were washed and incubated in cyanine (1:100 dilution in PBS) for 8 min. Cover slips were mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA) after which type I muscle fibers, type II muscle fibers and capillaries were examined using a Nikon E1000 fluorescence microscope (Nikon, Boerhavedorp, Germany). Photos of the slides were analyzed with ImageJ software (version 1.41, National Institutes of Health, USA). Only fibers with adequate cross-sections showing no signs of distortion or folding were counted. On average (± SD) 181 ± 73 fibers were analyzed per biopsy.

Western Blotting – Standard Western Blotting procedures have been described elsewhere [65]. Following homogenization and protein extraction of ~20 mg muscle tissue, 20-40 µg of proteins were loaded on sodium dodecyl sulphate polyacrylamide gels for electrophoretic separation. The proteins were then electro-transferred to a polyvinylidene difluoride membrane at 90 V for 100 min. Subsequently the membranes were blocked for 1 h in 0.1% Tween 20 Tris-buffered Saline (TBST) containing 5% fat free dry milk powder, after which they were emerged in one of the following primary antibodies for overnight incubation at 4°C: monocarboxylate 1 (MCT1; AB3538P, Millipore, Temecula, CA, USA), MCT4 (AB3316P, Millipore, Temecula, California), carbonic anhydrase III (CA3; AB135995, Abcam, Cambridge, UK) and Na^+/H^+ exchanger 1 (NHE1; AB126725, Abcam, Cambridge, UK). The membranes were then washed with TBST and incubated for 60 min at room temperature in the appropriate secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit; Sigma-Aldrich, Bornem, Belgium) for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genesnap and Genetools software (Syngene, Cambridge, UK),

respectively. Results are presented relative to a standard sample (pool) run on each blot and relative to GAPDH (cat no. 2118 14C10, Cell Signaling Technology, Danvers, MA) as a housekeeping gene which was unaffected by the experimental conditions.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 23.0 (SPSS, Chicago, Illinois). In order to compare the effects of training in normoxia (TR_{NOR}) *versus* training in hypoxia (TR_{HYP}) a 2-way repeated measures ANOVA (group x time) was performed, using TR_{NOR} and TR_{HYP} in the factor 'group'. The effect of the 'living high' intervention was evaluated using a 2-way repeated measures ANOVA (group x time) with CON and TR in the factor 'group'. Because pretest values in CON were similar between the legs for all variables measured ($P > 0.05$ paired Students' T-test), values from both legs were averaged and considered as one data point. A 3-way repeated measures ANOVA was used to assess the effects of iron supplementation over time (pretest vs. posttest) as a within subject variable and both iron supplementation (iron vs. no iron) and group (CON vs. TR) as between subjects variables. Violations of sphericity were tested by Mauchly's Test of Sphericity. Whenever violations of sphericity occurred, Greenhouse-Geisser correction was applied. Post-hoc Students' T-tests with Bonferroni correction were used for multiple comparisons whenever ANOVA yielded a significant main or interaction effect. Changes in performance and hemodynamics during RSA in CON were evaluated by paired Student's T-test. 95% confidence intervals (CI) are reported for significant results. In addition, effect sizes are given as Cohen's d or partial eta squared (η^2_p). Pearson correlation coefficients were calculated to evaluate the relationship between variables. A probability level $P < 0.05$ was defined as statistically significant. All data are expressed as means ± standard error of mean (SEM) unless stated otherwise. Typical percentage errors of measurements were calculated based on changes from the pretest to the posttest in CON as previously described [66].

Results

Effect of 'living high' on blood measurements and VO_2max

$\%SpO_2$, sEPO and sleep quality – Compared to normoxia morning $\%SpO_2$'s in TR during the 5-weeks 'living high' were consistently lower ($P < 0.05$, **Figure 2**). Furthermore, due to decreasing $\%F_iO_2$ from week 1 to week 5, $\%SpO_2$ was slightly lower in week 5 (90.1 ± 0.2%, $P < 0.05$) than in week 1 (92.6 ± 0.3%). sEPO responses were highly variable between individuals **(Figure 3)**. Compared to baseline, sEPO on average increased ~2-fold in week 1 ($P < 0.05$, CI: +4.4 to +9.8 $mIU \cdot mL^{-1}$) and this high level was maintained till week 5 ($P < 0.05$, CI: +4.9 to +11.0 $mIU \cdot mL^{-1}$). Subjective assessment of sleep quality was obtained from the Lake Louise Questionnaire, which rates sleep difficulty on a scale from 0 (slept as usual) to 3 (could not sleep at all). Sleep quality was similar between normoxia (0.1 ± 0.1) and hypoxia from week 1 (0.1 ± 0.0) to week 5 (0.3 ± 0.1). Sleep duration on average was 7.9 ± 0.1 h per night from the start to the end of the study. On average, scoring of acute mountain sickness by the Lake Louise Questionnaire in TR did not indicate incidence of AMS (total score of 3 or more) throughout the study. Nonetheless, compared to baseline values in normoxia (0.1 ± 0.1, range: 0-1), scores were significantly higher during hypoxia in week 5 (1.1 ± 0.2, range: 0-4, $P < 0.05$) with one subject testing positive for AMS.

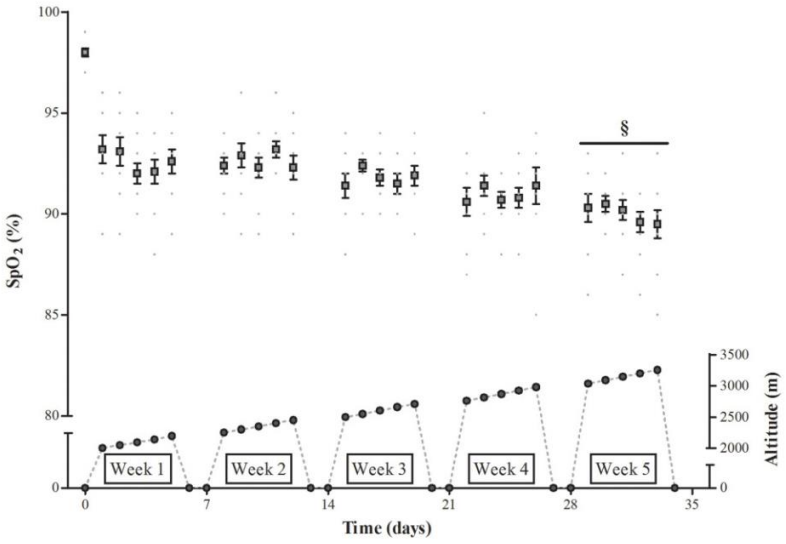


Figure 2 | Effect of incremental altitude on arterial oxygen saturation during 'living high'. %SpO2, arterial oxygen saturation. §, $P < 0.05$ compared to week 1 at altitude.

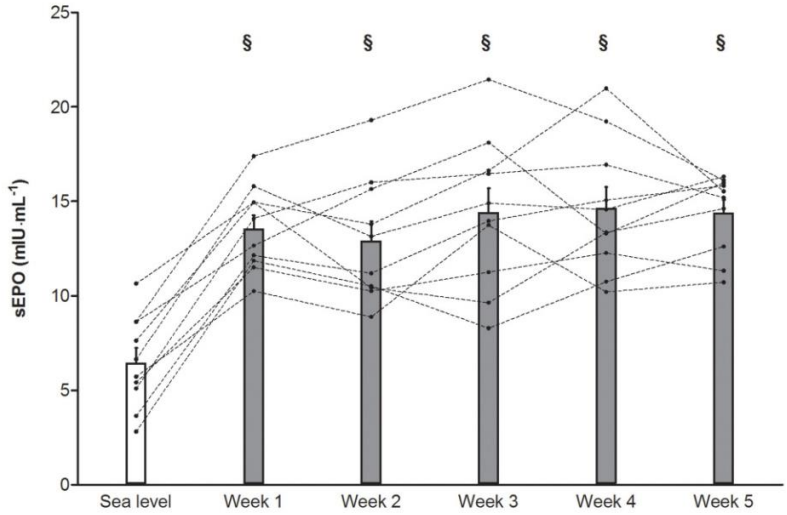


Figure 3 | Effect of 'living high' on serum erythropoietin concentration.
Data are means ± SEM (n = 10) and individual data (dotted lines) for serum erythropoietin concentration (sEPO) in subjects living in normobaric hypoxia with simulated altitude gradually increasing from 2000m in week 1 to 3250m in week 5. See Methods for further details.
§, $P < 0.05$ compared to sea level.

Serum ferritin and hemoglobin mass

Total Hbmass ($P > 0.05$, CI: -18 to +7 g, $\eta^2_p = 0.05$) and sFer concentrations in CON were constant throughout the study (**Figure 4**). Conversely, in TR Hbmass on average increased by 2.6% from the pretest to the posttest ($P < 0.05$, CI: +11 to +33 g, $\eta^2_p = 0.54$). In TR, in subjects not receiving supplementary iron because of high initial values (>100 $\mu g \cdot \mu L^{-1}$, n = 5), sFer decreased by ~30% from the pretest to the posttest ($P < 0.05$). Conversely, in subjects with low initial values who received iron supplementation (n = 5), sFer was constant at ~60-70 $\mu g \cdot \mu L^{-1}$ throughout the 'living high' period. The average increase in sEPO (ΔsEPO, mIU·mL−1) over 5 weeks in TR was closely correlated with ΔHbmass (g) ($r = 0.78$, $P < 0.05$, **Figure 5**). In fact, correlations between ΔsEPO and ΔHbmass gradually increased from week 1 ($r = 0.54$, $P = 0.09$) to week 5 ($r = 0.81$, $P < 0.05$). No correlations were found between $\%SpO_2$ and either absolute sEPO concentration or ΔsEPO or ΔHbmass at any time.

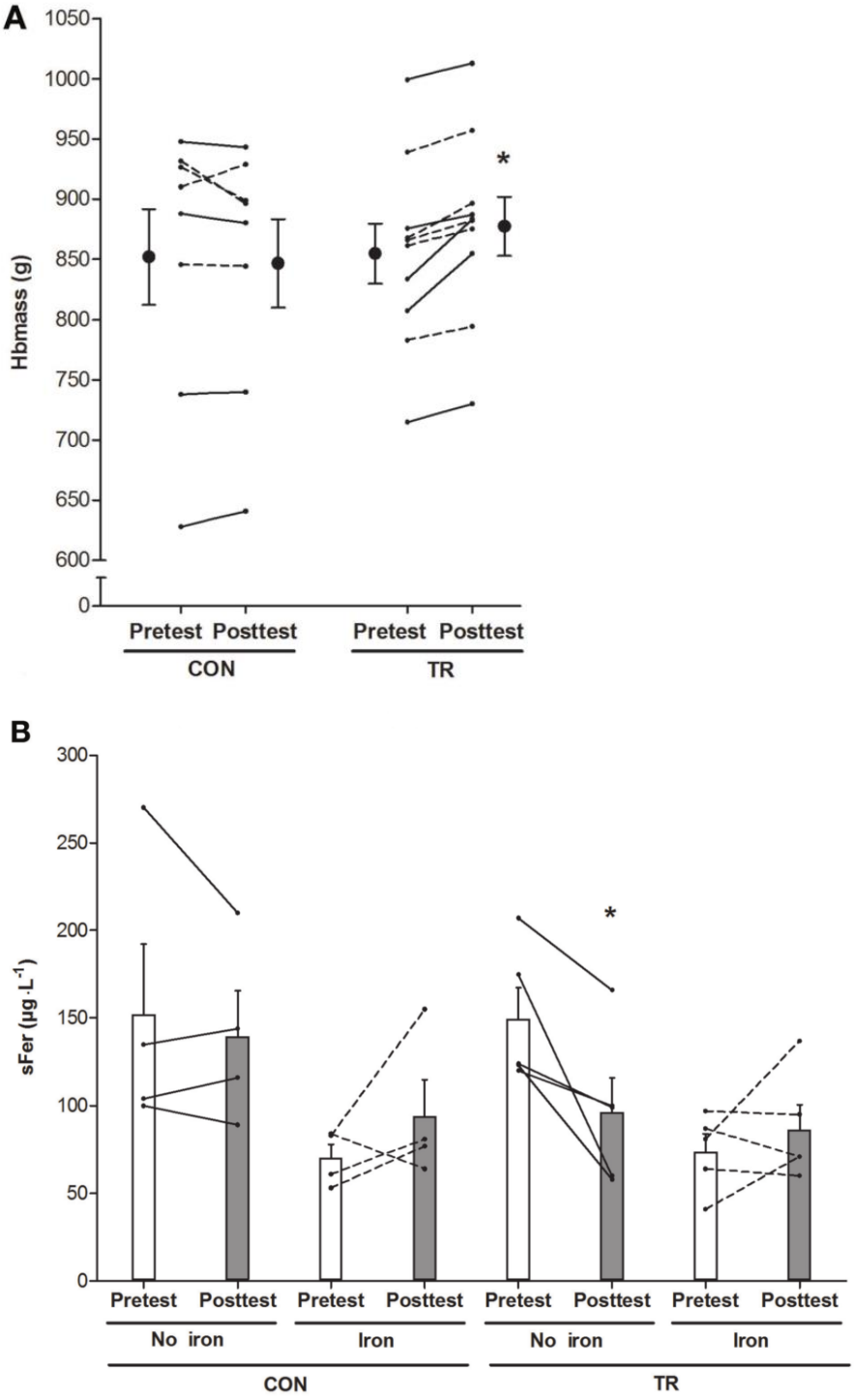


Figure 4 | Effect of 'living high' on blood hemoglobin mass and serum ferritin concentration.
Data represent means ±SEM (n = 10) and individual values (lines) for hemoglobin mass (Hbmass, **A**) and serum ferritin concentrations (sFer, **B**) before (Pretest) and after (Posttest) living 5 weeks in normoxia (CON) or in normobaric hypoxia (TR) with simulated altitude gradually increasing from 2000 to 3250m. Iron supplementation was provided in subjects with sFer below 100 $\mu g \cdot L^{-1}$ (dotted lines), but not in subjects with sFer above 100 $\mu g \cdot L^{-1}$ (solid lines). See Methods for further details.
*$P < 0.05$ compared to the pretest.

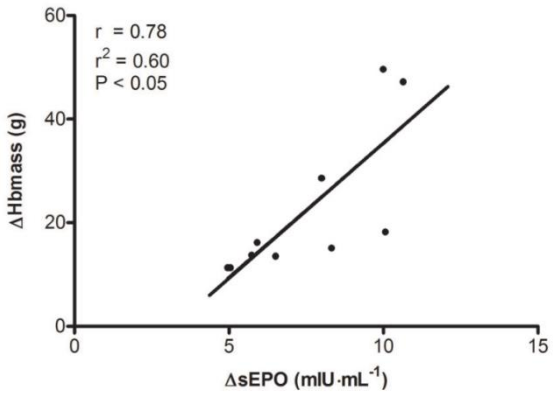


Figure 5 | Relationship between changes in serum erythropoietin concentration and changes in hemoglobin mass during 'living high'.
ΔHbmass, change in total blood hemoglobin mass; ΔsEPO, mean increase in serum erythropoietin concentration during 5 weeks of normobaric hypoxia with simulated altitude gradually increasing from 2000 to 3250m of simulated altitude.

VO_2max

VO_2max in CON was stable between the pretest (4.13 ± 0.21 L·min−1) and the posttest (4.11 ± 0.18 L·min−1). 'Living high' in conjunction with knee-extension training did not significantly change VO_2max either. VO_2max in TR was 4.14 ± 0.11 L·min^{-1} in the pretest vs. 4.18 ± 0.15 L·min^{-1} in the posttest, and 4.24 ± 0.15 L·min^{-1} 2 weeks after return to normoxia following the posttest. In both the pretest (r = 0.85) and the posttest (r = 0.77) total Hbmass in CON and TR(n=18) was closely correlated with VO_2max (P < 0.05 for both). In contrast, in TR hypoxia-induced ΔHbmass did not correlate with ΔVO_2max (r = 0.36, P > 0.05).

Effects of hypoxic (TR_{HYP}) vs. normoxic (TR_{NOR}) training on muscular performance and oxygenation status

Measurements during knee-extension training – Training workloads on the knee-extension apparatus were adjusted weekly to the actual 1RM. 1RM significantly increased from the first (TR_{NOR}, 40.7 ± 2.5; TR_{HYP}, 42.4 ± 2.6 kg) to the last (TR_{NOR}, 46.9 ± 2.4; TR_{HYP}, 47.8 ± 2.4 kg) training week in both the legs (P < 0.05). Hence training workloads also were similar between TR_{NOR} and TR_{HYP} at any time of the study. Workloads in the initial training sessions on average were ~8.3 kg (range: 5.3-11.3 kg), increasing by ~25% to ~11.8 kg (range: 7.8-14.0 kg) in the final sessions. Maximal isometric contraction forces measured on a dynamometer significantly increased from the pretest to the posttest in TR_{HYP} (pretest, 230 ± 17 Nm; posttest 248 ± 15 Nm; P = 0.03, CI: +2 to +35 Nm, η^2_p = 0.23) but not in TR_{NOR} (pretest, 236 ± 15 Nm; posttest 245 ± 14 Nm; P = 0.30, CI: -8 to +25 Nm, η^2_p = 0.06), without significant difference between the groups. Corresponding values in CON were 208 ± 8 Nm and 210 ± 9 Nm in the pretest and the posttest, respectively (P > 0.05, CI: -14 to + 17 Nm, d = 0.08). To evaluate the effect of hypoxia on muscular oxygenation status during training, tissue oxygenation index (TOI) was measured by NIRS in m. vastus lateralis at the start and at the end of the training period. TOI during both the contraction and the recovery episodes consistently was ~10-15% lower in TR_{HYP} than in TR_{NOR} (**Figure 6**; week 1, P < 0.05, CI: -7.7 to -7.0 TOI percentage unit, η^2_p = 0.13; week 5, P < 0.05, CI: -6.3 to -5.7 TOI percentage unit, η^2_p = 0.12). Furthermore, TOI's were lower in week 5 than in week 1 in both the legs (TR_{NOR}, P < 0.05, CI: -6.0 to -5.3 TOI percentage unit, η^2_p = 0.09; TR_{HYP}, P < 0.05, CI: -4.7 to -3.9 TOI percentage unit, η^2_p = 0.06). The exercise-induced changes in tHb during training were similar between TR_{NOR} and TR_{HYP} at any time. However, in TR_{NOR} values were lower in week 5 than in week 1 (P < 0.05, CI: -21 to -10, η^2_p = 0.003), whilst in TR_{HYP} values were higher in week 5 than in week 1 (P < 0.05, CI:+7 to+18, η^2_p = 0.002).

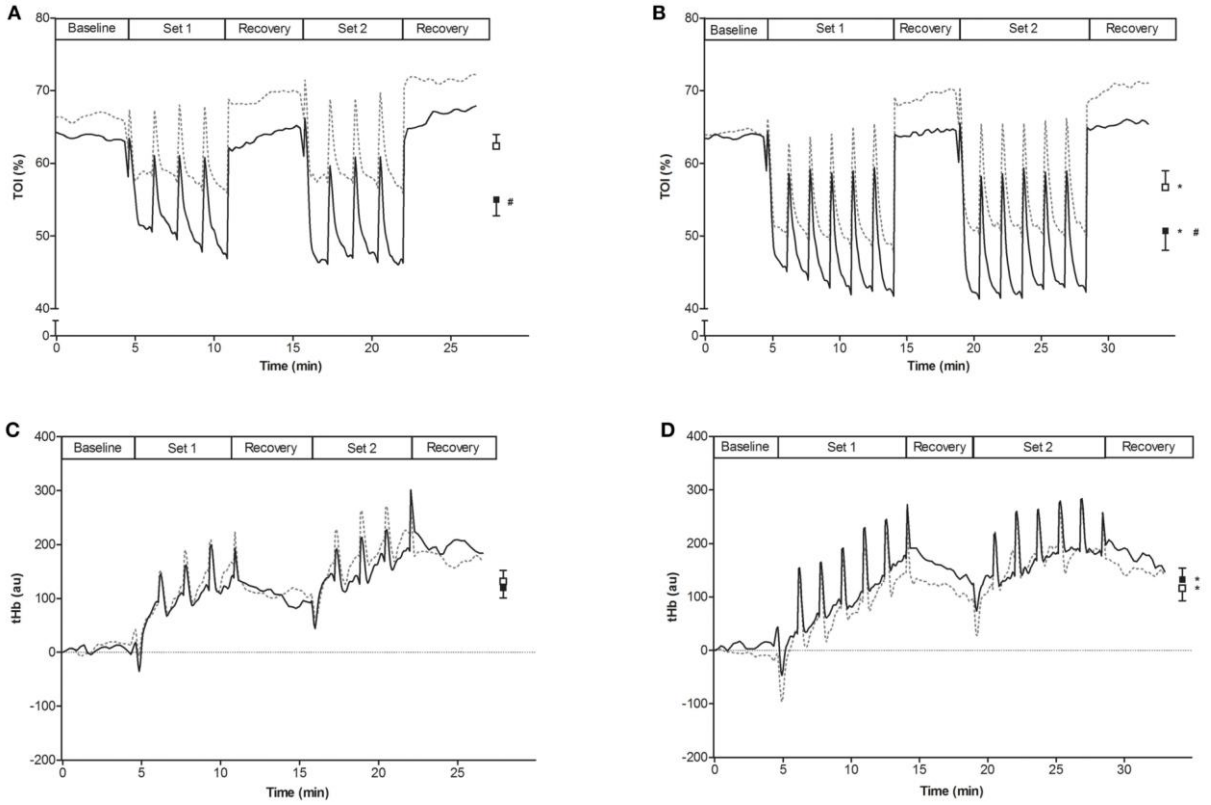
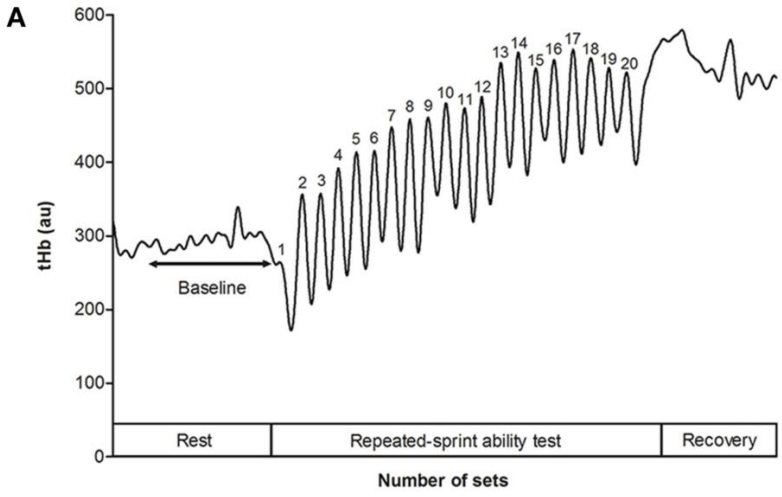


Figure 6 | Effect of knee-extension training in normoxia vs. hypoxia on muscle oxygenation status. Curves represent mean values (n = 10) for tissue oxygenation index (TOI, **A**, **B**) and muscle total hemoglobin (tHb, **C**, **D**) measured by NIRS in subjects living for 5 weeks in normobaric hypoxia with simulated altitude gradually increasing from 2000 to 3250 m. Meanwhile one leg was trained in 12.3% F_iO_2 (TR_{HYP}, ~4,300 m, full lines), whilst the other leg was trained in 20.9% F_iO_2 (TR_{NOR}, dotted lines). The training sessions consisted of two times 4 series of 30 knee extensions at 20% 1RM in week 1, increasing to two times 6 series of 30 knee extensions at 25% of 1RM in week 5. Mean values ± SEM are given at the right side of each graph (TR_{NOR}, □; TR_{HYP}, ■). Values were measured in week 1 (**A**, **C**) and week 5 (**B**, **D**) of the training period. See Methods for further details.
*P < 0.05 compared to week 1; # P < 0.05 compared to training in normoxic conditions.

Performance and hemodynamics in the RSA test – The RSA test consisted of 20 intermittent sets of 10 maximal unilateral knee extensions on an isokinetic dynamometer. Torque output in both TR_{NOR} and TR_{HYP} gradually decreased from set 1 to ~10 where after it was stable and there was no difference in percentage decrement score [67] between TR_{NOR} (pretest, 32 ± 3%; posttest, 34 ± 2%) and TR_{HYP} (pretest, 31 ± 3%; posttest, 32 ± 2%) at any time. Training increased mean torque production during the RSA test by ~8% in TR_{NOR} (pretest, 70 ± 3 Nm; posttest 76 ± 3 Nm, P < 0.05, CI: +3 to +9 Nm, $\eta^2_p = 0.47$). Corresponding pretest and posttest values in TR_{HYP} were 74 ± 3 Nm and 80 ± 3 Nm (P < 0.05, CI: +3 to +9 Nm, $\eta^2_p = 0.51$), respectively. By analogy with mean torques, training increased peak torque during the RSA test by ~8% from 85 ± 3 Nm to 92 ± 3 Nm in TR_{NOR} (P < 0.05, CI: +4 to +10 Nm, $\eta^2_p = 0.50$), vs. from 90 ± 3 Nm to 96 ± 4 Nm in TR_{HYP} (P < 0.05, CI: +3 to + 10 Nm, η^2_p = 0.49). In CON mean and peak torques values were constant at ~72 Nm (P > 0.05, CI: −4 to +2 Nm, d = 0.25) and ~87 Nm (P > 0.05, CI: -5 to +3 Nm, d = 0.18), respectively. During the RSA test tHb decreased during each series of contractions, where after it increased during the recovery intervals (**Figure 7**). ΔtHb's for both contraction and recovery phases were similar between TR_{NOR} and TR_{HYP} in the pretest,

and in CON values were stable till the posttest (sprints, P > 0.05, CI: -15 to +34, d = 0.31; recovery, P > 0.05, CI: -16 to+36, d = 0.33). However, training significantly increased ΔtHb's by ~80% during both the contraction and the rest episodes in TR_{HYP}. Indeed, in TR_{HYP} ΔtHb during sprints increased from 69 ± 12 in the pretest to 126 ± 21 in the posttest (P > 0.05, CI:+22 to+93, η^2_p = 0.39), whilst ΔtHb during recovery increased from 80 ± 13 in the pretest to 141 ± 22 in the posttest (P > 0.05, CI: +24 to +97, η^2_p = 0.40). Conversely, in TR_{NOR} ΔtHb during sprints remained constant from the pretest (59 ± 12) to the posttest (90 ± 21, P = 0.08, CI: −4 to +67, η^2_p = 0.16). Accordingly, also ΔtHb during recovery remained stable from the pretest (70 ± 13) to the posttest (103 ± 22, P = 0.08, CI: -5 to +69, η^2_p = 0.16) in TR_{NOR}. Still ΔtHb values in the posttest were not significantly different between the two experimental conditions (P = 0.15). Furthermore, ΔHHb's were similar between TR_{NOR} and TR_{HYP} in both the pretest and the posttest, and in CON values were stable from the pretest to the posttest (sprints, P > 0.05, CI: -47 to +38, d = 0.09; recovery, P > 0.05, CI: -48 to +37, d = 0.10). However, ΔHHb's for both the contraction and rest intervals increased from the pretest to the posttest in TR_{NOR} (+ ~15%) but not in TR_{HYP} (+ ~3%). Indeed, in TR_{NOR} ΔHHb increased during sprints (pretest, 204 ± 34; posttest, 235 ± 35; P < 0.05, CI: +1 to +61, η^2_p = 0.21) as well as during recovery (pretest, 203 ± 35; posttest, 235 ± 35; P < 0.05, CI: +1 to +61, η^2_p = 0.21). In TR_{HYP}, however, ΔHHb remained stable from the pretest to the posttest during both sprints (pretest, 215 ± 34; posttest, 220 ± 35; P > 0.05, CI: -24 to +36, η^2_p = 0.01) and recovery (pretest, 214 ± 35; posttest, 220 ± 35; P > 0.05, CI: -25 to + 35, η^2_p = 0.01). ΔO_2Hb also increased from the pretest to the posttest in TR_{NOR} (+ ~27%, P < 0.05) and TR_{HYP} (+ ~24%, P < 0.05), whilst it was stable in CON. ΔTOI was unaffected by training and was similar between TR_{NOR} and TR_{HYP} at any time (P > 0.05, data not shown).



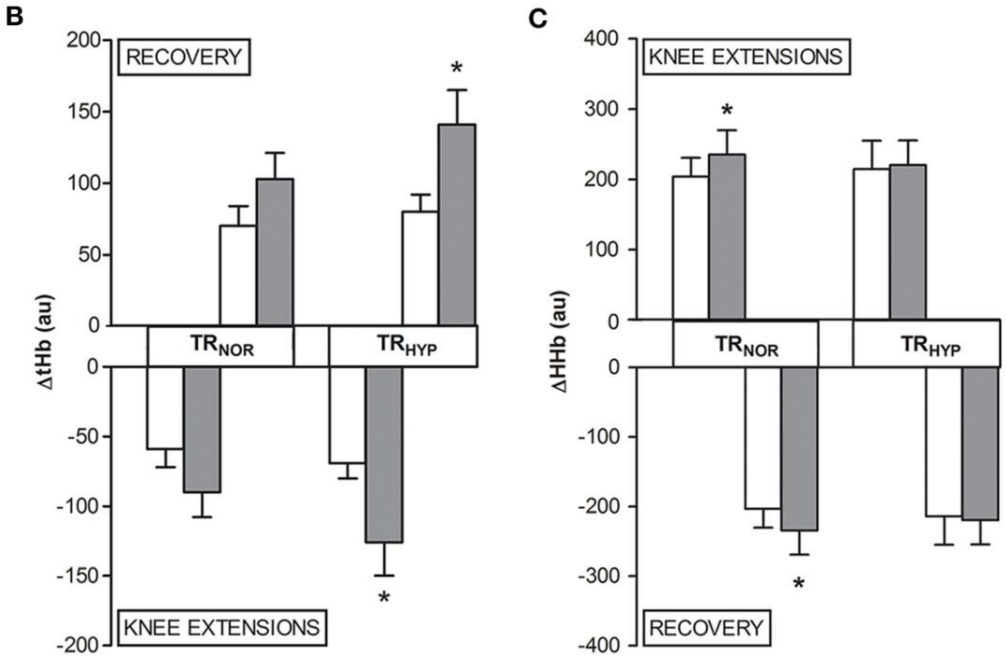


Figure 7 | Effect of knee-extension training in normoxia vs. hypoxia during 'living high' on muscle total hemoglobin content during the repeated-sprint ability test.
A represents the typical signal of muscle total hemoglobin (tHb) measured by NIRS during the repeated-sprint ability test (n = 1), with tHb decreasing during consecutive knee extensions and increasing during recovery intervals. **B** and **C** represent means ± SEM for the change in tHb (ΔtHb) and HHb (ΔHHb), respectively, during the consecutive knee extensions and during the recovery intervals of the repeated-sprint ability test before (open bars) and after (solid bars) subjects living for 5 weeks in normobaric hypoxia with simulated altitude gradually increasing from 2000 to 3250m. One leg was trained in 12.3% F_iO_2 (TR_{HYP}, ~4300 m), whilst the other leg was trained in 20.9% F_iO_2 (TR_{NOR}). See Methods for further details.
*P < 0.05 compared to the pretest.

Performance in MAX3′ – training improved MAX3′ performance by about 10-15% in both the legs. Mean power output values in the pretest were 40 ± 2 and 40 ± 3 Nm for TR_{NOR} and TR_{HYP}, respectively, increasing to 44 ± 2 and 46 ± 4Nm in the posttest (P < 0.05 for both groups; TR_{NOR}, CI: +2 to +6 Nm, η^2_p = 0.45; TR_{HYP}, CI: +3 to +8 Nm, η^2_p = 0.59). Corresponding values in CON were 40 ± 3 and 42 ± 3 Nm (P > 0.05, CI: -1 to +4 Nm, d = 0.45).

Effects of hypoxic (TR_{HYP}) vs. normoxic (TR_{NOR}) training on muscular adaptations

Muscle fiber type composition and capillarity (**Table 1**) – The relative number of type I and type II fibers was similar between the pretest and the posttest in both TR_{NOR} and TR_{HYP} (P > 0.05), and no overall training effect was found (P > 0.05). Type I and type II fiber cross-sectional areas (CSA) were constant from the pretest to the posttest in both TR_{NOR} and TR_{HYP} fibers. Capillary contacts per type I fibers increased similarly in TR_{NOR} (P < 0.05, CI: +0.3 to +1.2, η^2_p = 0.37) and TR_{HYP} (P < 0.05, CI: +0.2 to +1.1, η^2_p = 0.32). Accordingly, also capillary contacts per type II fibers increased similarly in both experimental conditions (TR_{NOR}, P < 0.05, CI: +0.4 to +1.3, η^2_p = 0.44; TR_{HYP}, P < 0.05, CI: +0.1 to +1.0, η^2_p = 0.28).

Muscle pH regulation – Total buffer capacity measured in muscle homogenates (βhm) was similar between TR_{NOR} and TR_{HYP} in the pretest (164 ± 4 mmol H+·kg dm^{-1}·pH^{-1} in both legs) and was unchanged in the posttest (TR_{NOR}, 164 ± 3 mmol H^+·kg dm^{-1}·pH^{-1}; TR_{HYP}, 167 ± 3 mmol H^+·kg dm^{-1}·pH^{-1}). The relative abundance of MCT1, MCT4, CA3 and NHE1 in muscle was not significantly altered by training and was similar between TR_{NOR} and TR_{HYP} in both the pretest and the posttest.

TABLE 1 | Effects of training and nitrate intake on muscle fiber composition.

	TR_{NOR}		TR_{HYP}	
	Pretest	Posttest	Pretest	Posttest
RELATIVE FIBER NUMBER (%)				
Type I	58 ± 3 (42–71)	54 ± 4 (36–71)	55 ± 5 (31–73)	57 ± 4 (34–72)
Type II	42 ± 3 (29–58)	46 ± 4 (29–64)	45 ± 5 (27–69)	43 ± 4 (28–66)
RELATIVE FIBER CSA (%)				
Type I	45 ± 1 (42–49)	46 ± 1 (42–49)	46 ± 1 (41–50)	46 ± 1 (41–56)
Type II	55 ± 1 (51–58)	54 ± 1 (51–58)	54 ± 1 (50–59)	54 ± 1 (44–59)
FIBER CSA (μm^2)				
Type I	4614 ± 298 (3526–5530)	4477 ± 276 (3460–5587)	4639 ± 371 (3495–5995)	4162 ± 288 (3119–5185)
Type II	5452 ± 239 (4638–6154)	5300 ± 423 (4036–7092)	5610 ± 446 (3824–6873)	5276 ± 541 (3732–6874)
CAPILLARY CONTACTS				
Type I	5.0 ± 0.3 (4.1–6.6)	5.7 ± 0.4* (4.4–8.2)	5.2 ± 0.3 (3.7–6.9)	5.8 ± 0.4* (4.4–8.6)
Type II	4.8 ± 0.3 (3.9–7.0)	5.6 ± 0.4* (4.3–8.0)	4.9 ± 0.3 (3.4–6.0)	5.5 ± 0.3* (4.4–7.5)

Data are mean ± SEM, and range in parentheses, before (Pretest) and after (Posttest) 5 weeks training in normobaric normoxia (TR_{NOR}, n = 10) or hypoxia (TR_{HYP}, F_iO_2: 12.3%, n = 10) while subjects lived in normobaric hypoxia with simulated altitudes gradually increasing from 2000m to 3250 m. See Methods for further details.

**P < 0.05 compared to the pretest.*

Discussion

In this study, young healthy volunteers were exposed to a 5-week intermittent 'living high' protocol during which altitude exposure, administered in the form of normobaric hypoxia, was gradually increased from ~2000 m (F_iO_2: 16.4%) to ~3250 m (F_iO_2: 14.0%). Meanwhile, subjects performed high-intensity interval-training (HIIT) with one leg in hypoxia equivalent to ~4300 m (TR_{HYP}, F_iO_2: 12.3%), whilst the contralateral leg trained in normoxia (TR_{NOR}). It was postulated that lower muscular oxygenation status during HIIT in hypoxia, via activation of hypoxia-sensing pathways, could induce unique muscular adaptations to eventually enhance exercise performance. The 5 weeks of 'living high' elicited a consistent elevation in serum erythropoietin (sEPO) levels which adequately predicted the eventual increment in total hemoglobin mass (Hbmass). Furthermore, compared with TR_{NOR}, training-induced adaptations in the amplitude of muscle blood volume changes during intermittent high-intensity muscle contractions and maximal isometric strength were more pronounced in TR_{HYP}. Nonetheless, HIIT during 'living high' in general produced similar enhancements in exercise performance regardless of whether the training was performed in normoxia or hypoxia.

The primary aim of the current study was to compare the effects of hypoxic *versus* normoxic HIIT during 'living high'. Training workload was identical between TR_{NOR} and TR_{HYP} at any time, indeed, and increased by ~25% from the start to the end of the training period. Nonetheless, compared to TR_{NOR}, muscle oxygen desaturation during exercise bouts was consistently exaggerated in TR_{HYP} (see

Figure 6). Although the possibility of collateral training adaptations via hemodynamic forces and humoral factors can not completely be excluded [68], such effects are limited to absent when exercise involves small muscle groups [45-47]. Still, training responses were largely similar between the two experimental conditions, except for the exercise-induced oscillations in muscle blood volume and the increase in isometric strength. Muscle tHb content, measured by NIRS, was used as a marker for vascular blood filling [72-73] during the RSA test. Muscle tHb content alternatingly dropped during exercise intervals, conceivably due to rise in intramuscular pressure, and increased during rest episodes (see **Figure 7**). Interestingly, however, the amplitude of tHb oscillations increased from the pretest to the posttest in TR_{HYP} but not in TR_{NOR}. Concomitantly, unlike TR_{NOR}, ΔHHb during contractions did not increase from the pretest to the posttest in TR_{HYP}. Taken together, these observations indicate that higher power productions in the posttest in TR_{NOR} resulted in higher fractional O_2-extraction, whilst in TR_{HYP} O_2-delivery conceivably was increased due to elevated perfusion, yet at constant fractional O_2-extraction rate. These findings corroborate recent observations [40,47-48] showing more pronounced increases in the amplitude of tHb shifts during intermittent whole-body sprinting in normoxia following a period of repeated-sprint training in hypoxia *versus* normoxia. A potential mechanism to explaining these greater amplitudes in ΔtHb's in TR_{HYP}, indicating greater blood volume shifts, is higher degree of arteriolar dilation in conjunction with increased capillary volume. Hypoxic training has previously been postulated to stimulate capillary growth by increased nitric oxide production in conjunction with increased vasodilation and endothelial shear stress [74-75] as well as by elevated HIF-1-induced VEGF gene expression [43,76-77]. However, literature data in this regards are equivocal with some studies showing hypoxic training to stimulate capillary density [30,31,33,78,79], *versus* others showing similar adaptation between normoxic and hypoxic training [27,28,80–82]. In the conditions of the current study HIIT raised the number of capillary contacts per fiber by ~15% in both type I and type II muscle fibers, and irrespective of hypoxia. Still, this measure does not exclude higher capillary volume due to higher capillary tortuosity following training [49-83].

It was also postulated that hypoxic training during 'living high' might promote muscular buffering capacity, which is pivotal in anaerobic performance. Stimulation of the HIF-1 pathway during hypoxic training is a potential mechanism to upregulate the expression of pH-regulating proteins such as monocarboxylate transporters, Na^+/H^+ exchangers and carbonic anhydrases [84]. However, regardless of whether HIIT was performed in normoxia or hypoxia, training altered neither the expression of muscle membrane proteins MCT1, MCT4, or NHE1, nor the abundance of cytoplasmic protein CA3. Furthermore, in vitro measured muscular total buffering capacity was also unaffected by training, independent of whether training was done in normoxia or hypoxia. Our current observations combined with literature data thus clearly indicate that short-term HIIT in hypoxia, by analogy with HIIT in normoxia [46,85–92], is ineffective to increasing myocellular buffering capacity during either 'living high' or 'living low'.

pH-regulating proteins are differentially expressed between muscle fiber types, i.e. higher MCT1 and CA3 in oxidative *versus* glycolytic muscle fibers [93-95] and higher MCT4 and NHE1 in glycolytic *versus* oxidative fibers [95-96]. Hence training-induced muscle fiber-type shifts *per se* could alter the MCT1:MCT4 ratio, as well as total buffer capacity in mixed muscle tissue. Five weeks of whole-body sprint interval training was recently demonstrated to decrease the fraction of type IIx fibers, irrespective of ambient oxygen concentrations [46]. However, in the present study we did not differentiate between type IIa and type IIx fibers, and the proportion of type I *versus* type II fibers, either expressed as fiber numbers or cross-sectional areas, was constant throughout the study. With regard to fiber cross-sectional areas, some studies have indicated that hypoxia may stimulate muscle anabolism during resistance training [97-98], whereas others did not [78-99]. However, in keeping with earlier findings [99], the

low-load resistance training program used in the present study stimulated muscle fiber hypertrophy neither in normoxia nor in hypoxia. In fact, literature data [99] taken together with our current observations indicate that if an exercise training program does not stimulate muscle mass accretion in normoxia, it probably will also fail to stimulate muscle hypertrophy in hypoxia. Still, maximal isometric strength increased by ~8% in TR_{HYP} *versus* a non-significant increase of ~4% in TR_{NOR}. Accordingly, heavy resistance training in hypoxia has recently been reported to stimulate adaptations in maximal strength despite not increasing lean mass [39].

Another primary aim of the current study was to evaluate the potential additive effects of 'living high' and hypoxic HIIT in stimulating high-intensity exercise performance. It is well established that 'living high' can enhance performance by increasing total hemoglobin mass and thereby O_2-delivery to muscles during exercise [4]. Low arterial PO_2 during sustained hypoxic exposure increases red blood cell production via HIF-2-induced stimulation and proliferation of renal EPO-producing and oxygen-sensing cells [13]. It is therefore surprising to note that literature mostly shows poor correlations between increased sEPO levels monitored during 'living high' and eventual changes in hematological measurements [9,15,23,24]. It is commonly postulated that sEPO represents the ratio between EPO synthesis and EPO degradation, and thus only represents an indirect marker of the erythropoietic signalling response. In addition, instantaneously measured sEPO levels may not adequately reflect sustained EPO action during prolonged 'living high'. For circulating EPO levels often gradually decrease during short-term hypoxic exposure [8,14–16], probably due to desensitization of the oxygen-sensing mechanism in EPO-producing cells consequent to HIF-α-induced induction of its negative regulators prolyl hydroxylase domain proteins 2 and 3 [21,22], decreased oxygen hemoglobin affinity [19-20], as well as increasing arterial oxygen content [17-19]. In fact, only one [8] out of multiple studies [9,15,23,24] found increments in both absolute and relative plasma EPO to be associated with changes in total red cell volume during 'living high'. 'Living high' in the conditions of the current study increased total Hbmass by ~2.6% on average. Moreover, this increase was highly correlated with the average sEPO increase over the 5-week study intervention ($r = 0.78$, $P < 0.05$). In fact correlations between ΔsEPO and ΔHbmass gradually increased from the start (week 1, r= 0.54) to the end (week 5, r=0.81) of the intervention period. This supports the rationale that maintaining high circulating EPO levels during prolonged 'living high' is crucial to increasing Hbmass at altitude. The current hypoxic protocol consistently elevated sEPO concentrations from week 1 to week 5 in all subjects. This is probably at least partly due to gradually increasing hypoxia from 16.4 to 14.0% F_iO_2 (~2000 to 3250 m), which resulted in even lower arterial oxygen saturations in week 5 than in week 1 (see **Figure 2**). Furthermore, within each week, 5 days in hypoxia were alternated with 2 days in normoxia. Follow-up studies are needed to evaluate whether intermittent hypoxia may be more effective than continuous hypoxia to stimulating EPO production for a given hypoxic dose.

According to a recent meta-analysis, 'living high' is expected to increase Hbmass by ~1.08% for every 100 h of altitude exposure above 2100 m [4]. This algorithm predicts a ~4% increment in Hbmass for 350-400 h at >2100 m in the current protocol. Accordingly, hypoxic dose expressed as 'kilometer hours' predicts an increase of ~3.4% in Hbmass (linear model, [5]). However, Hbmass increased by no more than 2.6% (range: 1.3–5.9%) in the present study. Correction for withdrawal of ~10 g of hemoglobin in ~65 mL blood during the study yields a net 3.7% increase in Hbmass. Nonetheless, one other study reported a 6.7% rise in Hbmass for only ~230 h of intermittent normobaric hypoxia, similar to the current protocol, yet at a constant altitude of 3000 m [100]. This could indicate that intermittent hypoxia may become more effective to increase Hbmass at higher degrees of hypoxia. However, in order to maintain high sleep quality, which is important during altitude training in athletes [101-102], initial altitude was set at only 2000 m, reaching 3000 m only in week 5 of the intervention. As a result, the magnitude of ΔHbmass probably was also insufficient to inducing a measurable change in aerobic

power, because ΔVO_2max is expected to rise by no more than 0.6-0.7% for each 1% increment of Hbmass [6].

There is some evidence to indicate that iron availability is a primary factor limiting altitude-induced erythropoiesis, and that iron supplementation during 'living high' may stimulate erythropoiesis [103–106]. In the present study, none of the subjects were diagnosed to be iron-deficient based on clinical criteria. However, given that retrospective observations in athletes indicate that both individuals with low (sFer < 20 $\mu g \cdot L^{-1}$) and normal (20-200 $\mu g \cdot L^{-1}$) sFer levels may benefit from iron supplementation to increase Hbmass during 'living high' [104,105], we decided to administer iron supplements (105 mg elemental iron per day) to all subjects exhibiting sFer concentrations lower than 100 $\mu g \cdot L^{-1}$ at baseline. Supplementary iron intake clearly abrogated the decrease in sFer in the subjects 'living high' (see **Figure 4**). However, this did not alter the response of Hbmass to 'living high'.

In the current study, 'living high' in conjunction with hypoxic HIIT was found to increase total Hbmass and to stimulate oscillations in muscular perfusion during intermittent muscle contractions. It is reasonable to postulate that these effects eventually should contribute to enhancing performance in high-intensity exercise by increasing O_2-delivery to muscles as well as by facilitating washout of metabolic end-products during rest episodes interspersing exercise bouts. However, performance in the repeated-sprint ability test was enhanced by HIIT, irrespective of whether training was performed in either normoxia or hypoxia. In this regard, however, it is important to note that the unilateral knee-extension exercise model chosen to assess exercise performance in the context of the current study may explain why the documented beneficial physiological adaptations did not translate into improved performance. Rather than blood flow and oxygen delivery, neuromuscular factors are believed to play the primary role in repeated-sprint performance [67], especially during repeated sprints with small muscle mass. Indeed, it is well established that muscle blood flow is many fold higher during exercise involving a small muscle mass compared to whole body exercise [50,107]. Therefore, extrapolations from the present study from single-leg HIIT in hypoxia, to whole-body HIIT in hypoxia should be performed with caution. Studies investigating whole-body exercise have produced equivocal results, with two studies showing improved intermittent cycling [40] or double-poling [47] sprint performance following a period of RSH, versus one other study showing no such ergogenic effects on repeated cycling sprint performance [48]. Clearly, more studies are needed to define the conditions wherein hypoxic training could enhance high-intensity intermittent exercise performance. It is also clear from the current and other studies (see above) that elevation of muscular buffering capacity is not a mechanism by which hypoxic HIIT could enhance anaerobic exercise performance.

In conclusion, this study demonstrates single-leg HIIT in hypoxia to stimulate adaptations in muscular perfusion during single-leg intermittent high-intensity muscle contractions, and to stimulate the increase in isometric strength, though without further inducing greater muscular and performance adaptations compared to similar training in normoxia. In addition, this study also shows that intermittent exposure to progressively increasing normobaric hypoxia elicits a stable increase in serum erythropoietin which adequately predicts the eventual increase in Hbmass. Follow-up studies are warranted to evaluate whether progressively increasing intermittent hypoxia may be more effective than continuous hypoxia to stimulate the increase in Hbmass.

Author contributions

The experiments were performed at the Exercise Physiology Research Group, KU Leuven, Leuven, Belgium and at Bakala Academy–Athletic Performance Center, KU Leuven, Leuven, Belgium. Conception and study design: SD and PH. All authors contributed to acquisition, analysis and/or interpretation of data for the work, and revised and approved the final manuscript for important intellectual content.

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Conflict of Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper 3

High-intensity interval training in hypoxia does not affect muscle HIF responses to acute hypoxia in humans

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Abstract

Purpose: The myocellular response to hypoxia is primarily regulated by hypoxia-inducible factors (HIFs). HIFs thus conceivably are implicated in muscular adaptation to altitude training. Therefore, we investigated the effect of hypoxic *versus* normoxic training during a period of prolonged hypoxia ('living high') on muscle HIF activation during acute ischemia.

Methods: Ten young male volunteers lived in normobaric hypoxia for 5 weeks (5 days per week, ~15.5 h per day, F_iO_2: 16.4-14.0%). Meanwhile one leg was trained in hypoxia (TRHYP, 12.3% F_iO_2) whilst the other leg trained in normoxia (TRNOR, 20.9% F_iO_2). Training sessions (3 per week) consisted of intermittent unilateral knee-extensions at 20-25% of the 1-repetition maximum. Before and after the intervention, a 10-min arterial occlusion and reperfusion of the leg was performed. Muscle oxygenation status was continuously measured by near-infrared spectroscopy. Biopsies were taken from m. vastus lateralis before and at the end of the occlusion.

Results: Irrespective of training, occlusion elevated the fraction of HIF-1α expressing myonuclei from ~54 to ~64% ($P<0.05$). However, neither muscle HIF-1α or HIF-2α protein abundance, nor the expression of HIF-1α or downstream targets selected increased in any experimental condition. Training in both TRNOR and TRHYP raised muscular oxygen extraction rate upon occlusion by ~30%, whilst muscle hyperperfusion immediately following the occlusion increased by ~25% in either group ($P<0.05$).

Conclusion: Ten min of arterial occlusion increased HIF-1α expressing myonuclei. However, neither normoxic nor hypoxic training during 'living high' altered muscle HIF translocation, stabilization, or transcription in response to acute hypoxia induced by arterial occlusion.

Keywords: altitude training, high-intensity interval training, human skeletal muscle, hypoxia-inducible factor, ischemia, near-infrared spectroscopy

Introduction

The muscular response to cellular hypoxia is primarily mediated by hypoxia-inducible factors (HIFs). These transcription factors regulate the activity of a wide spectrum of genes, amongst which a significant part is involved in the regulation of oxygen delivery and oxygen expenditure, aiming to protect muscle cells from hypoxic stress [1]. HIFs are heterodimeric complexes composed of an α- and a β-subunit. The stability of the α-subunit is predominantly controlled by prolyl hydroxylase domain containing proteins (PHD) 1-3. In the presence of oxygen PHDs hydroxylate two specific proline residues of the HIF-α subunit, thereby recruiting the von Hippel-Lindau tumor suppressor protein (pVHL). pVHL is part of an E3-ubiquitin ligase complex which targets HIF-1α for proteosomal degradation [2]. In hypoxia, however, HIF-α accumulates and translocates to the nucleus to heterodimerize with its constitutively expressed HIF-1β partner to act as a functional transcription factor. Intracellular trafficking of HIF-1α in and out the nucleus is mediated via nuclear importers and exporters, and interaction of HIF-1α with exporter CRM1 is regulated by a mitogen-activated protein kinase (MAPK)-dependent mechanism [3]. Furthermore, it is also important to note that also oxygen-independent factors such as reactive oxygen species (ROS), inflammatory cytokines and nitric oxide can stimulate the HIF-pathway [1,4,5].

Intracellular oxygen partial pressure (P_iO_2) in muscle cells is normally ~30 mmHg [6,7]. Environmental hypoxia reduces arterial blood oxygenation and likewise P_iO_2 downstream the oxygen cascade. For instance, P_iO_2 decreases from 34 to 23 mmHg in normobaric hypoxia at 10% F_iO_2 corresponding to ~5800 m altitude [6]. It has been speculated that activation of HIF-1α [8] and its target genes [9–11] due to low P_iO_2 (>5500 m) is at least partly responsible for the development of muscle atrophy at extreme altitude [12–16]. Passive exposure to moderate altitude on the other hand presumably is not sufficient to induce HIF-1α stabilization in skeletal muscle. The oxygen partial pressure threshold for HIF-1α to stabilize in muscle has been estimated around 8 mmHg [17]. Accordingly, hypoxia-specific muscle adaptations are generally not demonstrated following passive exposure to moderate or high altitude [16,18,19]. In addition, HIF-1α stabilization during hypoxic exposure might only be a transient phenomenon [20] because HIF-1α has been reported to stimulate the expression of PHDs and thereby lower its normoxic setpoint to the ongoing P_iO_2 [21,22]. A similar mechanism in kidney and liver HIF-2α regulation might at least partially be implicated in the biphasic erythropoietin (EPO) response during prolonged hypoxic exposure [23–25]. In that respect, it is important to note that HIF-α stabilization induced by ambient hypoxia is tissue-specific, and that HIF-1α and HIF-2α have distinct transcriptional targets, with the former typically coordinating the glycolytic pathway and the latter regulating EPO expression [26].

Muscle contractions are an even more potent stimulus than low inspired PO_2 to decrease P_iO_2 by stimulation of myocellular O_2 consumption [27]. For instance, gradual knee-extension exercise decreased P_iO_2 up to about 50% of maximal work rate, where after P_iO_2 plateaus at about 3-7 mmHg [27–30]. Addition of ambient hypoxia only marginally exacerbates such contraction-induced drop in P_iO_2 [27,30]. Thus, exercise in either hypoxia or normoxia similarly increased muscle HIF-1α mRNA [31] and protein content [32], as well as the extent of HIF-1α translocation to the nucleus and DNA binding activity [32]. These observations indicate that irrespective of arterial O_2 saturation, any exercise-induced drop in P_iO_2 is probably ample to fully activate HIF-1.

Nonetheless, contrary to the above opinion, short-term endurance [33,34] or repeated sprint training [35,36] in hypoxia elevated muscle HIF-1α mRNA levels more than similar training in normoxia. Literature data also indicate that training can alter the acute response of the HIF-1α pathway to exercise. Both low-resistance knee-extension endurance training [37] and downhill running [38] attenuate the acute

exercise-induced stimulation of muscle HIF-1α mRNA content [37], as well as HIF-1α nuclear protein abundance [38] and transcriptional activity [38]. This may indicate that repetitive activation of HIF-1α and/or peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), both of which are stimulated by hypoxic and metabolic stress [2,39–41], forestall muscle homeostatic disturbances due to contractile activity. In fact, both HIF [42] and PGC-1α [43] independently stimulate angiogenesis and thereby facilitate muscular oxygen extraction. Along this rationale and given the potential role of ROS in HIF-1α stabilization [4,44], it is worthwhile mentioning that both these transcription factors also activate genes involved in cellular protection against ROS [45–48]. However, whereas HIF stimulates glycolytic metabolism [49], PGC-1α enhances mitochondrial metabolism [50]. Alternatively, the blunted HIF response to an acute exercise bout might also result from increased expression of HIF inhibitors such as factor inhibiting HIF-1 (FIH-1) and sirtuin 6 in the trained state [51]. Indeed, the asparagine residues of HIF-1α escaping PHD-induced degradation are hydroxylated by O_2-dependent FIH-1, thereby preventing HIF-1α to interact with its transcriptional cofactors [52–54]. Accordingly, 6 weeks of endurance training increased PHD2 protein content in muscles of healthy volunteers concomitant with increased mRNA levels of PHD2, PHD3 and FIH-1 [51]. Because PHD2 is regulated in a HIF-dependent manner [55], these findings indicate that exercise-induced changes in myocellular hypoxia may at least partially contribute to training-induced attenuation in HIF-1 activity.

Imposing repeated cycles of circulatory occlusion and reperfusion of the limbs has been used as a strategy to precondition and protect local and remote organs to potential ischemia-reperfusion episodes occurring in a later stage. Remote ischemic preconditioning (RIPC) has found its practice mainly in the clinical setting [56], but recently it has also gained attention as a strategy to reduce altitude illness [57,58] and possibly also to enhance sea-level exercise performance [59]. The underlying molecular mechanism is complex and only partially understood [60]. Available evidence indicates that HIF activation probably plays a pivotal role in the effects of RIPC [61–63].

A series of recent studies has indicated that high-intensity interval-training (HIIT) may be more potent than endurance training to induce muscular adaptations that are beneficial to endurance exercise performance [64,65]. However, whether HIIT in hypoxia *versus* similar training in normoxia may alter HIF-1α pathway responses to an acute hypoxic stimulus is unknown. Therefore, and to exclude potential confounding effects of training-induced adaptations in muscle vascularization and ROS scavenging capacity, here we investigated the HIF-1α and HIF-2α pathway responses to a 10-min arterial occlusion of the leg, before and after 5 weeks of HIIT in either normoxia or hypoxia. Meanwhile, within the framework of the principal study, subjects were 'living high' in normobaric hypoxia. We hypothesized that 'living high' in conjunction with training attenuates HIF-1α pathway activation during arterial occlusion. We also hypothesized HIIT in hypoxia to enhance training-induced attenuation of HIF-1α activation during arterial occlusion more than similar training in normoxia

Materials and methods

Participants

The muscle biopsy samples used for this study come from a larger experimental design which has been extensively described elsewhere [66]. Briefly, 18 non-smoking healthy men (mean ± SD; 23.9 ± 3.0 y, 1.80 ± 0.07 m, 70.1 ± 6.6 kg, 59.2 mL $O_2 \cdot kg^{-1} \cdot min^{-1}$) were recruited for participation in the study. Volunteers exposed to altitude above 1500 m in the 6 months prior to the study were excluded for participation. None of the participants took medication or supplements 3 months prior to or during the

study. Subjects were well trained (4.8 ± 2.8 h per week), yet none were engaged in training or sport at competitive level during or in the 3 months prior to the study. Subjects were instructed to maintain their habitual physical activities during the full study period. The study was approved by the KU Leuven Biomedical Ethics Committee and conducted in accordance with the Declaration of Helsinki. All subjects provided written consent after being fully informed about the content of the study and the potential risks associated with participation.

Study design

The study involved two experimental groups, of which one group was enrolled in a 5-week controlled 'live high – train low or high' intervention (TR, n=10), whilst the other group served as an untrained control group living at sea level (CON, n=8) (**Figure 1)**. 'Living high' was included because this study was part of a larger project wherein we wanted to explore the interaction between chronic hypoxic exposure by 'living high' on the one hand, and the addition of HIIT in either normoxia or hypoxia on the other hand [66]. From Monday evening until Saturday morning TR lived in a hypoxic facility. They spent ~15.5 hours per day in the facility and accumulated 387 ± 5.5 h (SD) of hypoxic exposure in total. During the 'living high' period, the degree of normobaric hypoxia was gradually increased from the 16.4% F_iO_2 (~2000 m) on day 1 to 14.0% F_iO_2 at the end (~3250 m) of the intervention period, which corresponds to a hypoxic dose of 1.016 km·h [67]. Subjects in TR, but not in CON, participated in three training sessions per week. We chose to administer HIIT in the form of unilateral knee-extension exercise in order to allow comparison of identical training in normoxia *versus* hypoxia within one individual during a period of 'living high'.One leg was trained by knee-extension exercise in normobaric hypoxia (TR_{HYP}, 12.3% F_iO_2, ~4300 m) whilst the other leg was trained in normoxia (TR_{NOR}, 20.9% F_iO_2). Before (pretest) and at the end (posttest) of the intervention period, we administered an acute hypoxic stimulus in the form of an arterial occlusion rather than exercise, so to eliminate potential exercise-induced modulation of HIF independent of decrease in muscular O_2-tension. In the pretest and the posttest, four sessions involving 10-min arterial occlusions were organized with a 24-h interval in between. Days 1 and 2 served to evaluate muscular oxygenation status by NIRS in both the legs during occlusion in TR and CON, whilst on days 3 and 4 in TR but not in CON needle biopsies were taken from *m. vastus lateralis* before occlusion, and immediately prior to reperfusion. NIRS measurements could not be performed simultaneously with the muscle biopsies because bandage sealing off the NIRS probes hindered needle biopsy sampling. Muscle biopsies were taken 6 (right leg) and 7 (left leg) days after the last 'living high' day, and 9 (right leg) and 10 (left leg) days following the final normoxic/hypoxic training session. Muscle biopsies of the right and left leg were taken 4 days after the final normoxic high-intensity muscle contractions required for testing of muscle performance in the context of the principal study [66].

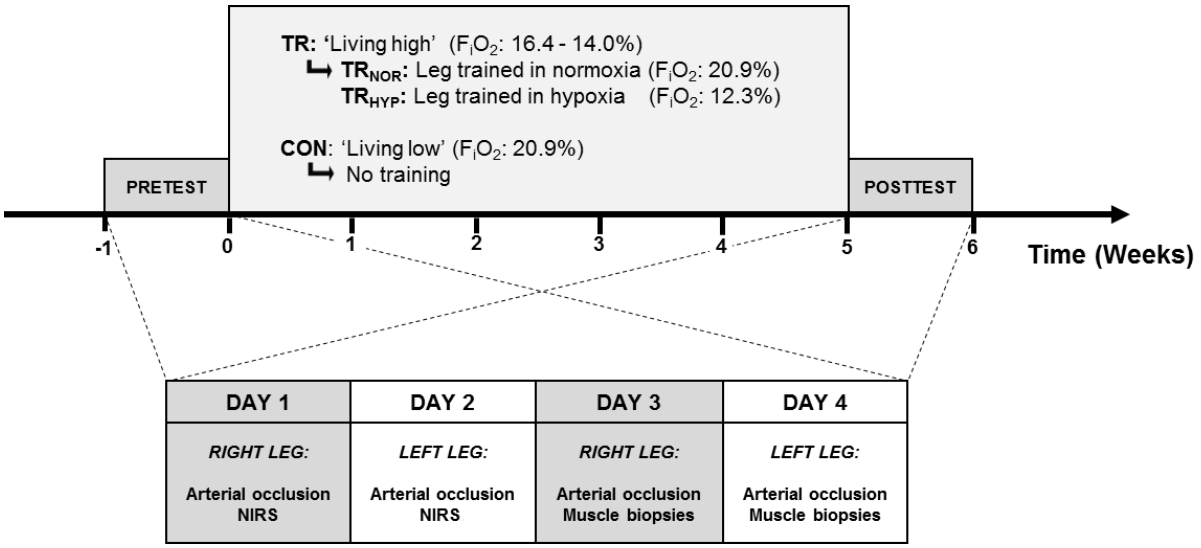

Figure 1 | Schematic presentation of the study protocol
The study involved two experimental groups, of which one group was enrolled in a 'live high' intervention using normobaric hypoxia (TR, n=10), whilst the other group lived at sea level (CON, n=8). In TR one leg was trained in 12.3% F_iO_2 (TR_{HYP}, ~4300 m), whilst the other leg was trained in 20.9% F_iO_2 (TR_{NOR}). Before (pretest) and after (posttest) the intervention period 10-min arterial occlusions were performed in both the legs. Before and during the occlusions muscular oxygenation status was measured by NIRS and needle biopsies were taken from *m. vastus lateralis*.

Hypoxic *versus* normoxic HIIT

TR performed unilateral knee extensions on a leg-extension apparatus (GLCE365, Body-Solid, Illinois, US) which was adapted to knee-extensions from 95° to 175° knee angle. Random allocation assigned the subjects to a group training with the dominant leg in hypoxia 12.3% F_iO_2 (~4300 m) and with the contralateral in normoxia (20.9% F_iO_2), or vice versa. The order of left *versus* right leg training was alternated between the sessions. The training consisted of 2 sets of knee extensions interspersed by 5-min recovery intervals. During each set 4-6 series of 30 unilateral knee-extensions were performed at a workload corresponding to 20-25% of the 1-repetition-maximum (1RM) which was measured as previously described [68]. Contraction series were interspersed by 30-s rest intervals. The number of series was increased from 4 in sessions 1-5, to 5 in sessions 6-10, and eventually 6 in sessions 11-14. Also the load was gradually increased: from 20% 1RM in sessions 1-7 to 25% of 1RM in sessions 8-14. 1RM was re-evaluated at the start of each week to adjust training workloads. Muscle contractions and relaxations were paced by both auditory and visual feedback signals to obtain a 3-s duty cycle with 1s concentric extension:1s eccentric flexion:1s rest.

Arterial occlusion and biopsy sampling

Subjects reported to the laboratory between 6 and 11 a.m. after an overnight fast. Following a 30-min rest period in the supine position, a biopsy of the right *vastus lateralis* muscle was taken using a Bergström-type needle through a single 5-mm incision in the skin (2% xylocaine without epinephrine, 1 mL subcutaneously). Subsequently an orthopedic cuff, wrapped as proximal as possible around the upper thigh, was inflated to 410 mmHg within 2-3 s by means of a compressor. Between the 10^{th} and 11^{th} min of the arterial occlusion, a second biopsy was sampled from the same incision site, where after the leg cuff was decompressed. The first biopsy was sampled with the needle pointing distally from the incision site whilst the second biopsy was sampled with the needle pointing proximally from the incision

site. Next morning an identical procedure was repeated for the left leg. Muscle samples were separated in two parts. One piece was rapidly frozen in liquid N_2 and stored at -80°C for further immunochemical and biochemical analyses. The remaining muscle was mounted in embedding medium (Tissue-Tek OCT), frozen in precooled isopentane, cooled to its freezing point in liquid N_2, and stored at -80°C until later histochemical analyses.

Near-infrared spectroscopy

A Niro-200 NIRS instrument (Hamamatsu, Japan) applying 3 different wavelengths of near-infrared light was used to measure tissue oxygen index (TOI) by spatially resolved spectroscopy, and to estimate changes in oxyhemoglobin (O_2Hb), deoxyhemoglobin (HHb) and total hemoglobin (tHb) content in the *vastus lateralis* muscle during arterial occlusions by using the Beer Lambert law. Considering the large variability in muscle fiber type composition and that uniform differential path length factor values are not available for peripheral skeletal muscles, no differential path length factor was used. Therefore raw NIRS data are reported. A NIRS probe was placed centrally on the belly of the *vastus lateralis* muscle and an elastic non-compressive bandage was wrapped around the probe to prevent movement and interference with external light. Spacing between the emitter and detector was fixed at 4 cm by an optically dense rubber holder, allowing a penetration depth of the NIRS voxel ~2 cm deep into the muscle [69]. A surgical pen marked the margins of the probe on the skin in order to allow for identical repositioning of the probe in later testing. The NIRS signal was recorded at 2 Hz. Before analysis, all data points were processed with a Butterworth filter (4^{th} order, cutoff frequency of 0.05 Hz) in a mathematical software program (Matlab R2011a, The Mathworks, Natick, MA, USA). A 3-min rest period prior to occlusion served as the baseline to which changes in O_2Hb, HHb and tHb are presented. Muscle oxygen extraction rate at rest was calculated from the rate of transformation of O_2Hb to HHb during the initial 60-s of occlusion [70].

Muscle analyses

Western blot

Details of the immunoblotting procedures have been described previously [71]. Briefly, frozen muscle tissue (approx. 20 mg) was homogenized 3 x 5 s with a Polytron mixer in ice-cold buffer (1:10, w/v) [50 mM Tris–HCl pH 7.0, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM glycerophosphate, 5mM sodium pyrophosphate, 50 mM sodium fluoride, 1mM DTT, 0.1% Triton-X 100 and a complete protease inhibitor tablet (Roche Applied Science, Vilvoorde, Belgium)]. Homogenates were then centrifuged at 10000 g for 10 min at 4 °C. The supernatant was collected and immediately stored at -80 °C. The protein concentration was measured using the DC protein assay kit (Bio-Rad laboratories, Nazareth, Belgium). 20–40 µg of protein was separated by SDS-PAGE (8–12% gels) and transferred to PVDF membranes. Subsequently, membranes were blocked with 5% non-fat milk for 1 h and afterwards incubated overnight (4 °C) with the following antibodies: AMP-activated protein kinase α (AMPKα) (#2532), phospho-AMPKα Thr172 (#2535), HIF-1α (#3716) and beta-actin (#4970) from Cell Signaling (Leiden, the Netherlands), HIF-2α (#ab109616) from Abcam (Cambridge, UK) and PHD2/EGLN1 (#NB100-37) from NovusBio (Abingdon, UK). The appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, Bornem, Belgium) were used for chemiluminescent detection of proteins. Membranes were scanned and quantified with Genetools and Genesnap software (Syngene, Cambridge, UK), respectively. Membranes were then stripped and reprobed with the antibody for the total form of the respective protein to ascertain the relative amount of the phosphorylated protein compared with the total form throughout the whole experiment. The results are presented as the ratio protein of interest/beta-actin or as the ratio phosphorylated/total form of the proteins when the phosphorylation status of the protein was measured. Data of TR_{NOR} and TR_{HYP}

are presented as fold change compared to the pretest biopsy taken prior to occlusion for the respective group.

RNA extraction and reverse transcription

The method used for reverse transcription is described in detail elsewhere [72]. Briefly, total RNA was extracted using TRIzol (Invitrogen, Vilvoorde, Belgium) from 17-20 mg of frozen muscle tissue. RNA quality and quantity were assessed by spectrophotometry with a Nanodrop (Thermo Scientific, Erembodegem, Belgium). One µg of RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Gent, Belgium) according to the manufacturer's instructions.

Real-time qPCR analysis

A SYBR Green-based master mix (Applied Biosystems) was used for real-time PCR analyses using the ABI PRISM 7300 (Applied Biosystems). Real-time PCR primers were designed for human, cyclophilin A (CycloA), regulated in development and DNA damage responses 1 (REDD1), ribosomal protein L19 (RPL19), vascular endothelial growth factor A (VEGFA), beta-2-microglobulin (B2M), PHD2, HIF-1α, von Hippel–Lindau tumour suppressor protein (pVHL), superoxide dismutase 1 (SOD1), nuclear factor of activated T-cells (NFAT), PGC-1α, pyruvate dehydrogenase kinase 4 (PDK4), mitochondrial transcription factor A (Tfam), cytochrome c oxidase subunit 4 (COX4), heat shock protein 70 (HSP70) and heat shock protein 27 (HSP27) (**Table 1**). Thermal cycling conditions consisted of 40 three-step cycles including denaturation during 30 s at 95 °C, annealing during 30 s at 58 °C and extension during 30 s at 72 °C. All reactions were performed in duplicate. To compensate for variations in input RNA amounts and efficiency of reverse transcription, CycloA and RPL19 mRNA were quantified, and results were normalized to these values. These genes were chosen out of three (CycloA, RPL19 and B2M) normalisation genes using the GeNorm applet according to the guidelines and theoretical framework described elsewhere [73]. Data are presented as fold change compared to the pre-occlusion biopsy in the pretest and the posttest.

TABLE 1 | Primer sequences used for real-time PCR

	Forward	Reverse
HIF-1α	GCCCCAGATTCAGGATCAGA	TGGGACTATTAGGCTCAGGTGAAC
REDD1	TGAGGCACGGAGTGGGAA	CAGCTCGAAGTCGGGCAA
PDK4	CAGCTACTGGACTTTGGTTC	CTAATTGGGTCGGGAGGATA
VEGFA	TTT CTG CTG TCTT GGG TGC ATT GG	ACC ACT TCG TGA TGA TTC TGC CCT
pVHL	CTCTCAATGTTGACGGACAG	GTAATTCTCAGGCTTGACTAGG
PHD2	AGGTGAAGCCAGCCTAT	CCTGATGCTAGCTGATACTTG
Tfam	AGCGTTGGAGGGAACTTCCTGATT	TTCTTTATATACCTGCCACTCCGCCC
PGC-1α	GGGATGATGGAGACAGCTATGG	CTCTTGGTGGAAGCAGGGTC
NFAT	CTACTCCTCTTTCCAGCACATC	AGGGTCGGGCTCATGTA
COX4	GAGAGCTTTGCTGAGATGAA	CCGTACACATAGTGCTTCTG
SOD1	TCGAGCAGAAGGAAAGTAATG	CCTGCTGTATTATCTCCAAACT
HSP70	GTGGCTGGACGCCAACACCTT	TTACACACCTGCTCCAGCTCCTTC

HSP27	GCTGACGGTCAAGACCAAGGATG	TGAAGCACCGGGAGATGTAGCC
RPL19	CGCTGTGGCAAGAAGAAGGTC	GGAATGGACCGTCACAGGC
CycloA	CTTCATCCTAAAGCATACGGGTC	TGC CAT CCA ACC ACT CAG TCT
B2M	ATGAGTATGCCTGCCGTGTGA	GGCATCTTCAAACCTCCATG

***HIF-1α**, Hypoxia-Inducible Factor-1α; **REDD1,** regulated in development and DNA damage responses 1; **PDK4**, Pyruvate dehydrogenase kinase 4; **VEGFA,** vascular endothelial growth factor A; **pVHL**, von Hippel–Lindau tumor suppressor protein; **PHD2**, prolyl hydroxylase domain containing protein 2; **Tfam**, mitochondrial transcription factor A; **PGC-1α**, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; **NFAT**, nuclear factor of activated T-cells; **COX4**; cytochrome c oxydase subunit 4; **SOD1**, superoxide dismutase 1; **HSP70**, heat shock protein 70; **HSP27**, heat shock protein 27; **RPL19,** ribosomal protein L19; **CycloA,** cyclophilin A; **B2M**, beta-2-microglobulin*

ATP and PCr assays

Muscle ATP, free creatine and phosphocreatine (PCr) concentrations were analysed from perchloric acid precipitated extractions of freeze-dried muscle samples (2-4 mg dm) using standard enzymatic fluorometric assays as previously described [74]. Data are expressed in mmol·kg^{-1} dry muscle (dm).

Immunofluorescence

Cross-sections (7 µm) of biopsies from *vastus lateralis* muscle were fixed in ice-cold acetone for 10 min. A solution containing 1% hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) was applied for 10 min to inactivate the endogenous peroxidase activity. Samples were then incubated with 1% bovine serum albumin (BSA) in PBS 0.2% Triton X-100 for 15 min, followed by incubation with PBS containing 3% BSA for 1 h at room temperature. Sections were incubated overnight at 4°C with a solution containing anti-rabbit laminin (Sigma, L9393) 1:300 and anti-mouse human HIF-1α (Novus Biologicals, NB-100-105) 1:100 antibodies in PBS with 1% BSA. Subsequently, samples were incubated for 45 min at room temperature with a solution containing goat anti-rabbit Alexa fluor 568 (Invitrogen, A11011) and goat anti-mouse Alexa fluor 488 (Invitrogen, A11029) secondary antibodies diluted 1:600 in PBS with 1% BSA. Nuclei were stained for 5 min with 0.5 µg·mL^{-1} Hoechst 33342 (Sigma) in PBS. Slides were visualized by fluorescence microscopy (Nikon E1000, Nikon, Boerhavedorp, Germany). Pictures of the slides were analysed by a blinded researcher with ImageJ software (version 1.41, National Institutes of Health, USA). An identical background subtraction was applied on all slides. HIF-1α positive nuclei were quantified as percentage of total nuclei. On average 737 ± 16 (SD) nuclei were analysed per biopsy.

Statistics

Statistical analysis was performed using IBM SPSS Statistics 23.0 (SPSS, Chicago, Illinois). A 2-way repeated measures analysis of variance (ANOVA) (group x time) was performed to test differences in NIRS-derived parameters, with CON, TR_{NOR} and TR_{HYP} in the factor 'group', and pretest and posttest in the factor 'time'. Because pretest values in CON were similar between the legs for all variables measured ($P > 0.05$, paired Students' T-test), values from both legs were averaged and considered as one data point. Differences in biochemical and histochemical data were tested using 3-way repeated measures ANOVA, with factor group (TR_{NOR} vs. TR_{HYP}) as a between subject variable and both factor time (pretest vs. posttest) and occlusion (before occlusion (0') *versus* end of occlusion (10')) as within subject variables. Post-hoc Students' T-tests with Bonferroni correction were used for

multiple comparisons whenever ANOVA yielded a significant main or interaction effect. A probability level $P < 0.05$ was defined as statistically significant. All data are expressed as means ± standard error of mean (SEM) unless stated otherwise.

Results

Effect of arterial occlusion on muscle oxygenation status (Figure 2) **–** We measured parameters of skeletal muscle oxygen status by NIRS during the 10-min arterial occlusion on *m. vastus lateralis*. During the initial 5 min of the occlusion TOI and O_2Hb rapidly decreased whilst HHb increased, where after values stabilized. Muscular oxygen extraction rate was calculated from the rate of conversion of O_2Hb to HHb during the initial 60-s of the occlusion. O_2-extraction rates were similar between the experimental conditions in the pretest. Training increased O_2-extraction rate by ~30% in both TR_{NOR} (pretest, 0.125 ± 0.012; posttest, 0.164 ± 0.012 $mL \cdot min^{-1} \cdot 100\ g^{-1}$; $P < 0.001$) and TR_{HYP} (pretest, 0.119 ± 0.018; posttest, 0.153 ± 0.012 $mL \cdot min^{-1} \cdot 100\ g^{-1}$; $P < 0.001$), whilst no change ($P = 0.27$) occurred in CON (interaction effect, $P = 0.04$). Accordingly, compared to the pretest, ΔO_2Hb values (from baseline to nadir) in the posttest were higher in TR_{NOR} ($P < 0.001$) and TR_{HYP} ($P < 0.001$), but not in CON ($P = 0.09$). Conversely, ΔHHb was similar between pretest and posttest in all conditions. TOI values in both pretest and posttest dropped from ~70% at baseline to ~24% during the occlusion, irrespective of the experimental conditions. tHb values typically dropped to bottom values by the end of the occlusion in all experimental conditions. However, compared to the pretest, in the posttest ΔtHb values during occlusion were higher in all groups ($P < 0.001$ for both TR_{NOR} and TR_{HYP}; $P < 0.01$ for CON), and values in the posttest were higher in TR_{NOR} compared to CON ($P < 0.05$).

Effect of arterial reperfusion on muscle oxygenation status (Figure 2) **-** Upon decompression of the cuff tHb precipitously increased to peak values within ~70 s in both the pretest and posttest. Apex tHb values were higher in the posttest than in the pretest in both TR_{NOR} ($P < 0.001$) and TR_{HYP} ($P < 0.01$), but no changes ($P = 0.53$) occurred in CON (interaction effect, $P < 0.01$). In the posttest apex tHb values were significantly higher in TR_{NOR} ($P < 0.001$) and TR_{HYP} ($P < 0.05$) as compared to CON, without differences between TR_{NOR} and TR_{HYP} ($P = 0.43$). By analogy, O_2Hb rapidly increased upon reperfusion and exceeded baseline values. Compared to the pretest, apex O_2Hb values in the posttest were 30-40% higher in TR_{NOR} and TR_{HYP} ($P < 0.001$), whilst no changes ($P = 0.96$) occurred in CON (interaction effect, $P < 0.01$). TOI values during reperfusion were similar between the experimental conditions in both the pretest and the posttest.

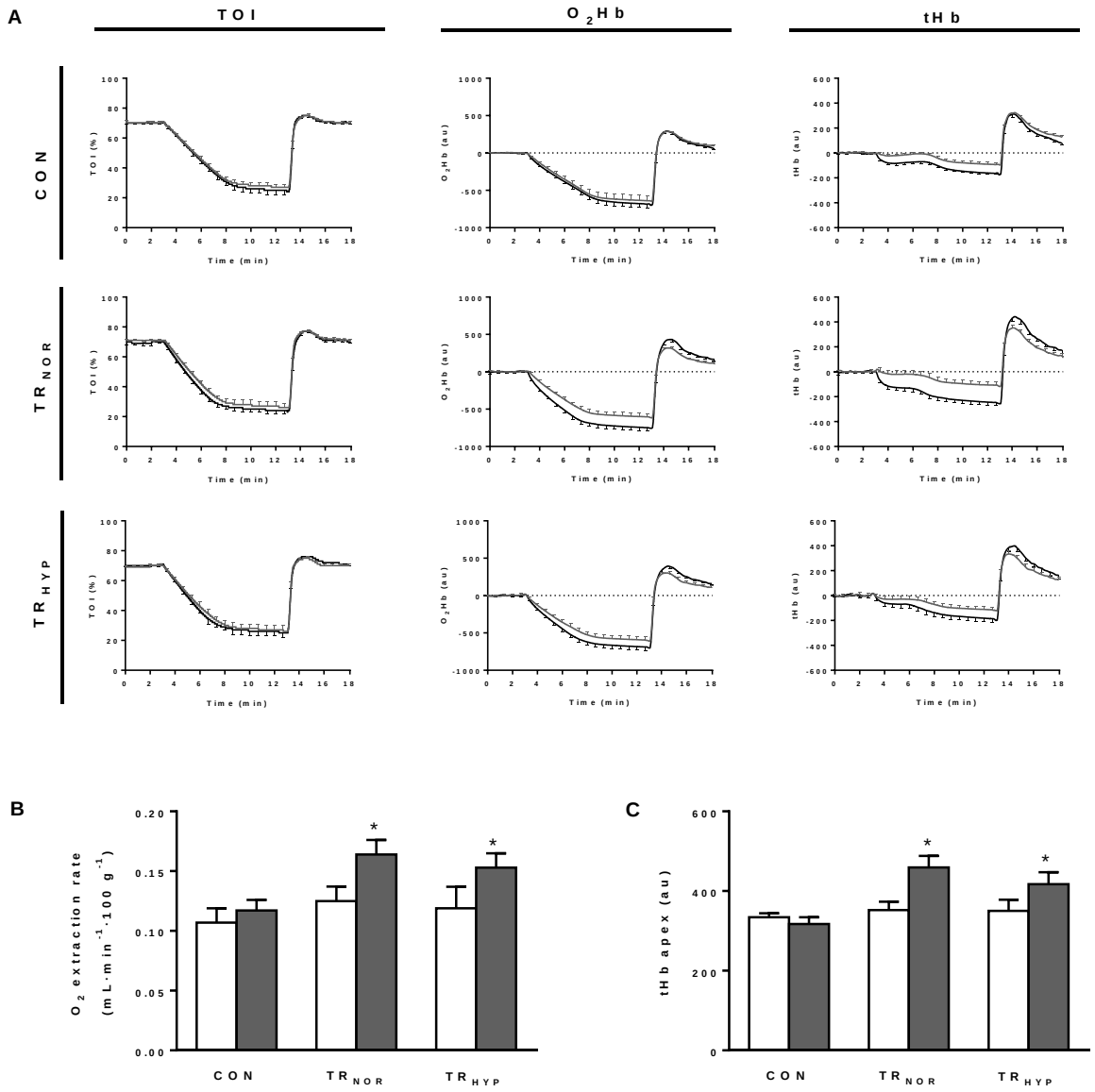

Figure 2 | Effect of knee-extension training in normoxia *versus* hypoxia during 'living high' on muscular oxygenation status in m. vastus lateralis during an arterial occlusion and reperfusion of the leg.

A: Data are means ± SEM and represent NIRS-derived parameters during a 10-min arterial occlusion of the leg before (grey lines) and after (black lines) 5 weeks of knee-extension training in normoxia (TR_{NOR}, 20.9% F_iO_2), or hypoxia (TR_{HYP}, 12.3% F_iO_2), or in normoxia without training (CON). TOI, muscle tissue oxygen index; O_2Hb, oxyhemoglobin; tHb, total hemoglobin content. Data presented in **B** and **C** are means ± SEM and represent oxygen extraction rate and peak tHb value during reperfusion, respectively, before (open bars) and after (solid bars) 5 weeks of knee-extension training.
*P < 0.05 vs. pretest

Effect of arterial occlusion on muscle high-energy phosphates and AMPK **-** We measured muscle ATP and PCr contents to assess the impact of the occlusions on muscular energy status. Muscle PCr content during occlusion on average decreased by ~25% from ~75 to ~57 mmol·kg^{-1} (P < 0.05) in both the pretest and the posttest, and irrespective of the experimental conditions. Muscle ATP content

was constant at ~21-25 mmol·kg^{-1} and was unaffected by the occlusions. Accordingly, phosphorylation status of muscle AMPK was constant, irrespective of the experimental conditions (**Figure 4**).

Effect of arterial occlusion on HIF-1α (Figures 3-4) - To evaluate the degree of activation of the HIF-1α pathway we first assessed the number of HIF-1α positive nuclei. Both in the pretest and in the posttest occlusion increased the number of HIF-1α positive nuclei from about 54 to 64% ($P < 0.05$). However, there were no differences between TR_{NOR} and TR_{HYP} at any time. Neither muscle HIF-1α and HIF-2α protein expression, nor HIF-1α mRNA levels were significantly altered by either the occlusion or the training intervention.

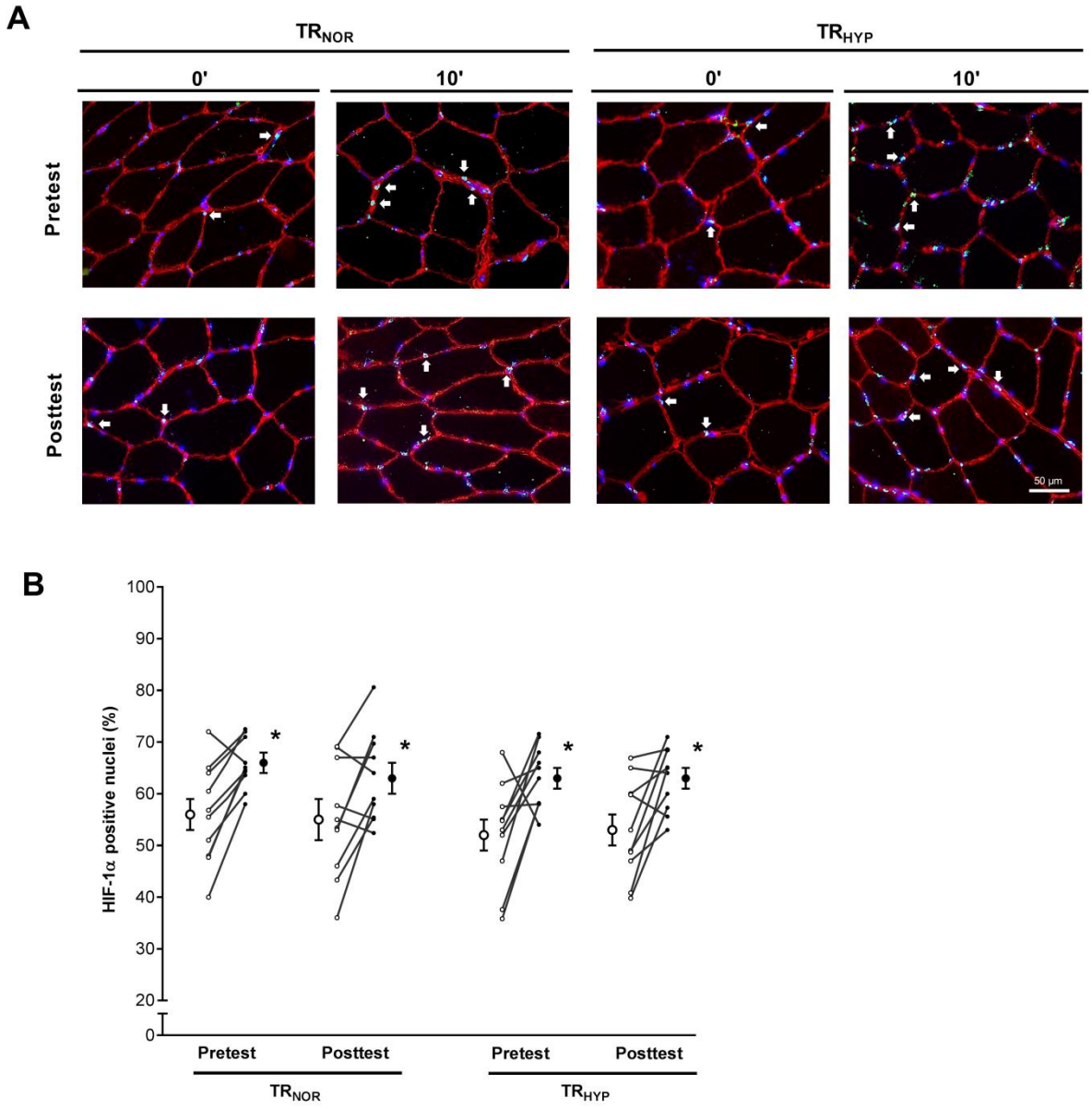

Figure 3 | Effect of knee-extension training in normoxia *versus* hypoxia during 'living high' on HIF-1α positive nuclei in m. vastus lateralis during an arterial occlusion of the leg.
A: Representative immunofluorescence images of vastus lateralis muscle sampled before (0') and at the end (10') of a 10-min arterial occlusion and before (Pretest) and after (Posttest) 5 weeks of knee-

extension training in normoxia (TR_{NOR}, 20.9% F_iO_2) or hypoxia (TR_{HYP}, 12.3% F_iO_2). Laminin (red), Hoechst (blue), HIF-1α (green). Arrows indicate HIF-1α positive nuclei. **B**: Data represent means ± SEM and individual values (lines) of HIF-1α positive nuclei before (open circles) and at the end (solid circles) of the occlusion in the pretest and posttest.

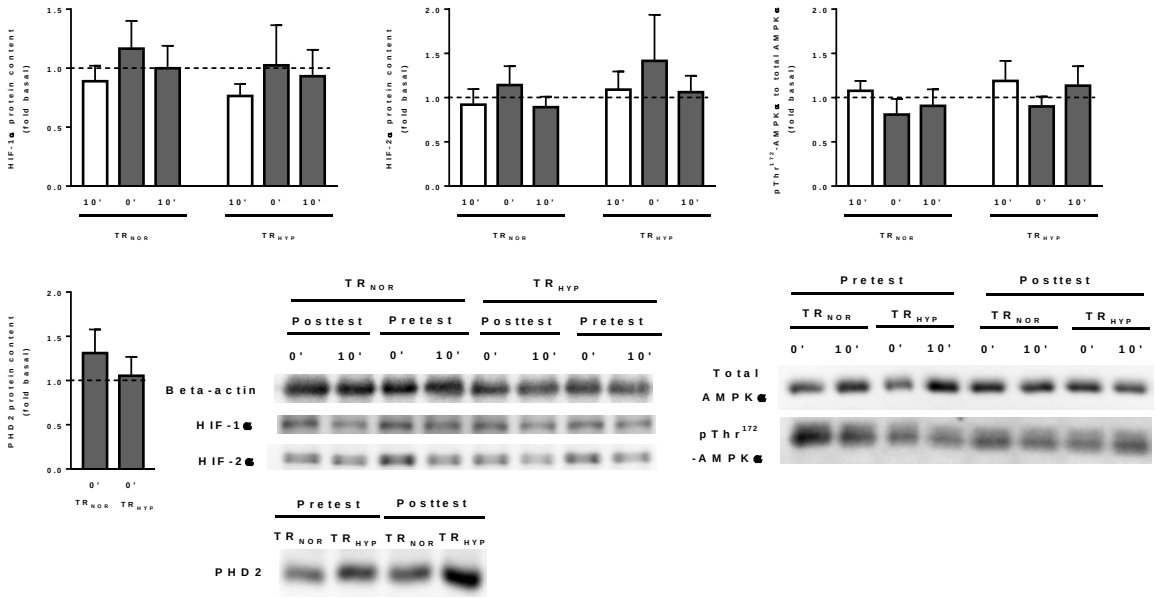

Figure 4 | Effect of knee extension training in normoxia *versus* hypoxia during 'living high' on protein content and phosphorylation status in m. vastus lateralis during an arterial occlusion of the leg.

Data are means ± SEM and are expressed as fold increase relative to biopsies taken prior to occlusion in the pretest. Before (open bars) and after (solid bars) 5 weeks of knee-extension training in normoxia (TR_{NOR}, 20.9% F_iO_2) or normobaric hypoxia (TR_{HYP}, 12.3% F_iO_2), muscle biopsies were sampled before (0') and at the end (10') of a 10-min arterial occlusion. HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; AMPK: AMP-activated protein kinase; PHD2, prolyl hydroxylase domain 2.

Transcriptional response to 10 min of arterial occlusion (Table 2) **-** To evaluate the effects of training in normoxia and hypoxia on the transcriptional response of HIF-1α and HIF-2α we also measured the mRNA content of a series of target genes and HIF regulators before and during the occlusion. Irrespective of the experimental condition, the transcriptional responses of markers involved in either muscular oxygen sensing, mitochondrial biogenesis, or cellular stress were negligible in both the pretest and the posttest.

TABLE 2 | Effect of knee extension training in normoxia *versus* hypoxia during 'living high' on fold change in muscle mRNA after 10 min of arterial occlusion

	Pretest		Posttest	
	TR_{NOR}	TR_{HYP}	TR_{NOR}	TR_{HYP}

Oxygen signaling	*HIF-1α*	0.28 ± 0.17	-0.10 ± 0.05	0.00 ± 0.10	0.33 ± 0.33
	REDD1	0.22 ± 0.10	0.64 ± 0.36	1.41 ± 1.45	0.45 ± 0.68
	PDK4	0.62 ± 0.27	1.05 ± 0.42	1.03 ± 0.30	0.30 ± 0.16
	VEGFA	0.01 ± 0.12	0.37 ± 0.18	0.11 ± 0.12	0.01 ± 0.33
	pVHL	0.95 ± 0.74	-0.08 ± 0.14	0.06 ± 0.18	-0.24 ± 0.08
	PHD2	0.48 ± 0.44	0.18 ± 0.16	-0.01 ± 0.09	-0.02 ± 0.22
Mitochondrial biogenesis and function	*Tfam*	0.12 ± 0.12	0.13 ± 0.11	-0.04 ± 0.14	-0.13 ± 0.16
	PGC-1α	0.38 ± 0.28	0.10 ± 0.14	0.14 ± 0.09*	-0.18 ± 0.19*
	NFAT	0.02 ± 0.18	0.31 ± 0.20	0.14 ± 0.09	0.38 ± 0.36
	COX4	-0.07 ± 0.12	0.40 ± 0.21	0.17 ± 0.11	0.23 ± 0.23
Oxidative stress	SOD1	0.02 ± 0.19	0.26 ± 0.18	0.13 ± 0.10	0.34 ± 0.34
Heat shock response	*HSP70*	2.09 ± 1.74	0.44 ± 0.34	0.03 ± 0.06	0.48 ± 0.53
	HSP27	0.39 ± 0.32	0.21 ± 0.16	0.14 ± 0.09	-0.21± 0.08

Data are means ± SEM and represent fold change in muscle mRNA after 10 min of arterial occlusion (Δ10' – 0') either before (Pretest) or after (Posttest) 5 weeks of knee-extension training in normoxia (TR_{NOR}, 20.9% F_iO_2) or normobaric hypoxia (TR_{HYP}, 12.3% F_iO_2).
** $P < 0.05$ compared to pretest*

Discussion

The HIF pathway plays a pivotal role in the modulation of cellular responses to hypoxia. In muscle, contractile activity is the primary mechanism to reduce intracellular O_2 tension due to stimulation of mitochondrial oxygen utilisation exceeding the rate of myocellular O_2 import [27]. Intermittent activation of HIFs during exercise conceivably is also important in early training adaptations, *i.e.* for training-induced angiogenesis [75]. Compatible with such mechanism, it has been demonstrated that a single bout of endurance exercise induces both the stabilisation, nuclear translocation and activation of HIF-1α [32]. However, consistent training eventually seems to attenuate HIF-pathway activation [37-38]. Accordingly, sustained cell exposure to a given degree of hypoxia *in vitro* resets HIF-1α activity to baseline level [21-22]. HIFs probably also are implicated in the specific physiological adaptations to altitude training. Over the last decades, 'living high – training low' has developed to be the predominant strategy in altitude training in athletic populations. However, it is still unclear whether the addition of hypoxic training to a period of 'living high' may alter HIF responsiveness, or stimulate physiological adaptations supposedly improving endurance performance. Therefore, in the present study, we examined muscular HIF-1α responses to an arterial occlusion before and after 5 weeks of normoxic *versus* hypoxic HIIT during short-term 'living high'. Muscle oxygenation during training as measured by TOI was 10-15% lower in hypoxic compared to normoxic training sessions [66]. The occlusions were successful to substantially decreasing muscular oxygenation saturation as evidenced by a ~46% drop of TOI measured by NIRS. Furthermore, muscle phosphocreatine content was decreased at the end of the occlusions, indicating significant energy stress. HIIT also increased muscular oxygen extraction rate during occlusion by ~30%, which indicates training-induced enhancement of muscular oxygen extraction capacity. Against this background, we found ischemia by

arterial occlusion to increase the abundance of myonuclei expressing HIF-1α. However, 5 weeks of 'living high' whilst training in either normoxic or hypoxic conditions did not alter this response.

It is the prevailing opinion that PHDs in normoxia quickly mark HIF-1α for proteosomal degradation. This is compatible with one earlier study showing very low myonuclear HIF-1α expression in humans at rest [32]. Here we quantified HIF-1α positive nuclei by immunofluorescence microscopy in muscle biopsies sampled before and at the end of a 10-min arterial occlusion, before and after 5 weeks of normoxic *versus* hypoxic knee-extension training. Interestingly, we found ~54% of myonuclei to yield a positive staining for HIF-1α in baseline conditions. The occlusion further increased the fraction of HIF-1α positive nuclei to ~64% in both the pretest and posttest, irrespective of normoxic *versus* hypoxic training. Furthermore, neither hypoxic nor normoxic training altered basal or ischemia-stimulated HIF-1α mRNA content, or HIF-1α, HIF-2α or PHD2 protein expression in muscle. Besides oxygen, also iron is an important regulator of PHD enzyme activity. Shortage of intracellular iron may increase HIF-α stability, indeed [76]. In this respect it is important to note that iron status was well controlled in the current study protocol. Serum ferritin concentrations were well above the cut-off criteria for iron deficiency in all subjects [66]. The amount of HIF-1α detectable in resting conditions is crucial to investigate the role of HIF-1α in muscle adaptation to physical activity. So far several studies in murine models identified quantifiable levels of HIF-1α protein in muscle tissues at rest, both in nuclear extracts [38,77,78] and in whole-cell extracts [79-81]. Conversely, in human studies baseline protein abundance of HIF-1α is still debated [32,71,82]. Ameln and colleagues showed an increment of HIF-1α at protein level after an acute exercise bout, while its expression was undetectable by both Western blot and histological analyses in muscles at rest [32]. On the contrary, we recently found detectable levels of HIF-1α protein in resting *m. vastus lateralis* in healthy volunteers [8]. Our analyses are also compatible with earlier observations indicating muscle-specific HIF-1α expression and showing *vastus lateralis* and *soleus* muscle to exhibit higher basal HIF-1α protein content than *triceps brachii* muscle [82]. It is important to emphasise that all earlier human studies measured HIF-1α protein by either Western blot [32,8] or sandwich enzyme-linked immunoassay [82] in whole-cell lysates, thus not distinguishing between HIF-1α-rich nuclear fractions from rather poor HIF-1α cytoplasmic protein fractions [78]. But by using immunofluorescence analyses here we were able to demonstrate high abundance of HIF-1α protein in nuclei in resting muscle, indeed, and even higher levels during hypoxia induced by arterial occlusion. Taken together, our current findings together with earlier observations indicate that PHD activity does not fully eliminate nuclear HIF-1α protein expression in human muscle at rest.

With regard to PHD activity, Lindholm *et al.* [51] have previously demonstrated that exercise training increases basal PHD2 protein content in muscle, which theoretically should allow for lower HIF-pathway activation. However, in the conditions of the present study, training while 'living high' did not alter PHD2 protein content, irrespective of whether the training was performed in normoxia or hypoxia. These discrepant findings may be at least partly due to different training modes. Lindholm *et al.* used moderate-intensity (70% VO_2max) whole-body exercise as a training stimulus, whilst we used high-intensity intermittent muscle contractions involving but a small muscle mass yet imposing higher mechanical and metabolic stress on the *vastus lateralis* muscle. The finding that HIF-1α levels are elevated in gastrocnemius muscle of mice following a period of HIIT also argues against HIIT-induced upregulation of PHD activity [81]. The effect of different training formats on muscle fibre-specific HIF-1α-pathway activity remains to be elucidated in further studies. Furthermore, because we did not include control groups 'living low', it is impossible to evaluate the effects of 'living high' *per se* on HIF-pathway regulation. Nonetheless, our current results taken together with previous research clearly indicate that regulation of human skeletal muscle HIF-1α activity is unaffected by short-term HIIT during 'living high'.

Nuclear HIF-1α translocation is controlled via different processes. Two separate nuclear localisation signals have been identified in HIF-1α, both of which are capable of interacting with specific nuclear import receptors [83,84]. Nuclear export on the other hand is modulated by p42-44 MAPK, which phosphorylates one or two HIF-1α serine residues in close proximity of a nuclear export signal (NES) [3]. Upon phosphorylation NES is masked for CRM1-dependent nuclear export, hence promoting nuclear accumulation. Despite a consistent increase in HIF-1α positive nuclei following 10 min of muscle ischemia in the present study, no increased protein abundance of either HIF-1α or HIF-2α was observed. We speculate that hypoxia, via the MAPK pathway [85], promptly phosphorylates HIF-1α, which in turn inhibits its nuclear export. Downregulation of PHD2 activity causing net degradation of HIF-1α protein to such degree that it can be detected by simple Western blot analysis in whole-cell extracts conceivably requires a hypoxic episode longer than 10 min. In addition, we took biopsies at the final min of the occlusion, but transcription and expression of HIF-α and its target genes still may have increased in the period following reperfusion [79].

For the current experiments we chose to use vascular occlusion rather than exercise to investigate the effects of hypoxia on HIF activation in muscle following training during 'living high'. Training might alter myocellular P_iO_2 during exercise via both increased blood flow [86] combined with higher capillary density [87], and elevated capacity for mitochondrial O_2 utilisation [88]. In addition, training-induced adaptations in ROS production and scavenging capacity may alter contraction-induced HIF-1α activation independent of changes in P_iO_2 [42,44,89]. However, muscular ROS production is limited during a brief period of ischemia as used in the current experiments [90-91].

Our NIRS measurements also for the first time show that short-term HIIT increases oxygen extraction rate in resting muscle during arterial occlusion, but again this effect was independent of whether the training was performed in normoxia or in hypoxia. This observation corroborates previous findings by our laboratory showing 2-fold higher muscular oxygen extraction rates in athletes at rest during a vascular occlusion identical to the current protocol [92]. There is ample evidence to indicate that short-term HIIT in young healthy volunteers is effective to increasing muscular oxidative capacity by virtue of elevated mitochondrial volume density and higher oxidative enzyme activities [65]. Here we add the novel observation that these beneficial myocellular adaptations translate into immediately higher oxygen extraction rates during sudden episodes of reduced muscular oxygen availability. Such mechanism is likely to contribute to reduced anaerobic energy contribution in any condition where oxygen delivery to muscles suddenly mismatches the oxygen requirement, *i.e.* like during blood flow limitation and/or high-intensity intermittent muscle contractions. Short-term HIIT also substantially increased the amplitude of the immediate post-occlusion hyperemic response (apex tHb, +~25%), but again this effect was independent of the oxygen supply status during training. This is compatible with one of our earlier findings showing muscle capillary density to increase to the same degree (+~15%) in the normoxically and hypoxically trained leg [66]. Our observations taken together with literature findings indicate that normoxic [93] and hypoxic exercise training involving small muscle mass similarly stimulate local vascular remodelling and vasodilator capacity in young healthy volunteers. Nonetheless, compared with normoxic training, 4 weeks of hypoxic moderate-intensity cycling training in sedentary males was found to stimulate the hyperemic response post arterial leg occlusion [94]. However, this discrepancy may be due to lower subjects' training status in conjunction with a different training mode potentially inducing central circulatory effects that are excluded in knee-extension exercise as was used in the current experiments.

NIRS-derived total hemoglobin content (tHb) is supposed to be constant during a full arterial occlusion. Nonetheless, tHb during occlusion decreased in the current study. However, this is unlikely to be due to incomplete occlusion because at an inflated cuff pressure of >400 mmHg any venous

outflow from the leg can be excluded, and arterial inflow if anything would increase tHb [95]. NIRS technology measures total heme oxygenation and thus cannot distinguish between changes in hemoglobin and myoglobin oxygenation. Therefore, the drop in tHb is probably largely, if not entirely explained by myoglobin desaturation, which decreases the O_2Hb signal without altering the HHb signal, leading to a 'false' drop of estimated ΔtHb.

Several confounding factors require attention for appropriate interpretation of the current findings. First, we did not include a sedentary group 'living high' in the protocol. Hence it is not possible to tease out the effects of 'living high' per se. Nonetheless, it is reasonable to assume that the role of 'living high' on the muscle HIF-pathway in the conditions of the present study was minimal. For in the absence of exercise, ambient hypoxia equivalent to just 2000-3250 m altitude is unlikely to induce HIF-α stabilisation [17]. Second, muscle biopsies were taken 6-7 days after the last 'living high' day, and even 9-10 days following the final normoxic/hypoxic training session. The acute exercise effects on HIF regulation thus were conceivably excluded, yet the unique effects of hypoxic *versus* normoxic training probably were partly abolished by the time muscle tissue was sampled. Third, one should keep in mind that a complete occlusion of muscle blood flow induces a different kind of physiological stress than acute exercise. Indeed, 10 min of arterial occlusion induces a higher degree of muscle tissue deoxygenation (current and principal [66] study), but presumably less formation of ROS and metabolic waste products [96] compared to high-intensity muscle contractions [97]. Finally, we used a single-leg model to allow comparison of identical training in normoxia *versus* hypoxia within one individual during 'living high'. However, both neuromechanical and metabolic stress largely differ between one-legged and whole-body HIIT. In addition, compared to whole-body exercise contracting muscle accommodates significantly larger volumes of blood (~oxygen supply) during exercise involving only a small muscle mass [98].

Conclusion

In conclusion, our current experiments for the first time demonstrate high nuclear expression of HIF-1α protein in human muscle at rest. In addition, nuclear HIF-1α abundance is further increased by acute hypoxic stress. However, these effects were unaffected by short-term HIIT in conjunction with 'living high'. Still, HIIT during 'living high' increased muscular oxygen extraction rate during acute ischemia and enhanced post-ischemic muscle hyperperfusion. However, none of these effects were differentially affected by training in normoxia *versus* hypoxia. Future research is warranted to investigate the unique effects of different hypoxic training strategies on muscle HIF responses to hypoxia, induced by either muscle contractions or by arterial occlusion.

Author contributions

Conception and design: SDS and PH. Biopsy sampling: RVT. Biochemical and histochemical analyses: GD, CP and EB. Data analysis: SDS, GD and CP. Drafting of the manuscript: SDS, GD and PH. All authors revized and approved the final version of the manuscript. SDS and GD contributed equally to this work.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Paper 4

Exercise intolerance, low arterial oxygen saturation, and vagal cardiac withdrawal in hypoxia in subjects at risk for AMS

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Abstract

Purpose: Acute mountain sickness (AMS) is a common issue during traveling at altitude beyond the level of acclimatization. Whether physiological responses to acute hypoxia can identify individuals at risk remains a matter of debate.

Methods: Sixteen young healthy adults participated in two VO_2max tests and two 24-h trials, once in normoxia (F_iO_2: 20.9%) and once in normobaric hypoxia (F_iO_2: 12.7%, ~4000 m). Attempting to identify predictors of AMS, a set of physiological measurements at rest and during intermittent submaximal cycling bouts (multiple 30 min bouts at 1.5 $W \cdot kg^{-1}$) was performed throughout the 24-h trials. AMS was diagnosed using Lake Louise scoring system (LLS).

Results: Nine subjects developed mild AMS (AMS_{LOW}) and seven subjects developed severe AMS (AMS_{HIGH}). Compared to AMS_{LOW}, early on during the hypoxic exposure AMS_{HIGH} exhibited lower minute ventilation, lower blood oxygenation, and a progressive decline in vagal heart rate variability. Subjects in AMS_{LOW} completed all cycling bouts in hypoxia. In contrast, five out of seven seven subjects in AMS_{HIGH} could not maintain the prescribed workload. Submaximal exercise at 2 h in hypoxia elicited higher blood lactate levels and greater arterial oxygen desaturation in AMS_{HIGH} than in AMS_{LOW}. AMS severity was unrelated to VO_2max measured in either normoxia or hypoxia. Stepwise multiple regression identified S_pO_2 during submaximal exercise in hypoxia to predict 53% of the peak LLS.

Conclusion: Individuals with severe *vs*. mild AMS are characterized by greater exercise intolerance, reduced vagal cardiac autonomic regulation, and lower blood oxygenation status at rest and during exercise in hypoxia. S_pO_2 drop during submaximal exercise following 2 hours of hypoxic exposure yielded the best prediction of subsequent AMS severity.

Keywords: Acute mountain sickness, normobaric hypoxia, heart rate variability, exercise capacity, arterial oxygen saturation

Introduction

Tourists, mountaineers, military staff, and high-altitude laborers often travel to altitudes beyond their current level of acclimatization. This is known to induce impairment in functional capacity and high-altitude illness in some, but not all individuals. High-altitude illness extends from acute mountain sickness (AMS) with mostly mild symptoms, to life-threatening high-altitude cerebral (HACE) and pulmonary edema (HAPE). AMS is the most common form of high-altitude illness. It affects approximately 25% of individuals traveling to ~2000-3000 m [2] and more than half the population traveling to ~4000-5000 m [3-6]. The primary symptom is headache, often in conjunction with non-specific symptoms such as gastro-intestinal distress, weakness, dizziness and insomnia [7,8]. Effective altitude, rate of ascent, altitude pre-exposure, and individual susceptibility are the main determinants of AMS development [1,5,9]. Estimations of susceptibility are currently based on previous history of AMS, since adequate physiological testing procedures to assess individual AMS predisposition is lacking. Nonetheless, valid screening procedures may be useful to identify individuals at risk prior to traveling.

A number of physiological events have been associated with the development of AMS. First, at the cerebral level, hypoxic exposure has been associated with increased cerebral arterial inflow [10-12], cerebral venous distention [12,13], and brain swelling [12,14]. These findings led to the 'tight-fit' hypothesis, suggesting that those with a 'tight' *versus* 'compliant' brain, the latter thus allowing greater asymptomatic increases in cerebral blood volume and/or edema, are more prone to the development of AMS [13,15-17]. However, the tight-fit hypothesis might be an oversimplification, given the absence of consistent evidence that hypoxia disrupts the blood-brain barrier or induces greater vasogenic edema in AMS positive subjects [14,18-21]. Nonetheless, a unique fluid redistribution from the extracellular to the intracellular (astrocytic) space has been reported in AMS [14,21]. Some data also indicate that cerebral oxidative and nitrosative stress in conjunction with local neurogenic inflammation may trigger the trigeminovascular system and so be involved in the pathogenesis of AMS [19,20,22,23].

Second, at the pulmonary level, hyperventilation serves to attenuate arterial deoxygenation in hypoxia. Lower hyperventilatory response during the initial hours or days at altitude has been reported in subjects developing AMS [24-26]. Accordingly, high pre-ascent hypoxic ventilatory response was found to be associated with lower incidence of AMS in some [25,27,28] but not the majority of studies [24,29-32]. Likewise, conflicting results have also been reported regarding S_pO_2 as a potential predictor of AMS. In a retrospective study, subjects with a history of AMS showed 5% lower S_pO_2 following 20-30 min of hypoxic exposure compared to non-susceptible subjects [33]. However, unfortunately, S_pO_2 values largely overlap between AMS susceptible and non-susceptible individuals [34-37].

Third, it is believed that alterations in autonomic nervous system activity may also play a role in the development of AMS. Greater hypoxia-induced elevations in sympathetic activity, as evidenced by higher urinary and blood catecholamine outputs, indicate sympathetic hyperactivity to be involved in AMS development [38-41]. Further support for such mechanism comes from the observation that pharmacological beta-adrenergic blockade may attenuate AMS development [42]. Heart rate variability (HRV) is being used as an indirect measure of cardiac autonomic regulation [43]. HRV studies indicate that hypoxic exposure may impair cardiac autonomic regulation, indeed [44-51]. Changes in specific HRV parameters indicating parasympathetic withdrawal at moderate altitude predicted AMS development at higher altitudes [52,53]. Furthermore, resilient *versus* susceptible individuals for AMS show normalisation of parasympathetic activity after the first night at high altitude [47]. However, again the validity of HRV changes to predict AMS is inconsistent [51,54,55].

Fourth, abnormalities in pulmonary nitric oxide (NO) metabolism may also play a role in the pathogenesis of AMS [56-59]. Exhaled NO (FENO) originates from NO production in the vast pulmonary

endothelium, epithelium of the airways' surface area, and from nonadrenergic noncholinergic nerves [60,61]. NO regulates ventilation-perfusion matching in the lungs by concerted bronchiolar and capillary vasodilation [61], which therefore are likely to prevent pulmonary hypertension. Studies have found FENO to be reduced in hypobaric hypoxia [62-65]. Although also normobaric hypoxia reduced FENO in some studies [66-68], others reported either no changes [57,63-65] or even an increase following 2 h of normobaric hypoxic exposure [56]. Still, human intervention studies identified low FENO measured in normoxia to predict AMS development during subsequent exposure to either hypobaric [58] or normobaric [56] hypoxia. Conversely, in a group of Chinese lowlanders, those with high FENO at sea level and high altitude (~3500 m) were more susceptible for AMS [59]. No association was found in one earlier hypobaric study [62], whilst only a trend for lower FENO in AMS susceptible individuals was reported in another recent normobaric experiment [57].

The above literature overview indicates that an adequate algorithm to predict AMS from physiological measurements is lacking. The development of AMS has been linked to a wide range of pathophysiological events, yet considerable discrepancies in literature findings exist and it remains difficult to adequately identify individuals at risk for AMS. Therefore, the aim of this study was to investigate whether the development of AMS can be predicted by a series of physiological measurements during an acute exposure to normobaric hypoxia equivalent to 4000 m altitude.

Methods

Participants

Seventeen healthy volunteers were recruited for participation in this study via social media and word of mouth. However, one subject was excluded from analysis due to sudden onset of acute low back pain during the sleepover in hypoxia requiring medical attention. The remaining sixteen subjects were on average (± SD) 24 ± 4 years old, 181 ± 6 cm tall and weighed 74.9 ± 8.9 kg. Participants were recreationally-active and on average participated 5.5 ± 2.3 h in exercise activities per week. All volunteers were medically screened prior to participation in this study in order to exclude any kind of cardiovascular or respiratory (*i.e.*, asthma) pathology. A 12-lead electrocardiogram was taken at rest to exclude subjects with cardiac arrhythmias. All subjects were none-smoking, neither did they use any kind of medication or supplements in the 3 months prior to the study. Furthermore, subjects exposed to altitude above 1500 m in the six-month period preceding the study were excluded from participation. The study was approved by the KU Leuven Biomedical Ethics Committee and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent after being fully informed about the content of the experiments and the risks involved.

Study design

Subjects participated in a non-randomized single-blinded study involving two preliminary sessions and two 24-h experimental sessions (**Figure 1**). All sessions were held in a normobaric hypoxic facility (b-CAT, Tiel, The Netherlands) at the KU Leuven Athletic Performance Center. This hypoxic facility contains 5 double bedrooms, an exercise room, and a major living room.

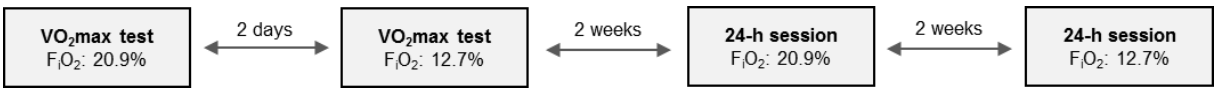

Figure 1 | Study design.
Two preliminary sessions served to measure maximal oxygen uptake (VO_2max) in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%), whereas two subsequent 24-h sessions in normoxia and hypoxia served to identify physiological parameters related to AMS development.

The first preliminary session was done in normoxia (F_iO_2 = 20.9%). Two rest days later, a second identical preliminary session was done in hypoxia (F_iO_2 = 12.7%). These sessions served to measure baseline values for some primary physiological responses to submaximal and maximal exercise in both normoxia and acute hypoxia. After an overnight fast, subjects reported to the laboratory between 8 and 11 a.m. for a maximal incremental cycling test (Avantronic Cyclus II, Leipzig, Germany). Initial workload was 70 W and was increased by 30 W every 4 min until volitional exhaustion was reached. Heart rate (Polar RS800cx, Kempele Finland), arterial oxygen saturation (Nellcor N-600x, Oxismart, Mallinckrodt, St Louis, Missouri), minute ventilation, oxygen consumption and carbon dioxide production (Cortex® Metalyzer IIIb, Leipzig, Germany) were continuously measured throughout the test. The highest oxygen uptake measured over a 30-s period was considered as the maximal oxygen uptake (VO_2max). Maximal power output was calculated as 30 W multiplied by the fraction of the final stage completed, added to the last workload step completed. A capillary blood sample was taken from the earlobe at the end of each step to measure blood lactate concentration (Lactate Pro 1, Akray). Power output and heart rate corresponding to 2 and 4 $mmol \cdot L^{-1}$ blood lactate were extrapolated on the power/heart rate-lactate curves.

The experimental sessions served to investigate the relationship between a series of physiological responses to standardized rest and submaximal cycling exercise at 1.5 $W \cdot kg^{-1}$ on the one hand, and the development of AMS during 24 h of hypoxic exposure on the other hand. The first experimental day was held in normoxia (F_iO_2: 20.9%). Two weeks later, the second experimental day was held in hypoxia (F_iO_2: 12.7%, ~4000 m).

The subjects spent the night before each experimental day at the athletic performance center in normoxia. Wake-up call was at 7 a.m. At 8 a.m. the subjects received a standardized light breakfast (~616 kcal, 74% carbohydrates, 11% fat, 15% protein). They eventually entered the hypoxic facility at 9 a.m. and left the hypoxic facility at 9 a.m. the next morning (see **Figure 2**). They received a standardized lunch (~641 kcal, 69% carbohydrates, 14% fat, 17% protein) at 1 pm. Also snacks (~917 kcal, 74% carbohydrates, 20% fat, 6% protein) and fluid intake (3.4 L in 24 h) throughout the day were standardized. The subjects were allowed to move freely around within the hypoxic facility, yet most of the time they rested in comfortable chairs and couches. However, between 11 a.m. and 5:30 p.m., the subjects intermittently performed five 30-min submaximal constant-load cycling bouts at 1.5 $W \cdot kg^{-1}$. Dinner (~962 kcal, 32% carbohydrates, 48% fat, 20% protein) was served at 8 p.m. and night's rest was between 11.30 p.m. and 7 a.m. Throughout the experimental day AMS symptoms were closely monitored, and a series of physiological measurements that were conceivably associated with the development of AMS were measured at regular intervals (see details below).

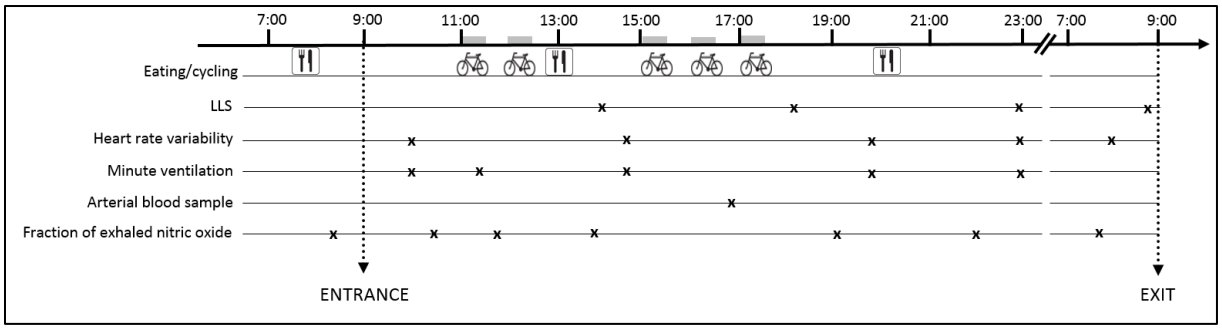

Figure 2 | Schematic design of the 24-h experimental session in normoxia and hypoxia.
Subjects were awaken at 7 a.m. and consumed a standardized breakfast. Fraction of exhaled nitric oxide was determined at 8.30 a.m. Subjects entered the normobaric hypoxic facility at 9 a.m. to stay till 9 a.m. next morning. F_iO_2 was either 20.9% (normoxia) or 12.7% (hypoxia). The subjects performed five submaximal cycling bouts (1.5 $W \cdot kg^{-1}$) between 1 and 8 p.m. Minute ventilation, heart rate variability, arterial blood gases, and fraction of exhaled nitric oxide were measured before and after exercise at different time-points throughout the day. The Lake Louise consensus scoring system (LLS) was used to diagnose AMS severity at regular intervals.

AMS severity - Self-reported Lake Louise consensus scoring system (LLS) was used to assess symptoms and severity of AMS [8]. Subjects were asked to rate 5 symptoms on a scale from 0 (no symptoms) to 3 (severe, incapacitating symptoms). Symptoms were 'headache', 'gastro-intestinal distress', 'fatigue/weakness', 'dizziness/light-headedness', and 'sleep difficulty'. Subjects completed the LLS after 5, 9, 14 and 24 h of hypoxic exposure. We considered LLS scores of 3-5 as *mild* AMS, and LLS scores ≥ 6 as *severe* AMS. Comparison was made between subjects scoring < 6 (AMS_{LOW}) *vs*. subjects scoring ≥ 6 (AMS_{HIGH}) on the LLS at some time point throughout the 24-h hypoxic trial.

Submaximal cycling exercises and concomitant measurements of ventilation and arterial oxygen saturation – The subjects performed two 30-min submaximal exercise bouts on a cycling ergometer (Avantronic Cyclus II, Leipzig, Germany) in the morning and three similar exercise bouts in the afternoon. The cycling workload of 1.5 $Watt \cdot kg^{-1}$ was chosen to correspond with an ascent rate of 300 elevation meters per hour in mountaineering [69]. S_pO_2 was continuously measured with an optode placed 2 cm above the eyebrow (Nellcor N-600x, Oximart, Mallinckrodt, St Louis, Missouri). Heart rate was recorded with a heart rate monitor (Polar RS800cx, Kempele Finland). A capillary blood sample from the earlobe for determination of lactate concentration (Lactate Pro 1, Akray) was taken before and at the end of each cycling bout. Minute ventilation (Cortex® Metalyzer IIIb, Leipzig, Germany) was recorded during the final 15 min of the first and final cycling bout of the day.

Arterial blood gases – At 8 h in the hypoxic facility, prior to the final cycling bout, an arterial blood sample (0.7 – 1.5 mL) was taken via puncture of the radial artery and analysed for pH, P_aO_2, S_aO_2, P_aCO_2, and HCO_3^- using an automated acid-base laboratory (ABL90 Flex blood gas analyser, Radiometer, Copenhagen, Denmark).

Heart rate variability - HRV was recorded 1, 6, 11, 14 and 23 h after entering the hypoxic facility. Polar RS800cx heart rate monitor (Polar Electro Oy, Kempele, Finland) was used for RR interval recordings at a sampling rate of 1000 Hz. Recordings from this monitor have previously been shown interchangeable with ECG-derived RR recordings and HRV analysis in men [70-73]. All measurements were performed in a quiet environment with the subjects lying in the supine position. Subjects were instructed to keep their eyes closed, yet sleeping was not allowed. HRV recordings were performed during 5 min of spontaneous breathing, and subsequently during 5 min of paced (auditory

signal) breathing at a rate of 12 breaths·min^{-1}. All HRV recordings, except at 23 h in the hypoxic facility, were performed in conjunction with measurements of minute ventilation (Cortex ® Metalyzer IIIB, Leipzig, Germany), which required the subjects to wear a face mask. VarCOr PF7 software was used to compute time-domain and frequency-domain HRV parameters as previously described elsewhere [74]. Data of interest from the time-domain were: the square root of the mean squared differences between adjacent normal RR intervals (RMSSD), the number of pairs of adjacent RR-intervals that differ more than 50 ms (NN50), and NN50 in relation to the total number of RR-intervals (pNN50). Frequency domain analysis was performed using Fast Fourier transform. Parameters of the frequency domain included low frequency power (LF, 0.04–0.15Hz), high frequency power (HF, 0.15–0.40 Hz), total power (0 to 0.40 Hz) and the LF/HF ratio. At 8 and 11 h in hypoxia, two subjects were withdrawn from the hypoxic environment because of severe AMS. Unfortunately, due to technical malfunctions, three measurements are missing in the hypoxic trial at 23 h (2 missing in AMS_{LOW}; 1 missing in AMS_{HIGH}).

Exhaled nitric oxide – The fraction of exhaled nitric oxide (FENO) was measured using a hand-held electrochemical analyser (NObreath, Bedfont Scientific Ltd., Kent, England) according to the ATS/ERS guidelines [75]. Measurements were performed before and 1.5, 2.5, 5, 10, 13 and 22 h after entering the hypoxic facility. Each measurement was performed in duplicate from which the mean was taken as the final result.

Statistical analysis

Statistical calculations were performed using SigmaStat (V3.5, Systat software, Chicago, IL), IBM SPSS Statistics (V23.0, Chicago, IL), and SAS (V7.13, SAS Institute, Cary, NC, USA) software. A two-way repeated measures ANOVA was used to analyse 'condition' (normoxia *vs*. hypoxia; within subject variable) and 'group' effects (AMS_{LOW} *vs*. AMS_{HIGH}; between subject variable), i.e. to test differences in parameters of the incremental exercise test, arterial blood gases, and minute ventilation. HRV differences between the AMS subgroups in normoxic and hypoxic conditions were analysed by two-way repeated-measures ANOVA (factor condition: normoxia *vs*. hypoxia; factor group: AMS_{LOW} *vs*. AMS_{HIGH}) run separately for each time point. To analyse divergent HRV responses over time between the AMS subgroups, two-way repeated measures ANOVA with factors group (AMS_{LOW} *vs*. AMS_{HIGH}) and time (1 *vs*. 6 *vs*. 11 *vs*. 14 *vs*. 23 h) were run separately for normoxia and hypoxia (n=11). Because of their skewed distribution, parameters of the frequency domain were normalized with natural logarithmic transformation. A three-way repeated measures ANOVA was used to analyse 'condition' effects, 'group' effects, and 'exercise' effects (rest *vs*. exercise, within subject variable) to test differences in S_pO_2 or blood lactate values before *vs*. during submaximal cycling. A Bonferroni post-hoc analysis was performed whenever appropriate. To evaluate whether physiological parameters assessed during early exposure to normoxia/hypoxia (up to and including the first submaximal exercise bout) could predict peak LLS scores, variables correlating with peak LLS scores were entered in a multiple linear regression model with stepwise selection ($P < 0.05$). A similar procedure was applied to identify predictors of the sum of 24-h LLS scores (∑LLS). In two subjects who were withdrawn from the hypoxic facility due to severe AMS, the ∑LLS was calculated by replacing their missing values by their last LLS score prior to leaving the hypoxic facility. Results are presented as means ± standard error of mean (SEM) unless stated otherwise. Significance level was set at $P < 0.05$.

Results

Incidence of AMS

LLS scores after 5, 9, 14 and 24 h of hypoxic exposure were 1.8 ± 0.4 (n=16), 4.9 ± 0.7 (n=16), 3.6 ± 0.4 (n=14) and 4.7 ± 0.6 points (n=14), respectively. Nine subjects were diagnosed with mild AMS (LLS score 3-5, AMS_{LOW}) at some point throughout the day. Seven subjects were diagnosed with severe AMS (LLS ≥ 6, AMS_{HIGH}), of which six with LLS scores ≥ 9. One subject in AMS_{HIGH} was withdrawn from the hypoxic facility after 8 h of hypoxic exposure because of neurological dysfunctions (ataxia, loss of hand-eye coordination), yet we included this measurement as a 9 h score. Another subject in AMS_{HIGH} requested to leave the hypoxic facility after 11 h due to incapacitating headache and gastro-intestinal distress (vomiting).

Effects of hypoxia during an incremental VO_2max test (Table 1)

In the preliminary sessions the subjects performed an incremental VO_2max test in both normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). In hypoxia at submaximal workloads power outputs corresponding to 2 and 4 mmol·L^{-1} blood lactate levels were ~30-40% lower than in normoxia while heart rate was reduced by ~14 beats·min^{-1} ($P < 0.05$). In the total group of subjects, hypoxia on average decreased VO_2max by ~25% (range: 11-35%, $P < 0.05$). Peak heart rate was 7-8 (range: +1 to -15) beats·min^{-1} lower ($P < 0.05$) while bottom arterial oxygen saturations on average dropped from 95% (range: 91-97) in normoxia to 73% (range: 65-80) in hypoxia ($P < 0.05$). Compared to normoxia peak pulmonary ventilation increased by ~15 L·min^{-1} ($P < 0.05$). Hypoxia also slightly increased peak lactate concentrations from ~9.4 to ~10.5 mmol·L^{-1} ($P < 0.05$). Values were significantly different between AMS_{LOW} and AMS_{HIGH} for none of the above variables.

Table 1 | Submaximal and maximal parameters during the incremental exercise test in normoxia and hypoxia

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
	n=16	*n=9*	*n=7*	*n=16*	*n=9*	*n=7*
SUBMAXIMAL						
2 mmol lactate·L^{-1}						
Power (W)	201 ± 11	205 ± 11	196 ± 22	120 ± 7*	128 ± 9*	110 ± 12*
	(94-255)	(136-252)	(94-252)	(70-174)	(94-174)	(70-154)
Power (W·kg^{-1})	2.7 ± 0.2	2.8 ± 0.1	2.6 ± 0.3	1.6 ± 0.1*	1.7 ± 0.1*	1.4 ± 0.2*
	(1.4-3.8)	(2.1-3.3)	(1.4-3.8)	(1.0-2.3)	(1.3-2.3)	(1.0-2.1)
Heart rate (bpm)	155 ± 3	155 ± 5	154 ± 5	141 ± 3*	141 ± 3*	142 ± 5*
	(133-171)	(133-170)	(136-171)	(120-165)	(128-157)	(120-165)
VO_2 (mL·kg^{-1}·min^{-1})	36 ± 2	37 ± 2	34 ± 3	24 ± 1*	26 ± 1*	21 ± 2*
	(24-48)	(32-47)	(24-48)	(16-32)	(21-32)	(16-28)
V_E (L·min^{-1})	67 ± 4	68 ± 5	64 ± 7	55 ± 3*	60 ± 4*	49 ± 4*
	(41-86)	(46-86)	(41-86)	(34-76)	(44-76)	(34-65)
S_pO_2 (%)	97 ± 0	97 ± 0	97 ± 1	74 ± 1*	74 ± 1*	73 ± 2*
	(95-100)	(96-100)	(95-99)	(64-81)	(64-79)	(68-81)
4 mmol lactate·L^{-1}						
Power (W)	251 ± 10	259 ± 7	242 ± 22	169 ± 7*	178 ± 7*	157 ± 14*
	(134-297)	(222-287)	(134-297)	(99-214)	(148-214)	(99-206)
Power (W·kg^{-1})	3.4 ± 0.2	3.6 ± 0.1	3.2 ± 0.3	2.3 ± 0.1*	2.4 ± 0.1*	2.0 ± 0.2*
	(2.0-4.5)	(3.2-4.2)	(2.0-4.5)	(1.5-2.9)	(2.1-2.9)	(1.5-2.8)

Heart rate (bpm)	174 ± 3	176 ± 3	170 ± 4	160 ± 2*	160 ± 2*	159 ± 5*
	(152-186)	(152-186)	(154-185)	(134-174)	(145-168)	(134-174)
VO_2 ($mL \cdot kg^{-1} \cdot min^{-1}$)	42 ± 2	44 ± 1	40 ± 3	31 ± 1*	32 ± 1*	29 ± 3*
	(30-55)	(39-51)	(30-55)	(20-39)	(28-37)	(20-39)
V_E ($L \cdot min^{-1}$)	88 ± 5	88 ± 5	89 ± 9	76 ± 4*	80 ± 4	71 ± 7*
	(56-122)	(67-109)	(56-122)	(46-98)	(61-93)	(46-98)
S_pO_2 (%)	96 ± 0	97 ± 0	96 ± 1	73 ± 1*	73 ± 1*	73 ± 2*
	(94-100)	(95-100)	(94-99)	(66-79)	(66-76)	(66-79)
MAXIMAL						
Power (W)	306 ± 10	314 ± 7	295 ± 20	232 ± 7*	238 ± 7*	222 ± 12*
	(191-344)	(284-344)	(191-343)	(152-271)	(223-271)	(152-242)
Power ($W \cdot kg^{-1}$)	4.1 ± 0.2	4.3 ± 0.2	3.9 ± 0.3	3.2 ± 0.1*	3.4 ± 0.1*	2.9 ± 0.2*
	(2.9-5.1)	(3.6-5.1)	(2.9-5.0)	(2.3-3.9)	(2.8-3.9)	(2.3-3.6)
Heart rate (bpm)	188 ± 2	189 ± 3	187 ± 4	182 ± 2*	183 ± 2*	180 ± 4*
	(171-203)	(173-203)	(171-196)	(162-190)	(172-190)	(162-190)
VO_2 ($mL \cdot kg^{-1} \cdot min^{-1}$)	52 ± 2	54 ± 2	49 ± 3	38 ± 1*	39 ± 2*	36 ± 2*
	(41-67)	(44-67)	(41-64)	(29-46)	(35-46)	(29-43)
V_E ($L \cdot min^{-1}$)	144 ± 7	147 ± 10	139 ± 9	159 ± 7*	158 ± 13*	152 ± 10
	(90-185)	(109-185)	(90-154)	(94-198)	(107-198)	(94-175)
S_pO_2 (%)	95 ± 0.4	95 ± 1	94 ± 1	73 ± 1*	72 ± 1*	74 ± 2*
	(91-97)	(92-97)	(91-97)	(65-80)	(65-80)	(65-79)
Lactate ($mmol \cdot L^{-1}$)	9.4 ± 0.6	9.4 ± 1.0	9.3 ± 0.6	10.5 ± 0.7*	10.6 ± 1.0*	10.5 ± 0.9
	(5.4-12.7)	(5.4-12.7)	(7.1-11.2)	(6.0-16.4)	(6.0-16.4)	(7.6-14.1)

*Data are mean ± SEM (range in brackets) and represent values corresponding to 2 and 4 $mmol \cdot L^{-1}$ and peak blood lactate levels during an incremental exercise cycling test to exhaustion in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects (All, n=16), as well as for subgroups AMS_{LOW} (LLS < 6, n=9) and AMS_{HIGH} (LLS ≥ 6, n=7). VO_2, oxygen uptake; V_E, minute ventilation, S_pO_2, arterial oxygen saturation. * P < 0.05 vs. normoxia.*

Arterial oxygen saturation, heart rate, and blood lactate during submaximal exercise

We separately report data for exercise bout 1 and exercise bouts 2-5 because (1) we used observations in exercise bout 1 to predict the development of AMS in a later stage, and (2) because exercise intolerance in hypoxia caused dropouts beyond bout 2.

Exercise bout 1 – In bout 1 S_pO_2 in normoxia on average was ~99% (range: 95-100%) both at rest and during exercise (P > 0.05). Hypoxia on average decreased S_pO_2 to ~85% at rest (range: 78-92%), and further to ~79% during exercise (range: 73-84%) (P < 0.05) (**Figure 2**). Compared to AMS_{LOW}, S_pO_2 was lower in AMS_{HIGH} during both rest (AMS_{HIGH}: ~82%, range: 78-86; AMS_{LOW}: ~87%, range: 81-92%) and exercise (AMS_{HIGH}: ~77%, range: 73-80%; AMS_{LOW}: ~82%, range: 78-84%) (P < 0.05). Exercise did not significantly raise blood lactate level (~1.0 $mmol \cdot L^{-1}$) in normoxia. Conversely, in hypoxia, blood lactate increased in both the groups (P < 0.05), increasing more in AMS_{HIGH} (~3.3 $mmol \cdot L^{-1}$, range: 1.2-5.4 $mmol \cdot L^{-1}$) than in AMS_{LOW} (~1.9 $mmol \cdot L^{-1}$, range: 1.1-3.2 $mmol \cdot L^{-1}$, P < 0.05) (**Figure 2**). Compared to normoxia (71 ± 2 bpm, range: 54-96 bpm), pre-exercise resting heart rate was higher in hypoxia (82 ± 2 bpm, range: 56-115 bpm, P < 0.05). Also exercise heart rates were higher in hypoxia (144 ± 2 bpm, range: 121-165 bpm, P < 0.05) than in normoxia (124 ± 3 bpm, range: 97-153 bpm), yet there were no significant differences between AMS_{HIGH} and AMS_{LOW} at any time.

Exercise bouts 2-5 – In normoxia, all subjects comfortably completed the 5 exercise bouts as prescribed by the study protocol (30 min at 1.5 $W \cdot kg^{-1}$). Conversely, in hypoxia all subjects were able to complete the bouts 1-2, indeed, yet from bout 3 either exercise intensity had to be reduced in some, whilst others abandoned. In AMS_{LOW} all subjects completed all cycling bouts as prescribed by the protocol. Conversely, 5 out of 7 subjects in AMS_{HIGH} were unable to maintaining the power output required (1.5 $W \cdot kg^{-1}$). One subject interrupted exercising in bout 3 after 5 min because of exacerbation of AMS-related symptoms (primarily headache) and soon after was exported from the hypoxic facility due to the development of neurological symptoms. One subject interrupted the exercise halfway bout 3 due to exacerbating headache and was unable to start in bouts 4-5. In one subject workload had to be reduced to 1.2 $Watt \cdot kg^{-1}$ from 10 min in bout 3, and in another subject from 5 min in bout 4, yet they both completed the exercise protocol at this lower intensity. One subject was afflicted by a syncope just prior to the start of bout 5. Because of this large dropout and different exercise durations and intensities performed, we only analysed the pre-exercise values. In normoxia mean SpO_2 in all subjects was constant at 98 ± 2%. In hypoxia pre-exercise S_pO_2 levels dropped to 87 ± 1% (range: 82-93%) in AMS_{LOW} and even further (83 ± 1%, range: 77-93%) in AMS_{HIGH} ($P < 0.05$). Mean pre-exercise heart rates on average were ~10-15 bpm higher in hypoxia (mean ~88 bpm) than in normoxia (mean ~75 bpm). In addition, compared with AMS_{HIGH} (94 ± 3 bpm, range: 57-118 bpm) heart rates prior to exercise bouts 2-5 in AMS_{LOW} were lower (bout 2, $P < 0.05$), or tended to be lower (bout 3, $P = 0.09$) in AMS_{LOW} (84 ± 2 bpm, range: 55-106 bpm). Pre-exercise blood lactate in normoxia was similar between the groups at all times (~1.0 $mmol \cdot L^{-1}$). Conversely, in hypoxia pre-exercise lactate in AMS_{LOW} increased in bout 2 (1.5 ± 0.1 $mmol \cdot L^{-1}$, $P < 0.05$) and 3 (1.4 ± 0.2 $mmol \cdot L^{-1}$, $P < 0.05$), but not in bouts 4 (1.0 ± 0.1 $mmol \cdot L^{-1}$) and 5 (1.0 ± 0.1 $mmol \cdot L^{-1}$). Compared with AMS_{LOW}, in AMS_{HIGH} blood lactate was even higher in bouts 2 (2.0 ± 0.21 $mmol \cdot L^{-1}$, $P < 0.05$) and 5 (1.5 ± 0.2 $mmol \cdot L^{-1}$, $P < 0.05$), but not in bouts 3 (1.5 ± 0.2 $mmol \cdot L^{-1}$) and 4 (1.2 ± 0.1 $mmol \cdot L^{-1}$).

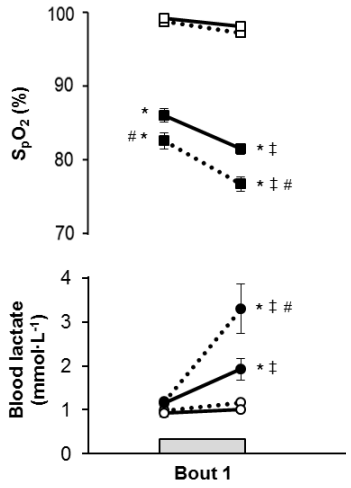

Figure 2 | Arterial oxygen saturation and blood lactate at rest and during submaximal cycling exercise in normoxia and hypoxia.

Data are mean ± SEM and represent arterial oxygen saturation (S_pO_2, squares) and blood lactate concentration (circles) before and after 30 min cycling at 1.5 $W \cdot kg^{-1}$ (bout 1) in either normoxia (open symbols, F_iO_2: 20.9%) or hypoxia (solid symbols, F_iO_2: 12.7%), in AMS_{LOW} (n=9, solid lines) or AMS_{HIGH} (n=7, dashed lines).

** P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; ‡ P < 0.05 vs. resting conditions.*

Arterial blood gases

Arterial blood was sampled at rest at 8 h in the hypoxic facility **(Table 2)**. Compared to normoxia, hypoxia decreased P_aO_2 from ~94 to ~43 mmHg (P < 0.05), irrespective of AMS. S_aO_2 dropped from ~98% in normoxia to ~83% in hypoxia. However, the decrease was greater in AMS_{HIGH} than in AMS_{LOW}: SaO_2 dropped to 80 ± 2% (range: 74-86) in the former *versus* 85 ± 1% (range: 77-90%) in the latter (P < 0.05 for interaction and post-hoc group effects). P_aCO_2 was ~43 mmHg in normoxia and dropped to ~39 mmHg (P < 0.05) in hypoxia. This drop in P_aCO_2 was more explicit in AMS_{LOW} than in AMS_{HIGH} (P < 0.05 interaction effect).

Table 2 | Arterial blood gases at rest after 8 h in normoxia and hypoxia

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
	n=16	*n=9*	*n=7*	*n=16*	*n=9*	*n=7*
P_aO_2 (mmHg)	94 ± 2	95 ± 3	93 ± 3	43 ± 1*	45 ± 2*	41 ± 2*
	(71-109)	(71-105)	(84-109)	(36-51)	(38-51)	(36-47)
S_aO_2 (%)	98 ± 0	98 ± 0	98 ± 0	83 ± 1*§	85 ± 1*	80 ± 2*#
	(95-99)	(95-99)	(97-99)	(74-90)	(77-90)	(74-86)
P_aCO_2 (mmHg)	43 ± 1	43 ± 1	43 ± 1	39 ± 1*§	38 ± 1*	41 ± 1*
	(39-49)	(41-49)	(39-47)	(36-51)	(34-44)	(38-43)
pH	7.412 ± 0.003	7.409 ± 0.003	7.415 ± 0.006	7.442 ± 0.004*§	7.448 ± 0.006*	7.435 ± 0.004*
	(7.395-7.439)	(7.395-7.422)	(7.397-7.439)	(7.415-7.477)	(7.415-7.477)	(7.421-7.451)
HCO_3^- (mmol·L^{-1})	26.5 ± 0.2	26.4 ± 0.3	26.5 ± 0.4	26.2 ± 0.2	26.0 ± 0.3	26.3 ± 0.3
	(25.1-28.0)	(25.4-28.0)	(25.1-27.8)	(24.4-27.4)	(24.4-27.0)	(25.0-27.4)

*Data are mean ± SEM and represent arterial oxygen partial pressure (P_aO_2), arterial oxygen saturation (S_aO_2), arterial carbon dioxide partial pressure (P_aCO_2), pH, and bicarbonate (HCO_3^-) at rest in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects (All, n=16), as well as for subgroups including AMS_{LOW} (LLS < 6, n=9) and AMS_{HIGH} (LLS ≥ 6, n=7). * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; § P < 0.05 group (AMS_{LOW} vs. AMS_{HIGH}) x condition (normoxia vs. hypoxia) interaction.*

Ventilation

Ventilation after 1 h – Minute ventilation (V_E) was measured in the supine position concurrent with the HRV recordings during both spontaneous and paced (12 breaths·$minute^{-1}$) breathing at rest, as well as during the final 15 min of exercise bout 1 (**Table 3**). Compared to normoxia, one hour of hypoxic exposure increased resting V_E during spontaneous breathing in AMS_{LOW} (+2.5 L·min^{-1}, P < 0.05) but not in AMS_{HIGH} (+1.3 L·min^{-1}, P = 0.16). Thus in hypoxia, but not in normoxia (P = 0.23), spontaneous V_E at rest was higher in AMS_{LOW} than in AMS_{HIGH} (P < 0.05). Compared to spontaneous breathing, pacing increased V_E in AMS_{LOW}, but only in normoxia (P < 0.05). Pacing did not alter V_E in AMS_{HIGH}. Compared to normoxia, hypoxia raised exercise V_E by ~21% (P < 0.05), irrespective of the degree of AMS.

Pulmonary ventilation after 6, 11, and 14 h – Pulmonary ventilation during spontaneous breathing in AMS_{LOW} increased from normoxia to hypoxia at 6 (10.2 ± 1.2 to 12.8 ± 0.8 L·min^{-1}, P < 0.05) and 11 h (11.5 ± 1.3 to 14.7 ± 1.9 L·min^{-1}, P < 0.05), but not at 14 h (12.5 ± 1.5 to 13.5 ± 1.6

L·min^{-1}, P > 0.05) in the hypoxic facility. In AMS_{HIGH}, ventilation during spontaneous breathing increased from normoxia to hypoxia at 6 h (9.2 ± 0.4 to 11.5 ± 0.8 L·min^{-1}, P < 0.05), but not at 11 h (9.9 ± 0.2 to 11.2 ± 0.9 L·min^{-1}, P = 0.31) and 14 h (9.8 ± 0.3 to 10.7 ± 0.5 L·min^{-1}, P > 0.05) in the hypoxic facility. Spontaneous ventilation did not significantly differ between groups at any time (P > 0.05). Pulmonary ventilation during paced breathing in AMS_{LOW} tended to increase from normoxia to hypoxia at 6 (13.8 ± 1.5 to 16.3 ± 1.8 L·min^{-1}, P = 0.07), 11 (15.8 ± 1.8 to 20.2 ± 3.5 L·min^{-1}, P < 0.05) and 14 h (15.3 ± 1.8 to 18.2 ± 2.9 L·min^{-1}, P = 0.07) in the hypoxic facility. Conversely, in AMS_{HIGH}, ventilation during paced breathing remained constant from normoxia to hypoxia at 6 (9.8 ± 0.6 to 10.9 ± 0.9 L·min^{-1}, P > 0.05), 11 (11.2 ± 0.7 to 10.9 ± 0.7 L·min^{-1}, P > 0.05), and 14 h (10.4 ± 0.9 to 10.4 ± 0.4 L·min^{-1}, P > 0.05) in the hypoxic facility. Therefore, in hypoxia, but not normoxia, paced ventilation was higher in AMS_{LOW} than AMS_{HIGH} at all times (P < 0.05). In normoxia, compared to spontaneous breathing, paced breathing increased minute ventilation at 6, 11, and 14 h in AMS_{LOW} (P < 0.05 at all times) but not AMS_{HIGH} (P > 0.05 at all times). Similarly in hypoxia, compared to spontaneous breathing, paced breathing increased minute ventilation in AMS_{LOW} at 6 (P = 0.08), 11 (P > 0.05), and 14 h (P > 0.05), but never in AMS_{HIGH} (P > 0.05 at all times).

Table 3 | Minute ventilation, tidal volume and breathing frequency in normoxia and hypoxia at rest and during submaximal cycling exercise.

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
	n=16	*n=9*	*n=7*	*n=16*	*n=9*	*n=7*
REST - SPONTANEOUS						
V_E (L·min^{-1})	10.5 ± 0.4	11.1 ± 0.5	9.7 ± 0.6	12.5 ± 0.8*	13.6 ± 1.1*	11.0 ± 0.8#
	(7.9-13.1)	(8.5-13.1)	(7.9-12.4)	(8.4-18.5)	(8.6-18.5)	(8.4-15.3)
V_T (mL)	823 ± 52	869 ± 84	762 ± 46	1008 ± 84*	1075 ± 119*	923 ± 118
	(573-1309)	(573-1309)	(588-941)	(603-1677)	(603-1677)	(611-1420)
f (breaths·min^{-1})	13.9 ± 0.9	14.1 ± 1.4	13.6 ± 1.3	14.2 ± 1.0	14.9 ± 1.3	13.3 ± 1.5
	(6.7-20.9)	(6.7-20.9)	(9.2-18.8)	(7.1-19.7)	(7.1-19.7)	(7.6-17.8)
REST - PACED						
V_E (L·min^{-1})	11.6 ± 0.8	13.2 ± 1.1†	9.5 ± 0.3#	12.4 ± 0.8	14.2 ± 1.1	10.1 ± 0.6#
	(8.6-19.2)	(9.9-19.2)	(8.6-11.0)	(8.5-18.1)	(9.3-18.1)	(8.5-12.8)
V_T (mL)	956 ± 66	1088 ± 95†	786 ± 27#	1029 ± 72	1188 ± 94	824 ± 46#
	(698-1601)	(760-1601)	(698-908)	(696-1500)	(774-1500)	(696-1071)
f (breaths·min^{-1})	12.2 ± 0.1	12.2 ± 0.2	12.2 ± 0.1	12.1 ± 0.2	12.0 ± 0.2†	12.4 ± 0.3
	(11.7-13.3)	(11.8-13.3)	(11.7-12.4)	(10.9-13.8)	(10.9-13.2)	(11.9-13.8)
EXERCISE						
V_E (L·min^{-1})	48.0 ± 1.6	48.2 ± 2.3	47.6 ± 1.6	57.9 ± 2.6*	58.8 ± 2.9*	56.4 ± 4.5*
	(38.6-62.2)	(38.6-62.2)	(42.8-53.1)	(40.2-77.5)	(42.6-77.5)	(40.2-69.6)
V_T (mL)	1886 ± 63	1818 ± 89	1973 ± 85	2109 ± 97*	2103 ± 132*	2117 ± 153
	(1500-2241)	(1500-2149)	(1640-2241)	(1564-2801)	(1680-2801)	(1564-2562)
f (breaths·min^{-1})	26.1 ± 1.0	27.3 ± 1.6	24.6 ± 0.9	28.1 ± 1.1*	28.9 ± 1.5	27.1 ± 1.7*
	(18.7-35.0)	(18.7-35.0)	(21.4-27.9)	(22.1-35.3)	(22.4-35.3)	(22.1-34.1)

Data are mean ± SEM and represent minute ventilation (V_E), tidal volume (V_T) and breathing frequency (f) after one hour at rest and during submaximal cycling at 1.5 W·kg^{-1} in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects (All, n=16), as well as for

*subgroups including AMS_{LOW} (LLS < 6, n=9) and AMS_{HIGH} (LLS ≥ 6, n=7). * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; † P < 0.05 vs. spontaneous breathing.*

Fraction of exhaled nitric oxide

Baseline FENO was measured just before entering the hypoxic facility for either the 24-h normoxic or hypoxic trial. Unexpectedly, baseline values were slightly different between the two 24-h trials (pre-normoxic trial: 14.1 ± 1.8 ppb; pre-hypoxic trial: 9.8 ± 1.9 ppb; P < 0.05). FENO values were significantly altered neither by the experiments in normoxia, nor in hypoxia, and there were no differences between AMS_{LOW} and AMS_{HIGH} at baseline prior to the hypoxic trial (11.9 ± 2.9 ppb, range: 2.0-28.0 ppb; 7.5 ± 1.7 ppb, range: 3.0-16.5 ppb, P = 0.24, respectively) or at any other time.

Heart rate variability

HRV during spontaneous breathing (**Table 4-5**) – Despite great inter-individual variability in HRV, a clear AMS-specific pattern became apparent. In AMS_{HIGH}, parameters indicating vagal heart rate modulation (RMSSD, NN50, pNN50, LnHF) were consistently reduced throughout the 24 h of hypoxic exposure (P < 0.05). Conversely, withdrawal of vagal activity was markedly less in AMS_{LOW}, and largely normalized within 14 h of the hypoxic exposure. To compare these time-dependent HRV responses between the AMS subgroups, a separate two-way repeated-measures ANOVA (factor time: 1 *vs.* 6 *vs.* 11 *vs.* 14 *vs.* 23 h; factor group: AMS_{LOW} *vs.* AMS_{HIGH}) was run for normoxia and hypoxia (n=11). The time course of LnHF, and LnTP in hypoxia was significantly different between the AMS subgroups in hypoxia, but not in normoxia (P < 0.05 interaction effect, **Figure 3**).

HRV during paced breathing (supplemental material) – A similar albeit less explicit hypoxia-induced drop of vagal activity was observed during paced breathing in AMS_{HIGH}. Hypoxia decreased RMSSD and LnHF in AMS_{HIGH} (P < 0.05), but not in AMS_{LOW}. Furthermore, NN50 and pNN50 were consistently reduced throughout the 24-h hypoxic experiment in AMS_{HIGH}, whilst values normalized within 6-11 h in AMS_{LOW}. Except for the LF/HF ratio, which in AMS_{HIGH} in hypoxia increased over time compared to AMS_{LOW} (P < 0.05), no other time x group interaction effects were found for the HRV measurements during paced breathing. In fact, compared to spontaneous breathing, paced breathing partially abolished the capacity of HRV analyses to distinguish between AMS susceptible and non-susceptible individuals.

Table 4 | Heart rate variability analysis in the time domain during spontaneous breathing in normoxia and hypoxia.

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
Heart rate (bpm)						
1 h (n=16)	61 ± 2	59 ± 3	65 ± 3	66 ± 2	63 ± 3	70 ± 4
	(42-77)	(42-74)	(54-77)	(51-81)	(51-75)	(56-81)
6 h (n=16)	54 ± 2	53 ± 3	55 ± 4	71 ± 3*§	66 ± 3*	78 ± 5*#
	(43-71)	(43-64)	(45-71)	(52-90)	(52-77)	(56-90)
11 h (n=15)	58 ± 3	56 ± 4	61 ± 4	73 ± 3*	71 ± 4*	77 ± 5*
	(43-78)	(43-70)	(50-78)	(53-92)	(53-85)	(57-92)
14 h (n=14)	61 ± 3	61 ± 5	61 ± 5	73 ± 3*	70 ± 3*	78 ± 6*

	(44-86)	(44-86)	(48-73)	(54-91)	(54-84)	(56-91)
23 h (n=11)	53 ± 3	52 ± 3	55 ± 5	69 ± 4*	64 ± 4*	78 ± 8*
	(43-65)	(43-65)	(46-65)	(48-90)	(48-78)	(54-90)
RMSSD (ms)						
1 h (n=16)	95 ± 17	103 ± 28	84 ± 14	73 ± 12*	91 ± 18	49 ± 10*
	(35-316)	(35-316)	(49-153)	(23-216)	(34-216)	(23-92)
6 h (n=16)	133 ± 19	129 ± 31	138 ± 18	67 ± 11*	72 ± 15	61 ± 17*
	(41-334)	(41-334)	(73-225)	(11-171)	(32-171)	(11-140)
11 h (n=15)	134 ± 26	131 ± 39	139 ± 30	52 ± 10*	59 ± 13	42 ± 17
	(5-396)	(5-396)	(62-247)	(17-136)	(17-136)	(17-125)
14 h (n=14)	105 ± 20	98 ± 27	117 ± 33	55 ± 17*	80 ± 23	19 ± 2*
	(21-287)	(21-287)	(44-234)	(11-216)	(19-216)	(11-24)
23 h (n=11)	100 ± 16	96 ± 24	107 ± 22	58 ± 18*§	84 ± 23	13 ± 2*
	(11-186)	(11-186)	(70-169)	(9-202)	(20-202)	(9-18)
NN50 (count)						
1 h (n=16)	136 ± 15	145 ± 22	124 ± 22	101 ± 17*	123 ± 22	72 ± 24*
	(32-258)	(32-258)	(73-228)	(4-204)	(37-204)	(4-168)
6 h (n=16)	189 ± 15	173 ± 23	210 ± 14	78 ± 15*	95 ± 20*	57 ± 20*
	(59-279)	(59-279)	(142-240)	(0-211)	(40-211)	(0-141)
11 h (n=15)	163 ± 21	152 ± 32	181 ± 24	55 ± 16*	69 ± 23*	34 ± 18*
	(0-279)	(0-279)	(95-243)	(3-220)	(4-220)	(3-117)
14 h (n=14)	137 ± 20	126 ± 27	157 ± 28	58 ± 20*	88 ± 27	6 ± 3*
	(4-237)	(4-232)	(68-237)	(0-207)	(1-207)	(0-17)
23 h (n=11)	132 ± 20	121 ± 30	152 ± 18	59 ± 22*§	93 ± 27	1 ± 1*#
	(4-223)	(4-223)	(118-199)	(0-192)	(8-192)	(0-3)
pNN50 (%)						
1 h (n=16)	45 ± 5	48 ± 7	41 ± 7	34 ± 6*	41 ± 7	24 ± 8*
	(11-86)	(11-86)	(24-76)	(1-68)	(12-68)	(1-56)
6 h (n=16)	63 ± 5	58 ± 8	70 ± 5	26 ± 5*	32 ± 7*	19 ± 7*
	(19-93)	(19-93)	(47-80)	(0-70)	(13-70)	(0-47)
11 h (n=15)	54 ± 7	51 ± 11	60 ± 8	18 ± 5*	23 ± 8*	11 ± 6*
	(0-93)	(0-93)	(32-81)	(1-73)	(1-73)	(1-39)
14 h (n=14)	46 ± 7	42 ± 9	52 ± 9	19 ± 7*	29 ± 9	2 ± 1*
	(1-79)	(1-77)	(23-79)	(0-69)	(0-69)	(0-6)
23 h (n=11)	44 ± 1	40 ± 10	51 ± 6	20 ± 7*§	31 ± 9	0.3 ± 0.3*#
	(1-74)	(1-74)	(39-66)	(0-64)	(3-64)	(0.0-1.0)

*Data are mean ± SEM (range in brackets) and represent heart rate, the square root of the mean squared differences between adjacent normal RR intervals (RMSSD), the number of pairs of adjacent RR-interval that differ more than 50 ms (NN50), and NN50 in relation to the total number of RR-intervals (pNN50) at rest during spontaneous breathing in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects, as well as for subgroups including AMS_{LOW} (LLS < 6) and AMS_{HIGH} (LLS ≥ 6). Analyses are performed with n=16 at 1 and 6 h, n=15 at 11 h, n=14 at 14 h, and n=11 at 23 h in the hypoxic facility. See METHODS for further details * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; § P < 0.05 group (AMS_{LOW} vs. AMS_{HGH}) x condition (normoxia vs. hypoxia) interaction.*

Table 5 | Heart rate variability analysis in the frequency domain during spontaneous breathing in normoxia and hypoxia.

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
LnLF (ms^2)						
1 h (n=16)	7.2 ± 0.2	7.0 ± 0.4	7.3 ± 0.2	6.8 ± 0.2§	7.2 ± 0.3	6.3 ± 0.3*
	(5.1-8.2)	(5.1-8.2)	(6.6-8.2)	(4.9-8.8)	(5.9-8.8)	(4.9-7.1)
6 h (n=16)	7.2 ± 0.2	7.0 ± 0.3	7.5 ± 0.3	6.9 ± 0.3	7.2 ± 0.3	6.4 ± 0.3*
	(5.6-8.5)	(5.6-8.5)	(6.5-8.5)	(4.9-8.6)	(5.9-8.6)	(4.9-7.7)
11 h (n=15)	6.9 ± 0.4	6.7 ± 0.7	7.2 ± 0.4	6.3 ± 0.3	6.3 ± 0.4	6.2 ± 0.4
	(2.0-9.4)	(2.0-9.4)	(6.0-8.3)	(4.9-8.4)	(4.9-8.4)	(5.2-7.8)
14 h (n=14)	6.8 ± 0.4	6.6 ± 0.6	7.3 ± 0.3	6.0 ± 0.5§	6.6 ± 0.7	4.9 ± 0.5*
	(3.5-9.6)	(3.5-9.6)	(6.7-8.2)	(3.2-9.1)	(3.2-9.1)	(3.5-6.3)
22 h (n=11)	7.3 ± 0.5	7.2 ± 0.8	7.6 ± 0.4	5.9 ± 0.5*§	6.7 ± 0.6	4.5 ± 0.6*#
	(2.8-9.4)	(2.8-9.4)	(6.8-8.5)	(3.1-8.5)	(5.0-8.5)	(3.1-5.8)
LnHF (ms^2)						
1 h (n=16)	7.6 ± 0.2	7.6 ± 0.4	7.6 ± 0.3	7.2 ± 0.3	7.6 ± 0.4	6.6 ± 0.5*
	(6.0-9.9)	(6.0-9.9)	(6.9-9.0)	(4.8-9.6)	(6.2-9.6)	(4.8-8.0)
6 h (n=16)	8.4 ± 0.2	8.2 ± 0.3	8.6 ± 0.3	7.0 ± 0.3*	7.3 ± 0.3	6.7 ± 0.7*
	(6.8-10.1)	(6.8-10.1)	(7.5-9.4)	(4.2-9.1)	(6.3-9.1)	(4.2-8.8)
11 (n=15)	7.9 ± 0.5	7.6 ± 0.8	8.4 ± 0.4	6.6 ± 0.3*	6.9 ± 0.4	6.2 ± 0.6*
	(1.9-10.5)	(1.9-10.5)	(7.1-9.7)	(4.4-8.8)	(5.4-8.8)	(4.4-8.7)
14 h (n=14)	7.7 ± 0.3	7.6 ± 0.4	8.0 ± 0.5	6.5 ± 0.4*§	7.2 ± 0.5	5.2 ± 0.4*#
	(6.2-9.7)	(6.2-9.7)	(6.2-9.5)	(3.6-9.2)	(5.6-9.2)	(3.6-5.8)
23 h (n=11)	7.4 ± 0.5	7.0 ± 0.8	8.0 ± 0.3	6.1 ± 0.6*§	7.2 ± 0.5	4.1 ± 0.3*#
	(3.1-9.0)	(3.1-8.9)	(7.4-9.0)	(3.3-9.5)	(5.2-9.5)	(3.3-4.6)
LnTP (ms^2)						
1 h (n=16)	8.4 ± 0.2	8.3 ± 0.4	8.5 ± 0.2	8.1 ± 0.2*§	8.5 ± 0.3	7.5 ± 0.3*#
	(6.8-10.2)	(6.8-10.2)	(7.9-9.3)	(6.4-9.8)	(7.1-9.8)	(6.4-8.3)
6 h (n=16)	8.8 ± 0.2	8.6 ± 0.3	9.1 ± 0.2	8.0 ± 0.3*	8.3 ± 0.3	7.6 ± 0.5*
	(6.7-10.1)	(6.7-10.1)	(8.4-9.8)	(5.7-9.7)	(7.5-9.7)	(5.7-9.2)
11 h (n=15)	8.4 ± 0.4	8.1 ± 0.6	8.9 ± 0.3	7.5 ± 0.3	7.6 ± 0.4	7.3 ± 0.5
	(3.6-10.5)	(3.6-10.5)	(8.2-9.9)	(6.3-9.4)	(6.3-9.1)	(6.3-9.4)
14 h (n=14)	8.4 ± 0.3	8.2 ± 0.4	8.7 ± 0.3	7.4 ± 0.4*§	8.0 ± 0.5	6.2 ± 0.2*#
	(6.5-10.4)	(6.5-10.4)	(7.9-9.7)	(5.8-9.5)	(5.9-9.5)	(5.8-6.9)
23 h (n=11)	8.4 ± 0.4	8.3 ± 0.7	8.7 ± 0.3	7.1 ± 0.5*§	8.1 ± 0.5	5.3 ± 0.4*#
	(4.7-9.8)	(4.7-9.8)	(8.1-9.5)	(4.4-9.9)	(6.0-9.9)	(4.4-6.3)
LF/HF						
1 h (n=16)	1.0 ± 0.2	0.7 ± 0.2	1.2 ± 0.5	1.5 ± 0.6	1.1 ± 0.3	2.1 ± 1.4
	(0.1-3.9)	(0.1-1.6)	(0.1-3.9)	(0.1-10.1)	(0.1-2.9)	(0.2-10.1)
6 h (n=16)	0.5 ± 0.1	0.6 ± 0.2	0.5 ± 0.2	1.1 ± 0.2*	1.2 ± 0.2*	1.1 ± 0.5*
	(0.0-1.3)	(0.0-1.3)	(0.1-1.2)	(0.3-3.5)	(0.4-2.4)	(0.3-3.5)
11 h (n=15)	0.9 ± 0.3	1.0 ± 0.5	0.7 ± 0.5	1.1 ± 0.4	0.7 ± 0.1	1.8 ± 0.8
	(0.0-4.3)	(0.0-4.3)	(0.0-3.1)	(0.1-5.3)	(0.1-1.4)	(0.3-5.3)
14 h (n=14)	0.9 ± 0.3	1.0 ± 0.5	0.8 ± 0.4	1.5 ± 0.5	1.4 ± 0.5	1.8 ± 1.0
	(0.0-4.5)	(0.0-4.5)	(0.1-2.4)	(0.1-5.5)	(0.1-4.8)	(0.1-5.5)
23 h (n=11)	1.3 ± 04	1.7 ± 0.6	0.7 ± 0.1	1.4 ± 0.4§	1.3 ± 0.6	1.7 ± 0.6*
	(0.3-4.7)	(0.3-4.7)	(0.4-0.9)	(0.1-4.9)	(0.1-4.9)	(0.9-3.4)

Data are mean ± SEM and represent the natural logarithmic transformation of total power (LnTP), low frequency power (LnLF), high frequency power (LnHF) and the ratio of LF/HF at rest during

*spontaneous breathing in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects, as well as for subgroups including AMS_{LOW} (LLS < 6) and AMS_{HIGH} (LLS ≥ 6). Analyses are performed with n=16 at 1 and 6 h, n=15 at 11 h, n=14 at 14 h, and n=11 at 23 h in the hypoxic facility. See METHODS for further details * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; § P < 0.05 group (AMS_{LOW} vs. AMS_{HGH}) x condition (normoxia vs. hypoxia) interaction.*

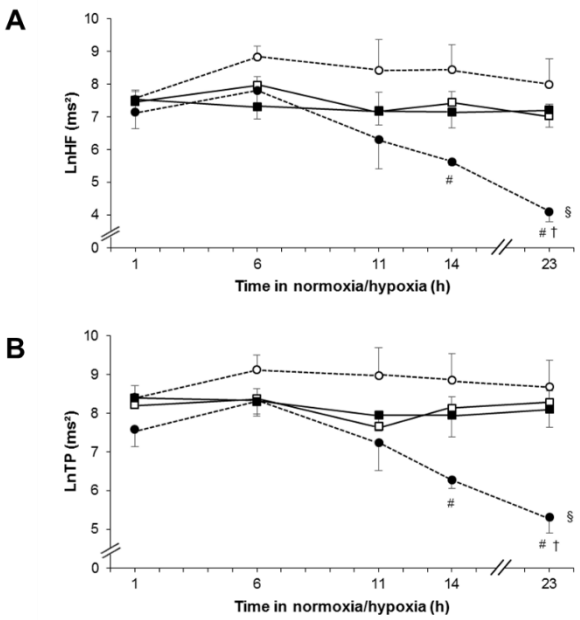

Figure 3 | HRV parameters showing a divergent response over time in hypoxia between AMS_{LOW} and AMS_{HIGH}
*Data are mean ± SEM and represent the natural logarithmic transformation of high (LnHF, **A**) and total frequency (LnTP, **B**) power at rest during spontaneous breathing in normoxia (F_iO_2: 20.9%, open symbols) and hypoxia (F_iO_2: 12.7%, solid symbols) in AMS_{LOW} (LLS < 6, n=7, squares with solid lines) and AMS_{HIGH} (LLS ≥ 6, n=4, circles with dashed lines). See METHODS for further details. # P < 0.05 vs. AMS_{LOW}; † P < 0.05 vs. 1 h; § P < 0.05 group (AMS_{LOW} vs. AMS_{HGH}) x time (1 vs. 6 vs. 11 vs. 14 vs. 23 h) interaction.*

Regression analyses to predict LLS scores

The regression analyses served to evaluate whether measurements during acute exposure to hypoxia (~2.5 h) and in the absence of severe AMS symptoms, can yield an adequate prediction of AMS development during sustained hypoxic exposure (24 h). In phase 1, to identify early predictors of peak LLS scores in hypoxia, all variables measured before (~rest) and during exercise bout 1, as well as VO_2max obtained from preliminary testing, were correlated with peak LLS scores. In phase 2, variables found to be differentiating in phase 1 (correlation P < 0.05) were integrated in a linear stepwise regression model. Hypoxic S_pO_2 at rest (r = -0.52) and during exercise (r = -0.73), hypoxic LnTP during spontaneous breathing (r = -0.54), as well as the normoxic-hypoxic difference in LnHF (r = 0.50) and LnTP (r = 0.66) during spontaneous breathing significantly correlated with peak LLS (P < 0.05). However, only S_pO_2 during submaximal exercise in hypoxia was retained in the stepwise regression model and predicted 53% of the peak LLS score according to the following formula:

Peak LLS score = 53.19 + (-0.59 * hypoxic S_pO_2 during submaximal exercise)

To account for the rate at which AMS developed, the same procedure was applied to identify predictors of the sum of LLS scores (∑LLS) throughout the 24-h hypoxic exposure. Hypoxic S_pO_2 at rest (r = -0.57) and during exercise (r = -0.54), the normoxic-hypoxic difference in LnTP during spontaneous breathing (r = 0.56), and lactate during submaximal exercise (r=0.53) significantly correlated with ∑LLS (P < 0.05). Still, only S_pO_2 at rest in hypoxia was retained in the stepwise regression model, which predicted 32% of ∑LLS according to the following formula:

∑LLS = 134.58 + (-1.39 * hypoxic S_pO_2 at rest)

Discussion

In this project young healthy volunteers were exposed to a 24-h episode of normobaric hypoxia equivalent to 4000 m altitude. A wide range of physiological measurements were performed in attempt to identify a physiological algorithm to predict AMS sensitivity. Compared to normoxia, the hypoxic stress induced by the current experimental conditions elicited the expected physiological responses such as hypoxemia, hyperventilation and tachycardia at rest and during exercise, reductions in HRV parameters reflecting vagal activity, exercise intolerance, and gradual AMS symptom development. We compared whether responses differed between subjects afflicted less (AMS_{LOW}) or more (AMS_{HIGH}) by AMS. We show differential responses between AMS_{LOW} and AMS_{HIGH} for pulmonary ventilation, blood oxygenation status, blood lactate concentration, exercise intolerance, and cardiac vagal activity. Regression analysis identified the magnitude of SpO_2 drop during submaximal exercise during early exposure to hypoxia to explain about half of the variability in the severity of AMS symptoms throughout the 24-h hypoxic exposure.

In order to identify potential predictive markers of AMS we first compared a wide range of physiological measurements at rest and during exercise between subjects exhibiting less (AMS_{LOW}) or more severe (AMS_{HIGH}) symptoms during 24 hours at 12.7% F_iO_2. From the perspective of preventive screening, we were specifically interested to explore whether physiological responses during the initial hours of hypoxic exposure could predict AMS development later in the experiment. Some acute physiological responses clearly differentiated AMS_{HIGH} and AMS_{LOW}, indeed. Within 2 h after entering the hypoxic facility, resting pulmonary ventilation was higher in AMS_{LOW} than in AMS_{HIGH}, which also resulted in greater S_pO_2 drop in the latter. Concurrently HRV parameters reflecting cardiac vagal input (RMSDD, NN50, pNN50, LnHF) acutely dropped in AMS_{HIGH}, but not AMS_{LOW}. Exercise effects were studied during a constant-load cycling bout of which the energy expenditure corresponds with hiking at an ascent rate of ~300 $m \cdot h^{-1}$ [69]. Exercise S_pO_2 was ~4% lower and blood lactate increased more in AMS_{HIGH} than in AMS_{LOW}. Literature data with regard to the relationship between the ventilatory response to hypoxia and AMS-sensitivity are equivocal [24-26,76,77]. Neither did S_pO_2 consistently differentiate between AMS-susceptible *versus* resistant subjects [33-37,78-82]. Our data corroborate the review findings of Burscher *et al.*, identifying S_pO_2 measured following 20-30 min of hypoxic exposure equivalent to 2300-4200 m as the most useful predictor of AMS susceptibility [83]. Furthermore, as was suggested in this aforementioned review, in the current setting lactate and HRV assessment indeed differentiated AMS resilient *versus* prone subjects for AMS. In line with our current findings, Botek *et al.* previously reported acute hypoxia to suppress HRV indices of cardiac vagal modulation, and this was correlated with the concurrent S_pO_2 drop [74]. We extend these findings by investigating the relationship with AMS severity.

Beyond exercise bout 1, which ended after 2.5 h in hypoxic facility, four additional 30-min cycling bouts at 1.5 $Watt.kg^{-1}$ were done during the next 6 h. We included exercise in the protocol because exercise is known to increase the incidence of AMS [55,84,85], as well as to increase the field validity of the study. The incidence of AMS symptoms was high, indeed, and Lake Louise scores (LLS) on average increased from ~2 to ~5 by the end of the 24-h protocol. Seven out of 16 subjects were diagnosed positive for severe AMS (LLS $\geq$ 6) with six subjects scoring LLS $\geq$ 9 (maximal observed LLS score = 10). All subjects easily completed the exercise protocol in normoxia, and AMS_{LOW} was also consistently successful in hypoxia. However, in AMS_{HIGH} endurance exercise capacity was markedly impaired. Five out of seven subjects in AMS_{HIGH} were unable to maintain the workload requested (1.5 $Watt.kg^{-1}$), either due to subjective feelings of fatigue/weakness (n=2), exacerbating headache/AMS (n=2), or even pre-exercise syncope (n=1). The degree of exercise intolerance and peak LLS scores in our study were unrelated to either 2 or 4 $mmol \cdot L^{-1}$ blood lactate thresholds, or to maximal

aerobic power (~VO_2max) measured in either normoxia or hypoxia, which corroborates earlier observations [30,86,87]. Very high VO_2max (>70 mL·kg^{-1}·min^{-1}) may predispose to systemic deoxygenation during exercise in hypoxia [88], and hence predispose to AMS [80,81]. However, within the range of VO_2max values considered in the present study (40-65 mL·kg^{-1}·min^{-1}), blood oxygen desaturation during submaximal exercise bouts was independent of VO_2max. In fact, the incremental VO_2max tests in acute hypoxia prior to the 24-h experimental sessions did not yield any useful parameter to estimate AMS-sensitivity.

As indicated above, a primary aim of the current study was to predict the later development of AMS from the initial responses to hypoxia. We found the acute hypoxia-induced responses of S_pO_2 at rest, LnTP and LnHF during spontaneous breathing at rest, and SpO_2 and blood lactate during submaximal exercise to significantly correlate with the degree of AMS development. In multiple regression analysis we found S_pO_2 at rest to predict 32% of ∑LLS over the 24-h hypoxic episode, whilst S_pO_2 measured during cycling at 1.5 W·kg^{-1} even predicted 53% of peak LLS. In line with these findings, Burtscher *et al*. reported S_pO_2 after 30 min at rest in acute normobaric or hypobaric hypoxia of varying degrees to adequately identify 86% of AMS susceptible mountaineers [33]. Savourey *et al.* previously identified end-tidal O_2 pressure and peripheral blood O_2 content ([Hb]*S_pO_2*1.34) after 30 min in normobaric hypoxia (F_iO_2: 12%) at rest to predict 77% of the variance in peak LLS during subsequent exposure to 2800-5800 m altitude [89]. In the same study, 71% of the mean LLS score was predicted by peripheral blood O_2 content, the normoxic-hypoxic increase in breathing frequency, and the hypoxic cardiac response (increase in heart rate per decrease in S_pO_2). Our data taken together with previous literature [33,38] thus indicate that the potential to maintain high blood oxygenation status upon acute exposure to hypoxia in both rest and exercise yields the better, yet still inaccurate prediction of the degree of sensitivity to AMS. In addition, it must be emphasized that AMS susceptibility is not a fixed trait. Peak LLS scores are poorly repeatable across repeated hypoxic exposures [57], and AMS history yields poor sensitivity to predicting future AMS development [90]. Furthermore, previous altitude acclimatisation protects against future AMS development following either short (1-3 weeks) [91,92] or long-term (5 months) 'living low' [93]. Therefore, interpratation of AMS susceptibility based on screening procedures such as that from the present study should be performed with caution. In fact, the algorithm to predict AMS development in the present study was obtained from retrospective analysis. Its validity remains to be demonstrated in a prospective analysis.

Regression analyses did not identify HRV as a factor to adequately predict AMS in the current study. Nonetheless the time course of some HRV parameters during the 24-h hypoxic episode clearly differentiated the AMS_{LOW} *versus* AMS_{HIGH} subgroups. RMSDD, NN50, pNN50, LnHF were not significantly altered by normoxic control conditions. However, vagal HRV was decreased throughout the 24 h of hypoxic exposure in AMS_{HIGH}, whereas only a mild and transient vagal withdrawal was observed in AMS_{LOW}. Furthermore, LnHF and LnTP remained stable in hypoxia in AMS_{LOW}, but progressively decreased over time in AMS_{HIGH}. Previous literature observations of higher urinary, arterial and venous catecholamine output in subjects developing AMS has led to the assumption that sympathetic hyperactivity may be implicated in AMS development [38-41]. However, the other way around, sympathetic hyperactivity may also result from AMS symptoms. Our results indicate a shift towards relative sympathetic dominance in severe AMS as evidenced by increased heart rate and LF:HF ratio at rest. However, this elevated net sympathovagal output emanated from substantial parasympathetic withdrawal rather than from elevation in sympathetic tone. These findings are in line with recent evidence showing hypoxic tachycardia after two weeks at high altitude to result from parasympathetic attenuation [94]. Whilst parasympathetic HRV indicators in the present study were continuously decreased during the hypoxic experiment in AMS_{HIGH}, in AMS_{LOW} vagal withdrawal was a temporary phenomenon

restricted to the first 11 h of hypoxic exposure. This finding is in line with recent observations by Yih *et al.* showing recovery *vs*. further deterioration (albeit non-significant) in RMSSD, NN50, pNN50 and HF following the first night in hypoxia in AMS resilient *vs*. AMS positive subjects [47]. Here we also show that LnTP, which reflects overall cardiac autonomic activity, was unaffected by hypoxia in AMS_{LOW}, whereas it decreased in AMS_{HIGH}. Thus, in agreement with the findings by Karinen *et al.* [52], our data probably indicate that preservation of both vagal and overall cardiac autonomic activity indicates adequate hypoxic acclimatisation and/or resilience against AMS.

To integrate breathing-related fluctuations in RR intervals into the 'vagal' HF band (0.15-0.45 Hz), paced breathing at ≥ 0.2 Hz (≥ $12 \cdot min^{-1}$) may be advised. However, paced breathing also causes slight hyperventilation in some individuals [95], which in turn alters arterial oxygen saturation and downstream mechanisms. Therefore, most published studies did not use paced breathing to assess the role of cardiac autonomic activity in AMS [47,51-55,96]. In the present study, paced breathing at 0.2 Hz increased minute ventilation in AMS_{LOW} but not AMS_{HIGH}. Furthermore, HRV changes were largely similar between paced and spontaneous breathing. This is presumably due to high spontaneous breathing frequencies in hypoxia already integrate respiratory modulation of vagal activity into the HF band. Literature data taken together with the current data clearly indicate that HRV evaluation during spontaneous breathing is the better approach to investigate HRV responses in hypoxia.

Arterial blood samples for evaluation of blood oxygenation status and acid-base balance were taken only at 8 h in hypoxia, which prevented us from using these data in our predictive algorithm for AMS. In line with literature data [77,97,98], hypoxia produced a respiratory alkalosis which, however, was similar between AMS_{HIGH} and AMS_{LOW}. Also the PaO_2 drop did not differentiate between AMS_{HIGH} and AMS_{LOW}. Nonetheless SaO_2 was ~5% lower in the former. This may be explained by the slightly higher P_aCO_2, decreasing O_2 affinity of hemoglobin and lowering S_aO_2 for a given P_aO_2 in AMS_{HIGH}.

It has previously been postulated that the rate of nitric oxide expiration (FENO) in normoxia may be used to evaluate AMS sensitivity [56-59]. In the conditions of the current study, baseline FENO was not significantly different between AMS_{HIGH} and AMS_{LOW}. Data variability was high and there was a large overlap between the groups. Previous literature findings have been inconsistent, with some studies reporting lower [56-58] and other studies reporting higher [59] FENO in individuals at risk. Overall, support for the use of FENO to predict AMS is poor [62].

Our and aforementioned literature data clearly indicate the importance of maintaining high blood oxygenation status to prevent AMS development upon acute exposure to hypoxia. Prophylactic use of acetazolamide (≥250 mg per day) is a valid strategy to improve blood oxygenation and acclimatization through stimulation of pulmonary ventilation secondary to metabolic acidosis induced by renal secretion of bicarbonate [99,100]. Recent data also indicate acetazolamide to stimulate vagal activity of the heart in AMS susceptible subjects at altitude [101]. Amongst other interventions, subjects prone to AMS should also be encouraged to follow a slow ascend profile, preferentially preceded by a period of preacclimatization to hypobaric hypoxia [102].

In conclusion, healthy physically fit individuals presenting low S_pO_2 at rest and during submaximal exercise within the first hours in hypoxia should be closely monitored for subsequent AMS development. Furthermore, those struggling to keep up the groups' exercise pace are more likely to be afflicted by AMS. Subjects developing AMS are characterised by early and growing withdrawal of vagal cardiac regulation.

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Conflict of Interest Statement:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplemental material:

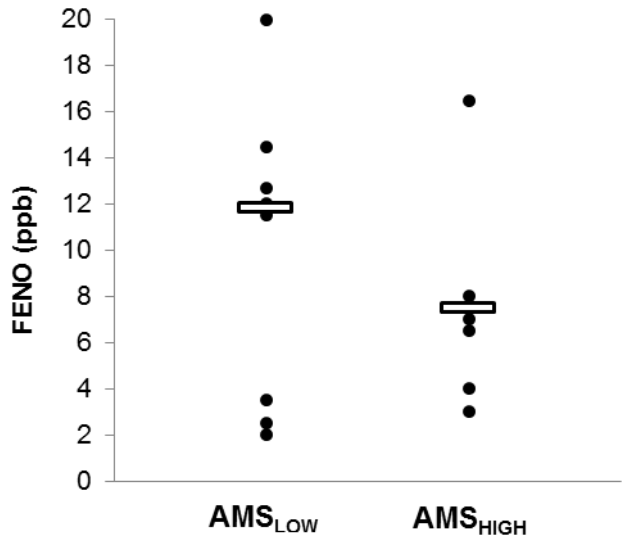

Figure 3 | Normoxic levels of fractional concentration of exhaled nitric oxide measured prior to 24-h hypoxic exposure.
Data represent individual (circles) and mean (rectangles) fractional concentration of exhaled nitric oxide (FENO) measured prior to 24-h hypoxic exposure in AMS_{LOW} (LLS < 6, n=9) and AMS_{HIGH} (LLS ≥ 6, n=7).

Table 6 | Heart rate and heart rate variability analysis in the time domain during paced breathing in normoxia and hypoxia.

	NORMOXIA			HYPOXIA		
	All	**AMS$_{LOW}$**	**AMS$_{HIGH}$**	**All**	**AMS$_{LOW}$**	**AMS$_{HIGH}$**
Heart rate (bpm)						
1 h (n=16)	60 ± 2	58 ± 3	63 ± 4	63 ± 3	60 ± 3	66 ± 6
	(42-77)	(42-74)	(49-77)	(49-97)	(49-71)	(51-97)
6 h (n=16)	56 ± 3	56 ± 4	56 ± 4	68 ± 3*§†	63 ± 3*	75 ± 6*#
	(42-77)	(42-74)	(43-77)	(50-95)	(50-72)	(56-95)
11 h (n=15)	60 ± 3	59 ± 4	61 ± 5	73 ± 3*	71 ± 4*	77 ± 6*
	(43-78)	(42-74)	(49-78)	(52-99)	(52-89)	(55-99)
14 h (n=14)	60 ± 4	60 ± 5	60 ± 6	72 ± 3*	70 ± 3*	76 ± 7*
	(44-86)	(44-86)	(46-76)	(52-96)	(52-80)	(53-96)
23 h (n=11)	56 ± 3†	54 ± 3†	58 ± 6	69 ± 4*	66 ± 4*	75 ± 7*
	(46-70)	(46-68)	(46-70)	(49-88)	(49-78)	(54-88)
RMSSD (ms)						
1 h (n=16)	103 ± 19	112 ± 31	91 ± 21	95 ± 13†	95 ± 18	94 ± 22†
	(40-331)	(40-331)	(41-182)	(40-214)	(40-214)	(40-181)
6 h (n=16)	131 ± 21	137 ± 35	123 ± 20	91 ± 15*	106 ± 18	75 ± 25
	(36-350)	(36-350)	(64-210)	(9-195)	(42-174)	(9-195)
11 h (n=15)	127 ± 23	118 ± 31	142 ± 36	62 ± 11*	64 ± 13	58 ± 23*
	(14-326)	(14-326)	(54-258)	(9-158)	(13-145)	(9-158)
14 h (n=14)	123 ± 26	121 ± 33	126 ± 45	49 ± 9*	55 ± 12	40 ± 17

	(24-305)	(24-293)	(53-305)	(7-127)	(23-127)	(7-101)
23 h (n=11)	114 ± 26	115 ± 37	112 ± 37	50 ± 12*	62 ± 18†	30 ± 4*
	(29-314)	(29-314)	(43-208)	(21-160)	(21-160)	(23-37)
NN50 (count)						
1 h (n=16)	140 ± 17	151 ± 23	125 ± 25	124 ± 16†	131 ± 22	114 ± 25†
	(55-272)	(62-272)	(55-229)	(41-229)	(50-229)	(41-221)
6 h (n=16)	173 ± 17	164 ± 27	184 ± 18	103 ± 18*§	129 ± 19	78 ± 30*
	(36-271)	(36-271)	(110-224)	(0-208)	(43-202)	(0-208)
11 h (n=15)	159 ± 17	145 ± 23	180 ± 22	71 ± 16*	87 ± 23*	47 ± 17*
	(2-255)	(2-223)	(97-255)	(0-215)	(1-215)	(0-96)
14 h (n=14)	146 ± 22	132 ± 30	172 ± 29	58 ± 17*	72 ± 24	30 ± 16*
	(10-260)	(10-226)	(81-260)	(0-197)	(8-197)	(0-75)
23 h (n=11)	130 ± 21	128 ± 32	133 ± 24	60 ± 19*	82 ± 26	22 ± 10*
	(9-249)	(9-249)	(65-167)	(3-199)	(6-199)	(3-46)
pNN50 (%)						
1 h (n=16)	47 ± 6	50 ± 8	42 ± 8	41 ± 5†	44 ± 7	38 ± 8†
	(18-91)	(21-91)	(18-76)	(14-76)	(17-76)	(14-74)
6 h (n=16)	58 ± 6	55 ± 9	61 ± 6	34 ± 6*§	43 ± 6	26 ± 10*
	(12-90)	(12-90)	(37-75)	(0-69)	(14-67)	(0-69)
11 h (n=15)	53 ± 6	48 ± 8	60 ± 7	24 ± 5*	29 ± 8*	16 ± 6*
	(1-85)	(1-74)	(32-85)	(0-72)	(0-72)	(0-32)
14 h (n=14)	49 ± 7	44 ± 10	57 ± 10	19 ± 6*	24 ± 8	10 ± 5*
	(3-87)	(3-75)	(22-87)	(0-66)	(3-66)	(0-25)
23 h (n=11)	43 ± 7	43 ± 11	44 ± 8	20 ± 6*	27 ± 9	7 ± 3*
	(3-83)	(3-83)	(22-56)	(1-66)	(2-66)	(1-15)

*Data are mean ± SEM (range in brackets) and represent heart rate, the square root of the mean squared differences between adjacent normal RR intervals (RMSSD), the number of pairs of adjacent RR-interval that differ more than 50 ms (NN50), and NN50 in relation to the total number of RR-intervals (pNN50) at rest during spontaneous breathing in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects, as well as for subgroups including AMS_{LOW} (LLS < 6) and AMS_{HIGH} (LLS ≥ 6). Analyses are performed with n=16 at 1 and 6 h, n=15 at 11 h, n=14 at 14 h, and n=11 at 23 h in the hypoxic facility. See METHODS for further details * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; § P < 0.05 group (AMS_{LOW} vs. AMS_{HGH}) x condition (normoxia vs. hypoxia) interaction.*

Table 7 | Heart rate variability analysis in the frequency domain during paced breathing in normoxia and hypoxia.

	NORMOXIA			HYPOXIA		
	All	**AMS_{LOW}**	**AMS_{HIGH}**	**All**	**AMS_{LOW}**	**AMS_{HIGH}**
LnLF (ms^2)						
1 h (n=16)	6.7 ± 0.2†	6.5 ± 0.4	6.9 ± 0.2	7.0 ± 0.3†	6.9 ± 0.3	7.3 ± 0.4†
	(5.1-8.8)	(5.1-8.8)	(5.8-7.8)	(5.9-8.7)	(5.9-8.7)	(6.1-8.6)
6 h (n=16)	7.2 ± 0.3	7.3 ± 0.3	7.0 ± 0.6	7.1 ± 0.3	7.4 ± 0.3	6.7 ± 0.4
	(4.0-8.8)	(5.7-8.7)	(4.0-8.8)	(4.8-8.7)	(6.4-8.7)	(4.8-8.2)
11 h (n=15)	6.4 ± 0.4	6.2 ± 0.7	6.7 ± 0.5	6.0 ± 0.3	6.0 ± 0.4	6.0 ± 0.5
	(1.4-8.3)	(1.4-8.0)	(4.6-8.3)	(4.0-8.0)	(4.1-8.0)	(4.0-7.5)
14 h (n=14)	6.6 ± 0.3	6.8 ± 0.4	6.3 ± 0.3	5.6 ± 0.3	5.6 ± 0.4	5.5 ± 0.7
	(4.9-8.4)	(4.9-8.4)	(5.4-6.9)	(3.4-7.9)	(4.6-7.9)	(3.4-7.0)

23 h (n=11)	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.9	6.0 ± 0.4	6.0 ± 0.5	6.0 ± 0.7
	(4.7-8.9)	(5.3-8.4)	(4.7-8.9)	(4.0-7.8)	(4.0-7.8)	(4.0-7.3)
LnHF (ms^2)						
1 h (n=16)	8.0 ± 0.3	8.1 ± 0.3	7.8 ± 0.4	7.9 ± 0.3†	7.9 ± 0.3	7.9 ± 0.4†
	(6.1-10.1)	(6.8-10.1)	(6.1-9.4)	(6.7-9.7)	(6.8-9.7)	(6.7-9.3)
6 h (n=16)	8.4 ± 0.3	8.3 ± 0.4	8.6 ± 0.2	7.5 ± 0.4*	7.9 ± 0.4	7.0 ± 0.7*
	(6.5-10.3)	(6.5-10.3)	(7.8-9.4)	(4.0-9.4)	(6.1-9.2)	(4.0-9.4)
11 h (n=15)	8.2 ± 0.4	7.9 ± 0.7	8.7 ± 0.4	7.1 ± 0.3*	7.3 ± 0.4	6.8 ± 0.6*
	(3.0-10.1)	(3.0-10.1)	(7.1-9.9)	(4.8-9.2)	(5.0-9.2)	(4.8-9.0)
14 h (n=14)	8.2 ± 0.4†	8.0 ± 0.5†	8.4 ± 0.5	6.7 ± 0.4*	7.1 ± 0.4	6.0 ± 0.8*
	(6.1-10.3)	(6.1-10.2)	(7.1-10.3)	(3.9-8.7)	(5.9-8.7)	(3.9-8.3)
23 h (n=11)	7.8 ± 0.4	7.7 ± 0.6	7.9 ± 0.3	6.8 ± 0.3*†	7.1 ± 0.4	6.2 ± 0.3*†
	(5.3-10.1)	(5.3-10.1)	(7.0-8.5)	(5.6-8.9)	(5.7-8.9)	(5.6-6.7)
LnTP (ms^2)						
1 h (n=16)	8.3 ± 0.2	8.4 ± 0.3	8.3 ± 0.3	8.5 ± 0.2†	8.5 ± 0.3	8.6 ± 0.4†
	(7.1-10.4)	(7.1-10.4)	(7.3-9.6)	(7.2-10.0)	(7.5-10.0)	(7.2-9.8)
6 h (n=16)	8.9 ± 0.2	8.8 ± 0.4	9.0 ± 0.2	8.3 ± 0.3*	8.7 ± 0.3	7.8 ± 0.6*
	(7.1-10.6)	(7.1-10.6)	(8.1-9.9)	(5.3-9.9)	(7.7-9.7)	(5.3-9.9)
11 h (n=15)	8.5 ± 0.4	8.2 ± 0.6	8.9 ± 0.4	7.7 ± 0.3	7.8 ± 0.3	7.5 ± 0.6
	(3.6-10.4)	(3.6-10.4)	(7.9-10.0)	(5.2-9.3)	(5.9-9.3)	(5.2-9.3)
14 h (n=14)	8.6 ± 0.3	8.5 ± 0.5	8.7 ± 0.4	7.3 ± 0.3*	7.5 ± 0.3	6.9 ± 0.7*
	(6.7-10.4)	(6.7-10.4)	(8.1-10.4)	(4.6-9.2)	(6.4-9.2)	(4.6-8.6)
23 h (n=11)	8.6 ± 0.3	8.7 ± 0.4	8.6 ± 0.5	7.5 ± 0.3*†	7.8 ± 0.4	7.1 ± 0.4†
	(7.1-10.3)	(7.1-10.3)	(7.2-9.5)	(5.8-9.2)	(6.4-9.2)	(5.8-7.8)
LF/HF						
1 h (n=16)	0.4 ± 0.1†	0.2 ± 0.0	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
	(0.1-1.9)	(0.1-0.5)	(0.1-1.9)	(0.1-1.6)	(0.1-1.3)	(0.2-1.6)
6 h (n=16)	0.5 ± 0.2	0.7 ± 0.4	0.3 ± 0.1	1.0 ± 0.2	0.8 ± 0.2	1.2 ± 0.5
	(0.0-3.8)	(0.1-3.8)	(0.0-0.5)	(0.2-3.9)	(0.2-1.8)	(0.3-3.9)
11 h (n=15)	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.9 ± 0.6*
	(0.0-0.6)	(0.1-0.6)	(0.0-0.6)	(0.1-3.7)	(0.1-0.8)	(0.2-3.7)
14 h (n=14)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1*§†	0.3 ± 0.1	0.6 ± 0.1*#
	(0.0-0.8)	(0.1-0.5)	(0.0-0.8)	(0.1-0.8)	(0.1-0.5)	(0.3-0.8)
23 h (n=11)	0.8 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	0.7 ± 0.2	0.4 ± 0.1	1.1 ± 0.4
	(0.1-2.5)	(0.1-2.5)	(0.1-1.6)	(0.1-2.0)	(0.1-0.8)	(0.2-2.0)

*Data are mean ± SEM and represent the natural logarithmic transformation of total power (LnTP), low frequency power (LnLF), high frequency power (LnHF) and the ratio of LF/HF at rest during spontaneous breathing in normoxia (F_iO_2: 20.9%) and hypoxia (F_iO_2: 12.7%). Values are reported for the total group of subjects, as well as for subgroups including AMS_{LOW} (LLS < 6) and AMS_{HIGH} (LLS ≥ 6). Analyses are performed with n=16 at 1 and 6 h, n=15 at 11 h, n=14 at 14 h, and n=11 at 23 h in the hypoxic facility. See METHODS for further details * P < 0.05 vs. normoxia; # P < 0.05 vs. AMS_{LOW}; § P < 0.05 group (AMS_{LOW} vs. AMS_{HGH}) x condition (normoxia vs. hypoxia) interaction.*

PART III

GENERAL DISCUSSION

1. General overview of main study results

Study 1. Effects of hypoxia and dietary nitrate supplementation on adaptations to sprint interval training

- ***Hypothesis 1:*** Sprint interval training in hypoxia did not stimulate improvements in endurance and/or sprint exercise performance, nor did it induce hypoxia-specific muscular adaptations.
- ***Hypothesis 2:*** Dietary nitrate supplementation during hypoxic sprint interval training did not increase power output during training. However, nitrate supplementation may have altered training-induced changes in musce fiber type composition.

Study 2. Muscular, haematological, and physiological adaptations to training 'low' *versus* 'high' whilst intermittent 'living high'

- ***Hypothesis 1:*** Hypoxic *versus* normoxic high-intensity interval training in the setting of 'living high' did not stimulate training-induced changes in muscle fiber cross-sectional area, capillarization, pH-handling capacity, or performance. However, training in hypoxia did stimulate changes in muscle blood volume oscillations during all-out repeated-sprint exercise.
- ***Hypothesis 2:*** Five weeks of intermittent 'living high' at progressively increasing altitude consistently increased serum EPO and total hemoglobin mass, but not maximal aerobic capacity.

Study 3. Effects of high-intensity interval training in normoxia *versus* hypoxia on muscular ischemic responses

- ***Hypothesis 1:*** High-intensity interval training in the setting of 'living high' did not attenuate the muscular response to a brief episode of ischemia-induced hypoxic stress, irrespective the ambient oxygen availability during training.

Study 4. Identification of markers associated with susceptibility to AMS

- ***Hypothesis 1:*** AMS-susceptible subjects display greater exercise intolerance, reduced vagal cardiac autonomic regulation, and lower blood oxygenation status in hypoxia.

2. Muscular adaptations to training in hypoxia

2.1. Rationale

The effectiveness of 'training high' to stimulate muscular adaptations and hence sea-level exercise performance has been a matter of debate for decades. Ambient hypoxia during training has been postulated to stimulate myocellular hypoxia-inducible factor (HIF) pathway and thereby gene expression involved in muscle pH-handling, glycolytic metabolism, and capillarization [1-6]. Second, reduced myocellular oxygen availability shifts metabolism from oxidative mitochondrial energy provision to glycolytic metabolism, conceivably stimulating adaptations in the latter [7]. Notwithstanding, endurance training improved adaptations in mitochondria and oxidative enzymes more following training in hypoxia compared to normoxia [3,8–13]. That is, however, on the condition that similar absolute workload is applied in normoxia and hypoxia [14]. Furthermore, recent evidence indicates greater amplitude of muscle blood volume changes during repeated-sprint exercise following hypoxic *versus* normoxic sprint training [6,15,16]. Finally, hypoxia-specific secretion and accumulation of hormones and waste metabolites, respectively, may stimulate the anabolic response required for muscle adaptation [17-21]. On the downside, however, hypoxia abolishes maximal absolute aerobic training workload that can be maintained for a given time [22-25]. Hence smaller oxygen fluxes and neuromechanical loading of muscles in hypoxia conceivably can abolish training adaptations [22]. To overcome this drawback, recent research has shifted focus from endurance to supramaximal high-intensity interval training.

In the present thesis, we investigated whether short-term training in hypoxia *versus* normoxia by either intermittent high-intensity knee-extension training or sprint interval training induces divergent responses on (i) muscle HIF pathway activation in hypoxia, (ii) muscle pH-handling capacity, (iii) muscle oxygenation and oxygen extraction during repeated-sprint exercise, and (iv) muscle fiber cross-sectional area and fiber type composition.

2.2. Arterial oxygenation, muscle oxygenation, and muscle HIF pathway activation

For the hypothesis of greater myocellular HIF pathway activation during training in hypoxia to hold true, one might raise the question whether intracellular PO_2 (P_iO_2) is indeed lowered by ambient hypoxia. Proton nuclear magnetic resonance spectroscopy has estimated severe environmental hypoxia (10% F_iO_2) to only modestly reduce P_iO_2 at rest, from ~34 mmHg in normoxia to ~23 mmHg in hypoxia [26]. In fact, contraction-induced drop in P_iO_2 during exercise in normoxia far exceeds the decrease due to passive hypoxic exposure. Indeed, high-intensity single-leg cycling exercise in normoxia decreases myoglobin-associated oxygen tension to ~3.1 mmHg [27]. Similar exercise in ambient hypoxia (12% F_iO_2) further decreases P_iO_2 to ~2.3 mmHg [27]. Whether P_iO_2 is likewise reduced by exercise and hypoxia during other types of training remains to be investigated. Although a threshold of 8 mmHg has been suggested for muscle HIF-1α to be stabilized [28], such threshold still needs to be experimentally confirmed, as does the assumption of a dose-dependent relationship between P_iO_2 and HIF pathway activation. In fact, exercise in either hypoxia or normoxia similarly increased muscle HIF-1α mRNA [29], protein content [30], nuclear translocation [30], and DNA binding activity [30]. In that respect, any exercise-induced drop in P_iO_2 seems ample to fully stabilize HIF-1α and activate the HIF-1 transcription machinery.

Proton nuclear magnetic resonance spectroscopy is limited to its application in the hospital setting. Conversely, the use of near-infrared spectroscopy (NIRS) enables non-invasive estimation of tissue oxygenation and changes in blood circulation by use of a portable device. Using NIRS, tissue oxygenation can be determined from the fraction of oxygenated hemoglobin and myoglobin within a

given voxel of muscle tissue. Nevertheless, extrapolation from the so-called tissue oxygenation index (TOI) to P_iO_2 should be performed with caution, given that NIRS estimates the degree of myoglobin and hemoglobin heme oxygenation in both the arterial and venous capillary bed [31]. As expected from the severe hypoxic training environment (F_iO_2: 12.3%) in **study 2-3**, TOI in *vastus lateralis* was markedly decreased during high-intensity single-leg knee extension training (intermittent 90-s knee extensions at 20-25% of 1RM) in hypoxia compared to normoxia (**Figure 1**). Likewise in **study 1**, arterial oxygen saturation (S_pO_2) was substantially lower in subjects performing sprint interval training (SIT) in hypoxia (F_iO_2: 15%; S_pO_2: ~86%) compared to normoxia (S_pO_2: ~98%).

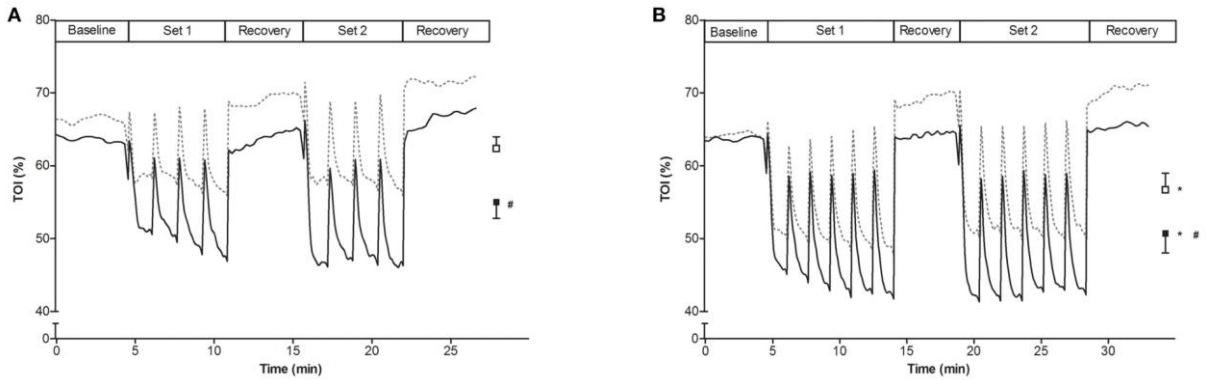

FIGURE 1 | Effect of knee-extension training in normoxia vs. hypoxia on muscle oxygenation status. Curves represent mean values (n = 10) for tissue oxygenation index (TOI) during week 1 (A) and week 5 (B) of the training intervention. One leg was trained in 12.3% F_iO_2 (solid lines), whilst the other leg was trained in 20.9% F_iO_2 (dotted lines). The training sessions consisted of two times 4 series of 30 knee-extensions (~90 s) at 20% 1RM in week 1, increasing to two times 6 series of 30 knee-extensions at 25% of 1RM in week 5. Mean values ± SEM are given at the right side of each graph (normoxia, □; hypoxia, ■). *P < 0.05 compared to week 1; # P < 0.05 compared to training in normoxic conditions.

We hypothesized hypoxic HIF pathway activation to be less pronounced following 5 weeks of high-intensity knee-extension training in normoxia, and even more so in hypoxia. After all, prolyl hydroxylase domain protein 2 (PHD2), known to initiate HIF-1α degradation, has been identified as a HIF-1 target gene [32]. Hence repetitive exercise-induced HIF-1 pathway activation may be expected to increase PHD2 expression and thereby reduce HIF-1 accumulation at a given P_iO_2. [33-34]. In support of this hypothesis, endurance training increased PHD2 protein content in healthy volunteers, and cross-sectional analysis showed higher protein abundancy of PHD2 and factor inhibiting HIF-1 (FIH-1) in elite endurance athletes compared to moderately active controls [35]. Furthermore, human and rodent studies applying short-term endurance training in the form of low-load knee-extension exercise or downhill running, respectively, reported an attenuation in acute exercise-induced HIF-1 pathway activation after short-term training [36-37]. However, the role of HIF-1α as a sensor of both metabolic (Krebs cycle intermediate α-ketoglutarate) and hormonal (inflammation, ROS) disturbances should be taken into account to put these findings in perspective [37-41]. To exclude potential confounding effects of training-induced adaptations in muscle vascularization, ROS scavenging capacity, and inflammatory responses to exercise, we investigated the muscle HIF pathway response during 10 minutes of ischemia-induced hypoxia as mediated by arterial occlusion of the leg. Arterial occlusion was successful in

decreasing muscle oxygenation, dropping TOI to ~24%, which is well beyond exercise-induced muscle deoxygenation in normoxia (TOI: 50%) or hypoxia (TOI: ~43%). Muscle biopsies were taken before and at the 10th minute of arterial occlusion.

For the first time, we provided an immunohistochemical analysis able to quantify nuclear abundancy of HIF-1α (**Figure 2**). Remarkably, we found ~54% of myonuclei to yield a positive staining for HIF-1α at baseline conditions. Furthermore, prior to the training intervention, arterial occlusion increased the fraction of HIF-1α-positive nuclei to ~64%. However, occlusion altered neither HIF-1α protein content nor target gene expression. Myocellular HIF-1α abundancy at rest has been debated. It was long believed that HIF-1α expression assessed by Western blot and histochemical analysis only reached detectable levels following exercise [30]. However, in line with our findings, recent studies reported detectable myocellular HIF-1α protein at rest in whole cell-extracts by means of Western blot [42] and enzyme-linked immunoassay [43]. Hypoxic inhibition of PHD2 activity causing net accumulation of HIF-1α protein to such degree that it would be detected by simple Western blot analysis in whole-cell extracts conceivably required a hypoxic episode longer than 10 minutes. Accordingly, increased HIF transcriptional activity evidenced by elevated mRNA levels of its target genes conceivably required substantially longer ischemia time and/or muscle sampling at some time after reperfusion.

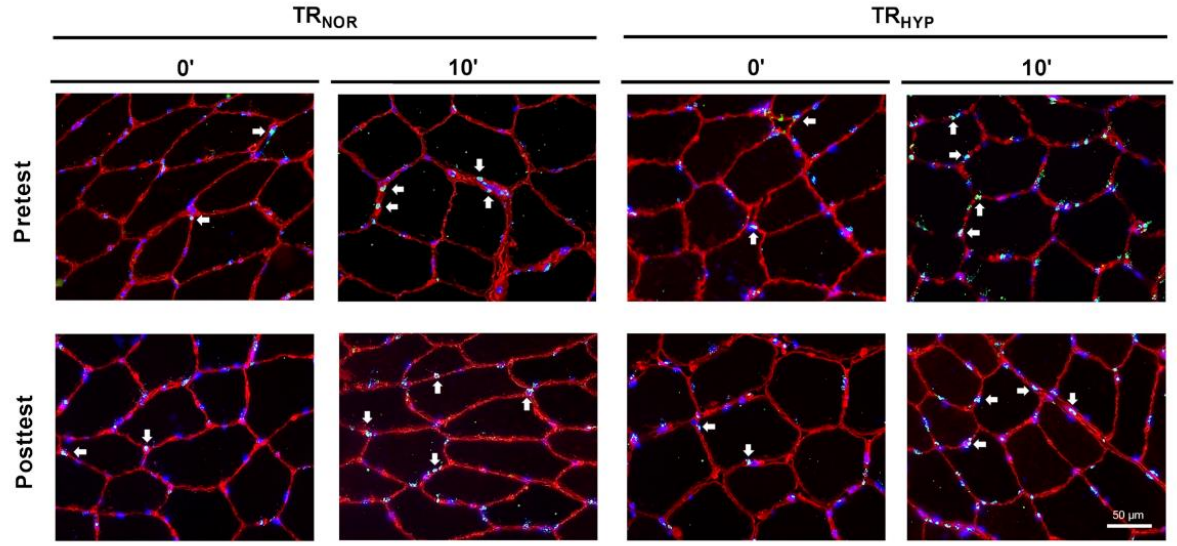

Figure 2 | Effect of knee-extension training in normoxia *versus* hypoxia during 'living high' on HIF-1α positive nuclei in *m. vastus lateralis* during an arterial occlusion of the leg.
Representative immunofluorescence images of vastus lateralis muscle sampled before (0') and at the end (10') of a 10-min arterial occlusion and before (Pretest) and after (Posttest) 5 weeks of knee-extension training in normoxia (TR_{NOR}, 20.9% F_iO_2) or hypoxia (TR_{HYP}, 12.3% F_iO_2). Laminin (red), Hoechst (blue), HIF-1α (green). Arrows indicate HIF-1α positive nuclei.

Against our hypothesis, 5 weeks of high-intensity knee-extension training did not alter the ischemia-induced increase in HIF-1α-positive nuclei, irrespective of oxygen availability during training. In keeping with this finding, HIF-1α, HIF-2α, and PHD2 protein content remained stable throughout the 5-week study intervention. Our observations are in contrast with previous reports of increased PHD2 abundancy and decreased exercise-induced HIF-1 pathway activation following short-term endurance training [35-37]. However, this discrepancy is most likely due to differences in the training loads applied.

Indeed, PHD2 mRNA expression was not increased following 2 weeks of repeated-sprint training in normoxia (RSN) or hypoxia (RSH) in the setting of 'living high' [5]. Furthermore, recent data show HIF-1α protein to remain stable or even increase following 12 weeks of resistance training in conjunction with either placebo or protein supplementation, respectively [42]. In mice, 6 weeks HIIT increased HIF-1α protein content in gastrocnemius muscle [44]. In fact, muscle-specific HIF-1α overexpression in rat induces a shift to the glycolytic phenotype [45], whereas muscle-specific HIF-1α knock-out mice portray the ultimate endurance phenotype [46]. At least in rodents, HIF-1α is more abundant in fast-twitch muscle fibers [47]. Training-induced changes in HIF-α and its regulators should therefore be interpreted along changes in fiber type distribution. In that respect, we did not observe any changes in type I *versus* type II fiber distribution following 5 weeks of high-intensity knee-extension training.

2.3. Muscle pH-handling capacity

It was postulated that high-intensity training in hypoxia *versus* normoxia would stimulate adaptations in glycolytic enzymes and protein involved in pH homeostasis. In support for this hypothesis, HIF-1 pathway activation is known to shift metabolism away from the mitochondrial towards the glycolytic system [48,49]. Furthermore, short-term endurance training in hypoxia, but not in normoxia, increased steady-state mRNA levels of monocarboxylate transporter 1 (MCT1), carbonic anhydrase 3 (CA3), glucose transporter 4 (GLUT4), and phosphofructokinase (PFK) [4], whereas short-term sprint training in hypoxia but not normoxia increased PFK activity [7] and steady-state mRNA levels of MCT4 and CA3 [6].

The content of myocellular proteins involved in H^+ transport over the cellular membrane, such as MCT1, MCT4, and the Na^+/H^+ exchanger 1 (NHE1) did not change following 5 weeks of high-intensity knee-extension training, irrespective of the ambient oxygen availability during training (**study 2-3**). Neither did CA3, a cytoplasmic protein catalyzing the reaction between H^+ and $HCO3^-$ towards H_2O and CO_2 or *vice versa*. Accordingly, in-vitro measured intracellular buffer capacity (βhm) was not affected by training in normoxia or hypoxia. In line with our findings, 4 months of high-intensity knee-extension training (6 sets of 25 maximal muscle contractions) was previously shown not to increase βhm [50]. Furthermore, we also showed sprint interval training (SIT) in either normoxia or hypoxia not to alter βhm (**study 1**). In fact, most [51-54] though not all [55] studies found SIT in normoxia not to affect βhm. Our data taken together with current literature support the idea of supramaximal-intensity training [50-54,56] to be less effective in enhancing βhm compared to training at intensities between the second lactate threshold and VO_2max [57-59]. Additionally, we now have shown that the addition of hypoxia to SIT or high-intensity knee-extension exercise does not alter this response. With regard to the effects of hypoxic training on protein abundancy of membrane H^+ transporters, current literature data are scarce and seem to contrast findings at the level of mRNA transcription. Three weeks of combined endurance (60% VO_2max) and interval (100% VO_2max) training increased neither MCT1 nor MCT4 content in trained cyclists, irrespective of whether the training was performed in normoxia or normobaric hypoxia (~3000 m) [60]. Accordingly, 5 weeks of SIT in either normoxic or hypoxic (F_iO_2; 14.5%) conditions did not affect MCT4 content and similarly increased MCT1 abundancy [7]. Short-term RSH and RSN in the setting of 'living high' did not affect MCT4 or CA3 protein levels and similarly increased MCT1 abundancy [5]. In summary, our data taken together with the current literature do not indicate training in hypoxia, compared to training in normoxia, to stimulate intracellular buffering capacity or the abundancy of proton-extruding muscle membrane proteins. Longer training periods and/or other training modalities may be required for such adaptations to occur.

2.4. Muscle blood volume changes and oxygen extraction during repeated-sprint exercise

Emerging data from two separate research groups suggest training in hypoxia to stimulate NIRS-derived changes in blood circulation during repeated-sprint exercise to exhaustion [6,15,16]. We evaluated whether such changes would likewise occur during repeated-sprint exercise of a fixed duration following 5 weeks of unilateral high-intensity knee-extension training in normoxia *versus* hypoxia (**Study 2**). Before and after the study intervention, subjects performed 20 sets of 10 maximal isokinetic knee extensions each (~15 s per set). Sets were interspersed by 15-s rest intervals. NIRS measurements during this repeated-sprint ability (RSA) test measured changes in total hemoglobin (tHb) as a marker of vascular blood filling [61-63]. Muscle tHb alternatingly dropped during exercise intervals, conceivably due to rise in intramuscular pressure, and increased during rest episodes (**Figure 3 A**). In accordance with previous findings [6,15,16], only in the hypoxically trained leg the amplitude of tHb oscillations increased from the pretest to the posttest (**Figure 3 B**). Oscillations in HHb only increased in the normoxically trained leg. Normoxic and hypoxic training similarly increased power production during the RSA-test. These findings indicate increased power production in the posttest to be accompanied by elevated perfusion in the hypoxically trained leg, whilst the absence of perfusion adaptations in the normoxically trained leg required elevated fractional oxygen extraction.

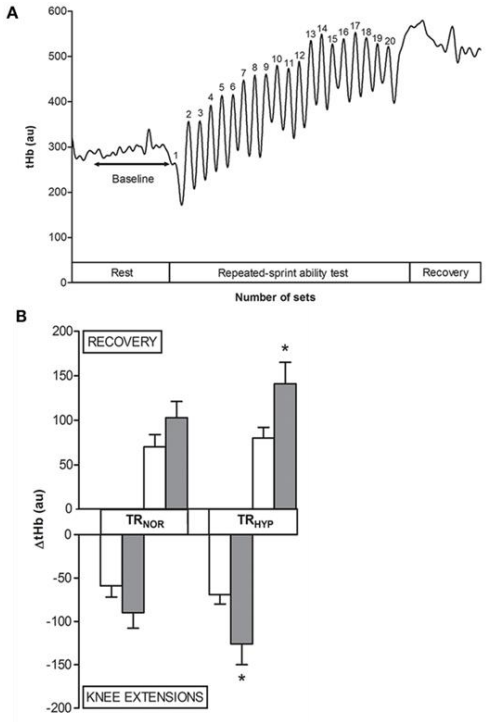

Figure 3 | Effect of knee-extension training in normoxia *versus* hypoxia during 'living high' on muscle total hemoglobin content during the repeated-sprint ability test.

A represents the typical signal of muscle total hemoglobin (tHb) measured by NIRS during the repeated-sprint ability test (n = 1), with tHb decreasing during consecutive knee extensions and increasing during recovery intervals. **B** represents means ± SEM for the change in tHb (ΔtHb) during the consecutive knee extensions and during the recovery intervals of the repeated-sprint ability test before (open bars) and after (solid bars) 5 weeks of knee-extension training in the setting of 'living high'. One leg was trained in 12.3% F_iO_2 (TR_{HYP}, ~4300 m), whilst the other leg was trained in 20.9% F_iO_2 (TR_{NOR}). $P < 0.05$ compared to the pretest.

These findings are conceivably due to the fact that hypoxic training stimuled muscle capillarization and vasodilatory function [64]. In our study, muscle capillarization increased irrespective of the training conditions. However, we measured capillary contacts per muscle fiber, on cryosections cut perpendicular to the muscle fibers. This widely used method is actually quite insensitive and has limited utility in accurate quantification of angiogenesis [65]. Hence this method typically lacks the capacity to detect divergent training responses between normoxic *versus* hypoxic training (recently reviewed in [64]). More sophisticated measurements of capillary length density (length of capillaries per muscle volume) allow detection of changes in tortuosity and branching of the capillaries. Training

studies integrating this method typically report greater gains in muscle capillarization following training in hypoxia *vs*. normoxia [64]. Additional studies are warranted to investigate (i) changes in muscle blood flow (i.e., indicator dilution and radiolabel tracer washout, Doppler ultrasound, MRI, or ultrasonography [66]) rather than changes in muscle blood volume, (ii) muscle angiogenesis (by capillary length density rather than capillary contacts [64]), and (iii) endothelial function following different modes of HIIT in hypoxia *versus* normoxia.

2.5. Muscle fiber type composition and fiber cross-sectional area

Muscle fiber type composition responds to several exercise-induced transcription factors. Fiber type transitions following endurance training have been well described. In short, elevated concentrations of cytosolic adenosine monophosphate (AMP) and Ca^{2+} during cyclic muscle contractions represent the major activators of a multifactorial transcription machinery that promotes the oxidative slow-twitch phenotype [67-69]. This transcription machinery includes, amongst others, activation of the following transcription factors (see abbreviation list for full forms): NFAT, MEF-2, AMPK, PGC-1α, and PPARβ. Less is known about transcription factors involved in slow-to-fast fiber type transition. In adults rats, HIF-1α overexpression induced a slow-to-fast fiber type transition in *extensor digitorum* and in *soleus* muscles [45]. Accordingly, in-vitro experiments showed HIF-1α transfected C2C12 myotubes to decrease and increase the relative mRNA expression of myosin heavy chain I and IIb, respectively [45]. In humans, a hyperactive polymorphism of HIF-1α is twice as prevalent in weightlifters compared to sedentary controls [70]. Based on the above literature, it is reasonable to postulate that training in hypoxia may stimulate a transition to the fast-twitch phenotype. However, irrespective of the intensity and speed of muscle contractions during training, an exercise-induced shift from slow to fast muscle fibers within type II muscle fibers (type IIa → type IIx) is highly unlikely to occur. In fact, within fast-twitch muscle fibers, even high-load resistance training in both trained [71,72] and untrained [73-75] subjects stimulates a fast-to-slow shift (type IIx → IIa), provided that any transition occurs. Accordingly, type IIx fiber proportions typically decrease following sprint training (reviewed in [76]). Most studies have found a bi-directional shift (I → IIa ← IIx), whereas others reported a unidirectional shift towards type I muscle fibers (I ← IIa ← IIx) [76]. The latter has been postulated to result from excessive or inappropriate protocols involving insufficient recovery between sprints and/or training sessions, sprints of too long duration, or involvement of too much aerobic work [76].

We found 5 weeks of high-intensity knee-extension training not to alter the distribution of type I *versus* type II (no dissociation was made between IIa vs. IIx) muscle fibers, irrespective of whether training was performed in normoxia or hypoxia (**Study 2)**. Accordingly, short-term sprint interval training in normoxia or hypoxia did not change the proportion of type I muscle fibers (**Study 1**). However, in accordance with previous studies [76], type IIx fibers' relative cross-sectional area and number decreased following SIT, irrespective of ambient oxygen availability. No changes occurred in type IIa fibers. However, when dietary nitrate was supplemented to hypoxic SIT, the decrease in type IIx fibers was accompanied by an increase in type IIa fibers. Blocking endogenous nitric oxide production in rodents has previously been demonstrated to inhibit fiber type transition to the slow-twitch phenotype [77-79]. It is thus somewhat surprising that we found type IIa fibers to be elevated when training in hypoxia was performed with dietary nitrate supplementation. On the other hand, nitrate supplementation primarily affects type II muscle fibers, by preferentially increasing blood flow [80] and elevating sarcoplasmic reticulum calcium stores and calcium-handling protein abundancy [81] in fast-twitch muscle fibers. Accordingly, nitrate supplementation stimulated rate of force development [81], muscle oxygenation [82], and power output [82,83] preferentially during high-intensity and high-velocity muscle contractions. We therefore speculate that nitrate intake during SIT in hypoxia stimulated type

IIa muscle fiber type recruitment and adaptations. Unpublished data from our laboratory showed that 6 weeks of endurance training at ~4-6 mmol·L^{-1} blood lactate in neither normoxia nor hypoxia changed type IIa muscle fiber proportions, irrespective of daily administration of placebo or nitrate-rich beetroot juice [84]. In normoxia, four weeks of SIT decreased type IIx fibers in subjects administered nitrate-rich beetroot juice, whereas type IIx fibers tended to increase in those receiving placebo [85]. Another normoxic training study found SIT not to alter type IIx fibers, but to increase type IIa fibers when beetroot juice but not potassium nitrate (KNO_3) was administered [86]. The role of dietary nitrate supplementation during training in either normoxia or hypoxia is not yet fully understood and requires further investigation. However, we here for the first time demonstrate that a dietary intervention as simple as dietary nitrate administration may potentially affect training-induced muscle fiber type transitions.

Resistance training in hypoxia *versus* similar training in normoxia has been postulated to enhance muscle anabolism [87,88]. However, conflicting findings have also been reported [20,89]. In our experiments, SIT (**Study 1**) nor high-intensity knee-extension training (**Study 2**) in either normoxia or hypoxia increased muscle fiber cross-sectional area. In line with current literature, SIT [90] and low-load high-repetition resistance training [89] that does not stimulate muscle mass accretion in normoxia probably also fails to stimulate muscle hypertrophy in ambient hypoxia.

2.6. Hematological adaptations to 'living high'

Performance enhancement following 'living high' is primarily driven by expansion in total hemoglobin mass (Hbmass). Hypoxia-induced HIF-2α activation and proliferation of renal erythropoietin (EPO) producing cells increase serum EPO (sEPO) in a dose-dependent manner [91-93]. However, the sEPO response is biphasic, reaching its peak within the first 48 h and gradually declining towards sea-level values over the following days and weeks **Figure 9**[93-95]. This suggest that a negative feedback loop decreases sEPO levels through either (i) decrements in HIF-α protein abundance consequent to elevated PHD protein expression (supra) [33,34,96], (ii) increased arterial oxygen content, (iii) facilitated oxygen extraction by elevated 2,3-diphosphoglycerate concentrations [91,97,98], (iv) suppressed intrarenal oxygen consumption increasing renal PO_2 [99], or (v) other unknown mechanisms occurring in acclimatizing lowlanders. Implementation of brief periods of 'living low', whilst gradually decreasing F_iO_2 during 'living high', could potentially attenuate desensitization of oxygen-sensing mechanisms in renal EPO producing cells and maintain elevated levels of sEPO during sustained intermittent hypoxic episodes.

In **Study 2-3**, subjects were exposed to gradually increasing normobaric hypoxia (~2000–3250 m) during five consecutive 5-day periods of 'living high' (**Figure 4 A**). Two days 'living low' interspersed each 5-day period of 'living high'. sEPO was measured after the first night of each 5-day period in hypoxia. Compared to normoxia, sEPO increased more than two-fold throughout the study intervention, from the initial to the final week (**Figure 4 B**). Hbmass increased by ~2.6% (range: 1.3-5.9%, $P < 0.05$) from the pretest to the posttest (**Figure 5 A**). In fact, after accounting for withdrawal of ~10 g Hb in ~65 mL blood during the intervention period, a 3.7% net increase in Hbmass was observed. The expansion in Hbmass was within the range of the expected ~3.4% increase predicted by the recent 'kilometer·hours' model proposed by Garvican-Lewis *et al.* [100]. However, another recent study reported a 6.7% rise in Hbmass for only ~230 h of intermittent normobaric hypoxia, similar to the current protocol, yet at a constant simulated altitude of 3000 m [101]. This could indicate that a higher degree of intermittent hypoxia is more proficient in increasing Hbmass. In addition, intermittent exposure to hypoxia may have stimulated erythropoiesis through maintenance of high sEPO concentrations. Indeed, administration of recombinant human EPO increases Hbmass in a dose-dependent manner [102-105], and

we showed the raise in endogenous EPO to correlate with Hbmass expansion (r=0.78). However, one must also beware of the role of low sEPO in neocytolysis. Upon re-exposure to normoxia, sEPO falls below its normoxic baseline level and induces destruction of newly formed young red blood cells [106,107]. In that perspective, intermittent 'living high' may inhibit rather than stimulate erythropoiesis. Unfortunately, the absence of a control group 'living high' does not allow us to tease out the effects of intermittent *vs*. continuous 'living high' at either fixed or gradually decreasing F_iO_2. At present, such alternative 'living high' models have not yet been compared to continuous 'living high' at fixed altitude and warrant further investigation.

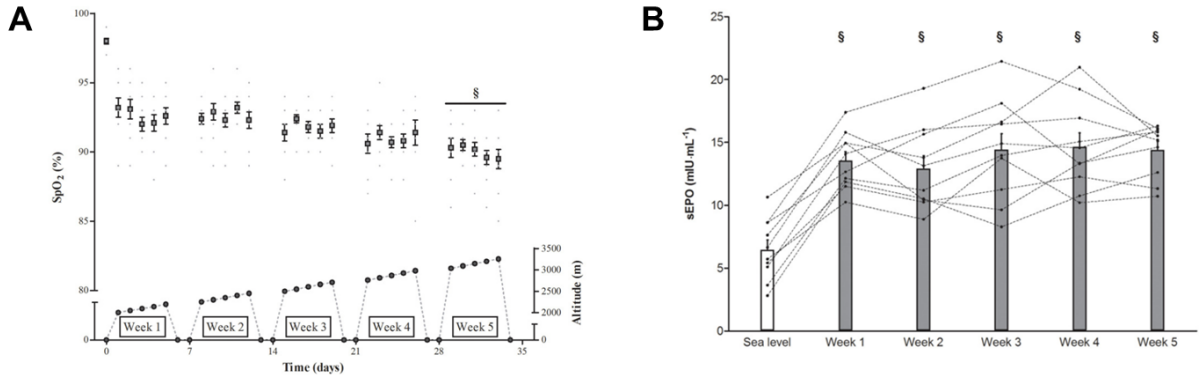

Figure 4 | Effect of incremental altitude on arterial oxygen saturation and serum erythropoietin concentrations during 'living high'.
Data are means ± SEM (n = 10) and individual data (dots) for (**A**) arterial oxygen saturation (S_pO_2) and (**B**) serum erythropoietin concentration (sEPO) in subjects living in normobaric hypoxia with simulated altitude gradually increasing from 2000 m in week 1 to 3250 m in week 5. §, P < 0.05 compared to sea level.

None of the subjects of **Study 2-3** were diagnosed with iron deficiency (serum ferritin < 15 µg·L-1 [108]) at baseline. Nonetheless, because iron supplementation was recently reported to increase the response in Hbmass during 'living high' in both athletes with low and normal (> 20–200 $\mu g \cdot L^{-1}$) serum ferritin (sFer) levels [109], and in fact considered essential to induce any erythropoiesis in the first place [110], we decided to administer iron supplements (105mg elemental iron per day) to all subjects exhibiting baseline sFer concentrations lower than 100 $\mu g \cdot L^{-1}$. Although Hbmass did not increase more in iron supplemented subjects in the present study (**Figure 5 A**), supplementation negated a ~30% drop in ferritin-based iron stores (**Figure 5 B**). In line with these results, non-iron supplemented women with low baseline serum ferritin levels (29 ± 16 $\mu g \cdot L^{-1}$) further decreased ferritin levels throughout 16 days at 5260 m (to 8 ± 5 $\mu g \cdot L^{-1}$) and increased their Hbmass by 5.5% [111]. Our data taken together with the current literature indicates iron supplementation not to be required to increase Hbmass during 'living high', yet to be advised to either (i) potentially stimulate the Hbmass response [109,110] and (ii) maintain adequate iron stores throughout the altitude intervention.

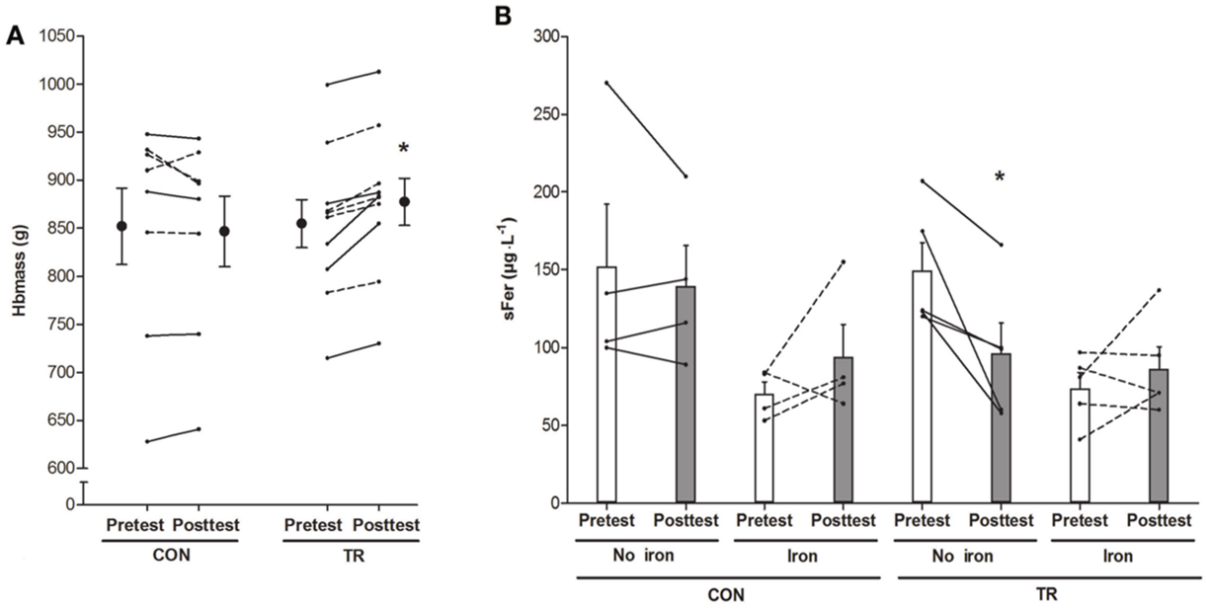

Figure 5 | Effect of 'living high' on blood hemoglobin mass and serum ferritin concentration. Data represent means ±SEM (n = 10) and individual values (lines) for hemoglobin mass (Hbmass, **A**) and serum ferritin concentrations (sFer, **B**) before (Pretest) and after (Posttest) living 5 weeks in normoxia (CON) or in normobaric hypoxia (TR) with simulated altitude gradually increasing from 2000 to 3250 m. Iron supplementation was provided in subjects with sFer below 100 $\mu g \cdot L^{-1}$ (dotted lines), but not in subjects with sFer above 100 $\mu g \cdot L^{-1}$ (solid lines). *P < 0.05 compared to the pretest.

3. Performance adaptations to altitude training

Sea-level performance enhancements following altitude training are mediated by hematological adaptations following 'living high', and/or muscular adaptations following 'training high'. In that respect, an increasing number of studies are investigating altitude training regimes combining both the strategies, i.e. 'live high plus train low and high' [5,112]. In the present thesis, it was hypothesized that SIT in hypoxia compared to normoxia would enhance sea-level aerobic and anaerobic capacity and performance (**Study 1**). Furthermore, it was postulated that high-intensity knee-extension training in hypoxia compared to normoxia in a setting of 'living high' would stimulate sea-level high-intensity endurance and repeated-sprint ability performance of the knee extensors whilst increasing total Hbmass and thus whole body VO_2max (**Study 2**).

In keeping with previous research [113,114], SIT in normoxia significantly increased sea-level VO_2max and 30-min time-trial performance (**Study 1**). However, similar training in ambient hypoxia did not further ameliorate these performances in the conditions of present study. Accordingly, citrate synthase activity, a marker of muscular oxidative capacity increased irrespective of the ambient oxygen level during training sessions. Our data corroborate previous literature findings showing equivalent improvements in VO_2max [7,115,116], time-trial performance [7], and time to exhaustion at 80% of VO_2max [116] following SIT in hypoxia *vs*. normoxia. Therefore, we can now conclude that, compared with similar training in normoxia, ambient hypoxia during short-term SIT does not stimulate adaptations in aerobic capacity or performance at sea level. In fact, neither SIT nor repeated sprint training (repeated sprints of 5-10 s interspersed by brief recovery periods of ≤ 45 s) in hypoxia ameliorated sea-level aerobic performance compared to similar training in normoxia [117]. Still, interindividual variability in training

responses may mask small but potentially relevant effects of hypoxic supplementation to training. Therefore, we compared the effects of normoxic *vs*. hypoxic high-intensity knee-extension training within the same individual (left leg *vs*. right leg) (**Study 2**). Again, in line with previous supramaximal intensity training studies, short-term high-intensity knee-extension training in hypoxia did not enhance 3-min all-out performance more than similar training in normoxia. Furthermore, despite that these subjects expanded their Hbmass by 2.6% from the pretest to the posttest, VO_2max increased neither one nor two weeks following the 5-week 'live high – train low or high' intervention. The magnitude of Hbmass increment probably was insufficient to detect changes in VO_2max, as an increase by 2.6% in Hbmass is expected to raise VO_2max no more than ~1.7%, which is within the error of measurement of the ergospirometry methodology.

Thirty-second sprint performance also increased to the same degree due to short-term SIT in normoxia and hypoxia (**Study 1**). Only one other study evaluated single 30-s sprint performance following SIT in normoxia vs. hypoxia and reported no additional benefits from training in hypoxia [116]. Accordingly, 10 and 30-s sprint performance similarly increased following repeated-sprint training in normoxia (RSN) and hypoxia (RSH) [118]. However, the number of sprints to exhaustion during a repeated-sprint ability (RSA) test increased more following RSH than RSN in studies using either ambient hypoxia [6,119] or voluntary hypoventilation [120,121] to induce hypoxemia during training, although these findings have been debated and require further investigation [16,122]. Mean power output, speed, or distance covered during RSA increased more after RSH than RSN in some [123,124] but not all [15,16,118,125,126] studies. We evaluated RSA before and after 5 weeks of high-intensity knee-extension training, but did not observe greater gains following training in hypoxia than in normoxia (**Study 2**). We conclude that supramaximal training in hypoxia does not consistently stimulate performance in single or repeated sprints, yet promising data on the number of sprints to exhaustion [6,119,120,121] indicate the need for further investigations.

4. Maladaptation to altitude: acute mountain sickness

Sixteen healthy volunteers were exposed to 24 h of normobaric hypoxia equivalent to 4000 m altitude. Five 30-min submaximal exercise bouts simulated the workload associated with normal ascend rates to alpine huts and summits [127]. A wide range of physiological measurements was performed and findings were compared to normoxia, as well as between subjects afflicted less (AMS_{LOW}) or more (AMS_{HIGH}) by acute mountain sickness (AMS). Early on during the hypoxic trial at rest, ~5% lower blood oxygenation, lower minute ventilation, and a progressive reduction in total and vagal heart rate variability (HRV) was observed in hypoxia in AMS_{HIGH} compared to AMS_{LOW}. Submaximal exercise following 2 h in hypoxia elicited greater blood lactate concentrations and ~4% arterial oxygen desaturation in AMS_{HIGH} compared to AMS_{LOW}. Subjects in AMS_{LOW} successfully completed all 5 submaximal cycling bouts in hypoxia. Conversely, 5 out of 7 seven subjects in AMS_{HIGH} could not maintain the prescribed exercise workload. Multiple regression analyses identified S_pO_2 after 2 h in hypoxia at rest to predict 32% of the average AMS symptoms, whilst S_pO_2 during the first submaximal exercise bout at 2 to 2.5 h in hypoxia predicted 53% of peak AMS symptom severity.

Some [128-130] but not all [131-132] earlier studies indicated relative hypoventilation within the initial hours in hypoxia in subjects prone to AMS. Also lower S_pO_2 in AMS susceptible subjects is a common observation [133-139]. However, its clinical relevance has been contested [140-143]. Nonetheless, Burtscher et al. reported S_pO_2 after 30 min at rest in acute normobaric or hypobaric hypoxia of varying degrees to adequately identify 86% of AMS susceptible mountaineers [134]. Savourey et al. identified end-tidal O_2 pressure and peripheral blood O_2 content ([Hb]*S_pO_2*1.34) at 30 min in normobaric hypoxia (F_iO_2:

12%) at rest to predict 77% of the variance in peak AMS symptom severity during subsequent mountaineering [144]. Furthermore, 71% of mean AMS symptom severity was predicted by peripheral blood O_2 content, the normoxic to hypoxic increase in breathing frequency, and the hypoxic cardiac response (increase in heart rate per decrease in S_pO_2). Our data taken together with previous literature findings [134,144] clearly show that subjects who are able to maintain high degree of blood oxygenation in acute hypoxia, both at rest and during exercise, are more resilient to AMS development.

Acute hypoxia has previously been shown to reduce cardiac vagal modulation in parallel with decrements in S_pO_2 [145]. Here, we showed a gradual reduction in total (total frequency power) and vagal (high frequency power) HRV in AMS_{HIGH}, whereas only a mild and transient vagal withdrawal was observed in AMS_{LOW} (**Figure 6**). At least at level of the heart, our results indicate an AMS-specific shift towards relative sympathetic dominance induced by substantial parasympathetic withdrawal rather than from elevation in sympathetic tone. Our observations corroborate the findings of Yih *et al.*, showing recovery *vs*. further deterioration (albeit non-significant) in vagal HRV following the first night in hypoxia in AMS_{LOW} *vs*. AMS_{HIGH}, respectively [146].

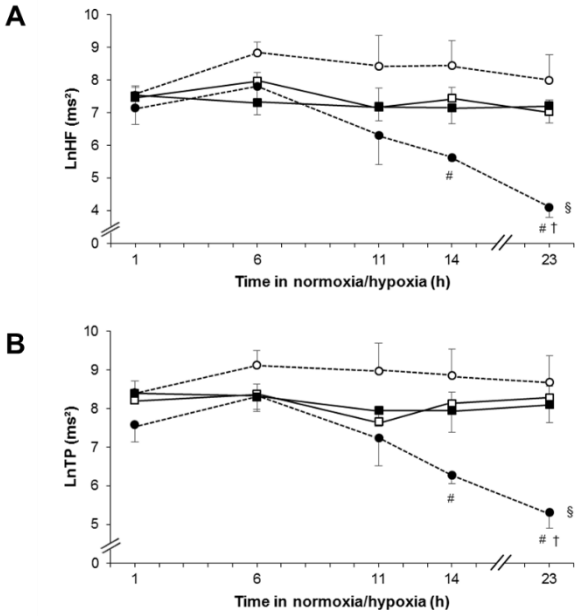

Figure 6 | HRV parameters showing a divergent response over time in hypoxia between AMS_{LOW} and AMS_{HIGH}

*Data are mean ± SEM and represent the natural logarithmic transformation of high (LnHF, **A**) and total frequency (LnTP, **B**) power at rest during spontaneous breathing in normoxia (F_iO_2: 20.9%, open symbols) and hypoxia (F_iO_2: 12.7%, solid symbols) in AMS_{LOW} (LSS < 6, n=7, squares with solid lines) and AMS_{HIGH} (LLS ≥ 6, n=4, circles with dashed lines). See METHODS for further details. # P < 0.05 vs. AMS_{LOW}; † P < 0.05 vs. 1 h; § P < 0.05 group (AMS_{LOW} vs. AMS_{HIGH}) x time (1 vs. 6 vs. 11 vs. 14 vs. 23 h) interaction.*

5. Methodological considerations and limitations

Several methodological considerations are warranted for appropriate interpretation of the present findings.

Normobaric hypoxia – In the present thesis all hypoxic experiments were performed in normobaric hypoxia. Normobaric hypoxia simulates terrestrial altitude by decreasing the fraction of ambient O_2 concentrations yet at constant barometric pressure equivalent to sea level. Minor differences exist in the physiological responses to normobaric and hypobaric hypoxia. Compared to normobaric hypoxia, hypobaric hypoxia is associated with a shallow rapid breathing pattern that leads to lower alveolar ventilation and blood oxygenation [147]. Consequently, inferior time-trial performance [148] and higher incidence and severity of AMS [149-150] have been reported in hypobaric compared to normobaric hypoxia. In contrast, no differences in erythropoietic adaptions were reported [151]. Taken together,

hypobaric hypoxia is associated with slightly different physiological responses and greater hypoxic stress. Therefore extrapolation of our data to terrestrial altitude warrants caution. Nonetheless, Burtscher et al. [134] and Savourey et al. [144] found S_pO_2 and other physiological parameters assessed in normobaric hypoxia to predict AMS susceptibility at terrestrial altitude. Therefore it is reasonable to postulate that our findings, at least for the major part, likewise predict physiological responses to hypobaric hypoxia.

NIRS –The use of NIRS allowed us to evaluate muscle oxygenation during training, i.e. the oxygenated fraction of hemoglobin and myoglobin in the area of interest. In addition, we estimated changes in muscle blood volume by analyzing changes in total hemoglobin content (ΔtHb). Using this method we confirmed earlier findings of greater muscle blood volume changes during repeated sprints following training in hypoxia *versus* normoxia [6,16]. However, it should be acknowledged that ΔtHb is not a surrogate of blood flow. Though still an interesting parameter, it only estimates relative changes in local blood volume compared to the start of the reading.

Timing of muscle biopsy sampling – Careful timing of muscle biopsy sampling is required to avoid acute effects of the last training sessions whilst also limiting the effects of detraining. Both in **Study 1** and **Study 2-3** of the present thesis, subjects were instructed to refrain from strenuous physical activity for at least 48 h prior to muscle sampling. In **Study 1**, muscle biopsies in the posttest were taken 3 to 4 days after the final training session. In **Study 2-3**, muscle biopsies were taken 4 days after the last exercise test in the posttest, which concur 6-7 days after the last day of 'living high' and 9-10 days after the final training session. Acute exercise effects were conceivably excluded, yet the unique effects of training in normoxia *vs*. hypoxia in **Study 2-3** may have partially faded by the time muscle tissue was sampled.

Study duration – Training in **Study 1** and **Study 2-3** was limited to 5 weeks in duration. Unique physiological and performance adaptations to training in hypoxia may have required similar training interventions of longer duration.

In-vitro muscle buffer capacity – Titrimetric determination of i*n-vitro* muscle buffer capacity assesses physico-chemical buffering but does not detect changes in metabolic buffering and H^+ extrusion capacity of the muscle cell. It is therefore possible that *in-vivo* buffer capacity, measured as the ratio of muscle lactate and pH differerences from rest to exercise, may have increased through normoxic or hypoxic exercise training in absence of observable changes in *in-vitro* buffer capacity.

Muscle fiber type composition – Changes in biopsy-derived muscle fiber type composition requires cautious interpretation. In human studies, only a small fraction of the *vastus lateralis* muscle is analyzed, i.e. ~200 fibers per biopsy of a muscle containing ~500000 fibers. Furthermore, fiber type composition varies across the transversal (i.e. deep *vs*. superficial) and sagittal (i.e. proximal *vs*. distal) plane of the muscle [152]. In fact, fiber type proportions may vary within single fascicles [153] and even within a single myofiber [154]. Therefore, care was taken to consistently sample muscle tissue at similar depth whilst limiting the distance across the sagittal plane between the pretest and the posttest. Nonetheless, interpretation of changes in muscle fiber type composition must be performed with caution.

Interindividual variance in response to training and hypoxic exposure – Great interindividual variance exist in response to short-term endurance training [155], resistance training [156], and exposure to hypoxia [130,157]. Small but potentially relevant effects of training in normoxic *versus* hypoxic conditions may remain undetected due to the noise induced by interindividual responses in studies applying a parallel study design. Cross-over designs are therefore considered golden standard, but carry-over effects and particularly practical and financial constraints limit the implementation of such designs in training studies. We therefore applied single leg training in normoxia *versus* hypoxia within one

individual (**Study 2-3**). Although the benefits of this model are clear, we can not fully exclude the possibility of contraleral training adaptations via hemodynamic forces [158], neural adaptations [159], and/or humoral factors excreted by the muscle [160] or other endocrine organs. Nonetheless, the single-leg training model has been extensively used in the past and provided valuable insights in unique muscular adaptations following hypoxic *versus* normoxic exercise training [8,9,161].

High-intensity knee-extension training – Our model to investigate high-intensity training in normoxia *versus* hypoxia within a single individual in **Study 2-3** was performed on a slightly adapted traditional strength training machine (leg-extension apparatus). The protocol involved 2 sets of 4-6 consecutive 90-s series of 30 muscle contractions (1 s concentric, 1 s eccentric, 1 s rest) at 20-25% of the subjects' 1-repetition maximum. This protocol involves too many repetitions to be classified as low-load resistance training, yet it is difficult to compare this training mode to other traditional high-intensity interval or sprint training protocols. Furthermore, extrapolations from single-leg to whole-body training protocols need to be performed with caution because contracting muscle during single-leg exercise may accommodate far larger quantities of blood circulation than during whole-body exercise.

Repeated-sprint ability – RSA was not measured in **Study 1**, whilst RSA across 20 all-out 'sprints' was investigated in **Study 2**. Additional performance gains following sprint training in hypoxia are generally not found in mean RSA power output but in the amount of sprints that can be performed to exhaustion [6,119,121]. It is reasonable to postulate that such ability may translate in better sport performance in team sports such as basketball, football, *etc*., but also track cycling and other sports requiring multiple consecutive sprints. We did not measure the amount of sprints to exhaustion. Therefore we can confirm nor exclude enhanced sprint ability to exhaustion following either SIT or high-intensity knee-extension training in hypoxia.

Blinding procedure – For practical considerations, subjects in **Study 1-3** were not blinded for the hypoxic training environment. In **Study 2-3**, to induce an expectancy effect, the control group 'living low' received daily placebo supplements which they were told would mimic the effects of altitude training. In **Study 4**, subjects were blinded for the hypoxic environment, but the development of AMS symptoms during the 24-h hypoxic experiment made blinding till the end of the study impossible.

Study participants – Study participants were recruited by word of mouth, social media, and announcements on notice boards (*ad valvas*) at the campuses of KU Leuven University. Only non-smoking, recreationally-active healthy men aged 18 to 30 years not taking any supplements or medication were considered eligible for participation. This is considered standard procedure in sport science and is applied to limit as many confounding factors as possible. However, caution is required for extrapolation of study findings to other populations, *i.e.* sedentary or elderly individuals requiring medication, or elite athletes potentially benefiting from the additional hypoxic stress during training. With regards to the studies' sample size, practical (time, accommodation, financial budget) considerations must be considered against the statistical power required to unmask discrepancies in responses to exposure and/or training in hypoxia. We acknowledge that the studies in the present thesis might have been underpowered, hence increasing the risk for false negatives (type II errors), low reproducibility, and false discovery rates (type I errors).Particularly in **Study 4**, many physiological parameters lacked the power to reach statistical significance (many P-values between 0.05 and 0.10). That being said, the sample size of the studies in the present thesis are in line with typical studies in the field of exercise physiology (minimum of ~n=8 per group).

6. Future directions

Despite decades of research in altitude training and AMS, many questions remain to be resolved.

Potential benefits of training in hypoxia on muscular adaptations are frequently attributed to activation of the HIF pathway. However, currently no clear myocellular oxygen threshold has been identified at which HIF-1α accumulates, translocates to the nucleus, and dimerizes with HIF-1β to form a fully active transcription complex. In fact, at present no human studies have investigated the dose-response relationship between HIF-pathway activation on the one hand and passive exposure to different magnitudes of hypoxia, muscle contractions of different duration, type, and intensity, and all possible combinations of hypoxia and muscle contractions on the other hand. If unique adaptations are indeed induced by training in hypoxia, and if these adaptations are indeed mediated through activation of the HIF pathway, such dose-response investigations would form a solid framework for future hypoxic training studies to build on. In that respect, immunohistochemical analyses, as reported in **Study 3,** can stimulate accurate quantification of HIF-1α translocation and, in conjunction with other techniques, activation of the HIF pathway. Electrophoretic mobility shift assays to quantify HIF binding activity may have provided interesting data in **Study 3** and should be considered in future investigations.

Certain polymorphisms in human HIF-1α have been associated with predominance in fast-twitch muscle fibers and hence explosive and glycolytic sports performance [170]. Future investigations are warranted to explore the role of HIF-1α isoforms in individuals' adaptation and maladaptation to hypoxia and altitude training.

To date it remains difficult to define strong evidence-based recommendations for hypoxic training. Much of the evidence propagating hypoxic training comes from studies comparing normoxic *versus* hypoxic high-intensity endurance training at similar absolute training intensities. This implies that subjects training in hypoxia exercised at higher relative intensity. In healthy athletes without contra-indications for high mechanical loading of the musculoskeletal system, it can be postulated that equal or greater muscular and performance adaptations would occur following training at similar relative intensities [14]. Sprint training protocols generally require each sprint to be performed as an all-out effort, thus both relative and absolute training intensities are equal in normoxic and hypoxic training conditions. Repeated-sprint training in hypoxia *versus* normoxia increased the number of sprints to exhaustion in two studies [6,15], whilst no additional benefits were found another study [16]. Two recent experiments investigating repeated-sprint training during voluntary hypoventilation corroborate the ergogenic effects found following RSH [120,121]. The underlying mechanism may involve hypoxia-specific gene transcription [151], but confirmation at the protein level is scarce, inconclusive [5], and requires further investigation. More compelling evidence exists for greater muscle blood volume oscillations after either repeated-sprint training [6,15,16] or high-intensity knee-extension training in hypoxia (**Study 2**). These findings now need to be confirmed by methodologically robust measurements of muscle blood flow during intermittent exercise.

Data from the present thesis together with previous literature indicate training at supramaximal intensities [50-54,56] to be less effective in enhancing βhm compared to training at intensities slightly below VO_2max [57-59]. Whether βhm increases more following training at such intensities in hypoxic *versus* normoxic conditions remains to be investigated.

We measured sEPO after the first night of each of the five 5-day periods in progressively increasing hypoxia (**Study 2**). sEPO was continuously increased during intermittent 'living high' and predicted changes in Hbmass. Future studies are warranted to investigate whether either progressively

increasing hypoxia, intermittent hypoxia, or a combination of both are more efficient strategies to stimulate erythropoiesis than continuous 'living high' at fixed simulated or terrestrial altitude. In addition, future work is warranted to identify the required hypoxic dose administered through intermittent 'living high' to attenuate or prevent a decline in Hbmass during re-exposure to normoxia.

In obese individuals, emerging data indicate that normobaric hypoxia *versus* normoxia at rest increases energy expenditure, whilst training in hypoxia appears to stimulate a decrease in body weight and blood pressure [162]. Furthermore, hypoxic training may improve glycemic control more than similar training in normoxia [163]. Implementation of hypoxic training as a non-pharmacological therapy to improve cardiometabolic health is a promising strategy that warrants further investigation. However, in this population, thorough medical screening prior to hypoxic training initiation is warranted to rule out any potential medical contraindications.

7. General conclusion

Altitude training through living and/or training at terrestrial or simulated altitude is widely used by athletes in attempt to stimulate hematological and muscular adaptations and exercise performance. We hypothesized that short-term training at supramaximal intensities in hypoxia *versus* normoxia, either or not in the setting of 'living high', would enhance muscular adaptations and performance. High-intensity knee-extension exercise in hypoxia clearly exacerbated muscle deoxygenation as compared to training in normoxia. Furthermore, short-term training in hypoxia induced greater changes in muscle blood volume oscillations during repeated-sprint exercise in normoxia, indeed. However, sprint interval training nor high-intensity knee-extension training in hypoxia stimulated adaptations in muscle buffering capacity, pH-regulating protein content, fiber cross-sectional area, or exercise performance compared to similar training in normoxia. Despite nitrate supplementation not increasing power output during sprint interval training in hypoxia, reductions in type IIx fibers were associated with an increase in type IIa muscle fibers only when hypoxic training was performed concurrent with dietary nitrate intake. We also hypothesized ischemia-induced HIF pathway activation in skeletal muscle to be less pronounced following high-intensity knee-extension training in normoxia, and particularly after training in hypoxia. A remarkably high presence of HIF-1α was detectable in myonuclei at baseline. Ten minutes of ischemia-induced hypoxia further increased myonuclear HIF-1α co-localisation. Against our hypothesis, however, high-intensity knee-extension training did not alter the ischemia-induced increase in HIF-1α-positive nuclei, irrespective of oxygen availability during training. At the hematological level, intermittent 'living high' at progressively increasing normobaric hypoxia elicited a stable increase in serum erythropoietin which adequately predicted the increase in total hemoglobin mass. Finally, we showed that exposure to normobaric hypoxia equivalent to ~4000 m induced mild symptoms of acute mountain sickness in some individuals, but severe AMS symptoms in others. Low arterial oxygen saturation at rest and during submaximal exercise during early exposure to hypoxia predicted acute mountain sickness in a later phase. Subjects most affected by acute mountain sickness displayed hypoxic exercise intolerance and greater withdrawal in vagal activity to the heart. Taken together, living in progressively increasing hypoxia efficiently increases erythropoiesis, but additional muscular adaptations through supramaximal intensity training in hypoxia are rather limited. Depending on the magnitude and duration of exposure hypoxia, environmental oxygen depreviation may also cause maladaptation. Susceptibility to acute mountain sickness differs greatly between individuals and is at least partially related to one's capabilty to maintain a high degree of blood oxygenation in acute hypoxia.

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9. Conflict of interest statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APPENDICES

Appositions – bijstellingen

Bijstelling 1

Intelligentiequotiënt (IQ) is de meest gebruikte stafmaat voor het meten van begaafdheid en voorspellen van toekomstig academisch of professioneel succes. Motivatie onder de vorm van passie en gedrevenheid voor het nastreven van (langetermijn-) doelstellingen, alsook de overtuiging dat je zelf bekwaamheden ontwikkelen kan, zijn echter minstens even doorslaggevend.

Bijstelling 2

Slechts ~33% van de robuuste wetenschappelijke evidentie wordt uiteindelijk geïmplementeerd in de klinische praktijk. Succesvolle implementatie kan gerealiseerd worden door de principes van 'implementation science' toe te passen, zoals bijvoorbeeld onder meer het uitvoeren van een grondige contextuele analyse, het betrekken van de stakeholders in de verschillende fases van het onderzoeksproces, het gebruik maken van implementatiestrategieën, het focussen op zowel implementatie als summatieve outcomes en het aanwenden van hybride designs.

Bijstelling 3

Fysieke inactiviteit is wereldwijd de vierde belangrijkste oorzaak van mortaliteit. Fysieke activiteit als curatieve en/of preventieve behandeling op voorschrift wordt onvoldoende erkend en benut.

Professional career of Stefan De Smet

PROFESSIONAL EXPERIENCE

10.2017 – Present	**Scientific co-worker** at 'Laboratory of abdominal transplantation surgery' – 'Transplantoux', Leuven, Belgium
10.2012 – 04.2017	**PhD candidate in exercise physiology**, Exercise Physiology Research Group, KU Leuven, Leuven, Belgium**.**
12.2011 – 10.2012	**Scientific co-worker** at 'TopsportABC' and 'Bakala Academy', Leuven, Belgium
10.2010 – 10.2012	**Physiotherapist** at 'BeFit', Meensel-Kiezegem, Belgium **Strength and conditioning trainer** at 'BeFit', Meensel-Kiezegem, Belgium

SCIENTIFIC EDUCATION

19.07.2017	**Diving Alert Network (DAN) Research Specialist.** DAN Europe, Malta.
24.11.2011	**Mountain Medicine Course, Nepal.** Academy of Wilderness Medicine, United Kingdom
02.07.2010	**Master of Rehabilitation Sciences and Physiotherapy** (Magna cum laude), KU Leuven, Leuven, Belgium

PUBLICATION LIST

Published papers in peer-reviewed journals:

De Smet S, D'Hulst1 G*, Poffé C, Van Thienen R, Berardi E, Hespel P (**2018**). High-intensity interval training in hypoxia does not affect muscle HIF responses to acute hypoxia in humans. *Eur J Appl Physiol. 118(4), 847-862*

De Smet S, van Herpt P, D'Hulst G, Van Thienen R, Van Leemputte M, Hespel P (**2017**). Physiological adaptations to hypoxic vs. normoxic training during intermittent living high. *Front Physiol. 8, 347*

Vandoorne T, **De Smet S**, Ramaekers M, Van Thienen R, De Bock K, Clarke K, Hespel P (**2017**). Intake of a ketone ester drink during recovery from exercise promotes mTORC1 signaling but not glycogen resynthesis in human muscle. *Front Physiol. 8, 310*

Hiroux C, Vandoorne T, Koppo K, **De Smet S**, Hespel P, Berardi E (**2016**). Physical activity counteracts tumor cell growth in colon carcinoma C26-injected muscles: an interim report. *Eur J Transl Myol. 26(2):5958*

De Smet S, Van Thienen R, Deldicque L, James R, Sale C, Bishop DJ, Hespel P (**2016**). Nitrate intake promotes shift in muscle fiber type composition during sprint interval training in hypoxia. *Front Physiol. 7, 233*

Botek M, Krejčí J, **De Smet S**, Gába A, McKune AJ (**2015**). Heart rate variability and arterial oxygen saturation response during extreme normobaric hypoxia. *Auton Neurosci.190:40-45*

Manuscripts under review in peer-reviewed journals:

De Smet S, Daucin C, Botek M, Van Thienen R, Hespel P (**2018**). Exercise intolerance, low arterial oxygen saturation, and vagal cardiac withdrawal in hypoxia in subjects at risk for AMS. *In preparation for review in High Alt Med Biol.*

Dalle S, **De Smet S**, Geuns W, Van Rompaye B, Hespel P, Koppo K (**2018**). Effect of gradual sodium bicarbonate loading on multiple all-out efforts. *Under review in IJSPP*

NON SCIENTIFIC QUALIFICATIONS

3* FD, Advanced Freediver (Nelos)

Advanced open water scuba diver (PADI)