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ORIGINAL ARTICLE

Training in the fasted state facilitates re-activation of eEF2 activity during recovery from endurance exercise

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Abstract Nutrition is an important co-factor in exercise-induced training adaptations in muscle. We compared the effect of 6 weeks endurance training (3 days/week, 1–2 h at 75% VO_{2peak}) in either the fasted state (F; $n = 10$) or in the high carbohydrate state (CHO, $n = 10$), on Ca^{2+}-dependent intramyocellular signalling in young male volunteers. Subjects in CHO received a carbohydrate-rich breakfast before each training session, as well as ingested carbohydrates during exercise. Before (*pretest*) and after (*posttest*) the training period, subjects performed a 2 h constant-load exercise bout ($\sim$70% of *pretest* VO_{2peak}) while ingesting carbohydrates (1 g/kg h^{-1}). A muscle biopsy was taken from m. vastus lateralis immediately before and after the test, and after 4 h of recovery. Compared with *pretest*, in the *posttest* basal eukaryotic elongation factor 2 (eEF2) phosphorylation was elevated in CHO ($P < 0.05$), but not in F. In the *pretest*, exercise increased the degree of eEF2 phosphorylation about two-fold ($P < 0.05$), and values returned to baseline within the 4 h recovery period in each group. However, in the *posttest* dephosphorylation of eEF2 was negated after recovery in CHO, but not in F. Independent of the dietary condition training enhanced the basal phosphorylation status of Phospholamban at Thr^{17}, 5′-AMP-activated protein kinase α (AMPKα), and Acetyl CoA carboxylase β (ACCβ), and abolished the exercise-induced increase of AMPKα and ACCβ ($P < 0.05$). In conclusion, training in the fasted state, compared with identical training with ample carbohydrate intake, facilitates post-exercise dephosphorylation of eEF2. This may contribute to rapid re-activation of muscle protein translation following endurance exercise.

Keywords Nutritional status · Metabolic adaptations · AMP-activated protein kinase · Ca^{2+}-calmodulin-dependent protein kinase · Eukarotic elongation factor 2

Introduction

It has been well documented that endurance exercise training promotes metabolic adaptations in skeletal muscle resulting in improved endurance performance. The rise in cytosolic Ca^{2+} concentration during muscle excitation and contraction probably is an important mechanism to alter muscle gene expression and mitochondrial biogenesis in response to training (Chin 2005; Wright 2007). In addition, it has been well established that 5′-AMP-activated protein kinase (AMPK), an ubiquitously expressed serine/thionine multisubstrate protein kinase, plays a pivotal role as a metabolic regulator in skeletal muscle cells (Jorgensen et al. 2006).

During contractions, intramyocellular Ca^{2+} level increases and initiates a signalling cascade in which calmodulin (CaM), a Ca^{2+} receptor protein, activates Ca^{2+}-calmodulin-dependent protein kinases (CaMK), such as eukaryotic elongation factor 2 (eEF2) kinase, CaMK I, II, and IV, and CaMK kinases (CaMKK) (Hook and Means 2001). It has been demonstrated that CaMKII is a pivotal CaMK in human skeletal muscle, and that phosphorylation of the CaMKII substrate Phospholamban at Thr^{17} (PLN^{17}) reflects in vivo CaMKII activity (Rose et al. 2006). Furthermore, next to LKB1, CaMKK is currently believed to

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act as an upstream kinase of AMPK (Jensen et al. 2007; Hurley et al. 2005; Hawley et al. 2005), which indicates a link between AMPK and Ca^{2+}-signalling. There are data to indicate that AMPK is implicated in the modulation of the protein elongation process by eEF2, at least in muscle cells at rest (Hong-Brown et al. 2007; Horman et al. 2002). On the other hand, Rose et al. (2009) recently showed that inhibition of protein synthesis during exercise in humans by phosphorylation of eEF2, due to the action of Ca^{2+}-CaM-eEF2 kinase, occurred independent of AMPK. Interestingly, recent findings also indicate that increased cytosolic Ca^{2+} in muscle cells also stimulates the activation of p38 mitogen-activated protein kinase (MAPK) by CaMKII. This in turn enhances peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) expression and thereby mitochondrial biogenesis (Wright et al. 2007; Akimoto et al. 2005). Taken together, the above findings indicate that Ca^{2+}-signalling pathways are implicated in metabolic adaptations of skeletal muscles to endurance training.

Evidence is also accumulating to indicate that nutrition is an important co-factor in endurance training-induced muscle remodelling (De Bock et al. 2008; Hansen et al. 2005; Yeo et al. 2008b; Hulston et al. 2010; Nybo et al. 2009). For instance, we have recently found that training in the fasted state during an episode of hypercaloric fat-rich diet in healthy young volunteers, compared with a similar training programme in the high carbohydrate state, increased the expression of muscle GLUT4, AMPKα, FAT/CD36, and CPT1, either at the protein or at the mRNA level (Van Proeyen et al. 2010). Similarly, both FAT/CD36 and membrane-bound fatty acid binding protein ($FABP_{pm}$) contents were elevated after short-term training in the fasted state, but this effect was negated by carbohydrate intake before and during the training sessions (De Bock et al. 2008). Furthermore, it also has been demonstrated that net muscle protein degradation is increased during prolonged exercise in a carbohydrate-deficient state (Wagenmakers et al. 1991; Jackman et al. 1997), which might in turn stimulate adaptation of signalling pathways implicated in post-exercise protein repair. Support for such assumption contention comes from our recent observation that $p70^{s6k}$ phosphorylation is elevated during recovery from a resistance exercise bout in the fasted state, indicating higher degree of activation of muscle anabolism than after an identical exercise bout in the fed state (Deldique et al. 2010).

It is well known that exercise changes the degree of activation of a wide range of signalling proteins in metabolic pathways involved in skeletal muscle adaptation. For example, consistent exercise training upregulates AMPKα phosphorylation status in human muscle (Frosig et al. 2004; Lee-Young et al. 2009; Benziane et al. 2008), whilst exercise-induced activation of AMPK is downregulated (McConell et al. 2005; McConell et al. 2008; Lee-Young et al. 2009; Benziane et al. 2008). Furthermore, after 6 weeks of aerobic training in mice, cardiomyocytes displayed elevated PLN^{17} phosphorylation in conjunction with increased CaMKII phosphorylation at Thr^{287} (Kemi et al. 2007). Rose et al. (2007) also previously reported that short-term endurance training elevated CaMKII activity, as well as CaMKII kinase phosphorylation at Thr^{287}. Still, in one other study endurance training was found to attenuate AMPK, but not CaMKII and p38 MAPK signalling, during exercise (Benziane et al. 2008). Whether the changes of eEF2 during exercise and recovery play a role in the adaptive response to endurance training is at present unknown. However, similar protein expression as well as phosphorylation status of eEF2 was found in resting muscle of endurance trained and untrained subjects (Roepstorff et al. 2005).

The dietary context of exercise is also believed to play a crucial role in the exercise-induced activation of AMPK as well as Ca^{2+}-dependent signalling. In this regard, studies by our (De Bock et al. 2005) and other laboratories (Akerstrom et al. 2006) have demonstrated higher AMPK activity in muscles during exercise in the fasted state than during exercise in conjunction with ample exogenous carbohydrate supply, which probably reflects greater challenge of energy balance in the former nutritional condition. Support for such assumption comes from observations showing higher degree of AMP accumulation in muscle for a given exercise intensity when carbohydrate availability is abundant than when it is limited (Spencer et al. 1991; McConell et al. 1999). It is also well established that failing energy homeostasis in muscle cells impairs sarcoplasmatic reticulum Ca^{2+} reuptake, resulting in elevated cytoplasmic Ca^{2+} concentration (Allen et al. 2008). If such mechanism also occurs during exercise in the fasted state, this is likely to enhance myocellular adaptations modulated via Ca^{2+}-dependent signalling pathways.

Against this background, the aim of the current study was to compare the effects of endurance training in the fasted state versus training with ample carbohydrate ingestion before and during exercise, on AMPK and Ca^{2+}-dependent signalling during exercise and subsequent recovery. Partial results of this study have been previously published elsewhere (De Bock et al. 2008).

Methods

Subjects

A total of 20 healthy, physically active men (age: 21.2 ± 0.4 years; body wt: 74.8 ± 2.0 kg) volunteered to

participate in the study, which was approved by the local Ethics Committee (K.U. Leuven). Subjects were instructed not to participate in any strenuous physical activity, except for the exercise sessions prescribed by the study protocol. Subjects gave their written, informed consent after they were informed in detail of all experimental procedures and risks possibly associated with the experiments.

Experimental protocol

The study protocol has been previously described in detail elsewhere (De Bock et al. 2008). Briefly, 2 weeks before the start of the study the subjects participated in a series of cycle exercise tests to determine VO_{2peak}. Subjects were then randomly assigned to one of two groups and participated in two experimental sessions (*pretest* and *posttest*), with a 6-week training period in between. On the morning of the *pretest*, they received a standardized carbohydrate-rich breakfast (722 kcal: 85% of total energy intake [En] carbohydrates, 4% En from fat, and 11% En from protein). After a 2-h rest period, a percutaneous needle biopsy sample was taken from the right vastus lateralis muscle. Thereafter subjects cycled for 2 h at $\sim$75% VO_{2peak} and received 1 g maltodextrin/kg body wt h^{-1} in a 15% solution. At the end of the exercise bout, another muscle biopsy was taken. During the following 4-h recovery period, the subjects rested in the laboratory and received 1.5 g maltodextrin/kg body wt (15% flavoured solution) during the initial and the last hour of recovery. Furthermore, between 2 and 3 h of recovery, they received a solid meal containing 1.5 g carbohydrates/kg body wt. At the end of the recovery period another muscle biopsy was taken. After the *pretest* the subjects were enrolled in a 6-week supervised training programme (3×/week, 60–120 min) combined with a dietary control regimen (65% En carbohydrates, 20% En fat, and 15% En proteins). One-half of the subjects trained in the fasted state (F; $n = 10$), whereas the others (CHO; $n = 10$) received a carbohydrate-rich breakfast and ingested a 15% maltodextrin solution during exercise. Subjects from F and CHO consistently trained as matched pairs at the same workload. At the end of the 6-week intervention period, subjects participated in the *posttest* which was identical to the *pretest*.

Analysis of muscle samples

After being freed from any visible non-muscle material, muscle samples were immediately frozen in liquid nitrogen. All samples were stored at −80°C for later analysis. Muscle specimens were freeze dried and dissected free of visible blood, connective, and fat tissues.

Muscle glycogen

Muscle glycogen content was measured as glucose residues after acid hydrolysis, in freeze-dried muscle tissue using a standard enzymatic fluorometric assay (Lowry and Passoneau 1972).

Muscle lysate production and Western blotting

Samples were homogenized in ice-cold buffer (20 mM Tris base, 50 mM NaCl, 2 mM DTT, 50 mM NaF, 1% Triton X-100, 250 mM sucrose, 5 mM Na-pyrophosphate, 4 μg ml^{-1} leupeptin, 6 mM benzamidine, 500 μM PMSF, 50 μg/ml soybean trypsin inhibitor, pH 7.4) for 20 s using a homogenizer (Polytron 2000, Kinematica, Littau, Switzerland). Homogenates were rotated end over end for 1 h at 4°C. Lysates were generated by centrifugation (17,500*g*) for 1 h at 4°C. Lysates were quick frozen in liquid nitrogen and stored at −80°C. Protein content in lysates was measured by the bicinchoninic acid method (Pierce Chemical Company, Ill., USA). Equal amounts of muscle extract protein were separated by SDS-PAGE, transferred to a polyvinylidene fluoride membrane, after which membrane was blocked at room temperature for 1 h in TBS-T (Tris-buffered saline containing 0.05% Tween 20, pH 7.4) comprising 2 or 5% skimmed milk powder. Blocked membranes were incubated with primary and secondary antibodies for optimized times and concentrations, and washed with TBS-T. Proteins were visualized by chemiluminescence (ECL plus, Amersham Biosciences, UK). The primary antibodies used were anti-phospho-Thr^{56}-eEF2 (Cell Signalling Technology Inc., USA; 2331), anti-phospho-Thr^{172}-AMPKα (Cell Signalling Technology Inc., USA; 2531), anti-phospho-Ser^{221}-ACCβ (Cell Signalling Technology Inc., USA, 3661), anti-phospho-p38 MAPK (Cell Signalling Technology Inc., USA, 9211), and anti-phospho-Thr^{17}-PLN (Cyclacel, UK, 010-13). Secondary antibodies were from DakoCytomation. Band density was calculated by using Kodak 1D image analysis software.

Statistical analyses

Treatment effects were evaluated using a repeated-measures ANOVA. Two-way ANOVA was performed to examine the main effects of treatment and/or time. In case the ANOVA yielded a significant effect, a planned contrast analysis was used for post hoc comparisons. In addition, contrast analysis was also used to evaluate specific preplanned comparisons. A probability level (P) < 0.05 was considered statistically significant. All data are expressed as mean ± SE.

Results

eEF2 (Fig. 1)

Compared with the *pretest*, baseline eEF2 phosphorylation status was markedly increased in CHO (+50%, $P < 0.05$), but not in F (+7%, $P = 0.66$). Still, changes from the *pretest* to the *posttest* were not significantly different between the groups ($P = 0.22$). The immediate exercise response was affected by training in neither group. However, in the *posttest*, eEF2 phosphorylation status returned to baseline within 4 h of recovery in F, whilst it remained elevated in CHO ($P < 0.05$). However, the change in the degree of eEF2 phosphorylation status during recovery was not significantly different between the groups ($P = 0.22$).

PLN (Fig. 2)

To evaluate the effect of training on Ca^{2+}-CaM-CaMKII signalling, we measured PLN phosphorylation at Thr^{17}, which is proposed to be indicative for in vivo CaMKII activity (Rose et al. 2006). In the *pretest*, baseline PLN^{17} phosphorylation status was similar between F and CHO. Compared with the *pretest*, baseline PLN^{17} phosphorylation status in the *posttest* was slightly increased both in CHO (+18%, $P < 0.05$) and in F (+15%, $P = 0.07$). Changes in the degree of PLN^{17} phosphorylation during exercise and recovery were similar between the groups in the *pretest* as well as in the *posttest*.

AMPKα and ACCβ (Fig. 3)

The training intervention significantly increased (~+35%) basal AMPKα phosphorylation status, whereas the exercise-induced increase in phosphorylation was blunted in both groups ($P < 0.001$). In fact, during the *posttest* the degree of AMPKα phosphorylation was slightly decreased following exercise in CHO ($P < 0.05$), but not in F. Still, the training-induced change in AMPKα phosphorylation status after exercise was not significantly different between the groups ($P = 0.30$). In parallel with the changes in AMPKα, pre-exercise ACCβ phosphorylation was higher in the *posttest* than in the *pretest*, whilst the rise in ACCβ phosphorylation immediately after exercise was negated by training in F as well as in CHO ($P < 0.05$). Nonetheless, in the *pretest* the exercise still slightly increased the degree of ACCβ phosphorylation in F ($P < 0.01$), but not in CHO ($P = 0.18$). However, again the changes from the *pretest* to the *posttest* were not significantly different between the groups ($P = 0.14$).

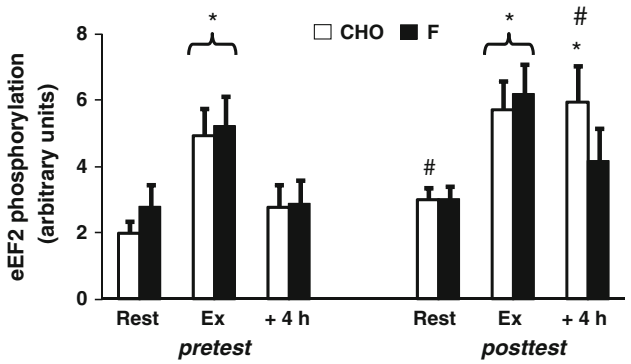

Fig. 1 Effect of 6 weeks training in the fasted state versus the high carbohydrate state on phosphorylation of muscle eEF2 during an acute exercise bout. Thr^{56} phosphorylation on eEF2 was measured by Western blotting on muscle samples obtained before (Rest), immediately after (Ex), and 4 h after (+4 h) a 2-h constant-load exercise bout. Data are given as mean arbitrary scanning units ± S.E.M. $^{*}P < 0.05$ indicates a difference compared with Rest. $^{\#}P < 0.05$ indicates a difference compared with the corresponding value in the *pretest*

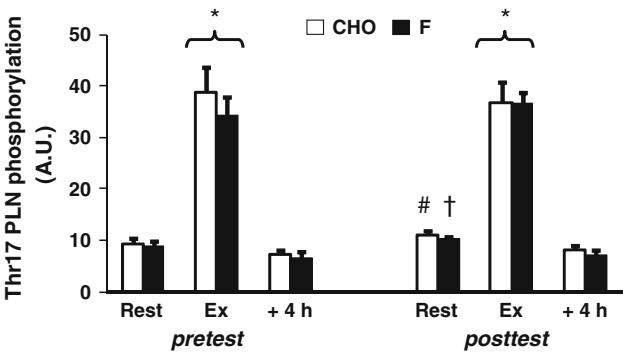

Fig. 2 Effect of 6 weeks training in the fasted state versus the high carbohydrate state on phosphorylation of muscle PLN^{17} during an acute exercise bout. PLN phosphorylation at Thr^{17} was measured by Western blotting on muscle samples obtained before (Rest), immediately after (Ex), and 4 h after (+4 h) a 2-h constant-load exercise bout. Data are given as mean arbitrary scanning units ± S.E.M. $^{*}P < 0.05$ indicates a difference compared with Rest. $^{\#}P < 0.05$ indicates a difference compared with the corresponding value in the *pretest*. $^{\dagger}P = 0.07$ versus the corresponding value in the *pretest*

p38 MAPK (Fig. 4)

As p38 MAPK lies downstream of CAMKII and mediates a variety of metabolic processes, we also measured the phosphorylation status of p38 MAPK. Baseline p38 MAPK phosphorylation was similar between the *pretest* and the *posttest* in both F and CHO. In the *pretest*, but not in the *posttest*, exercise stimulated p38 MAPK phosphorylation in each group ($P < 0.05$). Values were not significantly different between F and CHO at any time.

Muscle glycogen (Table 1)

During the *pretest*, muscle glycogen content was similar between the groups before and after exercise. Compared with the *pretest*, during the *posttest* initial glycogen content

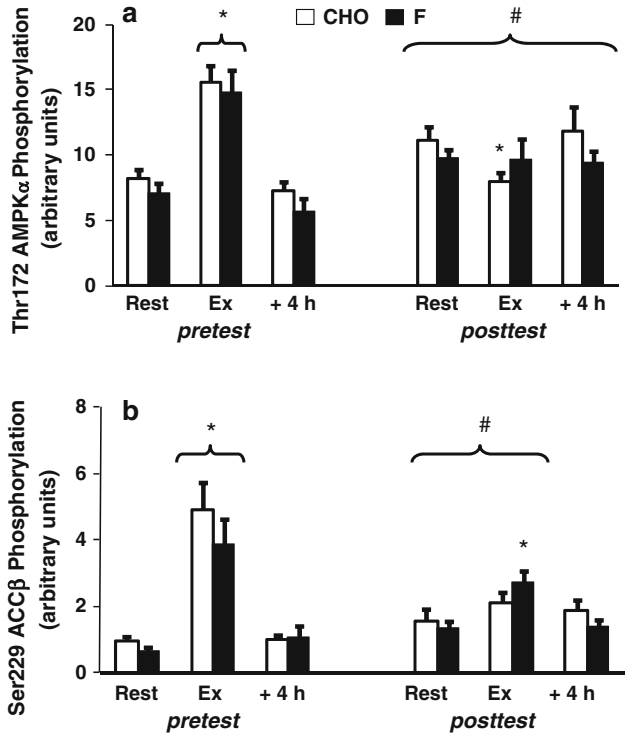

Fig. 3 Effect of 6 weeks training in the fasted state versus the high carbohydrate state on phosphorylation of muscle AMPKα and ACCβ during an acute exercise bout. Thr^{172} phosphorylation on AMPKα (**a**) and Ser^{221} phosphorylation of ACCβ (**b**) were measured by Western blotting on muscle samples obtained before (Rest), immediately after (Ex), and 4 h after (+4 h) a 2-h constant-load exercise bout. Data are given as mean arbitrary scanning units ± S.E.M. $^{*}P < 0.05$ indicates a difference compared with Rest. $^{\#}P < 0.05$ indicates a difference compared with the corresponding value in the *pretest*

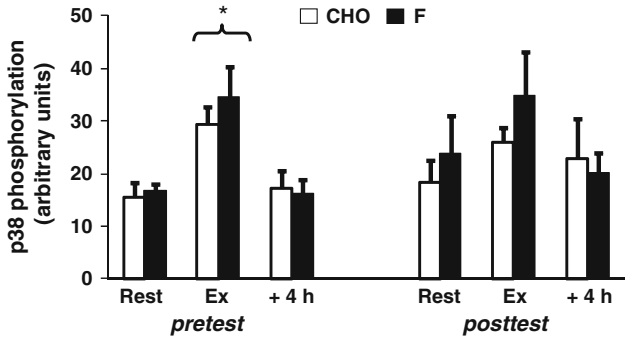

Fig. 4 Effect of 6 weeks training in the fasted state versus the high carbohydrate state on phosphorylation of muscle p38 MAPK during an acute exercise bout. p38 MAPK phosphorylation was measured by Western blotting on muscle samples obtained before (Rest), immediately after (Ex), and 4 h after (+4 h) a 2-h constant-load exercise bout. Data are given as mean arbitrary scanning units ± S.E.M. $^{*}P < 0.05$ indicates a difference compared with Rest

was increased in CHO ($P < 0.05$), but not in F ($P = 0.23$). The training intervention resulted in higher post-exercise glycogen levels in both groups ($P < 0.05$).

Table 1 Effect of 6 weeks training in the fasted state versus the high carbohydrate state on muscle glycogen content during an acute exercise bout

	F	CHO
Pretest		
Rest	378 ± 34	425 ± 49
Ex	167 ± 21*	158 ± 24*
+4 h	246 ± 23*	275 ± 28*
Posttest		
Rest	434 ± 33	545 ± 20#
Ex	295 ± 25*, #	241 ± 17*, #
+4 h	348 ± 26*, #	340 ± 26*, #

Muscle glycogen content was measured using a standard enzymatic fluorometric assay before (Rest), immediately after (Ex), and 4 h after (+4 h) a 2-h constant-load exercise bout. Data are given as mean mmol kg^{-1} dry weight ± S.E.M.

* $P < 0.05$ indicates a difference compared with Rest

# $P < 0.05$ indicates a difference compared with the corresponding value in the *pretest*

Discussion

Long-term training adaptations result from the sequential impact of acute responses to repeated exercise bouts. It is also well established that the metabolic responses of muscle cells to a given exercise mode depend on the nutritional status (De Bock et al. 2005; De Bock et al. 2007a; Deldique et al. 2010; Wagenmakers et al. 1991; Cluberton et al. 2005; Civitarese et al. 2005). Thus, long-term adaptations to training conceivably also depend on the nutrient intake before, during, and after the exercise sessions. It is well documented that exercise in the fasted state, compared with exercise in the fed state, generates a number of specific metabolic responses, most typically enhanced contribution of fat oxidation in energy provision, but also higher rate of muscle protein degradation (Gibala 2007; Koopman et al. 2004). Furthermore, net muscle protein breakdown is markedly enhanced during exercise when muscle glycogen stores are depleted (Wagenmakers et al. 1991; Jackman et al. 1997). In this regard, we have previously shown that net glycogen degradation is enhanced during exercise in the fasted state (De Bock et al. 2007a), which conceivably could result in premature glycogen depletion. Moreover, carbohydrate availability during the later stage of exercise in the fasted state is also limited due to liver glycogen depletion and thereby drop of blood glucose availability (Coggan and Coyle 1991). We recently have also demonstrated that $p70^{s6k}$ phosphorylation is enhanced following a resistance exercise bout in the fasted state, which indicates increased muscle anabolism (Deldique et al. 2010). Against this background and in line with the opinion that long-term training adaptations result

from the sequential action of acute exercise responses, we anticipated that consistent exercise training in the fasted state could trigger myocellular adaptations to facilitate post-exercise protein repair to compensate for the enhanced degradation during exercise. Consistent with such assumption, endurance training in the fasted state, compared with an identical training programme in the high carbohydrate state, facilitated post-exercise dephosphorylation (see Fig. 1) and thus activation of eEF2. This in turn probably contributes to rapid stimulation of net protein synthesis by virtue of facilitated protein translation in muscle cells during early recovery (Dreyer et al. 2006).

In keeping with earlier findings (Rose et al. 2005), acute exercise markedly increased eEF2 phosphorylation. Here, we add to this observation that the degree of exercise-induced eEF2 inactivation (∼phosphorylation) was not altered by short-term training in the fasted or high carbohydrate state (see Fig. 1). During recovery, however, activation of eEF2 by dephosphorylation (Dreyer et al. 2006) occurred more rapidly following training in the fasted state, than after training in the fed state with ample carbohydrate supplementation during exercise. It is also important to remind that the exercise test in the *pretest* as well as in the *posttest*, in contrast with the exercise sessions during the 6-week training period, was performed with ample carbohydrate supplementation before, during and after exercise. Such protocol was resolutely chosen because endurance athletes as a rule perform regular training in the fasted state aiming to improve performance during endurance competitions associated with ample carbohydrate supply before and during exercise. Therefore, our current findings indicate that acute high-dose carbohydrate intake does not negate the effect of prolonged training in the fasted state to stimulate rapid post-exercise eEF2 dephosphorylation and thereby re-activation of protein translation. Alternatively, we cannot exclude that this effect was at least partly due to an abrupt switch from habitual exercise in the fasted state to exercise with plenty of carbohydrate intake. The study protocol did not include an additional exercise trial with the participants fasted, which could have allowed to isolate the effects of training from the effect of diet on eEF2 activation. However, a number of arguments support the conclusion that the sustained post-exercise activation of eEF2 is likely due to the consistent fasted training. There is evidence to indicate that besides regulation by Ca^{2+} and AMPK, eEF2 activity can also be downregulated by cAMP-dependent protein kinase, versus upregulated via insulin-stimulated activation of the mammalian target of rapamycin complex 1 (Rose and Richter 2009). First, compared with exercise in the fasted state, during exercise with ample carbohydrate intake the facilitated energy balance (Spencer et al. 1991; McConell et al 1999) in conjunction with higher plasma insulin concentration (De Bock et al. 2005) may suppress exercise-induced inhibition of eEF2. This in turn may reduce the need for rapid eEF2 re-activation during early recovery. Thus, if no training adaptations were present one would rather expect a blunted eEF2 response after an exercise bout with high carbohydrate availability following a period of consistent fasted exercise. Second, given the greater rate of muscle protein degradation during exercise in the carbohydrate-depleted state than during exercise with high carbohydrate availability (Gibala 2007; Wagenmakers et al. 1991; Jackman et al. 1997; Koopman et al. 2004), rapid re-activation of eEF2 conceivably could contribute to facilitating post exercise protein repair during a period of consistently repeated exercise in the fasted state. Such mechanism is probably redundant during consistent exercise training with ample carbohydrate intake.

There are some literature data to suggest that the greater energy challenge during muscle contractions with limited carbohydrate availability, like exercise in the fasted state, also could facilitate activation of downstream targets of Ca^{2+} in muscle cells other than eEF2, such as AMPKα (De Bock et al. 2005; Akerstrom et al. 2006) and PLN^{17} (Rose et al. 2006). However, contrary to such assumption, independent of the dietary condition 6 weeks of training elicited similar adaptations of AMPKα, ACCβ as well as PLN^{17} activation. Before training (∼*pretest*) the 2 h constant-load exercise bout (171 ± 4 W) markedly activated AMPK, as evidenced by increased AMPKα phosphorylation status. The 6-week training programme expectedly negated this exercise-induced activation of AMPK (McConell et al. 2005, 2008; Benziane et al. 2008) (see Fig. 3a), which probably reflects facilitated energy homeostasis in muscle cells during a given absolute exercise intensity after a training period (Chesley et al. 1996; McConell et al. 2005). The effect of training to undo the exercise-induced activation of AMPK was independent of whether the training was performed in the fasted or high carbohydrate state. By analogy, the exercise-induced activation of ACCβ, an important downstream target of AMPK (Winder et al. 1997), was similarly reduced by training in both groups (see Fig. 3b). It has also been shown by some, but not by others (Rose et al. 2005; Rose et al. 2009; Miranda et al. 2008) that AMPK can phosphorylate eEF2, and thereby probably inhibit the process of protein elongation (Hong-Brown et al. 2007; Horman et al. 2002). However, in support of the latter opinion, we here clearly demonstrate that inhibition of the exercise-induced activation of AMPK by short-term endurance training (Fig. 3a), independent of the dietary condition, was not associated with downregulation of exercise-induced phosphorylation of eEF2 (Fig. 1). This again indicates that AMPK is probably not directly implicated in modulating training-induced adaptations of muscle protein translation

by regulation of eEF2 phosphorylation status. It has been shown that increased muscle glycogen content blunts exercise-induced activation of AMPK (Derave et al. 2000; Wojtaszewski et al. 2003). Basal muscle glycogen content was elevated following consistent exercise with high carbohydrate intake, but not after training in the fasted state. Still, 6 weeks of endurance training similarly decreased AMPK phosphorylation during exercise in both groups (Fig. 3a). This indicates a dissociation between initial glycogen level and exercise-induced AMPK activation after an episode of endurance exercise training, which corroborates some earlier observations (McConell et al. 2005; Yeo et al. 2008a; Van Proeyen et al., unpublished observations).

Phosphorylation of phospholamban at Th^{17} (PLN^{17}) reflects in vivo calmodulin kinase II (CaMKII) activity, which lies upstream of AMPK and is believed to play a pivotal role in Ca^{2+}-mediated intracellular signalling in muscle cells (Rose et al. 2006). Our current data demonstrate that in resting muscle the degree of PLN^{17} phosphorylation was increased by $\sim$15% following training in both experimental groups (Fig. 2). By analogy, Kemi et al. (2007) found higher PLN^{17} phosphorylation in mouse cardiac muscle after 6 weeks of training, which supports the conclusion that endurance training upregulates basal CaMKII activity in muscle cells. Still, training must probably be maintained for a sufficiently long period because training interventions of less than 3 weeks have been found ineffective to elevate baseline PLN^{17} phosphorylation in muscles (Benziane et al. 2008; Rose et al. 2007). Against the face of increased baseline PLN^{17} phosphorylation status, the current training intervention affected neither the exercise-induced rise in PLN^{17} activation nor the subsequent decline during recovery, which is compatible with the observations in one earlier short-term training study in humans (Benziane et al. 2008) and indicates that short-term training does not alter contraction-induced stimulation of CaMKII in muscle.

There is some evidence to indicate that p38 MAPK is probably implicated in Ca^{2+}-induced stimulation of mitochondrial biogenesis in muscle cells (Wright et al. 2007). Consistent with earlier findings, we found acute exercise to increase p38 MAPK, but this effect was not altered by training in either the fasted state or in the high carbohydrate state (Fig. 4), which is in line with one earlier study (Benziane et al. 2008). However, cross-sectional studies have found either unchanged (Nielsen et al. 2003) or decreased (Yu et al. 2003; Coffey et al. 2006) p38 MAPK signalling in endurance-trained subjects compared with sedentary controls. Furthermore, Coffey et al. (2006) found that p38 MAPK activation was blunted when trained subjects undertook their habitual exercise, but not when they participated in another activity mode. In this regard, switching from exercise in the fasted state to exercise with ample carbohydrate ingestion, and thereby increasing the contribution of carbohydrate oxidation in ATP production, was not a stimulus to significantly alter p38 MAPK signalling. In addition, other stress stimuli like for instance inflammatory cytokines (Raingeaud 1995), cell membrane stretching (Chambers 2009), or mitochondrial production of reactive oxygen species (Emerling 2005), which also can trigger p38 MAPK activation, probably were not affected by the nutritional status during training.

Finally, the current findings result from biochemical assays in mixed muscle extracts from m. vastus lateralis, which in healthy young men on average is composed of about 55% type I, versus $\sim$45% type II fibres (Lucia et al. 2002; De Bock et al. 2007b). Nonetheless, prolonged endurance exercise primarily involves recruitment of type I motor units, leaving a large fraction of the type II motor units inactive (Hultman 1995; Vollestad and Blom 1985). Therefore, such approach conceivably underestimates the true amplitude of contraction-induced regulation of intramyocellular signalling pathways.

In conclusion, the current study provides novel evidence to indicate that consistent endurance exercise training in the fasted state may contribute to facilitate rapid post-exercise re-activation of muscle protein translation by dephosphorylation of eEF2. This finding once more indicates that the nutritional status, i.e. exercise in the fasted state versus exercise in the carbohydrate repleted state, is an important factor to modulating muscular adaptations resulting from a given exercise training programme.

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Conflict of interest There is no conflict of interest.

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