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Effect of NOS-Compartmentalisation on Cerebral Perfusion and Oxygen Metabolism in Physiological and Pathological Conditions

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Helga Blockx, Uitgever

“I am among those who think that science has great beauty.
A scientist in his laboratory is not only a technician:
he is also a child placed before natural phenomena
which impress him like a fairy tale.”
Marie Curie (1867 – 1934)

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Curriculum Vitae

Helga Blockx (born 1981, Turnhout, Belgium) attended secondary high school (Matematics-Sciences) at the 'Sint-Jozef College' in Turnhout, Belgium from 1993-1999. She studied Biomedical Sciences at the 'Katholieke Universiteit Leuven' in Belgium and graduated in 2003. In 2004 she was awarded a 'Duane Bruley Student Award' at the 32nd International Society of Oxygen Transport to Tissue meeting in Bari, Italy; in 2006 she was awarded a 'Young Investigator Award' at the 14th National Scientific Conference of the Australian Vascular Biology Society in Brisbane, Queensland, Australia and in 2007 she was awarded a 'Dietrich Lübbers Award' at the 35th International Society of Oxygen Transport to Tissue meeting in Uppsala, Sweden. Her PhD training was fulfilled at the Division of Experimental Cardiac Surgery (Department of Cardiovascular Diseases, Faculty of Medicine, 'Katholieke Universiteit Leuven', Belgium) under guidance of Prof. W. Flameng and G. De Visscher (PhD).

List of Publications

Papers

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List of Abbreviations

[Hb]	deoxyhemoglobin concentration
$[Hb]_{blood}$	average hemoglobin concentration in arterial and venous blood
$[HbO_2]$	oxyhemoglobin concentration
[HbT]	total hemoglobin concentration
1400W	n-aminomethyl-benzyl-acetamidine
7NI	7-nitroindazole
ATP	adenosine-5-triphosphate
$avDO_2$	arterio-venous oxygen difference
BFI	blood flow index
BFIw	weight corrected blood flow index
BrR	breathing rate
CBF	cerebral blood flow
CBV	cerebral blood volume
cGMP	cyclic guanosine monophosphate
CHI	closed head injury
CI	confidence interval
DPF	differential path length factor
EDRF	endothelium derived relaxing factor
EEG	electroencephalography
eNOS	endothelial nitric oxide synthase
$EtCO_2$	end-tidal CO_2
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
fMRI	functional magnetic resonance imaging
HR	heart rate
i.c.v.	intracerebroventricular
ICG	indocyanine green
$ICMRO_2$	cerebral metabolic rate of oxygen index
ICP	intracranial pressure

iNOS	inducible nitric oxide synthase
l-NAME	nitro-l-arginine-methyl-ester
l-NIO	N5-iminoethyl-ornithine
l-NNA	N-nitro-l-arginine
l-NOARG	N-nitro-l-arginine
LPS	lipopolysaccharide
MABP	mean arterial blood pressure
mtNOS	mitochondrial nitric oxide synthase
NaCN	sodium cyanide
NADP	nicotinamide adenine dinucleotide phosphate
NIRS	near infrared spectroscopy
NMDA	N-methyl-D-aspartic acid
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
$PaCO_2$	arterial CO_2 pressure
PET	positron emission tomography
pHa	arterial pH
PKG	cGMP-dependent protein kinase
SaO_2	arterial oxygen saturation
SD	standard deviation
sGC	soluble guanylyl cyclase
$SmcO_2$	mean cerebral oxygen saturation
SvO_2	venous oxygen saturation
TBI	traumatic brain injury
T_{ear}	ear temperature
T_{rectal}	rectal temperature

Effect of NOS-Compartmentalisation on Cerebral Perfusion and Oxygen Metabolism in Physiological and Pathological Conditions

Helga Blockx

Introduction

Cerebral perfusion is an important physiological process, since neuronal activity and viability are highly dependent on constant and adequate delivery of oxygen and glucose[1]. This is illustrated by the fact that the brain requires 25% of all oxygen consumption of the body at a total cerebral blood flow (CBF) at rest of 15% to 20% of total cardiac output[2]. However, the mechanisms of cerebral circulation are still not known in detail due to unique anatomical and physiological characteristics, together with the difficulty of access due to the skull[3]. Today, many factors are thought to play a role. A close correlation exists between neuronal activity and CBF[4], while hypercapnia and hypoxia are known to be important cerebral vasodilators, but have little effect on peripheral circulation[5]. In addition, nitric oxide (NO) is introduced as a regulating factor[5].

NO, formerly known as endothelium derived relaxing factor (EDRF), is the smallest signaling molecule actively produced by cells. Besides being linked to different biological actions, NO is a major regulator of blood flow. Because of the complexity of regulatory and other mechanisms in which NO plays an important role, NO synthase (NOS), the enzyme responsible for NO production, is compartmentalised. This results in three NOS isoforms with 50-60% homology, each synthesized from a separate gene[6]: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). This compartmentalisation is important to define the function of each NOS isoform, because they each mediate exactly the same reaction, that is: the production of NO and l-citruline from l-arginine by oxidation[7]. Because of this identical reaction, the function is largely defined by spatial and temporal distribution of NOS.

In normal brain tissue, nNOS has been linked to the feedback mechanism by which the brain's oxygen consumption is linked to alterations in regional blood flow[8]. After activation of glutamate-receptors in post-synaptic neurons, a transient increase in $[Ca^{2+}]$ occurs, which binds to calmodulin, activating the constitutive nNOS[7]. eNOS is more associated to immediate shear stress induced changes in blood vessel calibre. NO diffuses from the endothelium to smooth muscle cells, and activates soluble guanylyl cyclase, which results in vasodilation[7]. Conversely, iNOS, as the name states, is inducible. This means that iNOS gene expression is induced by an insult, and protein expression is maximal 24 hours after the insult. Compared to nNOS and eNOS, iNOS can generate very high concentrations of NO as part of the inflammatory immune response[7]. In physiological circumstances iNOS is not present, however, it has been implicated in certain pathologies, such as septic shock and rheumatoid arthritis[9], where it can be produced either by the cells themselves or by infiltration of activated macrophages.

To date, although NO is known to be an important regulator of the cerebral circulation, the relative importance of eNOS, nNOS and iNOS remains elusive[10]. This effect of compartmentalisation can be studied by using specific NOS-inhibitors with different affinity for these NO synthases [11-13]. The inhibitors used in this study are nitro-l-arginine-methyl-ester (l-NAME), N5-iminoethyl-ornithine (l-NIO), n-aminomethyl-benzyl-acetamidine (1400W) and 7-nitroindazole (7NI). l-NAME, an l-arginine analogue, is a non-selective inhibitor of NOS, which is taken up by passive diffusion[14]. Administration of l-NAME normally results in both systemic and specific cerebral effects[15,16]. Although l-NAME can inhibit eNOS and nNOS *in vitro*, Traystman and co-workers[15] showed that l-NAME depressed nNOS activity in a dose- and time-related manner, but it requires over 30 min. to achieve this *in vivo*. l-NIO, another l-arginine analogue, is currently the most selective *in vivo* inhibitor of eNOS available. While l-NAME is an inhibitor of the NOS enzyme, l-NIO is also an inhibitor of l-arginine uptake[14]. However l-NAME is a more potent *in vivo* inhibitor compared to l-NIO[17]. In addition, l-NIO is only 4 times more selective for eNOS compared to nNOS[18]. On the other hand, 1400W is a very selective inhibitor of iNOS[9,19]: it is 4000 times more selective compared to eNOS and 32 times more selective compared to nNOS[11]. It penetrates cells and tissues and is a very slow reversible inhibitor of iNOS[11]. Finally, 7NI is a competitive inhibitor with l-arginine as well as with biopterin[11]. Although it is not selective for nNOS *in vitro*, 7NI is targeted specifically to neuronal cells, making it an *in vivo* selective inhibitor of nNOS[9,11].

Autoregulation

A very important feature of cerebral circulation is the ability to maintain constant blood flow despite blood pressure changes. This phenomenon is known as ‘autoregulation’ and next to the cerebral vasculature[20], it can also be found in the myocardium[21], kidney[22], retina[23] and others. Under normal conditions, the limits of cerebral autoregulation are mean arterial blood pressures (MABP) of 60 mmHg to 150 mmHg, respectively[24] (Figure 1). Between these limits, diameter of the blood vessels, thus cerebrovascular resistance, adapts, introducing a change in cerebral blood volume. If MABP decreases below the lower limit, more oxygen is extracted from the blood until oxygen supply is inadequate and cerebral metabolic rate of oxygen decreases[24]. On the other hand, if MABP increases above the higher limit, constriction cannot be maintained and blood-brain barrier damage occurs along with edema formation[24].

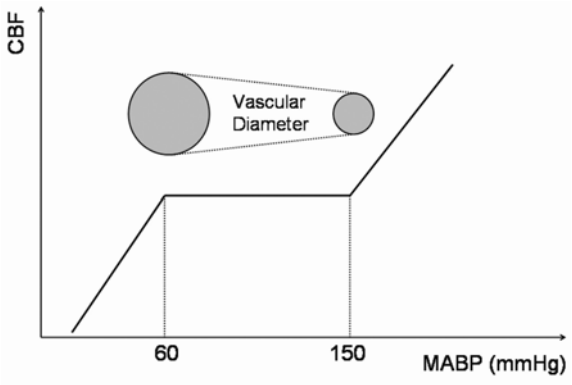


Figure 1: Schematic representation of cerebral autoregulation and the effect on blood vessel diameter.

Over time, different mechanisms were proposed to play a role in autoregulation: myogenic, neurogenic and metabolic. The myogenic mechanism is based on the phenomenon that smooth muscle cells can induce a vasoconstriction in response to an increase in transmural pressure[2,4]. This may be important in the upper limit of autoregulation, since at higher blood pressure, vascular diameter decreases. Possibly, a neurogenic mechanism might also play a role in autoregulation *in vivo*, but autoregulation was still seen in isolated intracerebral arterioles of the rat[25], decreasing the importance of this mechanism. Metabolic factors are a third possibility linked to autoregulation. Although controversial, adenosine is the only chemical factor leading to vasodilation with experimental support[26]. Lately, endothelial function is added to the possibilities. Besides endothelial hyperpolarizing factor, endothelin-1 and products of arachidonic acid metabolism, such as prostacyclins and prostaglandins, NO is one of the substances produced by the endothelium with effects on the vasculature[2].

Today, NO is thought to be an important regulator of the lower limit[27]. In isolated rat intracerebral arterioles, the non-selective NOS inhibitor l-NAME, inhibits autoregulatory vasodilation after pressure decrease, but not after pressure increase[25]. In addition, it has been shown *in vivo* that local NOS inhibition in the cerebral cortex by N-nitro-l-arginine (l-NNA, non-selective NOS inhibitor) suffusion, shifts the lower limit of autoregulation in the cortex of rats to higher blood pressures[28]. However, it is not clear which NOS isoform is responsible for the regulation of the lower limit of autoregulation. Investigation of the basilar artery during cerebral cortex suffusion with 7NI, an nNOS inhibitor, no effect on autoregulation was seen using laser-Doppler flowmetry, while suffusion with l-NNA increased the lower limit, suggesting that eNOS might be responsible[29]. In addition, nNOS is known to be responsible for the feedback mechanism coupling

cerebral oxygen consumption to CBF[8]. We therefore hypothesise that nNOS activity is responsible for the level of autoregulation, while eNOS plays a role in the lower part of the autoregulation curve.

Neurotrauma

Neurotrauma is known to decrease CBF early after injury. This decreased CBF is associated with cerebral ischemia and poor ultimate neurological outcome[30]. Even today, traumatic brain injury is still the leading cause of morbidity and mortality in individuals under the age of 45 in the world[30]. Twenty-four hours after neurotrauma, NO availability is massively increased due to iNOS activation[31] accompanied by inflammation. Due to this inflammation, injured and adjacent tissues are eliminated and scar tissue is synthesised[30].

The mechanism important in the early decrease of CBF is thought to be NO dependent[31]. This was shown in rats studying NO availability after head trauma with an NO-probe[32]. As soon as five minutes after an immediate transient increase in NO concentration, a decrease below baseline values occurred for at least three hours[32]. The observation that the early CBF decrease is dependent on NO availability, was endorsed by the observation that addition of l-arginine to restore NO concentrations, has a beneficial effect on CBF[33].

However, it is not certain which NOS isoform is important. Traumatic brain injury in eNOS deficient mice, showed a greater reduction in CBF compared to wild-type mice[34], which suggests that eNOS is still active. Interestingly, this protective role is not limited to neurotrauma, since mutant mice, deficient in eNOS, developed larger lesions after middle cerebral artery occlusion[35]. On the other hand, 24 hours after fluid-percussion brain injury, nNOS gene-expression and protein synthesis were not altered, while the activity of the nNOS enzyme was decreased[36]. Since iNOS is only maximal 24 hours after neurotrauma[7], it is improbable to play a role in the early phase after brain injury. Our hypothesis therefore states that nNOS activity is already, if not immediately, decreased early after neurotrauma.

To induce moderate neurotrauma in rats, we used an impact-acceleration closed head injury model. This model of neurotrauma induces an increased intracranial pressure (ICP) which remained stable for up to four hours[37]. In addition, it did not result in a compromised blood-brain barrier[38], nor in endothelial damage[39]. Because of the latter two properties, the impact-acceleration closed head injury model, is an ideal model to study compartmentalisation of NOS.

Since ICP measurements are very important in the assessment of severe head injury in humans and are commonly used for the confirmation of neurotrauma in animals, it is important to include these data into our neurotrauma experiment. Although fluid-filled ventricular catheters are believed to be the most accurate method in humans[40], intraparenchymal microtransducers are a good alternative. The Codman® microsensor probe is such an intraparenchymal microtransducer approved for use in humans with a maximal *in vitro* error of 5 mmHg from 60 mmHg onwards[41]. Previously, it was shown that cerebellar ICP measurements can be used as an alternative to cerebral ICP measurements in rats[42]. This way, the cortical area remains intact, presumably leading to a better assessment of cerebral parameters.

Cytochrome Oxidase

NO is not only known for its effects on cerebral perfusion. *In vitro* studies in isolated mitochondria and cells showed that cytochrome oxidase is reversibly inhibited by submicromolar levels of NO[43]. Although NO can inhibit cytochrome oxidase in its reduced as well as in its oxidised state[44], the most important inhibition is in the reduced state where NO competes with O_2 for the binding site[45]. Evidence for the latter includes that the K_m for oxygen of cells could be decreased with NOS inhibitors[46] and the intriguing finding that NOS inhibitors can increase oxygen consumption in the whole body[46]. This inhibition of cytochrome oxidase with NO is suggested to have physiological consequences. For example, NO may allow cytochrome oxidase to detect oxygen changes in the physiological sensing range[43]. In contrast, Palacios-Callender *et al.* state that this inhibition, within limits, only increases the flux of electrons through the uninhibited cytochrome oxidase population, resulting in the maintenance of cellular respiration[47].

However, to date, it is almost impossible to predict *in vivo* situations from *in vitro* data, since *in vivo* mitochondrial NO and oxygen tensions are very poorly characterised[43]. In addition, NO scavenging systems may be present *in vivo*, which may not be present in cell cultures. Taken together, although current *in vitro* data largely suggest an *in vivo* interaction between NO and cytochrome oxidase, actual *in vivo* measurements are indispensable.

One study is already performed *in vivo* in the brain: measurements of cytochrome oxidase redox status after NOS inhibition showed no effect of NO in physiological circumstances, nor in the hyperaemic phase after anoxia[48]. A minority of *in vitro* literature agrees with these data, suggesting that the NO amounts needed for inhibition of cytochrome oxidase might not be reached *in vivo*[49]. Because of this lack of effect in *in vivo* physiological conditions, we exclude an

effect of nNOS and eNOS. However, the question remains whether elevated NO concentrations can inhibit cytochrome oxidase *in vivo*[50] in very specific situations or not.

Since iNOS is able to induce large amounts of NO, lipopolysaccharine (LPS) was used to induce an inflammatory response including iNOS expression in the brain. In addition, because *in vitro* studies showed a longer-lasting inhibition with NO in low oxygen tension, compared to high oxygen tension[43], these high NO concentrations were combined with hypoxia. While considering the redox state of cytochrome oxidase in these circumstances, this study might be the first to demonstrate an effect of NO on cytochrome oxidase in the brain *in vivo*.

Near Infrared Spectroscopy

Because the difficulty of access due to the skull counteracts the study of cerebral circulation and metabolism[3], new non-invasive techniques, such as near infrared spectroscopy (NIRS), can be of great assistance. Jöbsis was the first to use light from the near infrared region of the spectrum to transilluminate brain through intact skull[51]. Together with this first use of NIRS in biological tissue, Jöbsis showed that cytochrome aa3 (part of cytochrome oxidase) oxidation status could be measured[51,52]. He found that cytochrome oxidase is partially reduced under normoxic conditions and could be oxidised by increasing oxygen availability to the brain[51], while Hoshi found that it only becomes more reduced when oxygen delivery is extremely impaired[53]. Even today, the possibility of measuring part of the mitochondrial electron chain *in vivo* is a unique feature of NIRS. Soon thereafter, the first article was published measuring blood oxygenation using the same technique[52]. Today, NIRS is able to study brain oxygen saturation and hemodynamics in a practical and non-invasive way[54].

In order to understand the mechanisms of NIRS, the basic principles are to be explained first. The underlying principle of all spectroscopy is the Beer-Lambert law. It states that when light is passed through a solution, part of the light is absorbed by the solution, thereby reducing the intensity of the emerging light (Figure 2). Using this principle, the concentration of a substance in the solution can be calculated according to following equation:

$$\text{Log } [I_0/I] = \alpha.c.d$$

Beer-Lambert equation where I_0/I is the ratio of the light intensity entering to emerging from the solution, α is the extinction coefficient, c the chromophore concentration and d the thickness of the solution.

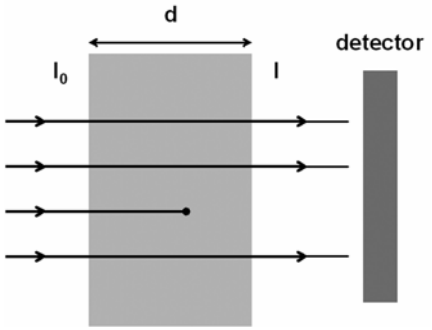


Figure 2: In a simple solution of a chromophore, light is absorbed and the detector measures the difference in intensity between incident (I_0) and emerging (I) light. When taking the distance (d) through which the light passes into account, the absolute chromophore concentration (c) can be calculated.

A first difficulty with this principle in biological tissues is the penetration of light. Due to the very strong absorption of water, visible light (450-700 nm) does not penetrate biological tissue sufficiently[55]. However, there is one part of the spectrum with low absorption of water and hemoglobins, allowing relevant penetration, called the “biological window”, ranging from 700 to 960 nm: the near infrared region.

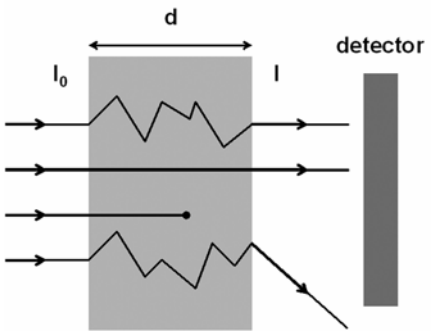


Figure 3: In a scattering solution of a chromophore, some photons are lost from the detector (like the lower one) and some photons travel a longer distance than the linear distance (like the upper one). For this reason, the unmodified Beer-Lambert law cannot be used.

A second difficulty is the fact that biological tissue is a highly scattering medium. This causes two major deviations from the Beer-Lambert law. A first is that the emerging light is not only attenuated by absorption, but also by scattering away from its original path (Figure 3). This can

be partially resolved by assuming that during a NIRS measurement, tissue scattering remains unchanged, making it possible to calculate the change in attenuation from the change in absorption, without knowing the absolute values[56]. A second consequence is that the light travels a longer distance than the straight line between the source and the detector (Figure 3). The actual distance the light travels is described by multiplying the distance, "d", by the "differential path length factor" (DPF). By knowing the DPF, the change in chromophore concentration in micromolar can be calculated.

The NIRS system and its algorithms used in our studies were developed at the Department of Medical Physics and Bioengineering, University College London (Figure 4). It allows us to measure absolute concentration deoxyhemoglobin ([Hb]) and absolute changes in oxyhemoglobine and oxidised Cu_A (Cu_A, part of cytochrome oxidase), because of characteristic absorption spectra in the near infrared range[55]. Terminal anoxia at the end of the *in vivo* experiments in order to obtain a desaturated spectrum, allows for back-calculation of the absolute oxyhemoglobin values ($[HbO_2]$). Additionally, two other variables can be derived: total hemoglobin ([HbT]), calculated by adding up [Hb] and $[HbO_2]$, and mean cerebral oxygen saturation ($SmcO_2$), using the formula: $SmcO_2 = ([HbO_2] / [HbT]) * 100$.

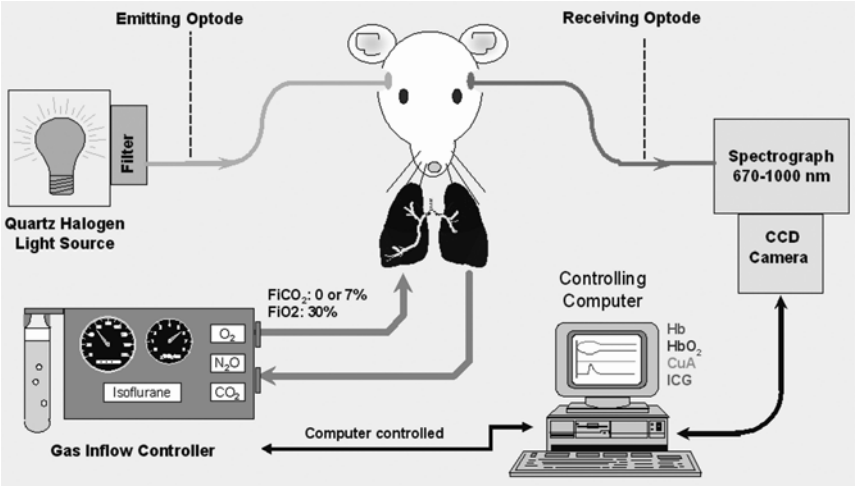


Figure 4: Schematic diagram of the NIRS system used, developed at University College London[57].

One of the ways to measure CBF is to calculate a blood flow index (BFI) from an indocyanine green (ICG) bolus transit curve. ICG has an absorption peak at 805 nm which makes it a suitable

tracer for NIRS[58]. Therefore, the absorption spectrum of ICG has been integrated into the algorithms of the NIRS system used. ICG is restricted to the intravascular compartment and is rapidly cleared from the blood by hepatic uptake and biliary excretion[59]. Kuebler *et al.*[58] used this tracer to measure BFI noninvasively which is proportional to CBF (Figure 5). His measurements of CBF in pigs were validated by comparison with a microsphere method which is a standard method to calculate blood flow through various organs. After introducing the ICG bolus transit curve to calculate BFI in rats[60], its use was again validated with microspheres[61]. In our studies, we corrected this BFI for individual differences in blood volume by multiplying BFI with the body weight expressed in grams (BFIw). Because blood volume is linearly proportional to body weight, the latter can be used as a valid correction factor.

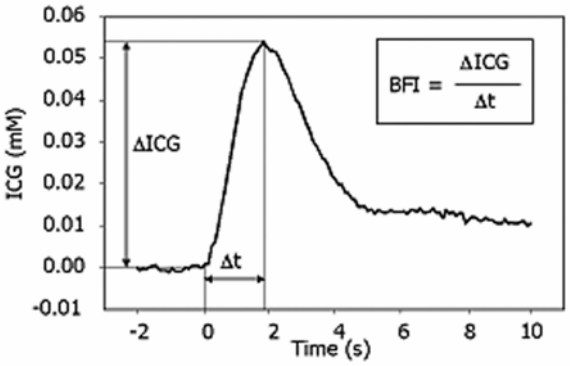


Figure 5: Calculation of blood flow index (BFI) using the tracer indocyanine green (ICG).

An additional parameter used in this work is arterio-venous oxygen difference ($avDO_2$). It is calculated from the oxygen content of an arterial and a sagittal sinus venous blood sample and the average of the hemoglobin concentrations of these two blood samples:

$$avDO_2 = 1.34 * [Hb]_{blood} * (SaO_2 - SvO_2)$$

$SmcO_2$ is shown to correlate well with SvO_2[57] and can therefore replace SvO_2 in this equation. Together with a BFIw measurement, $avDO_2$ can be used to calculate an index of cerebral metabolic rate of oxygen ($ICMRO_2$) as follows:

$$ICMRO_2 = avDO_2 * BFIw$$

NIRS in Mice

Today, transgenic mice models are often used to gain insight in physiological and pathophysiological processes like preconditioning[48] and Alzheimer[62,63]. In addition, eNOS and nNOS knockout mouse models are developed[64] in order to increase the understanding of NOS compartmentalisation. Receiving information about cerebral oxygenation, CBF and mitochondrial function of these mouse models, would be a great advantage. An interesting technique to measure these parameters is NIRS. We therefore want to test whether or not multi-wavelength NIRS can be used to examine cerebral oxygenation and hemodynamics in the anesthetised mouse. In addition, we have determined the optimal dose of ICG to calculate a cerebral blood flow index.

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Article 1

Endothelial and neuronal NO-synthase exert different roles in maintaining and regulating cerebral autoregulation

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Submitted

Condensed Abstract

Although NO has been shown to play a role in the lower limit of autoregulation, it is not known which NO synthase-isoform is responsible: endothelial or neuronal NOS. We studied the inhibitory l-NAME (non-selective) and 7NI (nNOS) on autoregulation during gradually reduced mean arterial blood pressure in rats using near infrared spectroscopy. Our study showed that nNOS and non-selective inhibition both decrease the level of autoregulation, while only non-selective inhibition shifts the onset of autoregulation to higher blood pressures. We conclude that nNOS links brain metabolism to cerebral perfusion, while eNOS is responsible for autoregulation during hypotension.

1. Abstract

Cerebral autoregulation is an important mechanism that maintains constant blood flow despite blood pressure changes. Although the exact mechanism of autoregulation remains elusive, nitric oxide (NO) has been proposed as a possible regulator of the lower limit. In our study, we aim to clarify the role of NO in the lower limit and normal level of cerebral autoregulation. We used two NOS inhibitors, l-NAME (non-selective) and 7NI (neuronal NOS), *in vivo* in rats and investigated cerebral autoregulation with near infrared spectroscopy. Because our experiments were performed in healthy rats, we can exclude an effect of inducible NOS.

During a step-wise reduction in mean arterial blood pressure, cerebral blood flow measurements were performed to obtain an autoregulation curve for each group. l-NAME induces a breakpoint shift to the right ($p < 0.001$ compared to saline, peanut oil and 7NI groups), while no shift in breakpoint occurs after 7NI injection. Heights of the saline and peanut oil group were not significantly different, as were heights of the l-NAME and 7NI groups. However, the level of the second linear part of the fitting of both the l-NAME ($p < 0.001$) and the 7NI group ($p < 0.03$) were significantly lower compared to their respective controls.

Of the two major contributors, we showed that nNOS inhibition decreases the height of the autoregulation curve, while eNOS inhibition shifts the lower limit of autoregulation to higher blood pressures. These data support the hypothesis that nNOS links brain metabolism to cerebral perfusion, while eNOS is responsible for the lower part of the autoregulation curve.

2. Introduction

Autoregulation is the ability to maintain constant blood flow despite blood pressure changes. Under normal conditions, the limits of cerebral autoregulation are mean arterial blood pressures (MABP) of 60mmHg to 150mmHg[1]. Although the mechanism of autoregulation remains elusive, several authors describe a role for nitric oxide (NO)[2,3]. NO is synthesised by three NO-synthase (NOS) isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS)[4]. In normal brain tissue, nNOS is important in the feedback mechanism linking blood flow to oxygen consumption[5], while eNOS maintains physiological vascular tone[6]. iNOS is not present in most normal tissue but can be induced during e.g. inflammation[6].

To date, NO has been proposed as a regulator of the lower limit of autoregulation[3]. Local NOS inhibition *in vivo* raises the lower limit of autoregulation in the cortex of rats measured by laser-Doppler flowmetry[3]. However, it is not clear which NOS isoform is responsible.

We, therefore, aim to clarify the role of NOS isoforms in the lower limit and normal level of cerebral autoregulation. Effects of iNOS were excluded, because our experiments were performed in healthy rats. We hypothesise that nNOS activity is responsible for the level of autoregulation, while eNOS is responsible for the lower limit of autoregulation during hypotension.

3. Materials and Methods

3.1. Animal treatment and preparation

Animal housing and treatment conditions complied with the European Union directive #86/609 on animal welfare. They were allowed free access to food and water (12/12-hour day-night cycle). Each rat (n=32, Sprague-Dawley, male, 380-430g, Charles River, Sulzfeld, Germany) was assigned to one of four groups in a fully randomised manner.

The procedure for preparing rats for Near Infrared Spectroscopy (NIRS) was described previously[7]. In short, rats were anaesthetised using isoflurane (4% in 30%O_2/70%N_2O) and endotracheally intubated. Isoflurane was switched to 2%. The right external jugular vein was cannulated towards the vena cava for ICG-bolus injection, the left femoral artery to monitor MABP and heart rate (HR) and the right femoral artery to induce hypovolemia. To administer l-NAME or saline, the left femoral vein was cannulated. 7NI and peanut oil were injected intraperitoneally.

The rats were fixed in a stereotaxic apparatus (Model 900, David Kopf, Tujunga, CA) with thermally isolated ear bars containing a thermistor. Body temperature was maintained at 37.0±0.5°C and tympanic temperature was measured. End-tidal CO_2 ($EtCO_2$) and breathing rate (BrR) were continuously monitored using an $EtCO_2$-monitor (Capnogard, Novametrix, Wallingford, CT). The parietal and temporal bones were exposed by removing the skin, periosteum and parts of the temporal muscles. Emitting and receiving optical fibres (3 mm diameter) were placed onto the right and left temporal bones, respectively (frontal pole of the fibres at the level of the bregma, angle of 10° relative to both the coronal and sagittal plane). Optical gel (Optical Gel code 0608, R.P. Cargille Laboratories Inc., Cedar Grove, NJ) was used to

prevent blood entering the space between the skull and the optical fibres. The skull was covered with black ink and black clay (Modelling Paste Softy, Darwy S.A., Wandre, Belgium).

The isoflurane concentration was then switched to 1.5%, which was maintained until the end of the experiment. A 10 min. stabilisation period was allowed.

3.2. Experimental protocol

Ten minutes after the start of the experiment, an arterial blood sample was taken, followed by a cerebral blood flow (CBF) assessment. Compounds were administered according to the protocol (at 15 min.): 0.9% saline (i.v. bolus, 1 ml), peanut oil (i.p. bolus, 1 ml), N-nitro-l-arginine methyl ester (l-NAME, 30 mg/kg i.v. bolus in saline, Sigma, Bornem, Belgium) or 7-nitroindazole (7NI, 25mg/kg i.p. bolus in peanut oil, Sigma). NIRS assessment of deoxyhemoglobin ([Hb]), oxyhemoglobin ([HbO_2]) and total hemoglobin ([HbT]) was continuous for the remainder of the experiments.

Forty-five minutes after the start of the experiment (30 min. after compound injection), MABP was lowered by steps of 10mmHg by drawing blood (hypovolemia). Depending on the starting MABP of the animal, CBF assessments were performed at MABP between 135mmHg and 35mmHg after a 3 min. stabilisation of the respective MABP. No pharmaceuticals were used to increase MABP. After hypovolemia, blood was reinjected and the animals were killed by terminal anoxia to obtain fully deoxygenated spectra.

3.3. NIRS equipment

The NIRS system and algorithms were developed at University College London[8,9] and allow assessment of absolute concentrations of oxyhemoglobin and deoxyhemoglobin. NIR spectra were collected contiguously with a period of 100 ms and 100 spectra were averaged to obtain a time resolution of 10 seconds (0.1 Hz). ICG can be measured by the same system. A sterile 1 mg/ml ICG (IR-125, laser grade, Acros, Belgium) solution containing 5% bovine serum albumin (BSA fraction V, Sigma) was used. For ICG-bolus transit detection, the sampling frequency was switched from 0.1 to 10 Hz (no averaging).

3.4. Data analysis

Blood Flow Index (BFI) was calculated as previously described[7] and corrected for individual differences in blood volume (BFIw) by multiplying BFI with the body weight expressed in grams. Arterio-venous O_2 difference ($avDO_2$) was calculated from arterial oxygen saturation (SaO_2) and

average hemoglobin concentration ($[Hb]_{blood}$) obtained from the same blood sample and mean cerebral oxygen saturation ($SmcO_2$) obtained by NIRS according to the following formulas:

$$SmcO_2 = ([HbO_2] / [HbT]) \times 100$$

$$avDO_2 = 1.34 \times [Hb]_{blood} \times (SaO_2\text{-}SmcO_2)$$

The index of the cerebral metabolic rate of oxygen ($ICmrO_2$) was calculated according to the following formula:

$$ICmrO_2 = avDO_2 \times BFIw$$

3.5. Statistical analysis

Results are expressed as mean ± standard deviation (SD). T-tests were used for between group and within group analysis. When measuring autoregulation, CBF is measured at selected absolute MABPs. No continues autoregulation curve is therefore available, making it necessary to use fittings of this curve. Since individual curves were not fitted due to variability, data of the autoregulation curves were averaged within every group and fitted with a linear and a linear-linear model (Prism 5, GraphPad, San Diego, CA) taking into account the frequency of observations for each MABP. Therefore one fitting per group is available, and estimated values and standard errors were used to perform t-tests to compare breakpoints and slopes, and to compare slopes to 0. A two-sided probability value of less than 0.05 was regarded as statistically significant.

4. Results

4.1. Baseline measurements

Before compound injection, no differences between groups were found in HR (365±46BPM), MABP (105.4±14.2mmHg), ear temperature (32.4±3.6°C), BrR (80.2±14.6BPM), $EtCO_2$ (48.1±3.2mmHg), arterial CO_2 pressure (46.3±5.9mmHg), arterial pH (7.37±0.03), [Hb] (21.9±3.5µM), $[HbO_2]$ (94.7±15.3µM), [HbT] (116.6±16.9µM), $SmcO_2$ (81.1±3.0%), $avDO_2$ (2.61±0.81ml/dl), $ICMRO_2$ (52.7±19.5) and BFIw (20.3±5.6). Data are mean ± SD of all groups together. Variables remained within physiological range for the entire experiment.

4.2. Effect compounds (data not shown)

After saline injection, MABP and [HbT] significantly decreased, while $EtCO_2$ increased. l-NAME induced a decrease in HR, [HbO_2], [HbT], $SmcO_2$ and ear temperature and an increase in MABP, [Hb] and $EtCO_2$. Peanut oil induced a decrease in MABP and $SmcO_2$. 7NI led to decreases in [HbO_2], [HbT], $SmcO_2$ and ear temperature.

Table 1. Data of the fittings of the autoregulation curves.

	Linear Fitting		Linear-Linear Fitting		
	Intercept	Slope	Slope 1	Breakpoint (mmHg)	Slope 2
Saline	4.177 ± 0.767	0.152 ± 0.012 *	0.326 ± 0.061 *°	58.3 ± 4.7	0.034 ± 0.046 °
l-NAME	6.404 ± 0.215 #	0.038 ± 0.003 *	0.062 ± 0.013 *	84.6 ± 13.0 *	0.008 ± 0.022
Peanut oil	5.823 ± 0.593	0.104 ± 0.009 *	0.264 ± 0.038 *°	58.4 ± 3.2	0.016 ± 0.023 °
7NI	4.482 ± 0.253	0.068 ± 0.004 *	0.120 ± 0.036 *	56.4 ± 10.5	0.048 ± 0.018 $

Values are expressed as mean ± SD. Linear-linear fitting: Slope 1: slope of the linear curve in MABP regions lower than breakpoint; Slope 2: slope of the linear curve in MABP regions higher than breakpoint. In the linear-linear fitting, slope 2 was significantly different from slope 1 within all groups. *: significantly different from all other groups, #: significantly different from saline and 7NI groups, $: significantly different from l-NAME and peanut oil groups, °: significantly different from the slope of the linear fitting within the same group.

4.3. Autoregulation (Figure 1, Table 1)

Two fittings were applied, a linear and a linear-linear fitting. As estimated from the R^2, the linear-linear fitting was in better agreement with the data in all groups than the simple linear fitting. This corresponds with the known shape of the normal autoregulation curve. We therefore mainly used the linear-linear fitting.

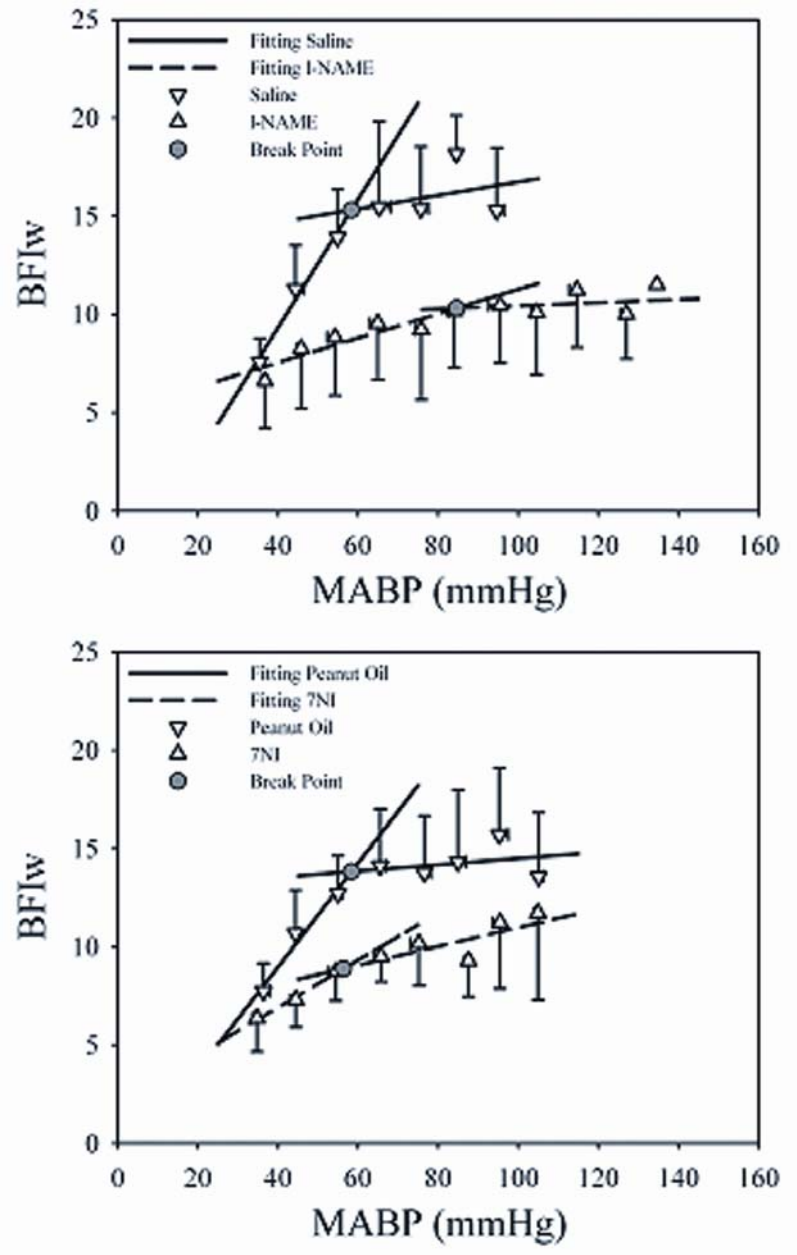


Figure 1: Linear-linear fittings of the autoregulation curves of saline, l-NAME, peanut oil and 7NI groups. BFIw: weight corrected blood flow index. Data are represented as mean ± standard deviation.

In this linear-linear fitting, l-NAME shifts the onset of autoregulation to higher blood pressures, while no shift occurs after 7NI injection. No difference in breakpoint was found between saline, peanut oil and 7NI groups. In addition, slope at lower blood pressures decreased after l-NAME or

7NI injection and were significantly different between saline and peanut oil groups and l-NAME and 7NI groups. Slope at higher blood pressures of 7NI group was different from peanut oil and l-NAME groups. The latter two were not significantly different from 0. Slope before and after breakpoint was significantly different in all groups. However, slope before and after breakpoint of l-NAME and 7NI groups were not significantly different from the slope of the linear fitting.

The autoregulation levels (the second linear part of the linear-linear fitting) of both l-NAME and 7NI groups were significantly lower compared to their respective controls. No difference in autoregulation level was found between saline and peanut oil groups and between l-NAME and 7NI groups.

5. Discussion

We have shown *in vivo* that nNOS inhibition decreases the level of autoregulation, but has no effect on the lower limit, while non-selective inhibition induces both effects. Our study, therefore, indicates that eNOS plays a role in autoregulation during hypotension, while nNOS is responsible for the level at which the autoregulation curve is maintained.

Non-selective inhibition with l-NAME had the expected effects systemically, since it induced a decrease in HR and an increase in MABP, while both vehicles (saline and peanut oil) induced a decrease in MABP. In addition, non-selective inhibition induced a decrease in [HbO_2], [HbT] and $SmcO_2$ and an increase in [Hb]. The same happened after nNOS inhibition, except for the increase in [Hb], possibly due to the smaller effect of nNOS inhibition. Both NOS inhibitors also induced a small decrease in ear temperature that related to a decrease in [HbT] and BFIw. Although saline induced a small decrease in [HbT] and peanut oil induced a small decrease in $SmcO_2$, we can state that both NOS inhibitors had the expected effects cerebrally.

To date, the contribution of NO to CBF autoregulation remains to be clarified. *In vitro* studies using intracerebral rat arterioles suggest a role for NO in autoregulatory dilatation after transmural pressure decrease[2]. Concomitantly, non-selective NOS inhibitors alter the autoregulatory response to hypotension *in vivo*[3,10,11]. Our data confirm the literature, since non-selective inhibition induced a shift in breakpoint to higher blood pressures. Although slopes before and after breakpoint are significantly different, this breakpoint might be artificial, since slopes of the linear-linear fitting after NOS inhibition do not differ from the single slope of the linear fitting. This would mean that NOS is responsible for the shape of the autoregulation curve at low blood pressures.

Interestingly, it is not established which NOS isoform is responsible. We, therefore, inhibited nNOS using 7NI, resulting in a decreased autoregulation level, but no shift in breakpoint. This suggests that endothelial NO regulates the cerebrovascular response to hypotension. This is to be expected, since eNOS is markedly present in the vasculature and therefore very close to the bloodstream.

Our study also shows that nNOS inhibition, as well as non-selective inhibition, induces a decrease in the autoregulation level, indicating an uncoupling of CBF and metabolism. Equally, nNOS inhibition with 7NI induced an uncoupling between electrical activity and evoked hemodynamic activity response measured with fMRI and EEG[12]. Our data, therefore, support the hypothesis that nNOS inhibition induces an uncoupling, effectively lowering the CBF set point at which autoregulation is maintained.

Conclusion

In this study, we aimed to clarify the role of NO in cerebral autoregulation during hypotension. We showed that nNOS and non-selective inhibition decrease the level of autoregulation, while only non-selective inhibition shifts the onset of autoregulation to higher blood pressures. These data support the hypothesis that nNOS links brain metabolism to cerebral perfusion, while eNOS is responsible for autoregulation during hypotension. This means that eNOS provides the mechanism and nNOS the set point.

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Article 2

Traumatic brain injury induces an early decrease in neuronal NOS activity

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Submitted

Condensed Abstract

Traumatic brain injury decreases early NO concentrations below baseline levels due to a decreased NOS activity. However, it is unknown which isoform is responsible, endothelial or neuronal NOS. The inhibitory effects of l-NAME (non-selective), l-NIO (eNOS) and 7NI (nNOS) cerebral hemodynamics of rats subjected to closed head injury (CHI) were studied. In sham animals, non-selective inhibition, selective eNOS and nNOS inhibition had the expected effects. In CHI animals, non-selective inhibition had generally shortened effects compared to sham animals, eNOS inhibition had some remaining effects, while nNOS inhibition had no effect whatsoever. We concluded a diminished nNOS activity early after neurotrauma.

1. Abstract

After traumatic brain injury, cerebral nitric oxide (NO) concentration drops below baseline values for several hours due to a sudden decrease in NO synthase (NOS) activity. It is, however, unknown to which NOS isoform this might be attributed. This study aims to define which isoform, the endothelial or neuronal, is responsible for this decrease.

Cerebral hemodynamics were assessed by means of near infrared spectroscopy in Sprague-Dawley rats subjected to experimental closed head injury (CHI). The NOS inhibitors l-NAME (non-selective NOS inhibitor), l-NIO (endothelial NOS inhibitor) and 7NI (neuronal NOS inhibitor) were administered *in vivo*.

Injection of l-NAME had the expected effects in sham animals (increased mean arterial blood pressure, deoxyhemoglobin concentration and arteriovenous oxygen saturation and decreased heart rate, oxy- and total hemoglobin concentration, cerebral venous oxygen saturation and cerebral blood flow index), but in CHI animals these effects are generally shortened. In sham animals, both 7NI and l-NIO resulted in largely the same cerebral effects compared to l-NAME, but in CHI animals, 7NI had no effect whatsoever, while injection of l-NIO attenuated the increase in oxyhemoglobin and total hemoglobin concentration induced by saline.

We, therefore, conclude that neuronal NOS activity is diminished early after traumatic brain injury and that this is a major contributor to the early NO decrease and consequent changes in cerebral perfusion after neurotrauma.

2. Introduction

NO, formerly EDRF, is the smallest signaling molecule actively produced by cells. Besides being linked to different biological actions, NO is a major regulator of blood flow. Because of the complexity of regulatory and other mechanisms in which NO plays an important role, NO synthase (NOS), the enzyme responsible for NO production from l-arginine, is compartmentalised. This resulted in three NOS isoforms with 50-60% homology, each synthesised from a separate gene[1]. Two calcium dependent isoforms were described, one attributed to endothelium (eNOS) and one to neurons (nNOS). However neither eNOS nor nNOS can exclusively be found in endothelium or neurons respectively: for example, eNOS also appears in brain and heart, while nNOS is also found in endometrium and skeletal muscle[1]. The third isoform, inducible NOS (iNOS), is calcium independent and mainly regulated by gene

expression. iNOS is induced upon inflammation[2] or, with respect to the brain, after ischemia/reperfusion and trauma[3].

The compartmentalisation is important to define the function of each NOS isoform, because they each mediate exactly the same chemical reaction, that is: produce NO and l-citruline from l-arginine by oxidation[2]. Because of this identical reaction, the function is largely defined by spatial and temporal distribution of the NOS. In normal brain tissue, nNOS has been linked to the feedback mechanism by which the brain's oxygen consumption is linked to alterations in regional blood flow[4]. eNOS is more linked to immediate shear stress induced changes in blood vessel caliber, where the endothelium senses the stress and releases NO to relax the smooth muscle cells in their vicinity[2]. Conversely, iNOS is not present in most normal tissue but has been implicated in certain pathologies like septic shock or rheumatoid arthritis[5] where it can be produced either by the cells themselves or by infiltration of activated macrophages.

The described situation is also valid for normal brain tissue, but neurotrauma has profound effects on both cerebral NO production and perfusion. Traumatic brain injury (TBI) induces an immediate transient increase in cerebral nitric oxide (NO) concentration. In less than five min., a rebound effect occurs: NO levels drop below baseline values for at least three hours, blood pressure decreases, and intracranial pressure (ICP) increases[6]. Because these effects were observed immediately after TBI and because the NO levels dropped rather than increased, we can exclude an effect by iNOS induction. Additionally, iNOS has only been found to be maximal 24 hours after trauma. Of the two major contributors, we hypothesise that nNOS activity decreases very early after neurotrauma causing decreased NO levels and consequent changes in cerebral perfusion.

3. Materials and Methods

3.1. Experimental protocol

The experimental protocol is depicted in Figure 1. The study comprises basically two groups: animals subjected to CHI (CHI-groups) or not (sham-groups). NIRS assessment of deoxyhemoglobin, oxyhemoglobin and total hemoglobin was continuous for the remainder of the experiments. Ten minutes after the start of the experiment, a first arterial blood sample was taken followed by an ICG-bolus CBF assessment and a first cerebral venous blood sample. After this CBF assessment, injections according to the protocol (at 15 min.) were started: there was either

no injection performed (CHI-control or sham-control), a 0.9% saline injection (CHI-saline or sham-saline), an l-NAME injection (30mg/kg i.v. bolus in saline; CHI-l-NAME or sham-l-NAME), an l-NIO injection (20mg/kg i.v. bolus in saline; CHI-l-NIO or sham-l-NIO) or a 7NI injection (25mg/kg i.p. bolus in peanut oil; CHI-7NI or sham-7NI). A specific control for 7NI was not included since no volume effect is possible after i.p. injection.

A second and third measurement (arterial blood sample, ICG CBF assessment, and venous blood sample) were performed 15 min. and 45 min. after injection, respectively. At the end of the experiment, the animals were killed by terminal anoxia to obtain fully deoxygenated spectra.

Figure 1

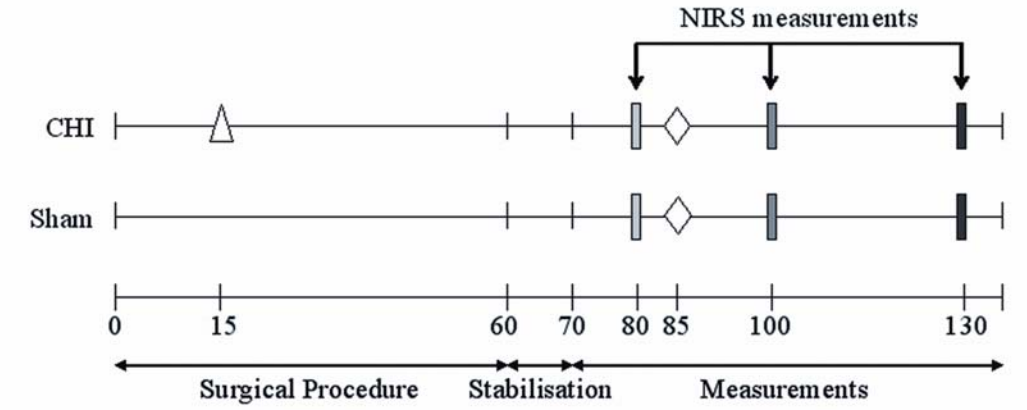


Figure 1: Illustration of the protocol used. CHI: closed head injury; □: CHI induction; ◊: infusion: no infusion (sham-control or CHI-control group), saline infusion (sham-saline or CHI-saline), l-NAME infusion (sham-l-NAME or CHI-l-NAME), l-NIO infusion (sham-l-NIO or CHI-l-NIO) or 7NI infusion (sham-7NI or CHI-7NI group).

3.2. Animal treatment and preparation

Animal housing and treatment conditions complied with the European Union directive # 86/609 on animal welfare. The study was performed on 131 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 380-430 g and each individual rat was assigned to one of the ten groups in a fully randomised manner. They were allowed free access to food and water (12/12-hour day-night cycle).

The procedure for preparing rats for near infrared spectroscopy (NIRS) measurement has previously been described in detail[7]. In short, the animals were anaesthetised using isoflurane (4%

in 30%O_2/70%N_2O) and endotracheally intubated. Isoflurane was then switched to 2%. The right external jugular vein was cannulated towards the vena cava for indocyanine green (ICG) bolus injection and the left femoral artery was cannulated to monitor mean arterial blood pressure (MABP) and heart rate (HR). For injection of N^{ω}-nitro-L-arginine methyl ester (l-NAME, Sigma, Bornem, Belgium) and l-N^5-(1-iminoethyl)ornithine (l-NIO, Calbiochem, San Diego, CA, USA), the left femoral vein was cannulated. 7-nitroindazole (7NI, Sigma, Bornem, Belgium) was injected intraperitoneally.

All rats received a 2 cm midline incision of the scalp and the periosteum was removed. The rats were transferred to the trauma device. To induce moderate closed head injury (CHI) a 400 g weight was dropped on the skull from a height of 51.5 cm. Control groups underwent all of the above, except the weight drop. Rats with skull fractures or major intracranial bleedings were excluded at this point.

The rats were fixed in a stereotaxic apparatus (Model 900, David Kopf, Tujunga, CA, USA) with thermally isolated ear bars. Body temperature was maintained at 37 ± 0.5°C and a thermistor inserted into the tip of an ear bar was used to measure the tympanic temperature, providing an accurate measurement of brain temperature. The end-tidal CO_2 ($EtCO_2$) and breathing rate (BrR) were continuously monitored with an $EtCO_2$-monitor (Capnogard, Novametrix, Wallingford, CT, USA). The parietal and temporal bones were exposed by removing parts of the temporal muscles and sites of possible bleeding were cauterised.

A small burr hole not penetrating the skull was made at the site of the sagittal sinus. A 23 gauge needle was used to open the burr hole and puncture the upper wall of the sagittal sinus. Immediately after the withdrawal of the needle a PE10 catheter was inserted into the sagittal sinus. A few drops of 2% (in saline) low meting point agarose (SeaPlaque, FMC BioProducts, Rockland, ME, USA) was applied at the site of entrance to avoid bleeding and catheter displacement. This cannula was used for cerebral venous blood sampling required for accurate measurement of the cerebral oxygen consumption[8].

To measure cerebellar intracranial pressure (ICP), the trapezial muscle was detached from the skull and possible bleedings were cauterised. A burr hole was drilled into the right part of the occipital bone, 2 mm caudal of the cranial edge and 2 mm lateral from the midline. The microsensor ICP probe (ICP Neuromicrosensor, Codman & Shurtleff Inc., Randolph, MA, USA) was inserted through this burr hole. The tip was positioned in the sagittal plane with an angle of about 35° relative to the horizontal plane and carefully inserted into the cerebellar parenchyma.

Emitting and receiving optical fibres (3 mm diameter) were placed onto the right and left temporal bones, respectively (frontal pole of the fibres at the level of the bregma, angle of 10° relative to both the coronal and sagittal plane). To prevent blood entering the space between the skull and the optical fibres, optical gel (Optical Gel code 0608, R.P. Cargille Laboratories Inc., Cedar Grove, NJ, USA) was used to fill in this space. The skull was covered with black ink and black clay (Modelling Paste Softy, Darwy S.A., Wandre, Belgium).

The isoflurane concentration was then switched to 1.5% in 30%O_2/70%N_2O, this level was maintained until the end of the experiment. A 10 min. stabilisation period was then allowed.

3.3. Exclusion criteria

Incorrect impact, skull fractures and subdural hematoma were set as CHI specific exclusion criteria. Rats showing prolonged apnoea without spontaneous recovery were also excluded, because they were suspected to have brainstem damage leading to a different pathology with severe regulatory disturbances. For control animals, NIRS specific criteria for exclusion were significant instability (variation > 20%) of a NIRS parameter or MABP during a 10 min. period before onset of treatment. When MABP was < 50mmHg at the end of the stabilisation period sham rats were excluded because of exceedingly abnormal systemic hemodynamics. When the aperture of the spectrograph had to be larger than 400 μm to achieve a minimal intensity peak of 50,000 counts, the animal was excluded, because this is indicative of intracerebral or ventricular bleeding. Finally, CHI rats with a posttraumatic ICP below 8 mmHg were excluded from the study.

3.4. NIRS equipment

The NIRS system and algorithms were developed at University College London[9,10] and allow the assessment of absolute concentrations of oxyhemoglobin and deoxyhemoglobin. ICG can be measured by the same system when its absorption spectrum is included in the algorithm. A sterile 1 mg/ml ICG (IR-125, laser grade, Acros, Belgium) solution containing 5% bovine serum albumin (BSA fraction V, Sigma, Bornem, Belgium) was used. NIR spectra were collected contiguously with a period of 100 ms and 100 spectra were averaged to obtain a time resolution of 10 seconds (0.1 Hz). For ICG bolus transit detection, the sampling frequency was switched from 0.1 to 10 Hz (no averaging).

3.5. Data analysis

Blood Flow Index (BFI) was calculated as previously described[7] and corrected for individual differences in blood volume (BFIw) by multiplying BFI with the body weight expressed in grams. Since blood volume is linearly proportional to body weight, the latter can be used as a valid correction factor.

The arterio-venous O_2 content difference ($avDO_2$) was calculated from the arterial (SaO_2) and venous (sagittal sinus) (SvO_2) oxygen saturations and the average hemoglobin concentration ($[Hb]_{blood}$) obtained from the same blood samples according to the following formula:

$$avDO_2 = 1.34 \times [Hb]_{blood} \times (SaO_2 - SvO_2)$$

The index of the cerebral metabolic rate of oxygen ($ICmrO_2$) was calculated from the $avDO_2$ and BFI_w according to the following formula based on the Fick-equation:

$$ICmrO_2 = avDO_2 \times BFI_w$$

3.6. Statistical analysis

All results are expressed as mean ± standard deviation (SD). A two-sided Wilcoxon-Mann-Whitney rank-sum test was used for between group analysis. Within group, analysis was performed with the Wilcoxon signed ranks test. A two-sided probability value of less than 0.05 was regarded as statistically significant.

4. Results

4.1. Inclusion rate

The overall inclusion rate of the animals was 49%. Most occurring incidents leading to exclusion were skull fractures (18%), apnoea (10%) and intracerebral or ventricular bleeding (8%).

4.2. Baseline measurements: the effects of closed head injury (Table 1)

To demonstrate the effect of CHI, the data of the first measurement period of the different CHI groups were pooled and compared to the pooled data of the first measurement of the control groups.

All brain oxygenation data derived from NIRS measurements showed significant changes after CHI. Indicative of vasoconstriction and reduced blood flow, the oxyhemoglobin concentration

([HbO_2]) decreased and a concomitant increase in deoxyhemoglobin concentration ([Hb]) was found, resulting in a decreased total concentration of hemoglobin ([HbT]). BFIw significantly decreased and $avDO_2$ significantly increased, while no difference in $ICMRO_2$ between sham and CHI animals was observed.

MABP, arterial pH (pHa), and SvO_2 decreased significantly after CHI and arterial CO_2 pressure ($PaCO_2$) increased significantly after trauma. Additionally, CHI induced a significant increase in ICP. HR, end tidal CO_2 ($EtCO_2$), breathing rate (BrR), and SaO_2 did not differ between sham and CHI animals.

Table 1: Baseline values derived from sham animals and animals subjected to closed head injury.

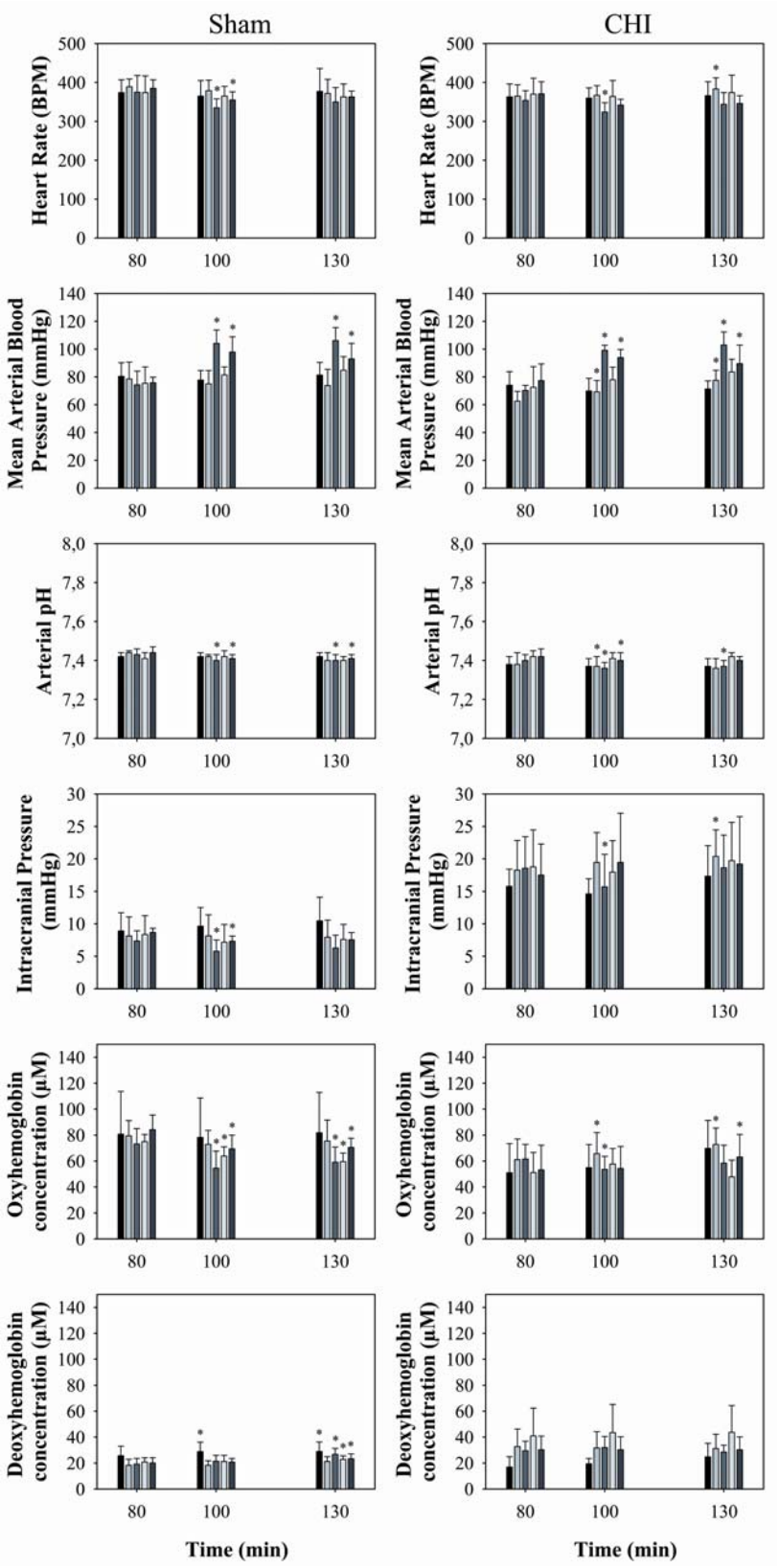
	Sham (n = 30)	**CHI (n = 30)**
Physiological data		
HR (BPM)	376 ± 38	363 ± 33
MABP (mmHg)	75.6 ± 9.9	70.2 ± 10.8 *
$EtCO_2$ (mmHg)	48.9 ± 3.5	50.0 ± 6.4
BrR (BPM)	82.8 ± 18.2	83.3 ± 13.2
$PaCO_2$ (mmHg)	41.4 ± 3.3	44.4 ± 5.9 *
pHa	7.43 ± 0.02	7.40 ± 0.04 *
SaO_2 (%)	94.9 ± 3.5	96.2 ± 1.6 *
SvO_2 (%)	78.5 ± 4.2	68.1 ± 9.0 *
Brain oxygenation data		
[HbO_2] (μM)	78.4 ±16.6	55.5 ±15.3 *
[Hb] (μM)	21.0 ± 5.1	29.5 ± 14.0 *

[HbT] (μM)	99.4 ± 20.0	85.0 ± 18.3 *
Cerebral blood flow, Intracranial pressure, and oxygen consumption		
BFIw	13.5 ± 3.0	9.2 ± 3.4 *
ICP (mmHg)	8.21 ± 2.30	17.50 ± 4.40 *
$avDO_2$ (ml/dl)	3.17 ± 1.01	5.20 ± 1.77 *
$ICMRO_2$	42.0 ± 13.9	44.8 ± 16.4

Values are expressed as mean ± SD. CHI: closed head injury; HR: heart rate; BPM: beats per minute; MABP: mean arterial blood pressure; Trec: rectal temperature; $EtCO_2$: end tidal CO_2; BrR: breathing rate; $PaCO_2$: arterial CO_2 pressure; pHa: arterial pH; SaO_2: arterial oxygen saturation; SvO_2: sinus sagittalis venous oxygen saturation; [HbO2]: oxyhemoglobin concentration; [Hb] deoxyhemoglobin concentration; [HbT]: total hemoglobin concentration; BFIw: weight corrected blood flow index; ICP: intracranial pressure; $avDO_2$: arterio-venous O_2 content difference; $ICMRO_2$: metabolic rate of oxygen index. *: significantly different from sham.

4.3. Groups without NOS inhibition (Figure 2)

In the sham-control and CHI-control groups and the sham-saline and CHI-saline groups, all variables remained within physiological range throughout the entire protocol. However, [Hb] increased over time in the sham-control group and saline injection in sham animals induced a late decrease in SvO_2 and a late increase in $avDO_2$. On the other hand, the effects of saline injection were clearly seen in the CHI-group: MABP, HR, ICP, [HbO_2], [HbT] and BFIw increased progressively, while a slight decrease in pHa was seen (see Figure 2).


Sham
CHI
Heart Rate (BPM)
Mean Arterial Blood Pressure (mmHg)
Arterial pH
Intracranial Pressure (mmHg)
Oxyhemoglobin concentration (µM)
Deoxyhemoglobin concentration (µM)
Time (min)
80
100
130

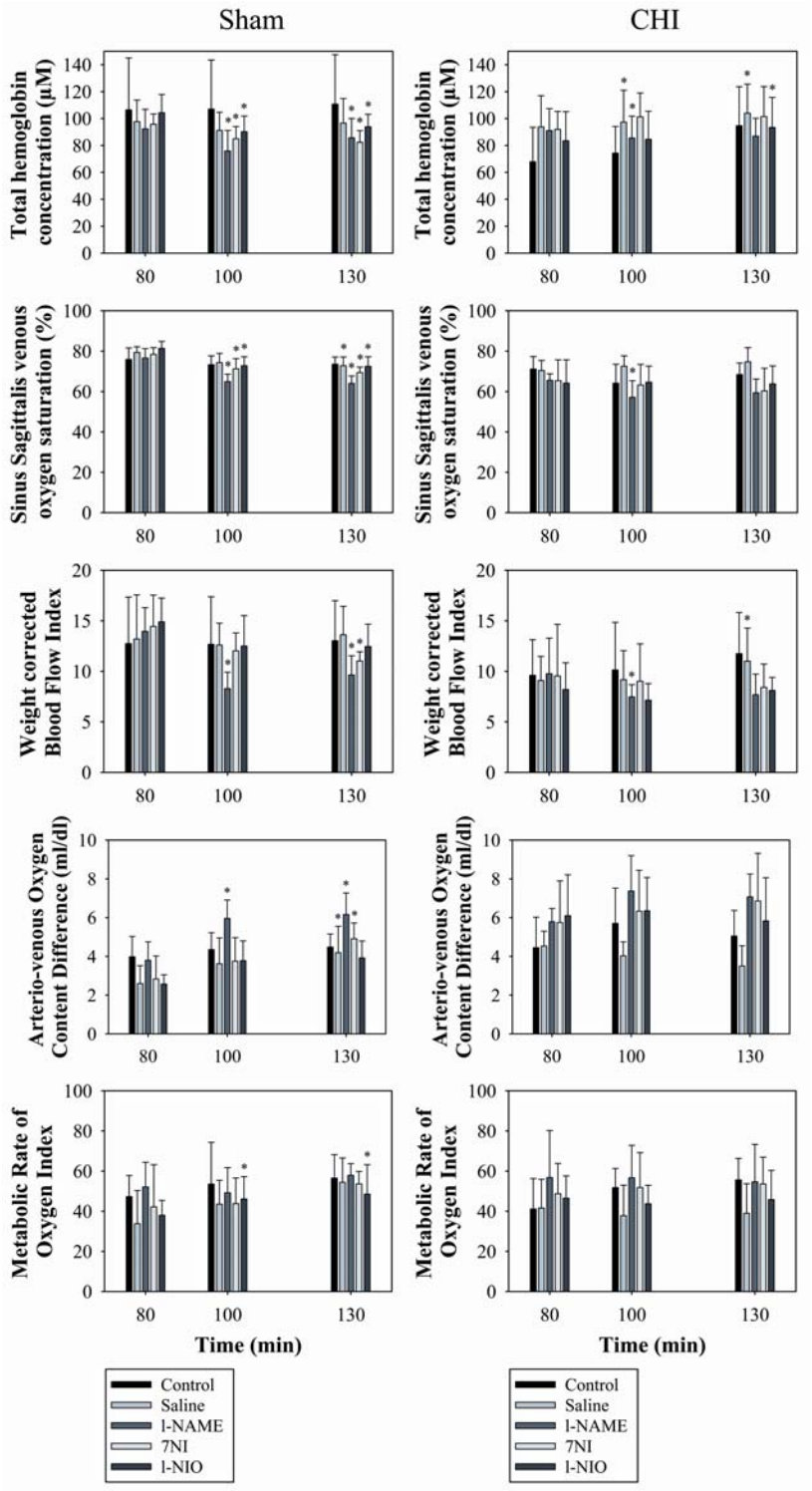


Figure 2: Effects of time and saline infusion and of the NOS inhibitors l-NAME, l-NIO and 7NI on systemic and cerebral parameters in sham animals and animals

subjected to closed head injury. Values are expressed as mean ± SD. CHI: closed head injury; BPM: beats per minute; *: significantly different from baseline within the same group.

4.4. Effects of NOS inhibition on sham and CHI animals compared to baseline (Figure 2)

After 15 min., l-NAME injection resulted in a decrease in HR, pHa, ICP, [HbO_2], [HbT], SvO_2, and BFIw in both sham and CHI animals. MABP increased significantly in both groups, whereas $avDO_2$ increased significantly in sham, but not in CHI animals. Forty-five minutes after l-NAME injection, MABP was still increased and pHa was still decreased significantly in both sham and CHI animals, while [HbO_2], [Hb], [HbT], SvO_2, BFIw and $avDO_2$ were still significantly altered only in sham animals.

Injection of 7NI resulted in significant decreases in [HbO_2], [HbT], and SvO_2 in shams after both 15 and 45 min. Additionally a significant increase in [Hb] and decrease in BFIw was observed 45 min. after 7NI administration. However, 7NI had no effect whatsoever in CHI animals.

After injection of l-NIO in sham animals, a significant decrease was found in HR, pHa, ICP, [HbO_2], [HbT] and SvO_2 at 15 min. and in pHa, [HbO_2], [HbT] and SvO2 at 45 min. A significant increase was found in MABP and $ICMRO_2$ at 15 min., and in MABP, [Hb], $avDO_2$ and $ICMRO_2$ at 45 min. In CHI animals, injection of l-NIO resulted in a decrease of pHa at 15 min., an increase in [HbO_2] and [HbT] at 45 min. and an increase in MABP both at 15 and 45 min.

5. Discussion

To investigate whether nNOS is responsible for the decreased NO levels very early after neurotrauma, we combined experimental neurotrauma with NOS inhibitors. First we evaluated the effectiveness of this study. The effects of the used impact acceleration closed head injury model showed to be in agreement with previous studies using the same neurotrauma model[8].

The results of the animals subjected to CHI receiving a saline injection markedly differed from those of similar animals which did not receive the saline injection. However, these effects were contrary to the effects of non-specific NOS-inhibition except for MABP (Figure 2). The observed effects of saline could not mask the clear effects of NOS-inhibition by l-NAME.

In order to study the effect of different NOS-isoforms, specific inhibitors with different affinity for these NO synthases were used[11-13]. The first, the l-arginine analogue l-NAME, is regarded a non-specific inhibitor of the different NO-synthases. Administration of l-NAME normally results in both systemic and specific cerebral effects[14,15]. The expected systemic effects (increase in MABP and decrease in heart rate) were found in normal animals and were maintained in animals subjected to CHI, clearly affirming that l-NAME still has its effects systemically. l-NAME also had the expected cerebral effects in sham animals, but in CHI animals some effects lasted shorter (decreased [HbO2], [HbT], SvO2 and BFIw) and others did not occur ([Hb], avDO2) (Figure 2). These observations confirm the altered status of NO homeostasis after neurotrauma and its subsequent effects in the cerebral vasculature, as was also concluded by Cherian and colleagues after direct measurements of NO by an NO-probe[6]. Although l-NAME can inhibit eNOS and nNOS *in vitro*, evidence exists that in our experiment its main effect is eNOS inhibition. l-NAME is taken up by passive diffusion[16], consequently since the endothelium[17] and the blood-brain barrier[18] are intact in our model, the natural barrier for l-NAME is still functional and impedes l-NAME from entering the brain. Additionally, Traystman and co-workers[14] showed that l-NAME depressed brain NOS activity in a dose- and time-related manner, but it requires over 30 min. to achieve this. Therefore, we assume that eNOS inhibition largely accounts for the systemic effects of l-NAME. Supporting this statement are the facts that l-NIO, an eNOS specific inhibitor, causes the same shifts in heart rate and MABP compared to l-NAME.

To further elucidate which NOS is responsible for this decrease in NO, we administered specific inhibitors for neuronal and endothelial NOS, 7NI and l-NIO, respectively. In sham animals, inhibition of nNOS or eNOS resulted in largely the same cerebral effects as aspecific NOS inhibition (Figure 2). However, nNOS inhibition had no effect on ICP and avDO2 and a postponed effect on BFIw and eNOS inhibition had no effect on BFIw and a postponed effect on $avDO_2$, suggesting that the effect of specific inhibition is smaller compared to aspecific inhibition. In addition, eNOS inhibition induces an increase in $ICMRO_2$. It is possible that this increase is induced by the decreased inhibition of cytochrome oxidase by NO, but this remains subject of further investigation.

In animals subjected to CHI the effects of nNOS inhibition, in contrast to eNOS inhibition, were, in accordance to our hypothesis, completely abolished.

l-NIO is currently the most selective *in vivo* inhibitor of eNOS available. While l-NAME is an inhibitor of the NOS enzyme, l-NIO is also an inhibitor of l-arginine uptake[16]. Nevertheless, l-NAME is a more potent inhibitor compared to l-NIO. In sham animals, l-NAME leads to an

increase in MABP of 40% and a decrease in HR, ICP, and [HbT] of 11%, 21%, and 18% respectively, while l-NIO causes an increase in MABP of 29% and a decrease in HR, ICP, and [HbT] of 8%, 16%, and 14%, respectively. Because of the differences in magnitude of the effects, it is likely that l-NAME will result in a larger effect in CHI-animals as well. Combining the discussion about l-NAME and l-NIO, we believe that eNOS is still active (Figure 2), although we cannot exclude that its activity is suboptimal.

In agreement with our hypothesis, the effects of 7NI are totally abolished by neurotrauma (Figure 2). In addition, $ICMRO_2$ remains the same after CHI when compared to sham, despite an uncoupling occurs resulting in a decreased CBF and consequent increased avDO2 (Table 1), a finding we described earlier[8]. Cholet and colleagues[4] found that this uncoupling of cerebral blood flow and metabolism is caused by nNOS inhibition. Based on our data and previous reports, we state that nNOS activity is diminished early after traumatic brain injury.

A possible mechanism regarding the decreased nNOS activity involves changed l-arginine concentrations. Due to high concentrations of glutamate after TBI, l-arginine is released from glial cells[19], temporarily resulting in high NO concentrations[20]. In less than five minutes, NO levels drop below baseline values for at least three hours[6]. We believe that this drop is caused by a reduced availability of the substrate of NOS due to excessive use within the first five minutes after trauma. This hypothesis is supported by Hlatky and coworkers[21], who found decreased l-arginine concentrations during the first three days after TBI in head-injured patients.

Although eNOS activity might be suboptimal after TBI, the decrease in NOS substrate is less problematic for eNOS because it is located near blood vessels, guaranteeing continuous replenishment. It is therefore able to preserve blood flow after neurotrauma and is consequently referred to as neuroprotective[20]. NO produced by nNOS and iNOS, on the other hand, may have detrimental effects[20]. Therefore, the inactivation of nNOS early after closed head injury might be part of an endogenous neuroprotective mechanism.

Conclusion

This study concerning cerebral blood flow and metabolism showed that aspecific NOS inhibition has largely the same effects in trauma animals compared to sham animals, although the effect is generally shortened, while eNOS inhibition has smaller cerebral effects in CHI animals compared to sham animals. In contrast, nNOS inhibition has no effect whatsoever in CHI animals. Also,

inhibition of nNOS is known to induce an uncoupling of cerebral blood flow and metabolism, which clearly occurs after induction of neurotrauma compared to sham animals.

We therefore conclude that nNOS activity is diminished early after traumatic brain injury and that this is a major contributor to the early NO-decrease and consequent changes in cerebral perfusion after TBI.

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Article 3

Nitric oxide has minor effects on cerebral cytochrome oxidase only at low oxygen tensions *in vivo* in rat

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Submitted

Condensed Abstract

NO is a competitive inhibitor of cytochrome oxidase (Cu_A) *in vitro*, but this was not confirmed *in vivo*. Since high NO and low oxygen concentrations can work synergistically to inhibit cytochrome oxidase *in vitro*, we studied the effect of this combination on the redox status of Cu_A in rats. We showed that elevated NO concentrations by themselves, are not sufficient to induce a change in cytochrome oxidase redox status but that combined with hypoxia small effects in Cu_A redox status at low oxygen tensions could be observed. Therefore NO only has effects on cytochrome oxidase *in vivo* in very specific conditions.

1. Abstract

Nitric oxide (NO) is known to be an important inhibitor of cytochrome oxidase *in vitro* by competing with the natural ligand oxygen. To date, *in vivo* studies have not been able to affirm this effect in physiological conditions, nor in the hyperaemic phase after anoxia. However, high NO concentrations and low oxygen tensions can work synergistically to inhibit cytochrome oxidase. We therefore studied the effect of high NO concentrations on the redox status of cytochrome oxidase during hypoxia using multi-wavelength near infrared spectroscopy in the brain of rats. High NO concentrations can be initiated by injecting lipopolysaccharide (LPS) inducing an inflammation, while cyanide can be used to completely reduce cytochrome oxidase. Since our technique only measures the oxidised form of the enzyme, we can find the baseline and back-calculate absolute values of oxidised cytochrome oxidase concentrations.

The study comprises three groups: control group (no injection), sham group (vehicle injection i.c.v.) and LPS group (LPS injection i.c.v.). Twenty-four hours later we subjected the rats to three subsequent hypoxias: baseline, after inducible NO-synthase inhibition and after non-selective NO-synthase inhibition. We also checked inducible NO-synthase gene expression and back-calculated absolute oxidised cytochrome oxidase concentrations after lethal sodium cyanide injection.

We showed that inducible NO-synthase gene expression was only present in the LPS group, while no difference in absolute oxidised cytochrome oxidase concentrations between groups was found. However, when high NO concentrations are combined with low oxygen tensions, we found small effects at mean cerebral oxygen saturations of $\leq 40\%$ in the LPS group compared to sham and control groups and after NO-synthase inhibition.

We therefore conclude that NO has effects on cytochrome oxidase *in vivo* in the brain of rats, only at very low oxygen saturations.

2. Introduction

Nitric oxide (NO) is the smallest signalling molecule actively produced by cells. Next to being a major regulator of blood flow and other biological actions, *in vitro* studies showed that NO can reversibly inhibit cytochrome oxidase in isolated mitochondria and cells[1]. While inhibition in the oxidised state is also possible[2], the most important inhibition of cytochrome oxidase is in its reduced state where NO competes with O_2[3]. This can be shown by the decreased K_m for O_2 of

cells after NO-synthase (NOS) inhibition[4]. Possibly, NO is able to allow cytochrome oxidase to detect oxygen changes[1]. However, since *in vivo* mitochondrial NO and O_2 tensions are very poorly characterised, it is difficult to predict *in vivo* situations from *in vitro* data[1]. Therefore, although *in vitro* data largely suggest an *in vivo* interaction between NO and cytochrome oxidase, actual *in vivo* measurements are indispensable.

Previously, it was shown *in vivo* that cytochrome oxidase redox status was not changed after NOS inhibition in physiological circumstances, nor in the hyperaemic phase after anoxia[5]. We therefore believe that neither neuronal NOS, nor endothelial NOS, have effects on cytochrome oxidase *in vivo*.

Later on, *in vitro* data showed that high NO levels together with hypoxia can work synergistically to induce neuronal death by inhibition of cytochrome oxidase[6]. Since it is known that iNOS is able to induce large amounts of NO, we induce a cerebral inflammation to increase iNOS expression in the brain. In addition, *in vitro* studies showed that inhibition lasted longer in low oxygen tension[1]. We therefore combined high NO concentrations with hypoxia, while considering the redox state of cytochrome oxidase.

We believe this study might be the first to demonstrate an effect of NO on cytochrome oxidase in the brain *in vivo*.

3. Materials and Methods

3.1. Animal treatment and preparation

Animal housing and treatment conditions complied with the European Union directive #86/609 on animal welfare. They were allowed free access to food and water (12/12-hour day-night cycle). Experiments were performed on male Sprague-Dawley rats (370-480g, Charles River, Sulzfeld, Germany), each assigned to one of three groups (control, sham or LPS) in a fully randomised manner.

Rats were anaesthetised using isoflurane (4% in 30%O_2/70%N_2O) and endotracheally intubated. Isoflurane was switched to 2%. The rats were fixed in a stereotaxic apparatus (Model 900, David Kopf, Tujunga, CA) with thermally isolated ear bars. Body temperature was maintained at 37.0±0.5°C. A hole was made in the skull located 2 mm lateral to the sagittal suture and 1 mm caudal to the coronal suture using a gimlet (Dremel Multipro Cordless 9.6V, Dremel Benelux, Breda, The Netherlands). A 25 µl Hamilton syringe with a 27-gauge needle was used to inject

LPS (0.25 µg in 5 µl saline, LPS E. coli O127:B8, Sigma, Bornem, Belgium) at a depth of 4 mm from the surface of the skull. The skin was then sutured and anaesthesia was discontinued. Sham animals underwent the same protocol but were injected 5 µl saline without LPS, while control animals did not undergo this protocol.

3.2. Experimental protocols

To determine iNOS upregulation, twenty-four hours after surgery rats (n=9, 3 in every group) were anaesthetised using isoflurane (4% in 30%O_2/70%N_2O) and killed by intracardial saline injection. The brains were removed, and small pieces were immediately transferred to 1.5 ml eppendorf tubes containing RNA stabilisation reagent (RNAlater, Qiagen, Hilden, Germany). Samples are put at 4°C and the following day at -20°C until RNA purification. RNA purification was performed using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In short, immediately before RNA purification, tissue is transferred to a test tube containing 1ml Qiazol and mixed using a rotor-stator homogeniser. 200 µl chloroform is added. After vortexing, samples are centrifuged 15 min. at maximum speed at 4°C. The top layer is transferred to a new tube and an equal volume of 70% ethanol is added. After vortexing, samples are transferred to a minicolumn and the manufacturer's protocol is followed accurately. RNA is collected from the column using RNAse-free water and stored at -80°C until PCR.

Two µg RNA of each sample was converted to cDNA with the reverse transcriptase polymerase chain reaction (RT-PCR) using 100 mM random primers (Roche Applied Science, USA) and 300 units of revertAid H Minus M-Mulv Reverse Transcriptase Enzyme (MBI Fermentas, Canada). The cDNA was used to execute a PCR reaction in the MJ Mini Gradient Therma Cycler (Bio-Rad, USA) using the forward and reverse primers for iNOS analysis 5'-AAAACCCCAGGTGCTATTCC-3' and 5'-TGGGTCCTCTGGTCAAACTC-3' (Gene Bank Acc. No. NM 012611) and the forward and reverse primers for β-actin analysis 5'-CAACCTTCTTGCAGCTCCTC-3' and 5'-ACCCATACCCACCATCACAC-3' (Gene Bank Acc. No. NM 031144). After a denaturation at 94°C for 2 min., 30 cycles were executed with every cycle consisting of 30 sec. at 94°C, 1 min. 65°C and 45 sec. 72°C. Temperature was then kept at 72°C for 10 min. whereafter samples were kept on ice until analysis.

Amplification samples were analysed using the Agilent 2100 Bioanalyser and DNA 1000 chips (Agilent Technologies, USA). Results are shown as gel-like visualisations of the electropherograms.

To determine the inhibition and absolute concentration of oxidised cytochrome oxidase, twenty-four hours after LPS or saline injection, all other rats were prepared for near infrared spectroscopy (NIRS) as described previously[7]. In short, rats were anaesthetised using isoflurane (4% in 30%O_2/70%N_2O) and endotracheally intubated. Isoflurane was switched to 2%. The right external jugular vein was cannulated towards the vena cava for ICG-bolus injection, the left femoral artery to monitor MABP and heart rate (HR) and the right femoral artery for blood sampling. To administer l-NAME or 1400W, the left femoral vein was cannulated.

The rats were fixed in a stereotaxic apparatus with thermally isolated ear bars containing a thermistor. Body temperature was maintained at 37.0±0.5°C and tympanic temperature was measured. End-tidal CO_2 ($EtCO_2$) and breathing rate (BrR) were continuously monitored using an $EtCO_2$-monitor (Capnogard, Novametrix, Wallingford, CT). The parietal and temporal bones were exposed by removing the skin, periosteum and parts of the temporal muscles. Emitting and receiving optical fibres (3 mm diameter) were placed onto the right and left temporal bones, respectively (frontal pole of the fibres at the level of the bregma, angle of 10° relative to both the coronal and sagittal plane). Optical gel (Optical Gel code 0608, R.P. Cargille Laboratories Inc., Cedar Grove, NJ) was used to prevent blood entering the space between the skull and the optical fibres. The skull was covered with black ink and black clay (Modelling Paste Softy, Darwy S.A., Wandre, Belgium).

The isoflurane concentration was then switched to 1.5%, which was maintained until the end of the experiment. A 10 min. stabilisation period was allowed.

For experiment 2 (n=24, 8 in every group), 10 min. after the start of the experiment, an arterial blood sample was taken followed by a cerebral blood flow (CBF) assessment. A first 70s hypoxia (0%O_2/100%N_2O) was performed at 15 min. followed by a 10 min. recovery. At 25 min., N-(3-[aminomethyl]benzyl)acetamidine (1400W, 5 mg/kg i.v. bolus in saline, Sigma, Bornem, Belgium) was injected intravenously. Fifty-five minutes after the start of the experiment, a second arterial blood sample was taken followed by a CBF assessment. A second 70s hypoxia was performed at 60 min. At 70 min., N-nitro-l-arginine methyl ester (l-NAME, 30 mg/kg i.v. bolus in saline, Sigma, Bornem, Belgium) was injected intravenously. One hundred minutes after the start of the experiment, a third blood sample was taken followed by a CBF assessment. A third 70s hypoxia was performed at 105 min.. At 115 min., the animals were killed by terminal anoxia to obtain fully deoxygenated spectra.

For experiment 3 (n=9, 3 in every group), 10 min. after the start of the experiment, an arterial blood sample was taken followed by a cerebral blood flow (CBF) assessment. A 70” hypoxia

($0\%O_2/100\%N_2O$) was performed at 15 min. followed by a 10 min. recovery. At 25 min. a lethal dose of sodium cyanide (NaCN, 5mg/kg in saline, Sigma, Bornem, Belgium) was injected to obtain absolute values oxidised cytochrome oxidase. NaCN binds to cytochrome oxidase, completely reducing cytochrome oxidase[8]. Since our technique only measures the oxidised form of the enzyme, we can find the baseline and back-calculate absolute values of oxidised cytochrome oxidase concentrations.

3.3. NIRS equipment

The NIRS system and algorithms were developed at University College London[9,10] and allow assessment of absolute concentrations of oxyhemoglobin and deoxyhemoglobin and absolute changes in oxidised cytochrome oxidase. Using NaCN, cytochrome oxidase becomes completely reduced, allowing for back calculation of absolute values oxidised cytochrome oxidase. NIR spectra were collected contiguously with a period of 100 ms and 100 spectra were averaged to obtain a time resolution of 10 seconds (0.1 Hz). ICG can be measured by the same system. A sterile 1 mg/ml ICG (IR-125, laser grade, Acros, Belgium) solution containing 5% bovine serum albumin (BSA fraction V, Sigma) was used. For ICG-bolus transit detection, the sampling frequency was switched from 0.1 to 10 Hz (no averaging).

3.4. Data analysis

Blood Flow Index (BFI) was calculated as previously described[7] and corrected for individual differences in blood volume (BFIw) by multiplying BFI with the body weight expressed in grams. Arterio-venous O_2 difference ($avDO_2$) was calculated from arterial oxygen saturation (SaO_2) and average hemoglobin concentration ($[Hb]_{blood}$) obtained from the same blood sample and mean cerebral oxygen saturation ($SmcO_2$) obtained by NIRS according to the following formulas:

$$SmcO_2 = ([HbO_2] / [HbT]) \times 100$$

$$avDO_2 = 1.34 \times [Hb]_{blood} \times (SaO_2 - SmcO_2)$$

The index of the cerebral metabolic rate of oxygen ($ICmrO_2$) was calculated according to the following formula:

$$ICmrO_2 = avDO_2 \times BFIw$$

Cu_A was plotted against $SmcO_2$ during hypoxia to obtain curves. These curves were fitted using a quadratic-linear model (NCSS, NCSS, Kaysville, Utah) (Figure 1):

Estimation equation:

$$Y = A + BX + CX^2 + (X - D)\ SIGN\ (X - D)\ [E - C\ (X + D)]$$

Common equation:

Quadratic part of the curve: $Y = a1 + b1*X + c1*X^2$ if $X \leq$ breakpoint

Linear part of the curve: $Y = a2 + b2*X$ if $X >$ breakpoint

With a1, a2: intercept; b1, b2: linear coefficient and c1: quadratic coefficient.

Parameter identities to calculate common equation:

$a1 = A - 2CD + DE$ $a = D$

$b1 = B - E$ $a2 = A + 2CD - DE$

$c1 = 2C$ $b2 = B + E$

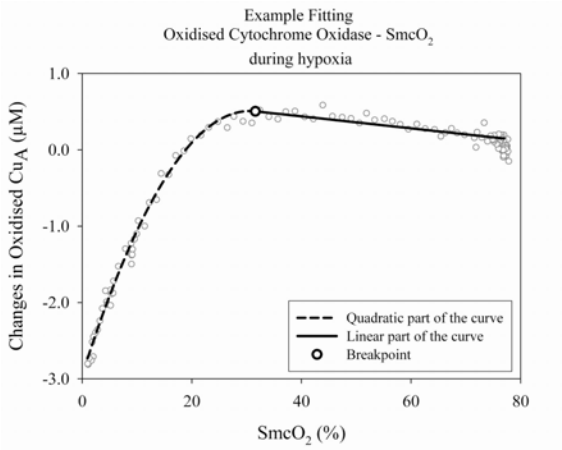


Figure 1: Example of a fitting of an oxidised cytochrome oxidase (changes in oxidised Cu_A) – mean cerebral oxygen saturation ($SmcO_2$) curve.

3.5. Statistical analysis

All results are expressed as median ± 95% confidence interval. A two-sided Wilcoxon-Mann-Whitney rank-sum test was used for between group analyses. Within group analysis was performed with the Wilcoxon signed ranks test. A two-sided probability value of less than 0.05 was regarded as statistically significant.

4. Results

4.1. iNOS gene expression

PCR showed that all samples were positive for the control gene β-actin as shown in Figure 2. No iNOS RNA was present in the control and sham samples, while all three LPS samples were positive for iNOS expression (Figure 2).



Figure 2: Gel-like visualisation of Agilent 2100 bioanalyzer electropherogram. β-actin is present in all samples, while iNOS is only present in LPS samples.

4.2. Baseline measurements

No difference in weight was found between groups at baseline (control: 418g [378, 452]). While the weight of sham rats (412g [377, 480]) remained constant (406g [379, 469]), the weight of LPS rats (411g [386, 463]) was significantly decreased 24 hours after LPS injection (388g [360, 442], p=0.016).

No differences between groups were found in breathing rate (77.9 bpm [55.8, 100.0]), end tidal CO_2 (49.2 mmHg [40.3, 58.1]), ear temperature (34.9 °C [34.0, 36.4]) and rectal temperature (37.6 °C [37.3, 38.5]) at the beginning of the experiment and these variables remained within physiological range for the remainder of the experiment. Data are expressed as median and 95% confidence interval of all groups together.

LPS injection resulted in an increased [HbO_2] and [HbT] as compared to control (p=0.015 and p=0.010, respectively) and sham groups (p=0.038 and p=0.028, respectively), while no effect on other parameters was seen (see also addendum 1).

4.3. Effect NOS inhibition

1400W significantly decreased [HbO_2] and $SmcO_2$ in the control group, heart rate, [HbO_2], [HbT], $SmcO_2$ and BFIw in the sham group and BFIw in the LPS group (see also addendum 1).

l-NAME, on the other hand, significantly decreased heart rate in the control group, significantly decreased [HbO_2], [HbT], $SmcO_2$ and BFIw in all three groups and significantly increased MABP, [Hb] and $avDO_2$ in all three groups (see also addendum 1).

4.4. Absolute Cu_A values

No differences were found between absolute oxidised Cu_A values (4.01 µM [3.70, 5.30], 4.02 µM [3.87, 4.89] and 4.51 µM [3.78, 4.80] for control, sham and LPS groups, respectively).

4.5. Effect of the combination of hypoxia and NO on cytochrome oxidase

In the quadratic part of the fitting (see also addendum 2), 1400W and l-NAME induce a significant increase in the quadratic coefficient in the LPS group, while LPS group at baseline is significantly increased in the linear coefficient compared to sham group and significantly decreased in the quadratic coefficient compared to control and sham groups.

While no difference in breakpoint was seen between groups at baseline, 1400W and l-NAME both significantly increase breakpoint in the LPS group as compared to baseline (see also addendum 2).

In the linear part of the fitting, 1400W and l-NAME both induce a significant increase in intercept in control and sham groups, while the intercept from the LPS group is significantly increased compared to control and sham groups at baseline. l-NAME induces an increase in the linear coefficient in all groups as compared to baseline (see also addendum 2).

5. Discussion

We first showed the presence of iNOS gene expression 24 hours after LPS injection in contrast to sham and control groups (Figure 2). We can therefore confirm high NO concentrations in the LPS group as compared to both control and sham groups. This resulted in increased oxyhemoglobin and total hemoglobin levels in the LPS group, as is expected during inflammation[11]. We also examined the effect of LPS injection on cytochrome oxidase redox status by back-calculating absolute oxidised cytochrome oxidase values after lethal cyanide injection. Cyanide binds to heme a3, preventing oxygen reduction by electrons leaving Cu_A and heme a, rendering both centres completely reduced[8]. In our experiment, no difference in absolute oxidised cytochrome oxidase concentration was found between groups. We can therefore conclude that elevated NO concentrations caused by inflammation are not sufficient to alter the oxidation status of

cytochrome oxidase *in vivo* in rats. This is in accordance to the *in vitro* data of Mander and colleagues[6], who showed that no neuronal death occurred under normal oxygen concentrations after the NO-donor DETA/NO, nor in mixed neuronal-glial cell cultures activated with LPS/IFN-γ. However, the addition of hypoxia to these high NO environments *in vitro*, induced large percentages of neuronal apoptosis and necrosis[6]. A possible explanation is that NO is a reversible inhibitor of cytochrome oxidase in competition with oxygen, markedly increasing the K_m for oxygen[6].

We thus studied the effect of high NO concentrations on cytochrome oxidase in combination with hypoxia in rats *in vivo*. Hypoxia itself does not change oxidised Cu_A early on, but after extremely compromised oxygen delivery, the Cu_A centre becomes more reduced[12,13]. From the hypoxia data, Cu_A was plotted against mean cerebral oxygen saturation[5], and we have examined the resultant curves under different NO conditions. Firstly, no differences were found between the control and sham groups (normal NO concentration). In addition, NOS inhibition in these groups only induced an increase in intercept of the linear part of the fitting. However, in the LPS group (high NO concentration), we found differences at baseline compared to control and sham groups in the quadratic part of the curve and in intercept of the linear part of the curve. In addition, 1400W and l-NAME induced changes in the quadratic part of the curve and induced a shift in breakpoint to higher mean cerebral oxygen saturations. A small effect of elevated NO concentration on cytochrome oxidase redox status during hypoxia therefore exists only at very low oxygen tensions ($SmcO_2 \leq 40\%$).

These data are in accordance to the *in vitro* work of Bellamy and colleagues who showed that even saturating NO concentrations of the guanylyl cyclase coupled receptor only affect respiration when the oxygen concentration is very low[14]. In addition, previous *in vivo* experiments showed no effect of NO on cytochrome oxidase in physiological conditions, nor in the hyperemic phase after anoxia[5]. Our data extend this *in vivo* knowledge to elevated NO concentrations, where minor effects were only found at low oxygen tensions.

Unexpectedly, l-NAME increased the slope of the linear part of the fitting in all three groups. This can probably be explained by the decrease in total cerebral hemoglobin after l-NAME injection due to vasoconstriction. Although it has been shown that it is possible to measure cytochrome oxidase redox changes in brain tissue using multiwavelength NIRS, even in the presence of large amounts of hemoglobin[15], oxidised Cu_A is known to have a very small NIRS signal compared to hemoglobin, making it prone to cross-talk[12]. By decreasing total hemoglobin, cross-talk reduces and the slope of the linear part of the curve flattens.

In our study, l-NAME had the expected results systemically (increased MABP) and cerebrally (increased oxyhemoglobine and total hemoglobin en decreased deoxyhemoglobin). As expected, 1400W did not have many systemic and cerebral effects, since it is known 1400W can be given up to 25 mg/kg, without apparent effect on hemodynamics[16]. In addition, LPS is known not only to induce iNOS gene expression, but also to induce a complete inflammation, while 1400W only has an effect on iNOS. On the other hand, 1400W did have effects in the LPS group, as compared with the control and sham groups, on the quadratic coefficient of the quadratic part of the curve and on breakpoint, confirming the activity of 1400W. Since no iNOS is present in control and sham groups, 1400W is likely to have more effect on neuronal NOS in these groups, as compared to the LPS group. We believe this can explain the hemodynamic effects in control and sham groups.

Conclusion

The aim of this study was to analyse the effects of high NO concentrations in combination with hypoxia on cytochrome oxidase redox status. We showed that elevated NO concentrations, by themselves, are not sufficient to induce a change in cytochrome oxidase redox status. Combined with hypoxia, however, high NO concentrations can induce small effects in cytochrome oxidase redox status only at very low oxygen tensions. We therefore conclude that NO only has effects on cytochrome oxidase *in vivo* in very specific conditions.

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Addendum 1

Table 1: Systemic and cerebral parameters in control, sham and LPS animals at baseline, after 1400W injection and after l-NAME injection.

	Baseline	1400W	l-NAME
Heart Rate (bpm)			
Control	377 [360, 402]	381 [344, 419]	347 [339, 400]*°
Sham	394 [374, 474]	386 [346, 418]*	378 [331, 421]
LPS	416 [326, 467]	407 [314, 462]	376 [324, 445]
Mean arterial blood pressure (mmHg)			
Control	82.8 [69.7, 99.1]	78.3 [66.9, 87.0]	116.4 [106.9, 126.7]*°
Sham	83.8 [67.2, 106.7]	78.6 [71.5, 91.1]	113.4 [105.6, 130.6]*°
LPS	87.9 [69.8, 92.1]	78.8 [73.0, 96.1]	113.6 [97.6, 118.3]*°
Oxyhaemoglobin concentration (µM)			
Control	82.2 [66.5, 111.9]	75.8 [56.2, 97.4]*	55.9 [44.7, 67.7]*°
Sham	86.3 [79.3, 103.3]	75.2 [70.1, 84.2]*	54.8 [48.8, 67.1]*°
LPS	104.0 [82.1, 125.2]$	94.8 [72.7, 108.4]	71.4 [54.9, 75.6]*°
Deoxyhaemoglobinconcentration (µM)			
Control	18.5 [15.5, 31.4]	20.1 [16.6, 30.2]	23.9 [20.9, 36.5]*°
Sham	21.5 [19.5, 30.1	24.4 [20.0, 31.2]	32.9 [27.1, 41.9]*°
LPS	23.9 [16.4, 25.3]	24.5 [23.1, 28.1]	30.0 [25.7, 33.5]*°
Total haemoglobin concentration (µM)			
Control	105.0 [83.2, 132.6]	99.7 [74.6, 120.8]	84.1 [68.7, 91.5]*°
Sham	110.4 [98.8, 124.0]	100.1 [93.6, 114.9]*	87.8 [75.9, 101.6]*°
LPS	127.2 [107.4, 148.9]$	121.0 [98.4, 133.7]	100.5 [86.0, 107.5]*°

Mean cerebral oxygen saturation (%)			
Control	82.2 [72.6, 84.4]	80.7 [71.3, 81.8]*	69.2 [59.7, 74.8]*°
Sham	80.5 [72.7, 83.9]	74.8 [70.6, 78.8]*	63.6 [57.1, 66.0]*°
LPS	82.4 [76.4, 87.4]	79.1 [73.9, 81.8]	70.9 [63.9, 73.2]*°
Weight corrected blood flow index			
Control	16.2 [12.9, 21.4]	14.3 [11.6, 22.6]	8.8 [5.1, 12.2]*°
Sham	20.4 [15.6, 25.9]	14.8 [7.1, 22.0]*	11.2 [7.8, 14.4]*
LPS	20.6 [15.6, 29.4]	16.1 [10.8, 22.8]*	10.4 [9.6, 14.0]*°
Arterio-venous O_2 content difference (ml/dl)			
Control	2.20 [1.06, 4.25]	2.96 [1.03, 4.36]	4.84 [1.04, 6.85]*
Sham	3.16 [1.89, 4.47]	3.90 [1.49, 4.48]	6.18 [4.57, 7.67]*°
LPS	2.80 [0.87, 4.46]	3.16 [1.53, 4.17]	4.94 [2.51, 6.23]*°
Metabolic rate of oxygen index			
Control	46.0 [16.2, 72.6]	48.9 [11.9, 60.0]	47.2 [8.7, 62.6]
Sham	56.3 [37.2, 91.6]	50.3 [24.6, 76.8]	67.5 [43.6, 110.2]
LPS	57.7 [20.2, 75.2]	48.2 [18.8, 66.6]	51.5 [24.3, 73.1]

Values are expressed as median [95% confidence interval]. Bpm: beats per minute. *: significantly different from baseline within the same group, °: significantly different from 1400W measurement within the same group, $^{\$}$: significantly different from control and sham groups at baseline.

Addendum 2

Table 2: Parameters of the quadratic-linear fitting on the Cu_A - $SmcO_2$ curves during hypoxia in control, sham and LPS animals at baseline, after 1400W injection and after l-NAME injection.

	Baseline	**1400W**	**l-NAME**
a1, intercept (at Cu_A, µM)			
Control	-4.63 [-5.95, -1.69]	-4.96 [-6.55, -2.29]	-5.28 [-5.60, -2.77]
Sham	-4.85 [-6.86, -2.56]	-4.88 [-6.19, -3.14]	-4.85 [-7.06, -3.27]
LPS	-4.98 [-6.05, -3.16]	-4.8 [-5.89, -3.85]	-4.76 [-5.56, -4.12]
b1, linear coefficient of the quadratic part of the fitting			
Control	0.226 [0.129, 0.3]	0.223 [0.124, 0.408]	0.233 [0.144, 0.269]
Sham	0.240 [0.097, 0.354]	0.265 [0.114, 0.354]	0.275 [0.165, 0.334]
LPS	0.261 [0.2, 0.378] ∫	0.257 [0.206, 0.341]	0.269 [0.154, 0.307]
c1, quadratic coefficient of the quadratic part of the fitting			
Control	$-3.70\text{x}10^{-3}$ [$-5.00\text{x}10^{-3}$, $-1.60\text{x}10^{-3}$]	$-3.18\text{x}10^{-3}$ [$-7.86\text{x}10^{-3}$, $-1.60\text{x}10^{-3}$]	$-3.25\text{x}10^{-3}$ [$-3.80\text{x}10^{-3}$, $-1.68\text{x}10^{-3}$]
Sham	$-4.16\text{x}10^{-3}$ [$-6.24\text{x}10^{-3}$, $-1.22\text{x}10^{-3}$]	$-4.46\text{x}10^{-3}$ [$-6.20\text{x}10^{-3}$, $-1.30\text{x}10^{-3}$]	$-4.48\text{x}10^{-3}$ [$-5.90\text{x}10^{-3}$, $-2.10\text{x}10^{-3}$]
LPS	$-4.58\text{x}10^{-3}$ [$-7.22\text{x}10^{-3}$, $-3.08\text{x}10^{-3}$] $	$-4.14\text{x}10^{-3}$ [$-5.70\text{x}10^{-3}$, $-3.12\text{x}10^{-3}$]*	$-4.02\text{x}10^{-3}$ [$-4.94\text{x}10^{-3}$, $-1.64\text{x}10^{-3}$]*
Breakpoint (at mean cerebral oxygen saturation, %)			
Control	31.6 [25.5, 45.7]	37.5 [25.3, 43.5]	37.4 [36.2, 50.2]
Sham	32.2 [25.5, 47.7]	30.7 [29.0, 49.7]	31.3 [28.3, 41.9]
LPS	29.8 [27.7, 36.3]	32.9 [31.1, 36.7]*	36.4 [32.0, 37.4]*°

a2, intercept (at Cu_A, µM)

Control	3.16 [2.48, 3.95]	3.65 [2.88, 4.65]*	3.92 [2.82, 5.5]*
Sham	3.27 [2.64, 4.06]	3.28 [2.78, 5.38]*	3.36 [2.66, 5.13]*
LPS	4.22 [3.07, 5.51] $	4.58 [3.13, 5.57]	4.96 [2.41, 5.41]

b2, linear coefficient of the linear part of the fitting

Control	-0.018 [-0.024, -0.008]	-0.015 [-0.028, -0.010]	-0.012 [-0.024, -0.005]*
Sham	-0.019 [-0.029, -0.008]	-0.010 [-0.016, -0.006]	-0.004 [-0.018, 0.001]*°
LPS	-0.026 [-0.031, -0.012]	-0.016 [-0.022, -0.011]	-0.012 [-0.015, -0.006]*

Values are expressed as median [95% confidence interval]. *: significantly different from baseline within the same group, °: significantly different from 1400W measurement within the same group, $: significantly different from control and sham groups at baseline, ∫: significantly different from sham group at baseline.

Article 4

Application of NIRS in mice: a study comparing the oxygenation of cerebral blood and main tissue oxygenation of mice and rat

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Condensed Abstract

The introduction of transgenic mice models in neuroscience requires miniaturisation of measuring systems such as near infrared spectroscopy (NIRS) to assess cerebral oxygenation and hemodynamics. Comparison of basic NIRS variables of mice and rats showed that cerebral blood and main tissue oxygenation can be adequately studied with NIRS without major adaptations. Additionally, cerebral blood flow can be calculated from the transit of an indocyanine green bolus of which 2.5 µl (1 mg/ml) was found to give a good signal-to-noise ratio with acceptable disturbances of the other NIRS measurements and minor variability of the blood flow index. We consider the application of this technique in mice a major step forward.

1. Introduction

In modern neuroscience, insight is gained in important pathophysiological processes underlying Alzheimer and Parkinson disease by the development of transgenic mice models[1-2]. Obtaining on-line information on the cerebral oxygenation, hemodynamics, cerebrovascular reactivity and mitochondrial function is crucial for the validation of these models, the understanding of the pathology itself, and the search for potential therapies. In addition, it would be very interesting to study cerebral blood flow and mitochondrial function in transgenic mice models of atherosclerosis[3] or cerebrovascular deformations leading to neurodegeneration[4].

A suitable technique for such measurement does not exist at the moment. Therefore, we tested if multi-wavelength near infrared spectroscopy (NIRS) technology, a minimally invasive technique, can be used as a tool to examine cerebral oxygenation and hemodynamics in the anaesthetised mouse.

In a first study we compare basic NIRS variables measured in mice with those obtained in rats and establish the possibility of NIRS measurement of cerebral blood and cellular oxygenation in mice. In a second study we determine the optimal dose of indocyanine green (ICG) providing a sufficiently large signal in the brain of mice and estimate the effect of the tracer injection upon the other NIRS variables.

2. Materials and Methods

2.1. Animal preparation and experimental protocol

Animal housing and treatment conditions complied with the European Union directive # 86/609 on animal welfare. The study was performed on 10 male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 380-430 g, 10 C57BL6 mice (CEHA, KULeuven, Leuven, Belgium) weighing 24-26 g and 12 NMRI mice (ANIMALIUM, St.-Rafael Proefdierencentrum, Leuven, Belgium) weighing 24-26 g. They were allowed free access to food and water (12/12-hour day-night cycle). The rat preparation for NIRS has previously been described in detail[5]. In short, anaesthetic induction was achieved by 4% isoflurane in 30%O_2/70%N_2O. Instead of endotracheal intubation, standard in rats, the C57BL6 mice were kept on 2% isoflurane by means of an anaesthesia mask and the NMRI mice underwent tracheotomy and were mechanically ventilated (7 µl/g body weight at 150 strokes/min.). In the NMRI mice a PE50 cannula was inserted into the right external jugular vein towards the vena cava for ICG injection and the aorta

descendens was cannulated to monitor mean arterial blood pressure (MABP) and heart rate (HR). All animals were positioned on a rectal temperature controlled heating pad and fixed into a stereotaxic apparatus (David Kopf, Tujunga, CA, USA). The skin was removed and combined with either temporal muscle resection or retraction for rats or mice, respectively. Possible sites of bleeding were cauterised and the skull was painted black. The receiving and emitting optode, identical in both studies, were placed onto the temporal bones by means of micromanipulators. The space between the skull and optodes was filled with optical coupling gel (R.P. Cargille Laboratories Inc., Cedar Grove, New Jersey, USA) and the skull was covered with black modelling clay (Eberhard Faber GmbH., Neumarkt, Germany). Anaesthesia was then lowered to 1.5% isoflurane.

After finalisation of the animal preparation, transillumination of the brain was started. The light entrance slit of the spectrograph was set at 350 µm and 160 µm for rat and mice, respectively. The measured variables were: concentration of deoxyhemoglobin ([Hb]), oxyhemoglobin ($[HbO_2]$), total hemoglobin ([HbT]) and oxidised cytochrome oxidase (Cu_A) as well as the optical pathlengths at 740, 840 and 960 nm. For the dose-finding study, the same variables were measured along with MABP and HR, and in each animal, boli of 0.5, 1, 2.5, 5 and 10 µl ICG were given at 5, 10, 15, 20 and 25 min. after the onset of the measurements, respectively. All animals were killed by terminal anoxia.

2.2. NIRS equipment

The NIRS system and algorithms have been developed at University College London and described in detail before[6-8]. The system allows the on-line assessment of absolute values of [Hb] and absolute changes in $[HbO_2]$ and Cu_A. Terminal anoxia at the end of an *in vivo* experiment allows back-calculation of the absolute $[HbO_2]$ and Cu_A. The [HbT] at each time point ($=[Hb]+[HbO_2]$) and the oxygen saturation of the hemoglobin in the cerebrum ($SmcO_2 =([HbO_2]/([Hb]+[HbO_2]))x100$) can be calculated.

A 1 mg/ml ICG (IR-125, laser grade; Acros, Geel, Belgium) solution was used containing 5% bovine serum albumin (BSA fraction V; Sigma, Bornem, Belgium) to bind the ICG. The solution was sterilised by filtration with a 0.22 µm filter unit.

NIR spectra were collected contiguously with a period of 100 ms and 10 spectra were averaged to obtain a time resolution of 1 second (1 Hz) throughout the entire experiment.

2.3. Data and statistical analysis

For each animal the data of the NIRS measured variables ([HbO_2], [Hb], [HbT], Cu_A and $SmcO_2$) were averaged over a 5-min. period starting 2 min. after the onset of the measurement for the first study and over a 3-min. period starting 1 min. after the onset of the measurement for the dose-finding study. For the first study we calculated the differential pathlength factor (DPF) by dividing pathlength by the interoptode distance[9].

ΔICG was calculated from the ICG bolus transit curve by subtraction of the baseline value before injection from the peak value after injection. The rise time (Δt) was measured from the first value where the ICG signal deviates from the baseline towards the peak value. A blood flow index (BFI) was then calculated by dividing ΔICG by Δt[10,5].

In order to evaluate cross-talk between the ICG signal and other NIRS signals during and after the ICG transit, we examined the duration of the disturbances of the different NIRS variables. We thus measured the time interval between the peak of the disturbance and the point of return to 10% of its amplitude (t_{90})[5] for [Hb], [HbO_2], Cu_A and ICG.

Results are expressed as median [95% confidence interval (CI)]. A two-sided Wilcoxon-Mann-Whitney rank-sum test and a two-sided Wilcoxon signed-ranks test were used for comparison of the mice and rat data and the dose-finding data, respectively. Two-sided probability values of less than 0.05 were regarded as statistically significant.

3. Results

3.1. Comparison study

Table 1 shows the results of the optical pathlengths and DPF at 740, 840 and 960 nm. Considering the absolute pathlengths, large differences were observed at every wavelength, clearly indicating a larger tissue volume measured in the rats. To normalise for these differences, we calculated the DPF, thereby correcting for the interoptode distance, corresponding to the width of the skull. The DPF at 740 nm is lower in mouse brain whereas the DPF at 840 nm and 960 nm are not different from rat.

Table 1: Near infrared spectroscopy assessed optical pathlength and DPF data.

	Mouse (n=10)	**Rat (n=10)**	**p-value**
Pathlength 740 nm (cm)	2.25 [1.74, 2.62]	6.31 [6.07, 6.69]	< 0.001
Pathlength 840 nm (cm)	2.62 [2.38, 2.73]	5.41 [4.98, 5.78]	< 0.001
Pathlength 960 nm (cm)	2.15 [2.01, 2.26]	4.21 [4.06, 4.38]	< 0.001
DPF 740 nm	2.51 [1.93, 2.91]	3.50 [3.37, 3.71]	< 0.001
DPF 840 nm	2.91 [2.64, 3.04]	3.01 [2.77, 3.21]	0.393
DPF 960 nm	2.39 [2.23, 2.51]	2.34 [2.26, 2.43]	0.280

Values are expressed as median [95% CI].

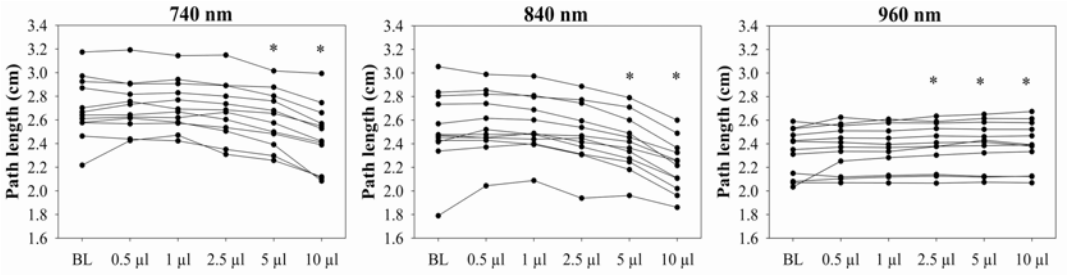


Figure 1: Pathlength at 740, 840 and 960 nm after the different ICG boli. Statistical significance was assessed by Wilcoxon signed-ranks test. *: significantly different from baseline values ($\alpha = 0.05$), BL = baseline.

3.2. Dose-finding study

The physiological data derived from the NMRI mice on the onset of the dose-finding study were stable (MABP: 71.9 [67.1, 89.5] mmHg; HR: 592 [543, 618] beats per min.; T_{rectal}: 36.8 [36.4, 37.0] °C; T_{ear}: 33.4 [33.0, 33.9] °C).

Figure 1 shows the pathlengths at 740, 840 and 960 nm in the mice brain. These data indicate that after larger ICG injection volumes the 960 nm pathlength significantly increases and both 740

and 840 nm significantly decrease. This is in accordance with the increased absorption of light in the lower end of the spectra where ICG is a major contributor to the general absorption spectrum.

Table 2: t_{90} (s) of NIRS signals after i.v. boli of different ICG dosages.

	ICG 0.5 µl	**ICG 1 µl**	**ICG 2.5 µl**	**ICG 5 µl**	**ICG 10 µl**
[Hb] (µM)	3.58 [1.10, 4.66]	3.22 [1.89, 9.22]	16.9 [10.4, 55.2]*	68.6 [24.0, 155.4]*	91.8 [65.9, 115.0]*
[HbO_2] (µM)	ND	1.84 [0.90, 2.88]	3.17 [1.98, 9.32]	8.82 [1.89, 70.3]	10.1 [3.66, 32.7]°
Cu_A (µM)	1.59 [0.79, 3.08]	2.18 [1.18, 3.57] *	39.8 [9.71, 93.1] *	113.5 [90.5, 246.0] *	235.0 [173.6, 308.1] *
ICG (µM)	11.8 [2.18, 60.0]	68.4 [52.1, 96.1] *	109.9 [82.1, 134.2] *	119.6 [96.4, 131.0] *	141.5 [112.4, 179.1] *

Values are expressed as median [95% CI]. ND: not determinable. *: significantly different from 0.5 µl values ($\alpha = 0.05$). °: significantly different from 1 µl values ($\alpha = 0.05$). t_{90} of Cu_A and ICG were all significantly different.

Table 2 presents a summary of the t_{90} data. After 0.5 µl ICG bolus injection, the change in the signal of [HbO_2] was too small to have a sufficient signal to noise ratio. T_{90} values of all variables rose with larger ICG bolus, except for t_{90} of Hb after 1 µl ICG bolus injection. T_{90} of [Hb] values of 2.5, 5 and 10 µl are significantly different from 0.5 µl value, t_{90} of [HbO_2] of the 10 µl bolus is significantly different from 1 µl and t_{90} values of [Cu_A] and ICG all significantly increased dose dependently.

To compare variability, we divided BFI by the injected volume (Figure 2). Significant differences were found between 0.5 and 1 µl, 2.5 and 5 µl, 2.5 and 10 µl and 5 and 10 µl.

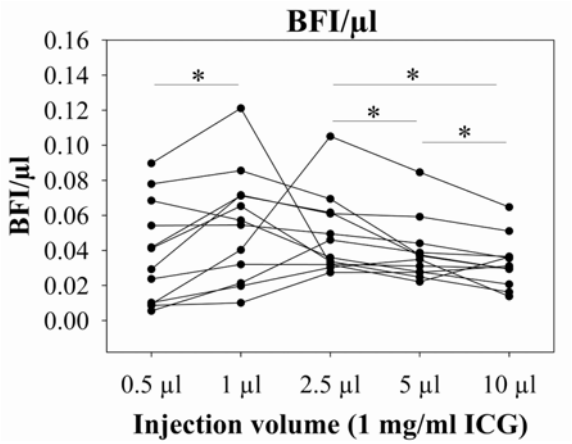


Figure 2. BFI/µl. Blood flow index divided by the amount of ICG injected. Statistical significance was assessed by Wilcoxon Signed Ranks test. *: significantly different from each other ($\alpha = 0.05$).

4. Discussion

At present, NIRS has been shown an accurate research tool for the study of cerebral hemodynamics and oxygenation in several species such as pig[10], piglet[8] and rat[11]. However, further miniaturisation of the system in order to obtain measurements in mice is not evident. To our knowledge, the present study is a first attempt to evaluate brain oxygenation in mice with multi-wavelength NIR spectroscopy.

A first prerequisite to allow absolute quantification of [Hb], [HbO_2] and Cu_A is the determination of the optical pathlength[6]. Pathlengths at 740, 840 and 960 nm are significantly smaller in mice compared to rats. The pathlengths are the actual average distance the light travels between the emitting and receiving optodes and are not very useful to compare between species since they are interoptode distance dependent[9] (9mm for mice and 18mm for rats). Calculation of the DPF enables comparison of both species. We showed a decreased DPF at 740 nm in mice compared to rats but equal DPF at 840 and 960 nm. Although shortening of the interoptode space theoretically shortens DPF and increases DPF *in vivo*[12], we find contradictory results. At this time we do not have a proper explanation for these data. The strong absorption peak of water at 960 nm[6] can be used to calculate and compare the actual DPF under the assumption that the water content of the brain is 80% in both mice[13] and rats[14]. Since the DPF at 960 nm are comparable between both species, we can conclude that they have similar scattering properties and that the algorithms allow to obtain absolute values for [Hb], [HbO_2] and Cu_A in mice.

Mice showed a significantly lower [HbT] than rats (data not shown). Only 72.3% of rats [HbT] was measured in mice, this compares to the value of 78.1% calculated from the average hemoglobin concentration published for rats and mice (14.2 and 11.1 g/100ml respectively[15]). The lower hemoglobin concentration was consistently found for oxy- and deoxyhemoglobin (71.2% and 79.1% respectively), resulting in comparable $SmcO_2$ between both species. For Cu_A in mice brain equivalent concentrations were obtained as in references 16 and 8, but validation still needs to be performed.

In the second study, we investigated the optimal ICG bolus to qualitatively measure cerebral blood flow. The aim was a sufficiently large signal in the brain of mice with only minor cross-talk effects. We evaluated the effect of ICG on the three pathlengths (Figure 1). Due to significant changes in pathlengths after large ICG boli, back-calculation of NIRS variables was incorrect. It is therefore not possible to simultaneously measure BFI and [Hb], $[HbO_2]$ and Cu_A. Most likely ICG accumulates in the blood and dominates the NIR spectrum. In rats this problem does not occur due to the small bolus to blood volume ratio (5 µl ICG/±20 ml blood) whereas the most appropriate volume for BFI calculation in mice, 2.5 µl, is only diluted in 2 ml of blood. To estimate the impact of ICG boli on the NIRS variables without taking the change in pathlength into account, we calculated their t_{90}. t_{90} of all variables rose with larger ICG bolus (Table 2), demonstrating the need to minimise bolus volume. On the contrary, while investigating variability by dividing BFI by the injection volume (Figure 2), we found that small injection volumes result in a large variability, indicating the need to increase the injected bolus.

To best fit all criteria, minimise bolus for minor disturbances in pathlength and t_{90} and maximise bolus to minimise variability, we find at present the optimal bolus to measure BFI in mice to be 2.5 µl ICG. Further attempts to increase reproducibility of the 1 µl bolus injection are a technical challenge but will be attempted.

Conclusion

We conclude that multi-wavelength NIRS can be used in mice to assess oxygenation of cerebral blood and the concentration of oxidised cytochrome oxidase in brain tissue. To our knowledge no previous report showing this possibility has been published. Monitoring of the transit of an indocyanine green bolus with NIRS can be used to qualitatively estimate cerebral blood flow in mice. A bolus with a volume of 2.5 µl of a 1 mg/ml ICG solution gives a good signal-to-noise ratio with acceptable disturbances of the other NIRS measurements and minor variability of the

blood flow index. At present combination of the assessment of the oxygenation of cerebral blood and the concentration of oxidised cytochrome oxidase with cerebral blood flow estimation is not yet possible due to technical difficulties. We consider the application of this technique in the study of transgenic mice models as a major step forward.

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General Discussion

NO and NO-synthases

Nitric oxide (NO) is one of the important players in the regulation of cerebral blood flow. It is a radical with an unpaired electron, but it is relatively less reactive than other oxygen radicals, like superoxide and the hydroxyl radical. It moves rapidly from cell to cell and has high biological activity, while it has a short half life of a few seconds or less[1]. The best studied effect of NO is the reaction with soluble guanylyl cyclase (sGC), an enzyme which contains heme and is activated by NO up to 300-fold. This triggers the increase of cGMP, which in its turn activates PKG resulting in a decreased intracellular calcium concentration. This mechanism mediates vessel relaxation, but also inhibition of platelet aggregation and others[2].

NO is synthesised by NO synthase (NOS), which forms a complex with several cofactors (tetrahydrobiopterin, FAD, FMN and heme) and catalyses the reaction of l-arginine, NADPH and oxygen into NO, citrulline and NADP[3]. For every mole of NO formed, 2 mole of oxygen and 1.5 mole of NADPH are consumed[4]. This generic reaction is mediated by three NOS isoforms. eNOS and nNOS were originally considered to be constitutively expressed[1], but both can be induced several-fold[5], and both eNOS and nNOS, but not iNOS, have an autoinhibitory loop[5]. They are active as dimers when bound to the calcium-calmodulin complex, while iNOS is only expressed after cellular stimulation. iNOS monomers can tightly bind calmodulin at very low calcium concentrations, making them virtually calcium independent and thus constantly active. The activity of this protein is therefore regulated at mRNA and protein levels[1]. While the homology of these three isoforms is about 50-60%, the homology of a given isoform between species can be as great as 85-92%[6].

Although controversial, it is suggested that another NOS isoform exists, namely mitochondrial NOS (mtNOS). mtNOS is believed to be a constitutive isoform of NOS associated with the inner mitochrondrial membrane[3,7]. Several suggestions about this isoform have been made. mtNOS seems to be a membrane-associated iNOS, since it was only recognised by antibodies against iNOS, not eNOS nor nNOS[3]. On the other hand, since nNOS knockout mice do not have mtNOS, mtNOS has been identified as an nNOSα isoform, likely coded by the same nNOS gene[7,8]. To date, the existence of mtNOS is still under debate[8].

Since all NOS isoforms synthesise NO in a similar way[9], spatial and temporal distribution of these isoforms is very important for the different functions of NO. To date, the relative

importance of nNOS versus eNOS in the control of cerebral blood flow is not known[10]. To investigate the effects of this compartmentalisation of NOS isoforms in different circumstances, we used different NOS inhibitors.

NO-synthase inhibitors

l-NAME is a non-selective potent NOS inhibitor, both *in vitro* and *in vivo*. It is a guanidine substituted analogue of l-arginine which is demethylated *in vivo* to yield active l-NOARG. This l-NOARG inhibits NOS by competition with l-arginine for the substrate binding site[11].

Nitroindazoles, like 7NI, are believed to bind to the heme prosthetic group of NOS, which results in a disruption of the electron flow through the enzyme and preventing NO formation[11]. In addition, 7NI is known to interfere with the binding of l-arginine and tetrahydrobiopterin[11,12]. 7NI was 30-40 times more potent in inhibiting constitutive NOS than iNOS[13], but *in vitro* no selectivity was found versus eNOS or nNOS. Surprisingly, 7NI does not inhibit acetylcholine-induced endothelium-dependent relaxation of rabbit aorta *in vitro* and has no effect on arterial blood pressure in e.g. mouse and rat[11]. In addition, 7NI did not affect the CBF response to oxotremorine, a muscarinic agonist that dilates cerebral vessels through eNOS[13]. This indicates that, although 7NI inhibits eNOS *in vitro*, no effects of eNOS inhibition is seen *in vivo*[11]. On the other hand, 7NI is known to reduce cerebral blood flow and prevent NMDA induced convulsions *in vivo*, suggesting an *in vivo* inhibition of nNOS[11]. Despite the absence of *in vitro* selectivity, 7NI may therefore be considered the first selective inhibitor of nNOS *in vivo*[12]. The mechanism of this selectivity may be due to a better uptake into the neurons versus endothelial cells, or to an extensive catabolism in the endothelium compared to neurons[12]. However, a small remark has to be made: since all three isoforms can be expressed in neurons, cell-type specificity is very different from isoform selectivity[3].

Although there still is a lack of specific eNOS inhibitors[5], l-NIO has been proposed as a relatively specific eNOS inhibitor[14]. It is an l-arginine analogue, which *in vitro* is a five times more potent inhibitor of the NOS enzyme compared to l-NAME[14], and it is also an inhibitor of l-arginine uptake[15]. On the other hand, l-NAME is ten times more potent than l-NIO to increase blood pressure *in vivo*[14]. Furthermore, l-NAME was able to inhibit the relaxation of aortic rings after acetylcholine completely, while l-NIO was only able to partially inhibit this relaxation[16]. l-NAME is therefore a more potent inhibitor compared to l-NIO *in vivo*. Additionally, l-NIO is a potent inhibitor of all three NOS isozymes with low selectivity[17]: it was found to be only four times more selective for eNOS versus nNOS[18].

1400W is *in vitro* a very potent inhibitor of iNOS, highly selective versus both eNOS (> 4000 fold) and nNOS (32 fold)[3]. It is an irreversible inhibitor or an extremely slowly reversible inhibitor of iNOS. In contrast inhibition of eNOS and nNOS with 1400W was relatively weak, rapidly reversible and in competition with l-arginine[19]. Additionally, it is able to penetrate cells and tissues[3] and can be given in a single intravenous dose to normal rats up to 25 mg/kg, without apparent effect on hemodynamics[19].

Anaesthesia

In our study, the inhalational anaesthetic isoflurane is used. Inhalational anaesthetics are known to increase CBF in a dose-dependent manner with a concomitant decrease in cerebral metabolism, but although flow is set to a higher level, local coupling of flow and metabolism is still present[20]. In addition, when using low isoflurane concentrations, cerebrovascular CO_2 reactivity and cerebral autoregulation are maintained[20]. When using nitrous oxide as a background of isoflurane, CBF is increased without changing metabolism[20].

Effect of NOS compartmentalisation on autoregulation

Autoregulation

The autoregulation concept was first reported in 1902 in the dog's denervated hindlimb[21]. Only in 1959, this concept was introduced in cerebral circulation[22]. To study cerebral autoregulation, we used hypovolemia to induce extreme hypotension. During hypovolemia, cardiac output is maintained by increasing heart rate and preload (through venoconstriction), and blood pressure is maintained by systemic arterial constriction[23]. This constriction does not apply to the cerebral and coronary circulations, preserving blood flow to brain and heart, even under severe hypotensive conditions[23]. Up to a certain point below the lower limit, when CBF decreases, the brain can maintain its oxygen metabolism by extracting more oxygen from the blood[24]. However, when blood flow and/or oxygen delivery becomes limited, intracellular oxygen tensions may fall below a critical value, diminishing cellular function. This may activate adaptive mechanisms aimed at restricting ATP consumption to life-sustaining processes only[23]. Likewise, studies in primates, cats and humans showed that spontaneous and evoked electrical activity ceases when CBF falls below 16-18 ml/100 g/min.[25], and an interruption of CBF for 8-10 seconds is generally sufficient to induce loss of consciousness[26].

Studying autoregulation

The arterial blood pressure data obtained during hypovolemia and concomitant CBF measurements are used to draw an autoregulation curve. This curve, usually used to explain cerebral autoregulation, is a rather simplistic representation of the physiologic phenomenon, since it can rapidly change to higher and lower blood flows and higher and lower blood pressures[20]. For example, in autoregulation, the starting level of CBF seems to be important, since at high resting CBF, the plateau is shortened and may even be abolished[24]. Chronic changes also exist: despite absence of differences in the absolute levels of CBF between normotensive and hypertensive subjects, chronic hypertension shifts the lower limit of the autoregulation curve to higher blood pressures[24]. This way, tolerance to high blood pressure improves, while tolerance to low blood pressure becomes impaired. This finding is a consequence of structural adaptation, since small arteries and arterioles are narrowed with thickened walls[24].

NO in autoregulation

Today, NO is suggested to be a mediator of cerebral autoregulation[25]. In isolated intraparenchymal rat arterioles, vascular NO release increases in response to a decrease in transmural pressure from 60 mmHg onwards, while arteriolar nitric oxide appears not to be involved in the autoregulatory vasoconstriction induced by elevating transmural pressure from 60 to 90 mmHg[27]. Also, local NOS inhibition *in vivo* with l-NNA raises the lower limit of autoregulation in the cortex to higher blood pressures[28]. Again, in eNOS deficient mice, the autoregulation curve was slightly shifted to higher blood pressures, suggesting a higher cerebrovascular resistance[29]. Consistent with the literature, we showed that non-selective inhibition using l-NAME induces a shift in lower limit towards higher blood pressures (Chapter 1, Figure 1). We therefore conclude that NO at least plays a role in autoregulation near the lower limit.

However, the effect of the compartmentalisation of NOS isoforms on autoregulation has not been investigated before. It is therefore not known which NOS isoform is important near the lower limit of autoregulation. In Chapter 1, we showed that nNOS inhibition does not shift the lower limit of autoregulation (Figure 1). These data, together with the facts that eNOS is markedly present in the vasculature and iNOS is not present in physiological circumstances, suggest that endothelial NO regulates the cerebrovascular response to hypotension. As a possible mechanism, shear stress has been introduced, since shear stress is known to be a major inducer of eNOS. Although hypotension is likely to result in a decrease of shear stress, the opposite might be true,

because of passive contraction of elastic elements in the vascular wall[28]. We therefore suggest that eNOS is important near the lower limit of autoregulation.

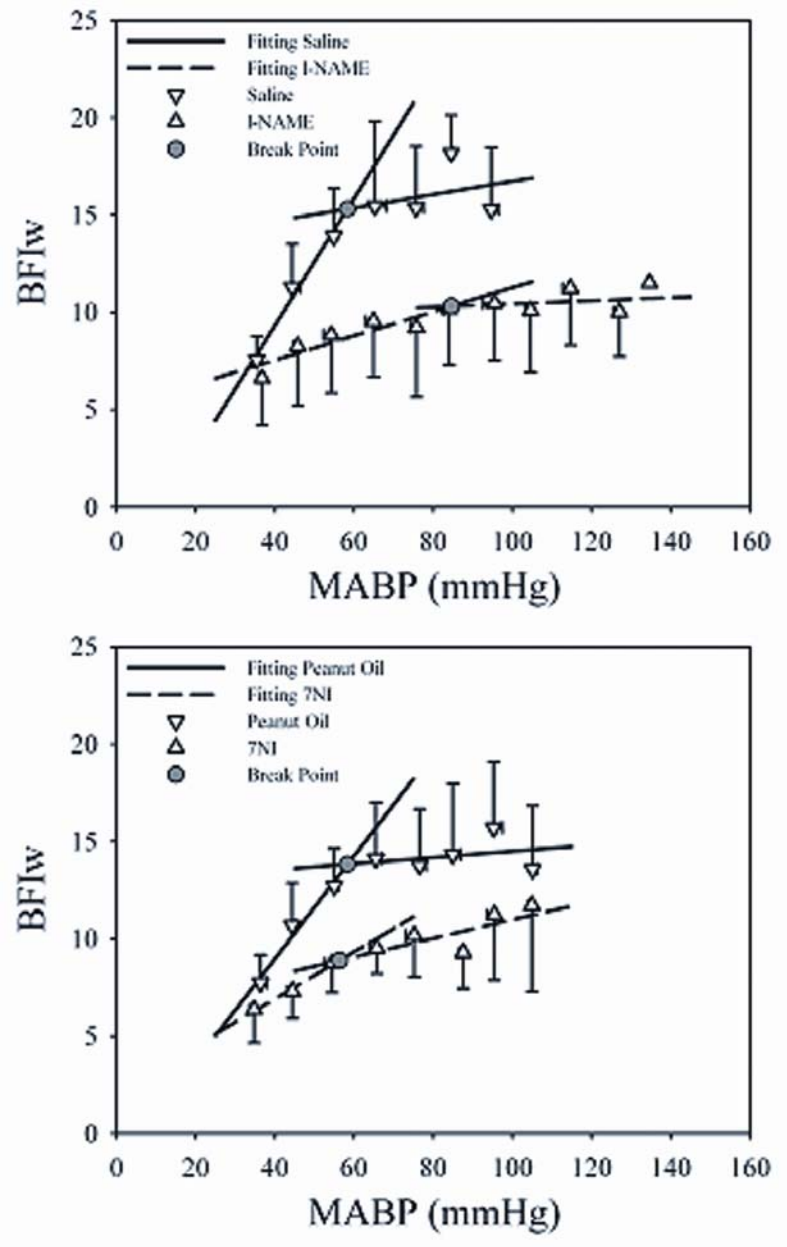


Figure 1 (Chapter 1): Linear-linear fittings of the autoregulation curves of saline, l-NAME, peanut oil and 7NI groups. BFIw: weight corrected blood flow index. Data are represented as mean ± standard deviation.

Furthermore, we showed that nNOS as well as non-selective inhibition, induced a decrease in autoregulation level (Figure 1), that is the constant level of CBF, while $CMRO_2$ remained the same (Chapter 1). We believe this indicates an uncoupling of CBF and metabolism, whereby the CBF set point at which autoregulation is maintained, is lowered. This hypothesis is supported by the literature, since nNOS inhibition with 7NI decreases blood flow compared with vehicle-treated rats, without affecting cerebral glucose use[30,31]. In other words, CBF and cerebral glucose use may vary independently under nNOS inhibition[30]. We therefore suggest that nNOS is responsible for the level of CBF autoregulation.

In summary, we believe that eNOS provides the mechanism of cerebral autoregulation, while nNOS is responsible for the set point of cerebral autoregulation.

Effect of NOS compartmentalisation early after neurotrauma

Neurotrauma incidence and experimental model

The incidence of hospitalisation with any kind of brain injury in the United States is 175-200 per 100 000 per year, with an average brain injury death rate of about 22 per 100 000 per year[32]. In the world, TBI even represents the leading cause of morbidity and mortality in individuals under the age of 45[33]. It is therefore very important to elucidate the early changes in the brain after neurotrauma.

To study brain injury, we used the impact-acceleration closed head injury model. It is a diffuse brain injury model, using a free falling guided weight together with a platform on springs with a known 'spring constant' to allow defined movement of the head after impact[34]. This model predominantly leads to diffuse shear forces. It produces coma as well as widespread axonal swelling with the severity related to the level of injury. Impact-acceleration TBI produced no endothelial damage, but was followed within 24 hours by the death or migration of vascular pericytes[35], while severe neurotrauma induces a significant impairment in brain oxygenation in the acute phase after TBI[36]. In addition, moderate neurotrauma using this model did not result in a compromised blood brain barrier[37]. Because of the ease of use, the absence of endothelial damage, the intact blood-brain barrier and the relevance to human trauma, we used this neurotrauma model in our experiments.

NO early after neurotrauma

Together with the characteristical decrease in CBF early after neurotrauma[38], most experimental models of trauma showed relative deficiencies in NO during this time[4]. For example, after impact injury, the concentration of NO in the brain, measured directly by an NO probe and indirectly using the microdialysis technique, increased immediately after TBI, but as soon as five minutes later, NO concentrations fell below baseline levels for at least three hours[39]. It is believed that a clear relationship exists between the decreased NO concentrations and decrease in CBF, since l-arginine restores CBF to near baseline levels in mice and rats, without the need to increase blood pressure[38,40].

In our study (Chapter 2), we showed that non-selective NOS inhibition has the expected cerebral effects in normal animals, while in neurotrauma animals, the same effects lasted shorter, or did not occur. As in the literature, this too suggests decreased NO concentrations after moderate neurotrauma.

Again, the role of the compartmentalisation of NOS in this decrease in NO concentrations has not been investigated before. It is therefore not known which NOS isoform is important in the decrease in NO concentrations early after neurotrauma. In Chapter 2, we saw that, while an effect of eNOS inhibition can still be seen in neurotrauma animals as compared to control animals, nNOS inhibition did not have any effect on neurotrauma animals. In addition, we observed an uncoupling of CBF and cerebral oxygen consumption when comparing baseline values of neurotrauma rats to those of normal rats. Since nNOS inhibition is known to uncouple CBF and cerebral metabolism[30], we believe this uncoupling is the result of nNOS inhibition. Consequently, the early decrease in NO and concurrent changes in CBF after neurotrauma are primarily attributed to a decrease in nNOS activity.

The mechanism of this diminished nNOS activity is not known today. Earlier, three possible mechanisms have been proposed: (1) the acute increase in NO immediately after trauma may inactivate nNOS by binding of NO to its heme iron, (2) the high calcium concentration immediately after trauma may activate protein kinases, resulting in nNOS phosphorylation and (3) finally nNOS inactivation or an increase in endogenous nNOS inhibitor may occur[41]. However, we suggest that the decreased nNOS activity involves changed l-arginine concentrations. Biomechanical injury to the brain induces an abrupt indiscriminant release of neurotransmitters and unchecked ionic fluxes. The binding of excitatory transmitters to their receptors leads to a further neuronal depolarisation with efflux of potassium and influx of calcium[42]. This sequence of events leads to massive NO production immediately after

neurotrauma. However, five minutes after trauma, all substrate is depleted, resulting in NO levels dropping below baseline values[39]. This hypothesis is supported by the fact that decreased l-arginine levels were found during the first three days after neurotrauma in head-injured patients[43]. eNOS activity might also be suboptimal due to this occurrence, but the decrease in NOS substrate is less problematic for eNOS compared to nNOS, since eNOS is located near blood vessels, guaranteeing continuous replenishment and first access. eNOS is therefore able to preserve its contribution to cerebral blood flow regulation after neurotrauma.

Today, it is believed that the NO produced by eNOS is neuroprotective because of this preservation of brain blood flow, while NO produced by nNOS may have adverse effects on the outcome[4]. The neuroprotective effect of eNOS can be seen in eNOS deficient mice: eNOS knockout mice show a lower CBF after controlled cortical impact injury compared to control, suggesting that NO produced by eNOS normally plays a role in preserving perfusion in the injured brain[44]. In addition, it was shown that eNOS is necessary for the positive effects of l-arginine after impact injury [44]. On the other hand, nNOS is believed to have detrimental effects. The intracerebral contusion area after fluid-percussion injury was reduced after pre-treatment with 3-bromo-7-nitroindazole (an nNOS inhibitor) or post-treatment with l-arginine (substrate of NOS)[45]. Accordingly, activation of the NMDA receptor plays a role in the cerebral damage that follows TBI[46]. In a moderate closed head injury model in mice, non-selective inhibition as well as nNOS inhibition after TBI resulted in an increased grip score[46]. However, TBI induced no changes in nNOS expression and protein synthesis compared to non- and sham-operated rats, while nNOS enzymatic activity was dramatically decreased 24 hours after trauma[41]. Since the nNOS enzyme is present, but not functionally active, the nNOS inactivation may represent an endogenous neuroprotective mechanism[41], while further inhibition of nNOS may provide additional protection.

In summary, we believe that nNOS is responsible for the decrease in NO concentrations early after neurotrauma and that this is part of an endogenous neuroprotective mechanism.

Effect of NOS compartmentalisation on cytochrome oxidase *in vivo*

NO and cytochrome oxidase

NO is not only important in cerebral perfusion, but it is also believed to play a major role in mitochondrial respiration. The *in vitro* interaction of NO with cytochrome oxidase is the most

important interaction within the respiratory chain, since it is fast and reversible, while interactions of NO with other complexes are slower and occur at higher, non-physiologic NO concentrations[8]. The most important inhibition within cytochrome oxidase is the binding of NO to reduced heme a3, forming nytrosyl[47] and inhibiting the enzyme fast and in competition with O_2[8]. On the other hand, NO is also able to inhibit the fully oxidised cytochrome oxidase by binding to Cu_B, but this occurs more than two orders of magnitude slower. In this reaction, NO is oxidised to nitrite[47], which binds to the binuclear site. However, after reduction of cytochrome oxidase, the binding is halted and the enzyme recovers activity[8].

To date, it is therefore known that respiration of mitochondria can be decreased when subjected to NO, but the degree of inhibition depends on both NO and oxygen concentrations[8]. Interestingly, in physiologic NO concentrations, cytochrome oxidase can be inhibited *in vitro*[8]. Also, in isolated enzymes, NO can reduce mitochondrial cytochrome oxidase at NO/O_2 ratios that do not inhibit oxygen consumption[48]. It was therefore proposed that NO could be a physiologic modulator of the oxygen affinity of cytochrome oxidase *in vivo*. However, actual *in vivo* measurements did not show major effects of NO on cytochrome oxidase redox status in physiological conditions nor in the hyperaemic phase after anoxia[49]. Concerning the compartmentalisation of NOS isoforms, we therefore believe that not endothelial derived NO, nor neuronal derived NO play a role in mitochondrial respiration.

In contrast, iNOS, the third NOS isoform which is not present in physiological circumstances, is able to induce much higher amounts of NO as compared to eNOS and nNOS[9], increasing the possibility of an effect on cytochrome oxidase redox status. Nevertheless, in our study, we did not find an effect of high NO concentrations on absolute oxidised cytochrome oxidase concentrations *in vivo* (Chapter 3).

NO, cytochrome oxidase and hypoxia

Interestingly, *in vitro* data show that NO and hypoxia can work synergistically to induce neuronal death[50]. Cytochrome oxidase has a K_m for oxygen below 1 μM, making it insensitive to all but the most extreme levels of hypoxia[50]. Oxygen-dependent redox changes of cytochrome oxidase therefore only occur during extremely compromised oxygen delivery[51]. In piglets, oxidised Cu_A did not change early after anoxia, but after a substantial fall in cerebral haemoglobin oxygenation, the Cu_A centre becomes more reduced, probably due to the falling of the mitochondrial electrochemical potential[52]. NO, being a reversible inhibitor of cytochrome oxidase in competition with oxygen, potentially sensitises the brain to hypoxia[53], markedly increasing the K_m

of cytochrome oxidase for oxygen[50]. However, when combining high NO concentrations with hypoxia *in vivo*, our study showed only small effects on cytochrome oxidase redox status only at very low oxygen saturation (Chapter 3). We therefore state that the effect of NO on cytochrome oxidase redox status might exist *in vivo*, but we do not believe it to be an important physiological principle *in vivo*.

These data are supported by the work of Bellamy and colleagues[54] who showed that the guanylyl cyclase coupled receptor is not saturated during maximal endogenous NO synthase activity and that even saturating NO concentrations only affect respiration when the oxygen concentration is very low *in vitro*. It is therefore believed that the NO signaling pathway has evolved to target the guanylyl cyclase coupled receptor without simultaneously influencing mitochondrial function[54].

In summary, we showed that high NO concentrations are not sufficient to influence cytochrome oxidase redox status, while in combination with hypoxia small effects are possible. We therefore believe that, although NO showed to be a very important regulator of mitochondrial respiration *in vitro*, NO only has effects on cytochrome oxidase in very specific conditions *in vivo*.

Measuring cerebral blood flow and cytochrome oxidase redox status

Near infrared spectroscopy

Near infrared spectroscopy (NIRS) is clinically a non-invasive and continuous technique to measure cerebral hemodynamics. It uses near infrared light that, in human, penetrates the cerebral cortex by only 2 or 3 mm due to skin and skull. However, contamination of the signal due to extracranial tissue can be a confounding factor[20]. Main advantages of NIRS are the non-invasive nature of the technique, the ability to bring the equipment to the patient in stead of taking the patient to the machine, the fact that no radionuclides are needed and the fact that the data are provided in real-time. Although the spatial resolution of NIRS is of lesser functional anatomic value, it has a good temporal resolution, a high specificity as to the parameters monitored, and rather undemanding technical requirements[55]. In addition, the potential to map changes in a parameter of cellular metabolism, cytochrome oxidase redox status, may be a potential "exclusive strength" of NIRS[55]. However, in rats, this technique is limited invasive since we want to eliminate the effect of extracranial tissue by removing scalp and muscle. In addition, due to the small volume of rat brain, the complete brain is measured, returning global measurements of hemodynamics and oxidised cytochrome oxidase.

Three assumptions have to be made when working with NIRS: (1) scatter is high but changes negligibly during the measurement, (2) the medium in which changes are monitored, is homogeneous (one of the reasons of low spatial resolution) and (3) the change in volume sampled is homogeneous within the sampling volume[55]. Once a tissue spectrum has been collected, the individual effects of all the chromophores present, need to be identified. The technique of least squares multilinear regression from known absorption spectra allows the identification of each chromophore, including tissue water (peak at 975 nm). If the concentration of water in tissue is known (assuming a water content of the brain of 80%), the optical path length can be determined, allowing the quantification of the other chromophores[56].

It is known that deoxyhemoglobin has a peak absorption at 760 nm and oxyhemoglobine at 920 nm[57], with a significant relationship between optical path length and hemoglobin concentration in a brain mock up as well as in piglet experiments[58]. In addition, the unique Cu_A centre of mitochondrial cytochrome oxidase has a strong absorbance in the NIR region (830 nm) and is responsible for over 80% of the spectral changes in this region, with the remainder being due to cytochrome c, and the other three metal centres of cytochrome oxidase[57]. When oxygen becomes limiting, electrons cannot be transferred from Cu_A to the other downstream metal centres of the enzyme, and thus remain at the Cu_A site, placing it in a reduced state[57]. This reduction results in a disappearance of its 830 nm absorption band[57]. The spectrum of cytochrome oxidase in the near infrared was identical in the neonatal pig, the adult rat and in the purified enzyme[59]. In addition, it was shown that NIRS can accurately measure changes in the cerebral cytochrome oxidase redox status, even in the presence of much higher concentrations of hemoglobin[59]. In addition, when necessary, absolute concentration oxidised cytochrome oxidase can be measured in animals when using cyanide. The brain is especially sensitive to cyanide, since it contains only small amounts of rhodanese, the enzyme that metabolises cyanide[60]. Cyanide binds to heme a3, and as a consequence, electrons cannot leave Cu_A and heme a to reduce oxygen, leading to completely reduced Cu_A and heme a centres[60], which can be used to back-calculate absolute oxidised cytochrome oxidase. In short, the technique is able to measure small changes in cytochrome oxidase redox status if they occur, however, when studying hypoxia, these changes only occur during very low oxygen tensions.

NIRS can be used to measure hemodynamics in both adults and neonates. In neonates, the thin skull and small head readily allows light to be shone through the whole head, making transmission spectroscopy possible[61]. In adults, this is not possible, requiring reflectance spectroscopy, where the transmitting and receiving optodes are placed a few centimetres apart,

normally 4 cm[61]. In this case, when the distance between light source and detector on the scalp is sufficiently large, it is believed that changes in scalp blood flow have no effect on NIRS measurements of cerebral hemodynamics[61]. In addition, changes in [HbT] and the absorbance at 805 nm (point at which the absorbance of hemoglobin is independent of oxygenation), are believed to reflect changes in cerebral blood volume (CBV), except when cytochrome oxidase was reduced[51]. However, these changes are at least six times smaller compared to CBV measurements with PET[62]. Nevertheless, it is accepted that NIRS can be used to measure global cerebral blood flow and volume in healthy adult volunteers[63].

Because venous blood contributes about 70% of intracranial blood volume, the majority of contribution to the NIRS signal is likely to be from venous blood[61]. It is suggested that the relative contributions of arterial and venous compartments are a fixed ratio of 25:75. However, during validation, this ratio *in vivo* was averaged at 85% venous and 15% arterial, not differing significantly between normoxia, hypoxia and hypocapnia[64].

Since NIRS has the advantage of being a non-invasive technique, it has several clinical applications[61]. A first one is the measurement of changes in cerebral oxygenation, by calculating the ratio of HbO_2 and HbT. Also cerebral blood flow can be measured using NIRS by using changes in HbO_2 as a tracer. After a stable 5% reduction in HbO_2, a breath of 100% oxygen creates a tracer in the arterial circulation. The measured increase in HbO_2 can be assumed to be entirely the result of arterial inflow of the tracer. As an alternative, we use the tracer indocyanine green (ICG) in a similar way[61]. ICG has an absorption peak at 805 nm and is restricted to the intravascular compartment[65], since it is not cleared by the kidney and peripheral tissue uptake of the dye is negligible[66]. It has a rapid clearance from blood by the hepatic uptake and biliary excretion, which makes it a suitable tracer for repetitive measurements[65]. The BFI calculated using ICG correlated significantly with cortical blood flow measured using microspheres, making it a useful tool for non-invasive estimation of CBF by NIRS[65]. ICG combined with NIRS can be used in patients to measure CBF, as compared to perfusion-weighted MRI[67] and can also be used in rats[68].

With respect to clinical use of NIRS only a limited number of commercial set ups is available. A first category of commercially available NIRS equipment for use in humans, measures the ratio of light absorption by HbO_2 and HbT to calculate mean cerebral saturation[61,69]. This technique uses a two wavelength light source and two detectors. The first detector measures largely non-cerebral tissue (skin and bone), whereas the second measures cerebral as well as non-cerebral tissue. By subtracting the first from the second, a mean value for cerebral oxygen saturation can be

given[61,69]. A second category of commercially available equipment does allow for the full quantification of changes in chromophore concentration, although baseline remains unknow[61]. One of them is the spatially resolved spectrometer, which estimates the pathlength of light by measuring the slope of light attenuation. This is done by placing multiple detectors at several points from the light source[57], thus allowing calculation of the relative concentrations of Hb and HbO_2[61]. In addition, This system uses this multidistance approach with four wavelengths, allowing for the absolute measurement of cerebral haemoglobin oxygen saturation[69]. A benefit of this technique is that it is not dependent on pathlength[69], decreasing the effect of movement artefacts. However, no specific adjustment is made for extracerebral blood[70]. Another example of equipment which allows for full quantification of changes in chromophore concentration, uses a phase shift technique[61]. This technique uses laser light oscillating at a known frequency. The emerging light is out of phase with the incident light, enabling quantification of absorption and scattering. This way, with this technique, it is possible to calculate absolute changes in HbO_2 and Hb[64]. However, the clinical use of NIRS is still difficult on a daily basis. Problems like a lack of absolute values make comparisons between patients or groups difficult. Also movement artefacts decrease the clinical applications, however, the newer techniques, like spatially resolved spectroscopy have promising results.

Near infrared spectroscopy in mice

In order to provide additional information regarding the compartmentalisation of NOS isoforms, NOS knockout mice are an interesting way to increase insight into the physiology and pathology of this subject. To date, NIRS has been used to accurately study cerebral hemodynamics and oxygenation in several species such as human[69,70], pig[65], piglet[52] and rat[49]. In order to measure hemodynamics in mice, it is necessary to further miniaturise our multi-wavelength NIR spectroscopy system.

In the first part of our study (Chapter 4), we showed that mouse and rat have similar scattering properties and that the algorithms allow for measurement of [Hb], [HbO_2] and Cu_A in mice. In addition, the measured values of both mice and rats were comparable to the literature[52,59,71]. We therefore conclude that multi-wavelength NIRS can be used in mice for the assessment of cerebral blood oxygenation and cerebral concentration of oxidised cytochrome oxidase.

Concerning CBF measurements in mice, we found the optimal ICG bolus to be 2.5 µl of a 1 mg/ml ICG solution. This gives a good signal-to-noise ratio, with acceptable disturbances of the other NIRS measurements and minor variability of the blood flow index. However, for now, it is

not possible to combine CBF measurements with the assessment of oxygenation of cerebral blood and the concentration of oxidised cytochrome oxidase. Further attempts will be made to decrease the variability of the 1 µl bolus. Nevertheless, we believe the application of this technique in the study of mice models is a major step forward.

Final conclusion

The compartmentalisation of NOS isoforms makes it possible for NO to have different actions. We showed that, in autoregulation, eNOS is important near the lower limit of autoregulation, while nNOS is important for the set point of the autoregulation level at which CBF is constant (Chapter 1). On the other hand, in neurotrauma, we showed that mainly nNOS is responsible for the early decrease in NO concentrations, which is probably part of an endogenous neuroprotective mechanism (Chapter 2). While eNOS and nNOS have no effect on cytochrome oxidase redox status *in vivo*[49], we showed that iNOS has only small effects on cytochrome oxidase redox status at very low oxygen saturations *in vivo* (Chapter 3). Finally, we showed that multi-wavelength NIRS can be used to measure cerebral hemodynamics in mice (Chapter 4).

In conclusion, NOS compartmentalisation is important to control the different actions of NO in space and time and in physiological and pathological conditions.

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Summary

Cerebral perfusion, cerebral oxygen metabolism and nitric oxide

Cerebral blood flow is an important physiological process, since the brain is highly dependent on constant and adequate delivery of oxygen and glucose. Nitric oxide (NO) is believed to be an important player in the regulation of cerebral blood flow. Next to this function, NO is also believed to be a physiological regulator of oxygen metabolism, since it is able to inhibit cytochrome oxidase *in vitro*. NO is synthesised by three NO-synthase (NOS) isoforms with different spatial and temporal distribution. This compartmentalisation is important to define the function of each NOS. However, the specific functions of endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) in cerebral perfusion and cerebral oxygen metabolism are to be elucidated.

Effect of NOS compartmentalisation on autoregulation (Article 1)

Autoregulation is an important feature of cerebral circulation. It is the ability to maintain a constant blood flow despite blood pressure changes between 60 to 150 mmHg. While NO is believed to be a regulator of the lower limit, it is not known which NO-synthase isoform is responsible. To study this, we decreased arterial blood pressure using hypovolemia and performed a cerebral blood flow measurement for every 10 mmHg drop in arterial blood pressure. From the drawn autoregulation curve, consistent with the literature, it was shown that non-selective NOS inhibition induces a shift in lower limit towards higher blood pressures. We therefore confirm that NO plays a role in autoregulation near the lower limit.

In our study, we showed that nNOS inhibition did not shift the lower limit of autoregulation. Consequently, eNOS is responsible for the shift in lower limit of autoregulation. In addition, we showed that nNOS inhibition, as well as non-selective inhibition, induced a decrease in autoregulation level, constant level of cerebral blood flow. We therefore believe that nNOS is responsible for the level of autoregulation.

In summary, we conclude that eNOS provides the mechanism of cerebral autoregulation, while nNOS is responsible for the set point of cerebral autoregulation.

Effect of NOS compartmentalisation early after neurotrauma (Article 2)

Neurotrauma is known to decrease cerebral blood flow early after injury. Additionally, it was shown that NO concentration decreases during this time. Our study is in agreement with the

literature, since we showed that non-selective NOS inhibition has the expected cerebral effects in normal animals, while in neurotrauma animals, the same effects lasted shorter, or did not occur, suggesting decreased NO concentrations.

However, it is not known which NOS isoform is responsible for this decrease in NO concentrations. An effect of eNOS inhibition can still be seen early after neurotrauma as compared to control animals, while no effect of nNOS inhibition is present. In addition, neurotrauma induced an uncoupling of cerebral blood flow and cerebral metabolism, which, in literature, is believed to be the result of nNOS inhibition.

In summary, we believe that nNOS is responsible for the decrease in NO concentrations early after neurotrauma and that this is part of an endogenous neuroprotective mechanism.

Effect of NOS compartmentalisation on cytochrome oxidase *in vivo* (Article 3)

Next to its role in cerebral hemodynamics, NO is also believed to play a major role in mitochondrial respiration. *In vitro* evidence suggests a physiological role as a regulator of oxygen metabolism through inhibition of cytochrome oxidase in competition with oxygen. However, a first *in vivo* experiment did not show any effect of NO on cytochrome oxidase redox status in physiological conditions, excluding an effect of eNOS and nNOS. The question remains whether iNOS could induce high enough NO concentrations to inhibit cytochrome oxidase *in vivo*.

Our experiments showed that high NO concentrations, induced by lipopolysaccharide injection, did not have any effect on absolute oxidised cytochrome oxidase concentrations *in vivo*. However, hypoxia can work synergistically with NO to inhibit cytochrome oxidase *in vitro*. We therefore combined high NO concentrations with hypoxia *in vivo* in rat. Our study showed only small effects on cytochrome oxidase redox status only at very low oxygen saturation.

In summary, we showed that high NO concentrations are not sufficient to influence cytochrome oxidase redox status; while in combination with hypoxia small effects are possible. We therefore believe that although NO showed to be a very important regulator of mitochondrial respiration *in vitro*, NO only has effects on cytochrome oxidase in very specific conditions *in vivo*.

Measuring cerebral hemodynamics and cytochrome oxidase redox status in mice (Article 4)

In rats, near infrared spectroscopy (NIRS) is an interesting technique, since it can be used to measure cerebral hemodynamics in a limited invasive and continuous way. In addition, the possibility of measuring part of the mitochondrial electron chain *in vivo* is a unique feature of

NIRS. In order to provide additional information regarding the compartmentalisation of NOS isoforms, NOS knockout mice are an interesting way to increase insight into the physiology and pathology of this subject. We therefore checked whether we could use the multi-wavelength NIRS system in mice.

Our study showed that brains of mouse and rat have similar scattering properties and that the algorithms allow for measurements of hemoglobin, oxyhemoglobin and oxidised cytochrome oxidase. When checked, the measured values of both mice and rats were comparable to the literature. In addition, in order to measure cerebral blood flow in mice, we found the optimal ICG bolus to be 2.5 µl of a 1 mg/ml ICG solution. This gives a good signal-to-noise ratio, with acceptable disturbances of the other NIRS measurements and minor variability of the blood flow index. However, for now, it is not possible to combine CBF measurements with the assessment of oxygenation of cerebral blood and the concentration of oxidised cytochrome oxidase.

In summary, we conclude that multi-wavelength NIRS can be used in mice for the assessment of cerebral blood oxygenation and cerebral concentration of oxidised cytochrome oxidase, and we believe that the application of this technique in the study of mice models is a major step forward.

Final conclusion

The spatial end temporal compartmentalisation of NOS isoforms makes it possible for NO to perform different actions. We showed that, in autoregulation, eNOS is important near the lower limit of autoregulation, while nNOS is important for the set point of the autoregulation level at which CBF is constant (Article 1). On the other hand, in neurotrauma, we showed that mainly nNOS is responsible for the early decrease in NO concentrations, which is probably part of an endogenous neuroprotective mechanism (Article 2). While NO produced by eNOS and nNOS has no effect on cytochrome oxidase redox status *in vivo*, we showed that NO produced by iNOS has only small effects on cytochrome oxidase redox status at very low oxygen saturations *in vivo* (Article 3). Finally, we showed that multi-wavelength NIRS can be used to measure cerebral hemodynamics in mice (Article 4).

In conclusion, NOS compartmentalisation is important to control the different actions of NO in space and time and in physiological and pathological conditions.

Samenvatting

Cerebrale perfusie, cerebraal zuurstofmetabolisme en stikstofmonoxide

Cerebrale doorbloeding is een belangrijk fysiologisch proces, omdat de hersenen zeer sterk afhankelijk zijn van voortdurende en voldoende aanvoer van zuurstof en glucose. Er wordt aangenomen dat stikstofmonoxide (NO) één van de belangrijke spelers is in de regulatie van de cerebrale doorbloeding. Daarnaast wordt aangenomen dat NO een fysiologische regulator van het zuurstofmetabolisme is, omdat NO cytochroom oxidase kan inhiberen *in vitro*. NO wordt gesynthetiseerd door drie NO-syntase (NOS)-isovormen die verschillend voorkomen in plaats en tijd. Deze compartimentalisatie is belangrijk om de verschillende functies van elke NOS te bepalen. Tot op vandaag zijn de specifieke functies van endotheliale NOS (eNOS), neuronale NOS (nNOS) en induceerbare NOS (iNOS) in cerebrale doorbloeding en cerebraal zuurstofmetabolisme nog niet opgehelderd.

Effect van NOS-compartimentalisatie op autoregulatie (Artikel 1)

Autoregulatie is een belangrijk kenmerk van de cerebrale doorbloeding. Ze biedt de mogelijkheid om een constante doorbloeding te behouden ondanks bloeddrukveranderingen tussen 60 en 150 mmHg. Hoewel wordt aangenomen dat NO een regulator is van de lage limiet, is niet geweten welke NOS-isovorm hiervoor verantwoordelijk is. Om dit na te gaan, hebben we de arteriële bloeddruk stapsgewijs verlaagd door middel van hypovolemie. Bijkomend hebben we de hersendoorbloeding gemeten voor elke daling in arteriële bloeddruk van 10 mmHg. Deze data werden vervolgens gebruikt om een autoregulatiecurve op te stellen. In overeenkomst met de literatuur hebben we aangetoond dat niet-selectieve NOS-inhibitie een shift in de lage limiet naar hogere bloeddruk induceert. We bevestigen daarom dat NO een effect heeft op de lage limiet van autoregulatie.

Toch is het effect van de compartimentalisatie van NOS-isovormen nog niet gekend. In onze studie toonden we aan dat nNOS-inhibitie geen shift in lage limiet veroorzaakt. We besluiten daarom dat eNOS verantwoordelijk is voor de shift in de lage limiet. Bovendien hebben we aangetoond dat zowel nNOS-inhibitie als niet-selectieve inhibitie, een daling in het autoregulatie-niveau induceert, waardoor we besluiten dat nNOS hiervoor verantwoordelijk is.

Samengevat kunnen we stellen dat eNOS verantwoordelijk is voor het mechanisme van cerebrale autoregulatie, terwijl nNOS verantwoordelijk is voor de hoogte van het autoregulatieplateau.

Effect van NOS-compartimentalisatie vroeg na neurotrauma (Artikel 2)

Het is geweten dat neurotrauma een daling in cerebrale doorbloeding induceert in de vroege fase na trauma. Bovendien is geweten dat NO-concentraties gedaald zijn gedurende deze periode. Onze studie komt daarin overeen met de literatuur, omdat we aantoonden dat niet-selectieve NOS-inhibitie de verwachte cerebrale effecten had in normale dieren, terwijl in neurotrauma-dieren dezelfde effecten korter duurden of zelfs niet voorkwamen, wat een verlaagde NO-concentratie suggereert.

Momenteel is niet geweten welke NOS-isovorm verantwoordelijk is voor deze daling in NO-concentraties. In onze studie bestond er nog steeds een effect van eNOS-inhibitie vroeg na neurotrauma vergeleken met controledieren, terwijl helemaal geen effect van nNOS-inhibitie meer bestond. Daarbij hebben we aangetoond dat neurotrauma een ontkoppeling induceert van cerebrale doorbloeding en cerebraal metabolisme, wat in de literatuur wordt aanzien als nNOS-inhibitie.

Samengevat kunnen we stellen dat nNOS verantwoordelijk is voor de daling in NO-concentraties vroeg na neurotrauma, en dat dit zeer waarschijnlijk een onderdeel is van een endogeen beschermend mechanisme.

Effect van NOS compartimentalisatie op cytochroom oxidase *in vivo* (Artikel 3)

Er wordt aangenomen dat NO een belangrijke rol speelt in mitochondriale respiratie. *In vitro*-studies suggereren zelfs een rechtstreekse fysiologische rol voor NO als regulator van het zuurstofmetabolisme door competitieve inhibitie van cytochroom oxidase. Anderzijds toonde een eerste *in vivo*-experiment geen enkel effect aan van NO op de cytochroom oxidase redox status in fysiologische omstandigheden, waardoor effecten van eNOS en nNOS kunnen worden uitgesloten. Toch blijft de vraag bestaan of iNOS voldoende hoge concentraties NO kan produceren om cytochroom oxidase te inhiberen *in vivo*.

Onze experimenten toonden aan dat een hoge NO-concentratie, geïnduceerd door lipopolysaccharide-injectie, geen effect had op de absolute geoxideerde cytochroom oxidase concentratie *in vivo*. Anderzijds kan hypoxie samenwerken met NO om cytochroom oxidase te inhiberen *in vitro*. We hebben daarom een hoge NO-concentratie gecombineerd met hypoxie *in vivo* in de rat. Onze studie toonde aan dat enkel kleine effecten op cytochroom oxidase redox status waargenomen kunnen worden bij een zeer lage zuurstofsaturatie.

Samenvattend kunnen we stellen dat hoge NO-concentraties niet voldoende zijn om cytochroom oxidase redox status te beïnvloeden, terwijl in combinatie met hypoxie kleine effecten mogelijk

zijn. We besluiten dat, hoewel NO een belangrijke regulator van de mitochondriale respiratie leek *in vitro*, NO *in vivo* enkel effecten heeft op cytochroom oxidase in zeer specifieke omstandigheden.

Cerebrale hemodynamica en cytochroom oxidase redox status meten in muizen (Artikel 4)

In ratten is 'nabije infrarood spectroscopie' (NIRS) een interessante techniek omdat het gebruikt kan worden om de cerebrale hemodynamica te bestuderen op een weinig-invasieve en continue manier. Daarbij komt dat de mogelijkheid om een deel van de mitochondriale electron-overdrachtketen te meten *in vivo* een unieke eigenschap is van NIRS. Om bijkomende informatie te verkrijgen in verband met de compartimentalisatie van NOS-isovormen, zijn *knockout*-muizen een zeer interessant hulpmiddel om een beter inzicht te krijgen in de fysiologie en pathologie van dit onderwerp. We zijn daarom nagegaan of we het NIRS-systeem kunnen gebruiken bij muizen.

Onze studie toonde aan dat hersenen van muizen en ratten gelijkaardige verstrooiingseigenschappen hebben, waardoor de algoritmes van het NIRS-systeem dus gebruikt kunnen worden om hemoglobine, oxyhemoglobine en geoxideerd cytochroom oxidase te meten. Deze gemeten waarden van zowel muis als rat zijn bovendien vergelijkbaar met de literatuur. Om verder de cerebrale doorbloeding te meten in muizen, hebben we de optimale ICG-bolus bepaald. Deze bedraagt 2.5 µl van een 1 mg/ml ICG-oplossing en geeft een goede signaal-ruisverhouding met aanvaardbare afwijkingen van de andere NIRS-parameters en weinig variabiliteit van de doorbloedingsindex. Toch is het momenteel niet mogelijk om de hersendoorbloeding gelijktijdig te meten met cerebrale oxygenatie en met de concentratie geoxideerd cytochroom oxidase.

We kunnen dus stellen dat NIRS gebruikt kan worden in muizen voor het bestuderen van de oxygenatie van cerebraal bloed en van de concentratie geoxideerd cytochroom oxidase. We besluiten dat de toepassing van deze techniek in de studie van muismodellen een belangrijke stap voorwaarts is.

Finaal besluit

De compartimentalisatie van NOS-isovormen maakt het mogelijk voor NO om verschillende functies uit te oefenen. We toonden aan dat, tijdens autoregulatie, eNOS belangrijk is nabij de lage limiet van autoregulatie, terwijl nNOS verantwoordelijk is voor de hoogte van het autoregulatieplateau (Artikel 1). Anderzijds zagen we dat vooral nNOS verantwoordelijk is voor de vroege daling in NO-concentraties na neurotrauma en dat dit waarschijnlijk een onderdeel is van een endogeen beschermend mechanisme (Artikel 2). Terwijl NO, geproduceerd door eNOS

en nNOS, geen effect heeft op cytochroom oxidase redox status *in vivo*, konden we besluiten dat NO, geproduceerd door iNOS, enkel kleine effecten heeft op de cytochroom oxidase redox status bij zeer lage zuurstofsaturaties *in vivo* (Artikel 3). Tot slot toonden we aan dat NIRS gebruikt kan worden om cerebrale hemodynamica te bestuderen in muizen (Artikel 4).

We besluiten daarom dat NOS-compartimentalisatie belangrijk is voor de controle over de verschillende functies van NO in plaats en tijd en zowel in fysiologische als in pathologische omstandigheden.