MEDICAL UNIVERSITY OF VIENNA

Effect of Heme Oxygenase-1 Induction on Ischemia-Reperfusion-Injury in Skeletal Muscle

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Submitted by

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Zusammenfassung

Einleitung

Hämarginat (HA) induziert die Expression des Enzyms Hämoxygenase – 1 (HO-1). HO-1 kann den Ischämie-Reperfusionsschaden (IRS) reduzieren. Die Magnetresonanz- tomographie (MRI) und -spektroskopie (MRS) sind Methoden, die bereits häufig zur Beschreibung des oxidativen Muskelstoffwechsels angewendet wurden. Diese Techniken können daher möglicherweise auch für die Evaluierung des IRS herangezogen werden.

Die Ziele dieses Projekts waren die Entwicklung von MR - spezifischen Methoden zur Darstellung und Evaluierung des IRS sowie die Evaluierung des ischämischen Präkonditionierens und der Induktion von HO-1 zur Reduktion des IRS.

Methoden

Insgesamt wurden 35 Probanden in drei randomisierte Studien eingeschlossen. Das ischämische Präkonditionieren und die Induktion von HO-1 wurden mittels MRI und MRS beschrieben. Zusätzlich wurde in einer Studie die Muskelkraft gemessen.

Die Ischämie am Bein wurde mittels Aufblasen einer Oberschenkelmanschette für 20 Minuten erzeugt. Anschließend folgte eine reduzierte Reperfusion für einen Zeitraum von 5 Minuten. Ischämisches Präkonditionieren wurde 4 oder 48 Stunden vor der längeren Ischämie angewandt. HA (1 mg/kg Körpergewicht) oder Placebo wurde 24 Stunden vor der Ischämie infundiert. Veränderungen in den ^{31}P MRS Messwerten und den BOLD (blood oxygen level-dependent) MRI Signalen wurden aufgezeichnet.

Ergebnisse

Das Phosphokreatin (PCr) Signal sank deutlich während der Ischämie und erholte sich rasch in der Reperfusionsphase. Das BOLD Signal sank ebenfalls in der Ischämie, zunächst rasch und nach einigen Minuten nur mehr gering, und stieg im Rahmen der postischämischen Hyperämie an. Ischämisches Präkonditionieren 4 Stunden vor der Ischämie könnte das PCr Reperfusionssignal im Vergleich zu den anderen Gruppen deutlich erhöhen und reduzierte das BOLD MRI Signal.

Nach der Infusion von HA war das maximale BOLD MRI Signal in der Reperfusion höher und wurde früher erreicht als in der Placebogruppe.

Rückschlüsse

Ischämisches Präkonditionieren und HA können den Muskelstoffwechsel nach der Ischämie positiv beeinflussen. Ischämisches Präkonditionieren erhöhte die PCr Produktion und damit auch den Sauerstoffverbrauch, was zu einer Reduktion des BOLD MRI Signals führte. Eine einzelne Dosis von HA könnte, wahrscheinlich durch eine schützende Wirkung auf die Endothelzelle, die Reperfusionseigenschaften deutlich verbessern.

Abstract

Introduction

Heme arginate (HA) induces heme oxygenase-1 (HO-1), which protects tissue against ischemia-reperfusion injury (IRI). Magnetic resonance (MR) imaging and spectroscopy have been applied to assess skeletal muscle oxidative metabolism. Therefore, MR may enable the characterization of IRI in skeletal muscle. The goals of this project were the establishment of MR measurements for the detection of IRI and the evaluation of ischemic preconditioning (IPC) and HO-1 induction as therapeutic approaches of IRI in healthy subjects.

Methods

Thirty-five participants were included in three randomized crossover protocols in which the effects of IPC and HO-1 induction were measured by MR and muscle force assessments. Leg ischemia was administered over 20 minutes with or without a subsequent slow reperfusion for 5 minutes, followed by full reperfusion. IPC was administered 4 or 48 hours prior to ischemia. HA (1 mg/kg body weight) or placebo was infused 24 hours prior to ischemia for the induction of HO-1. Changes in ^{31}P MR spectroscopy and blood oxygen level-dependent (BOLD) functional MRI signals were recorded.

Results

The phosphocreatine (PCr) signal decreased robustly during ischemia and recovered rapidly during reperfusion. The BOLD signal intensity decreased during ischemia and increased during hyperemic reperfusion. IPC 4 hours prior to ischemia significantly

increased the maximal PCr reperfusion signal and mitigated the peak BOLD signal during reperfusion.

Peak reactive BOLD functional MRI signal was significantly increased and occurred earlier after HA compared to placebo.

Conclusions

IPC and HA positively influenced muscle metabolism after IRI. IPC resulted in an increase in PCr production and higher oxygen consumption, thereby mitigating the peak BOLD signal.

A single high dose of HA improves reperfusion patterns during ischemia reperfusion injury in humans.

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Publications

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Heme arginate improves reperfusion patterns after ischemia: a randomized, placebo-controlled trial in healthy male subjects.

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Re-establishment of normal blood flow is mandatory to restore intramuscular high energy phosphate levels after transient ischemia

Andreas M, Schmid AI, Doberer D, Meyerspeer M, Moser E, Roden M, Wolzt M

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The effects of post-conditioning on intramuscular high energy phosphate levels

Andreas M, Schmid AI, Doberer D, Meyerspeer M, Moser E, Roden M, Wolzt M

Oral presentation – Cardiovascular Research Days 2008, Weissensee (Austria)

Chapter 1: Introduction

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease affecting arterial vessels (1). The underlying pathologic substrate consists of arterial plaques, which change their structure and appearance during disease progression. Atherosclerotic lesions contain lipids, macrophages, foam cells and other inflammatory cells. They may calcify during disease progression. The plaques can generally be categorized as stable or vulnerable (2). Stable plaques have a higher content of fibrous tissue whereas vulnerable plaques have a higher partition of foam cells and lipids.

Atherosclerotic lesions may cause end-organ damage depending on their size and eventual plaque rupture. An atherosclerotic plaque, which is increasingly narrowing the vascular lumen, leads to decreased perfusion of the downstream vascular bed. As soon as organ perfusion falls below a distinct threshold, tissue ischemia will become clinically evident. Claudication is a typical symptom of malperfusion in patients with peripheral artery disease and angina pectoris may occur in patients with coronary artery disease.

Further, plaque rupture can cause acute ischemia (3). Highly thrombogenic material is present in the plaque. Rupture of the covering endothelium brings it into contact with the blood stream. This results in a strong activation of the coagulation cascade and acute thrombus formation. The fresh thrombus may block the affected vessel, leading to myocardial infarction in coronary arteries or peripheral ischemia in peripheral artery disease. On the other hand, the thrombus may also embolize in the

downstream vascular bed, leading to stroke (carotid artery lesions), myocardial infarction, peripheral ischemia or death (4).

Another consequence of atherosclerosis may be the slow but complete occlusion of the vascular lumen. Collateral vessels are able to build an additional support of the downstream tissue, inhibiting acute ischemia. However, reduced tissue perfusion may still be present in these patients.

The development of artherosclerosis is driven by the deposits of low-density lipoproteins (LDL) in the vascular wall (1). LDL particles leave the blood stream and accumulate in the vascular wall. Hypertension, endothelial lesions and dyslipidaemia may foster this. Several mechanisms, mainly driven by the risk factors of atherosclerosis, lead to the oxidation of LDL. Oxidized LDL cannot be processed by macrophages, which invade the vessel wall to clean it from lipid deposits. Therefore, these macrophages transform into foam cells, which later disrupt and form a necrotic core in the atherosclerotic plaque (5).

Risk factors driving this process are numerous and are acting on different steps of this pathophysiologic process. Common risk factors include smoking, high cholesterol diet, dyslipidaemia, hypertension, diabetes mellitus and a family history of atherosclerotic diseases.

Mitochondria

Mitochondria are intracellular organelles, which are responsible for the cellular energy production in eukaryotic cells. They consist of an outer lipid membrane, an intermembraneous space, an inner mitochondrial membrane, which is rich of proteins and also forms cristae to increase its surface and an intramitochondrial matrix. According to the endosymbiosis theory, ancient prokaryotes formed a symbiotic relationship with other cells, which were thereby able to perform oxidative phosphorylation. One piece of evidence for this theory is the distinctive appearance of mitochondria, including a cellular membrane, mitochondrial DNA, RNA and ribosomes. The amount of mitochondria varies according to the cell type. Cells with a high energy demand like muscle cells need a very high number of mitochondria. Oxidative phosphorylation is a complex process, which enables the production of a proton gradient between the mitochondrial matrix and the intermembraneous space by reducing O_2 to CO_2 in several steps. This proton motive force drives ATP synthase and enables the phosphorylation of ADP to ATP, which is the main energy source for cellular processes.

Mitochondria play a crucial role in several diseases due to their central role in cellular energy metabolism. Some distinct pathologies (mitochondriopathies) are induced by mutations of the mitochondrial DNA and are inherited diseases, which are always transferred from the mother to the child. However, mitochondria are also suggested to play a role in several other diseases, which are not a result of a mitochondrial defect. For example, diabetic metabolism may lead to the so-called diabetic cardiomyopathy by disturbed metabolization of fatty acids by cardiac mitochondria.

Ischemia

Ischemia is the status of any tissue which is subjected to reduced or totally blocked blood supply. This is characterized by a disrupted supply of all necessary supplements for cell survival. Ongoing ischemia leads to tissue damage and cell death. Compared to hypoxia, a state of intact perfusion with low oxygen saturation, all nutrients are lacking and cellular waste products accumulate. However, the depletion of oxygen may be the most striking component; it forces the reduction of most energy consuming cellular processes. Some processes are still immanent for cell survival. Membrane stabilizing functions and ion gradients are central and therefore further consume energy. Some pathologic stimuli may activate the inherent apoptotic program due to the ischemic stimulus, while other more pronounced cellular insults directly lead to cell death (6).

In the case of myocardial infarction, location of vessel occlusion and duration of ischemia are the most important predictors of infarct size. Different cell types across the body have a distinct potential to resist ischemia. Brain cells and myocardial cells have a very short period of tolerance, which is related to their very intense energy metabolism and an ongoing high energy demand during ischemia. This reflects the different cellular potentials to avert ischemic injury across the ischemic tissue. Three different consecutive time frames can be characterized after the onset of ischemia, which are also highly depended on the tissue subjected to ischemia. During the first period, ischemia is too short to induce cell death after reperfusion. During the second period, a various amount of tissue damage is produced according to the duration of ongoing ischemia. In the third time period, no significant amount of cells are alive in the affected area after reperfusion. Maruyama et al. support this hypothesis with their

recent findings, reporting that ischemic postconditioning could only prevent myocardial damage in rat hearts after 30 minutes of total ischemia, but not after 45 or 60 minutes (7).

Reperfusion itself, although crucial for cell survival, imposes an additional stress on the ischemic tissue. The terminus ischemia–reperfusion injury implies two different mechanisms of injury. First, an injury is produced by ischemia itself, as described above. A second tissue injury is produced by reperfusion. Several mechanisms of this complex process have been discussed in the past and will be summarized in the following section. It is important to stress that reperfusion injury may only be of significance in the second of the above described periods. Ischemia during the first time period does not produce enough cellular injury for cell death after reperfusion. Reperfusion during this time frame leads to full recovery of the ischemic tissue. However, this is unlikely in the clinical routine following myocardial infarction, stroke or other previously described diseases. On the other hand, several medical procedures leading to tissue ischemia have to be performed in this time frame. Examples include cardiac surgery procedures requiring cardioplegic cardiac arrest, surgical procedures in deep hypothermic circulatory arrest (e.g. aneurysm repair) and transplantations. The second time period is characterized by a varying amount of cell death throughout the ischemic tissue. Reperfusion should stop this process but reperfusion injury may increase the amount of tissue damage.

The third period insufficient vital tissue left to gain significant benefits from the treatment of reperfusion injury. However, systemic effects of reperfusion injury may still favor therapeutic approaches to reperfusion injury in this third time period. Furthermore, the amount of tissue injury in border zones of perfusion is always very hard to define and may almost always indicate a therapeutic approach.

Reperfusion injury

Due to ischemia-reperfusion injury's central role in medicine, it has been addressed in several preclinical and clinical trials in the past. The rapid reestablishment of perfusion is essential to salvage ischemic tissue. The key finding of Jennings et al. was that reperfusion itself results in additional cell damage of ischemic tissue, which is known as ischemia-reperfusion injury (8-10). He experimentally ligated the left anterior descendent artery (LAD) of a canine heart. Compared to no reopening, myocytes showed a faster structural deterioration when reperfusion was performed after prolonged ischemia. However, this worsening of the histological findings may also be due to accelerated necrosis of cells, which are already obliged to death due to ischemic damage. Therefore, further research was performed in a widespread setting of experiments (11). Murry et al. first described the concept of ischemic preconditioning, which consists of the repeated application of short ischemic periods prior to a prolonged ischemia. Thereby, he finally proved the existence of additional damage due to reperfusion injury by applying ischemic preconditioning as the first therapeutic approach against it (12). In his experiments, he occluded the LAD of a dog's heart for three periods of five minutes prior to a 40-minute ischemia. This pre-treatment significantly reduced myocardial infarct size. Several cellular processes may be involved in the detrimental cascade of cell damage and necrosis due to reperfusion injury, but there is still no comprehensive understanding of the underlying mechanisms.

There are two phases of reperfusion injury. During the first time period, fresh blood, which has high oxygen saturation and oxygen partial pressure, promotes the production of reactive oxygen species (ROS). A small amount of ROS, which are

comprised of the superoxide anion radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), are produced during healthy cell metabolism. They are mainly formed in the mitochondria during the process of oxidative phosphorylation. The mitochondrial respiratory chain complexes, in particular complexes I and III, are in a reduced state during early reperfusion and contribute to the production of ROS. Furthermore, as a second source of ROS in the cytoplasm, the xanthine oxidase represents a major ROS contributor during reperfusion injury. After the onset of reperfusion, the xanthine oxidase produces a significant amount of free oxygen radicals (13).

Furthermore, endothelial cells are a source of toxic products during the reperfusion period. Nitric oxide (NO), a volatile gaseous molecule, is the product of different nitric oxide synthases (NOS). The three NOS isoforms are neuronal NOS, which may also be present in mitochondria, inducible NOS and endothelial NOS. Tetrahydrobiopterin (BH_4) is the essential cofactor for the production of NO.

NO has several protective patterns in the healthy state, such as vasodilation and the inhibition of platelet aggregation and leukocyte adhesion (14, 15). However, during reperfusion it may react to the highly toxic peroxynitrite ($ONOO^-$) in the presence of ROS (16). Peroxynitrite is a very aggressive molecule which leads to the irreversible nitration of proteins (17). The final role of NO during reperfusion has to be determined. Although it may react to peroxynitrite and thereby cause cell damage, a lack of NO during reperfusion probably also contributes to reperfusion damage due to the protective effects of NO listed above and the detoxifying reaction of NO with peroxinitrite to N_2O_3. Taken together, it is likely that physiologic concentrations of NO have a protective effect but excessive NO production may increase peroxynitrite concentration and induce mitochondrial permeability transition pore (mPTP) opening (18).

In addition, NOS may also contribute to ROS production under pathologic conditions like substrate depletion present during reperfusion (19). All molecules with oxidative capability may damage cellular organelles by the chemical alteration of proteins, lipids, carbohydrates and nucleic acids. As the reactions are random, the effect on cellular metabolism cannot be predicted. They may consequently lead to cell death or apoptosis (20).

Therefore, several cellular strategies exist for the scavenging of ROS. The superoxide dismutase (SOD) is able to reduce two superoxide anion radicals to O_2 and H_2O_2, which in turn can be further detoxified by catalase to H_2O and O_2. Further antioxidative substances are glutathione, the vitamins C and E as well as bilirubin.

Glycolysis is the only cellular energy source in the absence of oxidative phosphorylation. The accumulation of lactic acid due to prolonged anaerobic glycolysis and the absence of circulation lead to a cellular pH decrease.

Na^+, Ca^{2+} and hydrogen accumulate in the cytoplasm during ischemia. Na^+ concentration increases due to the decreased function of the Na^+/K^+ ATPase as well as the action of the Na^+/H^+ exchanger. The enforced activation of the Na^+/H^+ exchanger is due to decreased pH during ischemia. Further, intracellular accumulation of Na^+ reverses the Na^+/Ca^{2+} antiporter and increases the intracellular Ca^{2+} concentration (21). Ca^{2+} has very low intracellular concentrations under physiologic cellular conditions. Increased cellular concentration activates several pathways and cellular functions.

After reperfusion, the pH is normalized and ion transporters lead to a further increase of cellular Na^+ concentration. This promotes the release of Ca^{2+} from the sarcoplasmatic reticulum in muscular cells. Intracellular Ca^{2+} mediates muscular

hypercontractility, thereby inducing damage to the contraction band, disruption of the sarcolemma and further reducing scarce cellular energy (22).

Mitochondria play a central role in the process of reperfusion injury. They are the place of oxidative phosphorylation and are therefore the main contributors to cellular energy metabolism. The enzymes of the respiratory chain produce a small amount of ROS as a byproduct of oxidative phosphorylation. However, the amount of these byproducts can be significantly increased during reperfusion. Further, mitochondria play a significant role in the process of cellular apoptosis. ROS, increased Ca^{2+} and pH change induce prolonged opening of the mPTP which leads to cell death (23). Although ROS and increased Ca^{2+} are also present during ischemia, cellular acidosis prevents mPTP opening prior to reperfusion (24). The proton gradient is reestablished during reperfusion. This leads to an increased uptake of calcium in the mitochondria, which consumes the scarce energy and inhibits early ATP production. As stated above, the mPTP is activated by several stimuli, including oxidative stress, rapid pH changes and high intracellular Ca^{2+} concentrations (23, 25, 26). This results in the opening of a channel between the inner mitochondrial matrix and the cytoplasm. Thereby, it neutralizes the hydrogen gradient across the inner mitochondrial membrane by nonselective transport of several molecules. Cellular oxidative phosphorylation, the crucial process of life, is stopped and cellular ATP is further reduced by the reversed action of the F_1F_0ATPase. In addition, a high amount of ROS and cytochrome C is released into the cellular plasma (27, 28). During reperfusion, the initial increase of ROS may therefore trigger a much larger increase by stimulating the mPTP opening, functioning as a positive feedback loop (27).
The mPTP probably consists of three main components, which are the adenine nucleotide translocase (ANT), cyclophilin D (Cyp-D) and the voltage-dependent anion

channel (VDAC). Whereas ANT and Cap-A are located at the inner mitochondrial membrane and in the inner matrix, VDAC is part of the outer mitochondrial membrane. Due to their effects on ANT, adenine nucleotides are able to inhibit the mPTP opening (29). The duration and degree of mPTP activation seems to discriminate between apoptosis and necrosis (6). Apoptosis is an energy demanding process. It is therefore limited to cells with a residual mitochondrial activity for ATP production. On the contrary, mitochondrial swelling and membrane rupture in cellular necrosis inhibits further energy production and by that cellular apoptosis. Therefore, it seems reasonable that apoptosis is concentrated on border zones of ischemia or that it occurs after shorter periods of ischemia compared to necrosis (30).

During early reperfusion, a residual impairment of blood flow due to microthrombus formation or plugging of the microvasculature by activated neutrophils may limit recovery. An inflammatory response is activated throughout the reperfused tissue in parallel, which introduces the second phase of reperfusion. This leads to endothelium activation and the expression of inflammatory surface markers. Further, inflammatory cells are activated and start to extravasate in the area of reperfusion injury. Although several cell types may take part in this process, the main contributors to later damage are neutrophils. These cells cause tissue damage by free oxygen radicals and several other inflammatory molecules in this second step of reperfusion injury. Macrophages may also play a significant role by producing ROS or performing autophagic processes (31). Furthermore, endothelial dysfunction is present in this phase of reperfusion. Compared to animal models applying vessel ligation, thrombotic masses may block peripheral vessels and by that worsen reperfusion injury.

Ischemia reperfusion injury in current medicine

Ischemia-reperfusion injury (IRI) plays a crucial role in daily clinical practice and current medical research. It accounts for several acute or subacute illnesses, which represent a major part of morbidity and mortality in the western world.
Acute ischemia leads to tissue damage and cell necrosis in myocardial infarction, stroke, peripheral embolism or traumatic interruption of blood supply. Furthermore, subacute or chronic ischemia accounts for end organ damage in several circumstances. Examples include myocardial hypertrophy due to aortic stenosis, peripheral artery occlusive disease and ischemic cardiomyopathy. In addition to these pathologies, ischemia plays a significant role in organ transplantation. The tissue-dependent resistance to ischemia limits the time from organ harvesting to restoration of blood flow. Therefore, early clinical outcome is strongly influenced by organ transportation and implantation time. This significantly limits the expansion of organ transplantation programs. Furthermore, ischemic injury during organ transportation seems to be a significant predictor of long-term graft performance (32). In addition to transplantation, some complex surgical procedures require a prolonged tissue ischemia. These are for example cerebral aneurysm exclusion, surgical replacement of the aorta due to thoracoabdominal aneurysms and cardiac surgery with the use of cardiopulmonary bypass.

Concept of ischemic Pre- and Postconditioning

Ischemic preconditioning (IPC) is an established method to avoid ischemia-reperfusion injury in different vascular beds (12, 33, 34). It was first described by Murry et al. in an experimental setting using the ligation of a dog's coronary artery for three periods of 5 minute ischemia prior to a prolonged ischemia (12). A significant amount of myocardial tissue could be protected using this approach. Until now, it has proved beneficial on different surrogate endpoints in several smaller human trials applying IPC during coronary angioplasty or cardiac surgery (35). The effects of IPC can be classified into an early phase of protection, which occurs during the first hours after IPC, and a late phase of protection, which is observed approximately 24 hours after IPC (36, 37). Previous data suggest that there is great variation in the amount of protection conferred by this mechanical intervention (36, 38, 39), and different protocols have been used for IRI attenuation in clinical studies. The controlled, repeated application of short periods of ischemia preceding a prolonged ischemic episode could also protect remote tissue against ischemia-reperfusion injury (39). The signaling pathways of this so-called remote ischemic preconditioning are still unknown. It is less than, or equally effective to, IPC directly applied to the ischemic area. The amount of tissue subjected to remote ischemic preconditioning, age and concurrent illnesses like diabetes and hyperlipidemia seem to play an additional role in determining the effectiveness of this approach (40-43).

Furthermore, short periods of ischemia applied during an ischemia (ischemic perconditioning) or directly after an ischemic insult (ischemic postconditioning) proved beneficial. These therapeutic approaches seem to be mediated by similar pathways to those activated after IPC (44). Postconditioning has now been applied in

several clinical settings and subjected to intensive research. Its application in clinical routine appears feasible due to the initiation of therapy after the onset of symptoms. However, data from large clinical trials are still scarce and underlying mechanisms have to be defined.

Ischemic preconditioning - molecular mechanisms

Several signaling pathways were described to be involved in mediating the protective effects of IPC. Most of these pathways ultimately target mitochondrial metabolism. The key event leading to massive ROS generation and cell death is the prolonged formation and opening of the mPTP. Most pathways inhibit this event. A limited formation of the mPTP occurs also during normal cell physiology, for example to reduce mitochondrial Ca^{2+} content (45). ROS are known to cause damage to several cellular structures. However, a limited increase of cellular ROS may activate several pathways later protecting against high ROS levels. A possible source of the protecting stimulus conferred by ROS may be the short opening of the mPTP, in this case induced by IPC, which leads to the protective signaling inhibiting the prolonged mPTP opening (46-48).

IPC activates G-protein coupled receptors and the soluble guanylyl cyclase, which induces the reperfusion-injury salvage kinases (RISK) pathway. Several other stimuli were shown to activate the RISK pathway, which finally mediates its protective action by Akt, Erk1/2 and NOS (49).

In addition, the survivor activated factor enhancement (SAFE) pathway, which acts via tumor necrosis factor – alpha, JAK and STAT3, may similarly be involved in the signal cascade of IPC (50). The final common action of both the RISK and the SAFE pathway on the mPTP is mediated by GSK-3beta (51). The RISK pathway seems to be less important in mammals (52).

Adenosine is a vasodilating agent which plays a crucial role in ischemic pre- and postconditioning. The increased concentration during IPC mediates preconditioning effects via the A1- receptor (53). Its signaling inhibits mPTP opening (54).

Intracoronary administration of adenosine has beneficial effects on IRI (55). However, systemic administration during myocardial infarction failed to improve mortality (56). Pharmaceutical adenosine receptor blockage also inhibits the effects of IPC (57). Therefore, adenosine plays a crucial role in the cascade of pre- and postconditioning, but its actions are time-dependent and not just related to the administration of adenosine. The continuous measurement of high energy phosphates by 31P-MRS allows the estimation of cellular adenosine concentration changes during ischemia and reperfusion. This technique may therefore elucidate some of adenosine's manifold functions during IRI.

Ischemic postconditioning - molecular mechanisms

Ischemic postconditioning is defined as the interruption of reperfusion in short cycles immediately after the onset of reperfusion. A fast onset of postconditioning after initial reperfusion is essential for the protecting mechanisms of postconditioning (58). Beneficial effects of postconditioning were shown in numerous preclinical studies and also in a number of clinical trials conducted during percutaneous coronary intervention or cardiac surgery (59, 60). There are still profound knowledge gaps in the understanding of postconditioning. It seems surprising that the damage induced by ischemia could be partially abolished by an intervention started after the onset of reperfusion. However, some facts could behave been elucidated and are well presented in a recent review by Vinten-Johansen (61). The exact timing of mechanical interventions during early reperfusion is the crucial factor enabling protection against the reperfusion injury. The activation of G-protein-coupled receptors is believed to be one of the first events necessary for intracellular signal transduction (62). Several activators, like adenosine and opioids, are known. As previously described, however, adenosine revealed conflicting results as a cardioprotective agent in clinical trials. The distinct mechanism of cardioprotection mediated by adenosine during reperfusion is unclear, as adenosine is also present abundantly at the onset of reperfusion without any conditioning. However, reduced washout kinetics of adenosine during postconditioning is one hypothesis which could explain the protective effects of adenosine and the conflicting results of externally administered adenosine (63).

Mitochondrial PTP opening induces mitochondrial disruption and cellular necrosis or apoptosis. The inhibition of this event is believed to be the main target of intracellular signal cascades, which were described here in detail for ischemic preconditioning.

Pathways for the inhibition of mPTP opening seem to be similar to those of ischemic preconditioning (44). ERK1/2, PI3K/Akt and the RISK pathway are known to be activated by G-protein-coupled receptors like the adenosine A2A receptor (64, 65). This could also be shown in the human heart (66). Although the intracellular pathways of ischemic preconditioning and ischemic postconditioning seem to be somewhat similar, the current knowledge about the distinct processes involved in tissue protection is too vague to define the crucial differences between these two therapeutic approaches, if any are present at all.

Platelet – monocyte complexes as surrogate marker for IRI

Two surface markers, CD62P and CD42b, were chosen as surrogate markers for systemic activation of the inflammatory system due to IRI. CD62P is a cell adhesion molecule, which enables the adherence of leukocytes to the endothelium. This molecule has a long extracellular portion, which enables leukocyte rolling on the endothelial surface. It is also called P-selectin and it is located in the Weibel-Palade bodies. Inflammatory stimuli activate the Weibel-Palade bodies and induce their conflation with the cell membrane (67).

Local inflammatory processes therefore activate the transfer of P-selectin to the endothelial surface in the blood stream. This mechanism brings leukocytes close to intercellular gaps and enhances extravasation to the area of inflammation. CD62P is not only stored in endothelial cells but also in platelets and is an important factor for the formation of platelet-monocyte complexes (PMC) (68). Platelets are able to form complexes with monocytes when they are activated (69). We suggest that the detection of PMCs by CD62P FACS analyses gating monocytes may represent a valid marker for the systemic inflammatory reaction induced by IRI (67).

CD42b is part of the GPIb-IX-V complex that acts as the receptor for the von Willebrand factor and thrombin on the surface of platelets. It therefore also serves as a platelet marker as it is known to mediate platelet interaction with the endothelium and to activate platelets by intracellular signaling (70). Furthermore, the interaction of CD42b with P-selectin may be a key factor for PMC rolling on activated endothelial cells (71, 72).

Therapeutic options of ischemia – reperfusion injury

Cyclosporine A

Cyclosporine A is an important immunosuppressive drug. It is currently in the routine therapeutic regimen for several chronic inflammatory diseases, some cancer chemotherapeutic regimens as well as for the immunosuppressive therapy after transplantation. It inhibits calcineurin and thereby modifies the expression of immunogenic proteins, especially in T cells. The first step of its intracellular action is the binding to Cyclophilin D. This complex later inhibits calcineurin. Intriguingly, Cyclosporine A is also believed to reduce reperfusion injury by inhibiting mPTP opening (73). This could also be shown in a small clinical trial in patients undergoing percutaneous coronary artery intervention (74). Although this drug is one of the most suitable therapeutic approaches according to the literature, it was not chosen for assessment in this project. This was due to the potential side effects. Further, a large scale clinical trial is currently being undertaken to evaluate this therapeutic approach.

Adenosine

Adenosine plays a crucial role in the signal transduction mediating the preconditioning effect. Therefore, several clinical trials have been undertaken to investigate the protective effect of adenosine administration in the setting of acute myocardial infarction. The AMISTAD I trial analyzed the protective effects of adenosine on ischemia – reperfusion injury in patients undergoing thrombolysis (56). Although infarct size could be reduced in the treatment group, an increase in adverse events was observed. The AMISTAD – II trial failed to reach a significant difference in the primary endpoint, which was congestive heart failure or death within 6 months, but did show an improved infarct size combined with decreased adverse events in the high – dose treatment group with timely reperfusion (75). Further analysis of the AMISTAD II trial revealed that an early reperfusion was associated with beneficial effects of adenosine treatment, whereas adenosine treatment applied with reperfusion 3 hours or more after the onset of ischemia could not protect cardiac tissue (76). Due to the numerous but conflicting clinical results concerning adenosine in the treatment of IRI, we did not include this approach in our trial.

Vitamin C

Previous clinical trials showed a systemic protective effect of high-dose intravenous vitamin C application in the ischemia – reperfusion setting. One study administered vitamin C during bilateral knee surgery with tourniquet ischemia. Patients had a reduced burden of ROS, measured by malondialdehyde levels. Interestingly, this resulted in a lower postoperative level of Troponin I, indicating a decreased cardiac affection of this remote ischemia – reperfusion injury (77). Likewise, pharmacologic therapies administered after the onset of reperfusion might prevent tissue injury. We have recently shown that high concentrations of exogenous vitamin C abrogate experimental IRI of the forearm vasculature in patients with peripheral artery disease and in healthy subjects.

Heme arginate

Pharmacologic concept

A promising concept for the reduction of IRI is the induction of heme oxygenase 1 (HO-1). Heme oxygenases are expressed in the cellular microsomes. HO-2 is the constitutively expressed form in humans. In contrast, HO-1 can be expressed in response to several stimuli, which are generally pro-inflammatory. As a new therapeutic approach in humans, the induction of the enzyme HO-1 may be employed to mitigate IRI (78). HO-1 is the rate-limiting enzyme for the degradation of heme B (79). This degradation reaction produces biliverdin, carbon monoxide (CO) and iron. Recently, the role of HO-1 as a protective enzyme was proposed due to its anti-inflammatory, antioxidant, anti-apoptotic and antiproliferative actions (78, 80). HO-1 is expressed in several organs, including endothelial and smooth muscle cells, in response to cellular stress conditions (81-83).

Heat shock proteins' transcriptional regulation responds to several chemical and biological stimuli. Although it has been shown that heme is such an inducer of HO-1 (84), the molecular steps and signal transduction pathways underlying HO-1 up-regulation in general, and by heme, in particular, remain largely unclear (85, 86).

IRI may be attenuated by pharmacological HO-1 induction with heme arginate (HA) as shown in a rodent hemorrhagic shock model (78, 87-89). Previous data have confirmed dose-dependent induction of HO-1 mRNA and protein by HA in venous blood of healthy subjects (90, 91).

There are two heme-containing drugs for intravenous human application available. Hemin is the ferriheme chloride. Heme arginate is the conjugation of hemin with L-arginine in a solution of propylene glycol, ethanol and water. HA is commercially available from Orphan Europe Ltd. as Normosang® which is the approved form of heme in Europe for episodes of acute intermittent porphyria (AIP), variegate porphyria (VP) and hereditary coproporphyria (HCP). The usual dose of HA is 3 to 4 mg/kg body weight given as intravenous infusion once daily over 4 to 7 days.

Safety and tolerability

HA has the same advantages as hemin with lesser side-effects. Most important, heme arginate is less harmful to the endothelium and thereby reduces vasculopathy and thrombotic side effects (92-94). Further, no adverse effects on hemostasis are observed (95). Acute toxicity occurs at doses of 48 to 70 mg/kg body weight (depending on animal species).

Table 1-1: Known adverse reactions to heme arginate (96)

frequency	adverse reaction
very common (> 10 %)	difficult venous access (with repeated administration)
common (1-10 %)	injection site pain, injection site swelling, phlebitis at injection site
uncommon (0.1-1 %)	elevated serum ferritin concentration
rare (0.01-0.1 %)	fever, anaphylactoid reaction, allergic reactions (dermatitis medicamentosa, tongue edema)

Pharmacokinetics

Intravenously administered HA is bound to hemopexin and albumin and as heme-hemopexin-complex transported to the liver. There it is degraded by HO-1 and biliverdin reductase to bilirubin and excreted into the bile.

Table 1-2: Relevant pharmacokinetic parameters of heme arginate (97)

Half-life (single dose)	10.8 hours
Volume of distribution	3.37 litres
Total plasma clearance	3.7 ml/min

Functional magnetic resonance imaging

Blood oxygen level dependent functional MRI

Blood oxygen level dependent (BOLD) functional magnetic resonance measurements (fMRI) are based on the local influence of the hemoglobin molecule on the magnetic field. Deoxygenated hemoglobin has four unpaired electrons in each heme group. This leads to a distortion of the magnetic field and influences signal intensity (98, 99). If the hemoglobin molecule is fully saturated with oxygen, the magnetic pattern changes to diamagnetic. Compared to paramagnets, diamagnetic molecules have a much weaker influence on the magnetic vicinity. The magnetic influence of hemoglobin is especially important for the relaxation time in water proton based MR sequences. It changes the transverse relaxation time (T2). This allows for oxygenation status susceptible imaging. T2 weighted acquisition is suited for these effects, but T2* acquisition is optimal.

The first MR protocols for the measurement of hemoglobin saturation status were described in 1982 (98). BOLD fMRI has been previously applied to measure local blood oxygenation to evaluate cerebral blood flow as well as skeletal muscle and myocardial perfusion (99, 100). In patients with peripheral artery occlusive disease, BOLD imaging can characterize post-ischemic hyperemia and the effects of percutaneous transarterial angioplasty (101, 102).

^{31}P magnetic resonance spectroscopy

The ^{31}P magnetic resonance spectroscopy (MRS) is a validated technique to investigate ischemia and the effects of physical exercise (103-105). This method allows for the relative quantification of adenosine diphosphate (ADP), adenosine triphosphate (ATP), inorganic phosphate (Pi) and phosphocreatine (PCr) with a high temporal resolution (106). Therefore, the effects of ischemia and exercise as well as the recovery after these states can be observed. PCr and Pi changes are most dramatic and allow for an extrapolation of mitochondrial function. Further, changes in pH can be measured simultaneously by ^{1}H MRS. PCr functions as a cellular energy storage molecule. A high-energy phosphate group can be transferred to ADP in order to restore the ATP concentration during physical exercise. Therefore, PCr concentration decreases during exercise and creatine concentration increases. Under physiologic conditions, creatine is rephosphorylated by ATP, which is produced in the mitochondria by oxidative phosphorylation. However, the increase of oxidative phosphorylation during exercise requires adaptation of perfusion and metabolism and therefore presents with a time delay. If ischemia is present, the adaptation is not possible and PCr cannot be restored. A further decrease of PCr and a maximal reperfusion with a strong increase of PCr after ischemia can be observed in this model.

The measurements obtained using this technique show a low variability in repeated testing and correlate well with those determined for the whole body maximal oxygen uptake (107). ^{31}P spectroscopy provides a marker of mitochondrial function and the oxidative capacity of tissues (108).

Project hypothesis

This project aims to investigate the therapeutic application of HA for the treatment of IRI. The strong induction of HO-1 by HA is a clinically feasible mechanism. However, HA had not been tested in this indication before and no human data on its effectiveness exists. Valid experimental IRI models to test HA in healthy human subjects prior to the clinical setting are lacking. Therefore, we decided to develop fMRI surrogate markers for the detection and quantification of protective effects of an intervention aiming to reduce IRI. IPC appeared to be a suitable test model as it has been evaluated in several human trials before.

In the first clinical trial, we hypothesized that the effects of IPC and the reperfusion pattern in the ischemic calf muscles could be detected and further analyzed using high-field magnetic resonance. Therefore, 3-Tesla ^{31}P MRS was applied in healthy subjects to quantify the levels of ATP, PCr and Pi as well as intracellular pH. BOLD fMRI was also performed in these subjects to study reperfusion patterns regarding blood flow and oxygen extraction.

In the second clinical trial, a therapeutic exploratory study was performed. We aimed to evaluate the effects of HA on skeletal muscle IRI in healthy humans. BOLD fMRI was used to measure alterations in tissue oxygenation with a high spatial and temporal resolution (103, 109). Our results in the first clinical trial showed an effect of IPC on BOLD fMRI and we suggested that the effects mediated by HO-1 induction may be more directly assessable by BOLD fMRI than by ^{31}P MRS. ^{31}P MRS may be more suitable to detect mitochondria – dependent processes. However, compared to IPC, the induction of HO-1 is protective against ROS and other cellular insults but

may not directly affect mitochondrial function. On the contrary, BOLD fMRI data seems to reflect a combined assessment of reperfusion patterns, including perfusion, oxygen saturation and tissue metabolism. We have learned from previous experiments that endothelial function is a very sensible marker of IRI (110). In our opinion, HO-1 induction may preserve endothelial function after IRI. This effect should be assessed by BOLD fMRI, which combines the effect of endothelial function dependent end organ perfusion with a combined metabolic assessment.

Chapter 2: General Methods

Clinical trials

All clinical trials described in this thesis were planned and conducted by the Group of Cardiovascular Pharmacology at the Department of Clinical Pharmacology at the Medical University of Vienna. MR sequences and methods were developed and adapted by the MR Center of Excellence at the Medical University of Vienna. Only healthy young men were invited to participate in the trials. All protocols were approved by the Ethics Committee of the Medical University of Vienna and conformed to the principles outlined in the Declaration of Helsinki, including current revisions and the Good Clinical Practice guidelines.

The effects of preconditioning on intramuscular high-energy phosphate levels during ischemia

EK 119/2008

ClinicalTrials.gov: NCT00883467

The effects of preconditioning on muscle force after short period ischemia

EK166/2009

The effects of intravenous heme arginate on functional magnetic resonance imaging during ischemia

EK 697/2008

EudraCT: 2008-006967-35

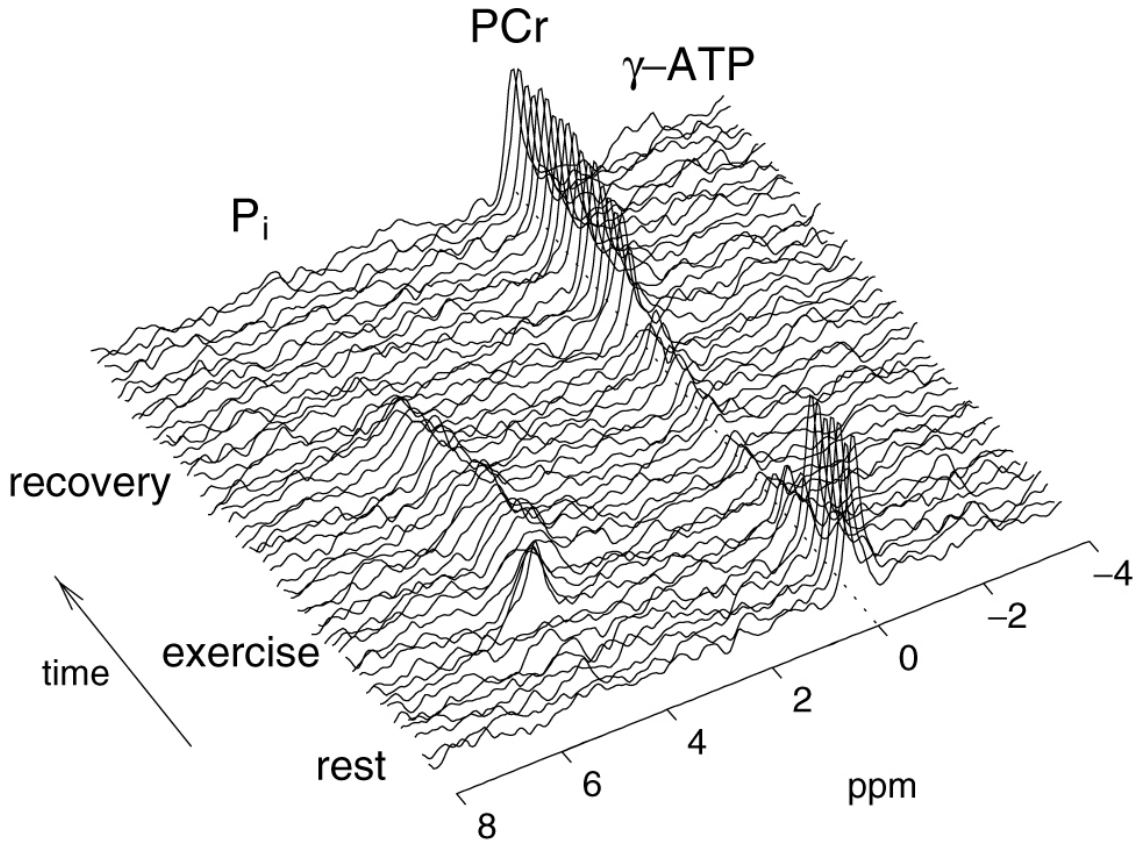
ClinicalTrials.gov: NCT01461512

Ischemia model

Experimental ischemia was previously used in humans to evaluate the physiologic and pathologic processes following the interruption of blood flow. It is essential to investigate ischemia and the therapeutic approach to this condition in humans to avoid any species – specific differences and enable reliable research. However, time of ischemia for research purposes is limited in healthy subjects to avoid any sustained tissue damage. Therefore, special measurements are necessary to allow the assessment of new therapeutic approaches without tissue damage.

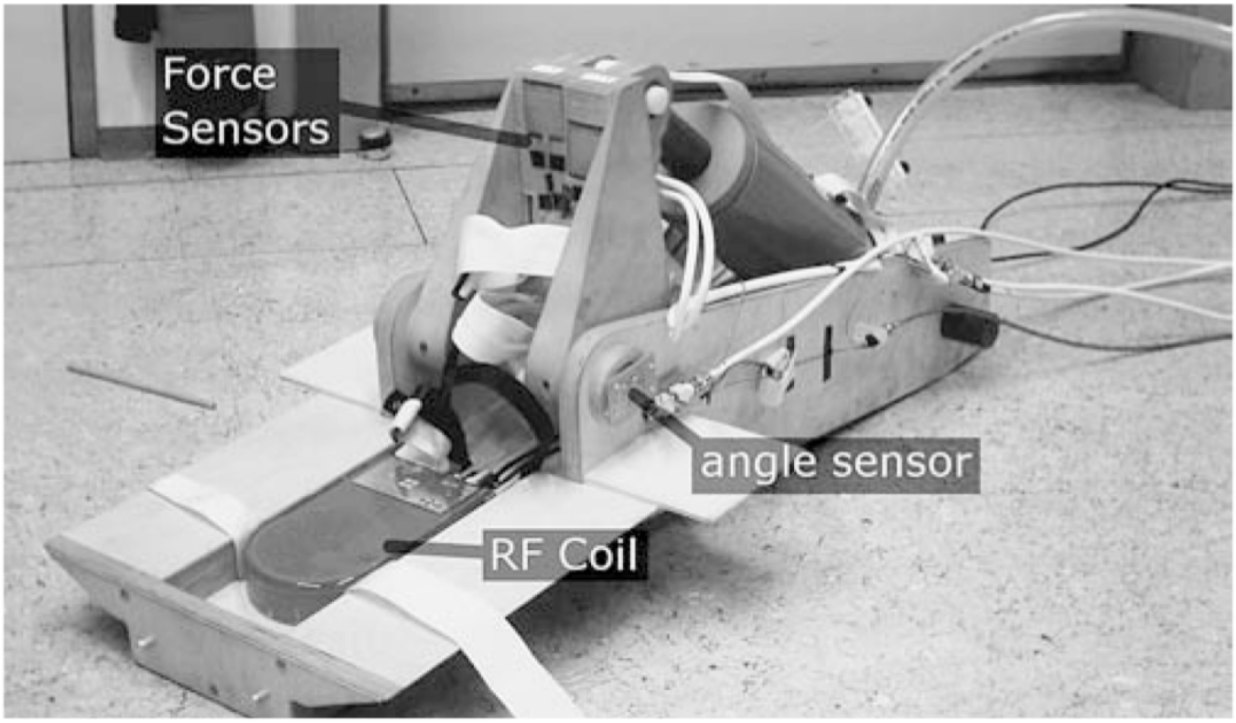
Strain gauge plethysmography was previously used at our department to assess the effect of vitamin C treatment on endothelial function after upper extremity ischemia (110). However, we aimed to assess the effects of therapeutic approaches directly in the muscular tissue, including not only the endothelial function but also the inherent cellular energy metabolism and tissue specific alterations. Models for the detailed assessment of energy metabolism during exercise and ischemia were previously developed at the MR Center of Excellence at the Medical University of Vienna (103, 109). A special research focused on the measurement of high energy phosphate changes during ischemia and reperfusion. High energy phosphates reflect the cellular energy status and allow for the assessment of mitochondrial function. The decline of PCr during ischemia and/or exercise reflects the cellular energy demand without further oxidative phosphorylation or energy supply (Figure 2-1). The regeneration of PCr during reperfusion is not only dependent on the full reestablishment of perfusion but also on the mitochondrial function.

Figure 2-1: Effect of exercise on phosphocreatine and inorganic phosphate (109)



We performed a modification to the routine ischemia setting in order to increase the capability of our ischemia model without prolonging the ischemic time. A wooden exercise rig, which was developed for the assessment of PCr changes during exercise, was included in our experimental setting (Figure 2-2). Due to technical reasons, the leg was used instead of the arm for ischemia application. The ischemia was induced by a special cuff for the thigh, which was inflated by pressurized air to 200 mmHg.

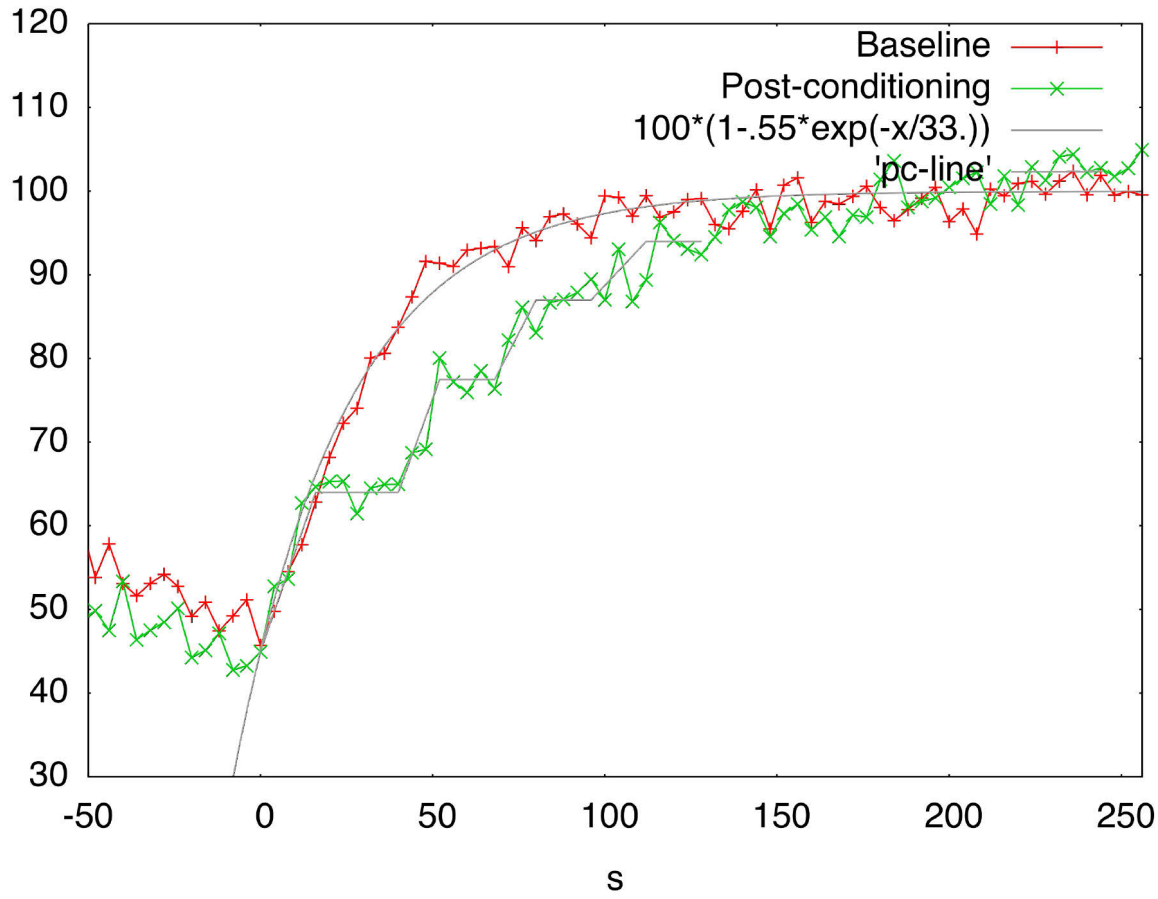
Figure 2-2: Wooden exercise rig for intra-MRI use (109)



Stenosis model

We investigated the effect of ischemic postconditioning prior to this project (data under preparation for publication). Our early data revealed a rather fast recovery of PCr during reperfusion. The recovery sequence compared to ischemic postconditioning is depicted in figure 2-3.

Figure 2-3: Reperfusion period with and without ischemic postconditioning

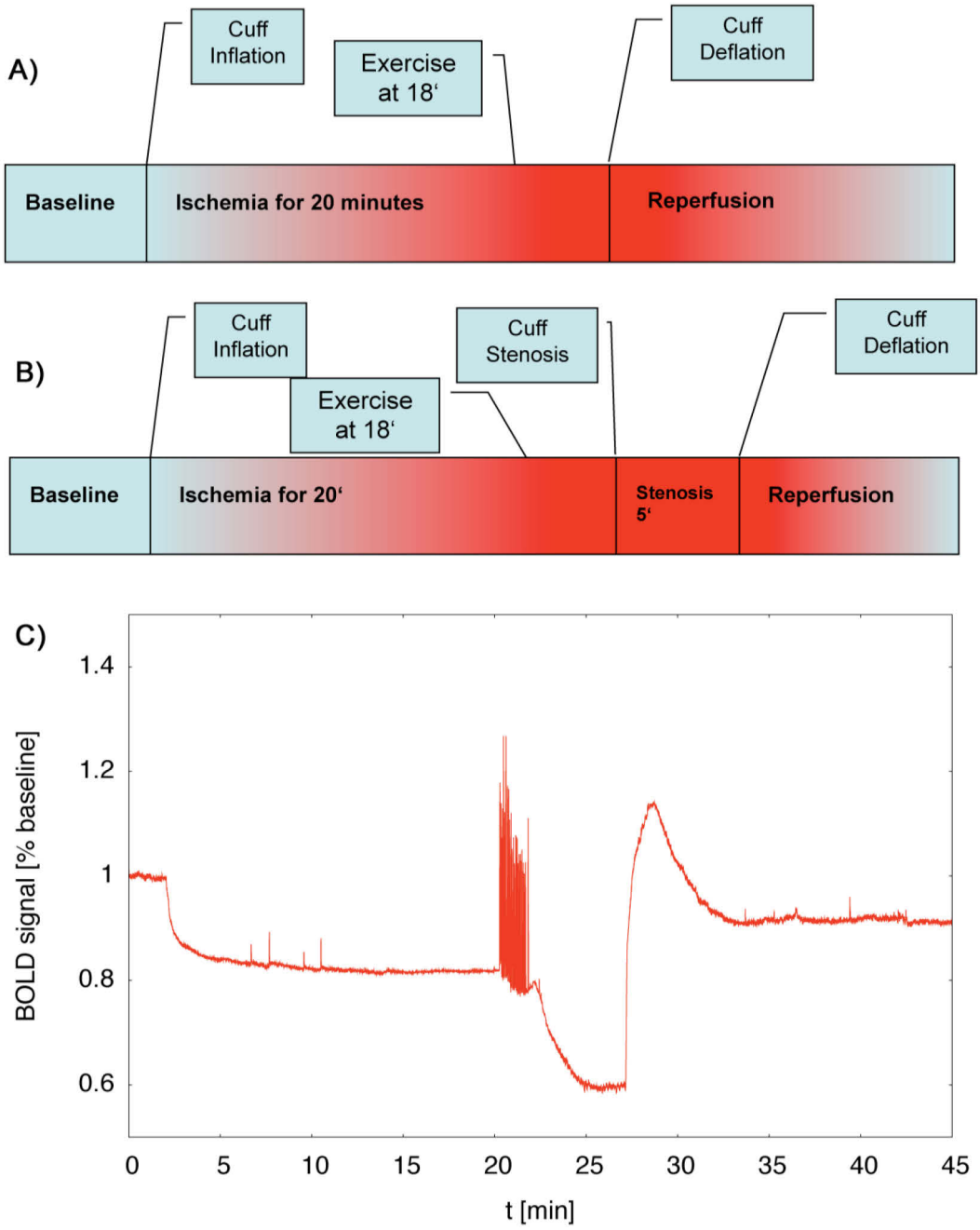


x-axis: time in seconds to the start of reperfusion; y-axis: PCr signal in percentage of the initial pre-ischemic signal. Grey line: interpolated PCr course.

Therefore, we aimed to bring our ischemia model closer to clinical reality. In addition to the exercise performed in the last two minutes of ischemia, a post-ischemic stenosis was included in our experimental setting. We suggest that a limited early reperfusion is present in several clinical settings. This may be, for example, due to guide wire reopening during percutaneous coronary intervention prior to balloon angioplasty and stent implantation. Further, a rather slow reopening may be present during thrombolysis. Even if no anatomical stenosis is present after vessel reopening, a reduced perfusion may be present due to vasoconstriction induced by endothelin,

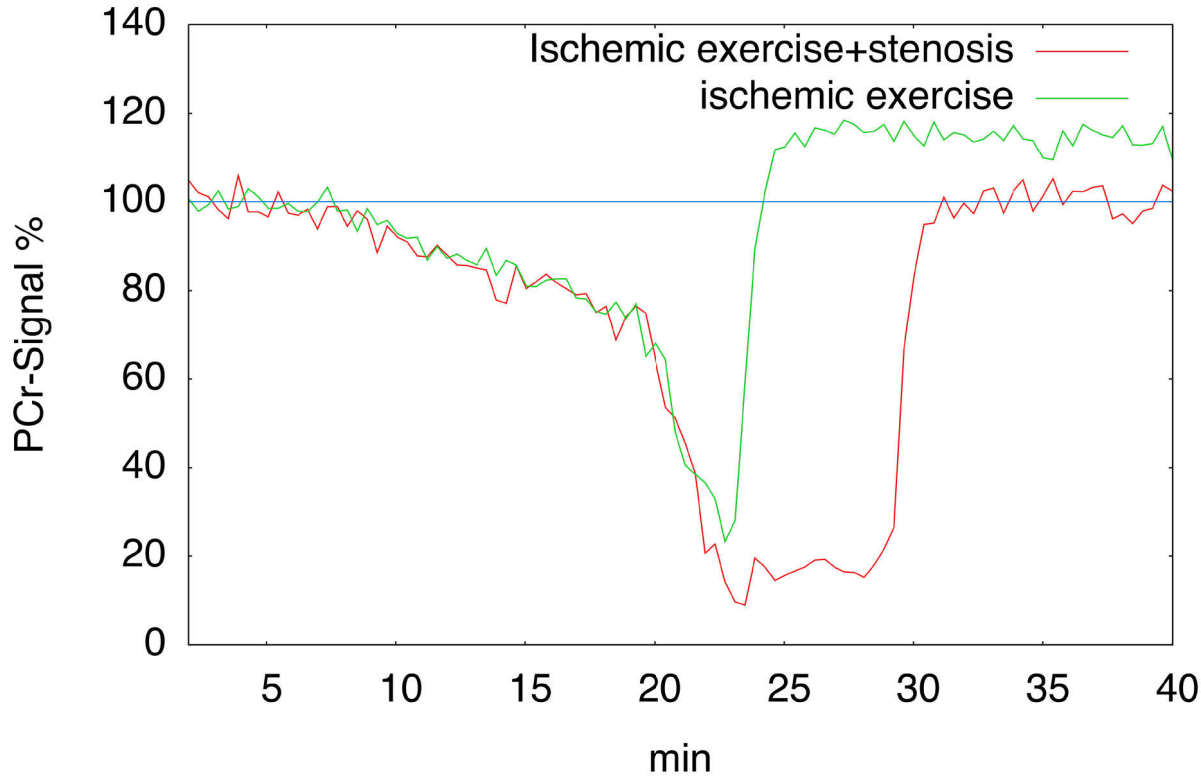
which is washed out of thrombotic material (111). All these circumstances cannot be simulated with external cuff inflation and simple deflation without the presence of thrombotic material. Therefore, we decided to apply a post-ischemic stenosis for five minutes prior to full reperfusion. This approach was implemented to adapt our setting to clinical reality and enable a real assessment of therapeutic approaches without increased ischemic time. The stenosis model impeded PCr recovery during stenosis and further decreased BOLD fMRI signal until reperfusion (Figure 2-4 and 2-5).

Figure 2-4: Stenosis sequence and the influence on BOLD signal



A) Experimental Setting Day A (Baseline)
B) Experimental Setting Day B (Stenosis)
C) Example for BOLD signal intensity in comparison to baseline for day B; further decrease of BOLD signal during increased venous filling takes place from minute 22 to minute 27

Figure 2-5: Effect of stenosis on PCr recovery



x-axis: time in minutes to MRS measurement start; y-axis: PCr signal in percentage of the initial pre-ischemic signal.

Chapter 3: Effects of ischemic preconditioning assessed by functional magnetic resonance imaging

Introduction

IPC is an established method to avoid IRI in different vascular beds (12, 33, 34). Effects of IPC can be separated into an early phase of protection, affecting the first hours after IPC, and a late phase of protection, present approximately 48 hours after IPC (36, 37). Previous data suggest a great variation in the amount of protection conferred by this mechanical intervention (36, 38, 39) and different protocols have been used in clinical studies (112, 113).

We aimed to establish an experimental setting that allows the detection of the protective effect conferred by ischemic preconditioning. We hypothesized that IPC effects and reperfusion pattern in ischemic skeletal muscle of the lower leg can be detected and further analysed by high-field magnetic resonance. Therefore, three Tesla magnetic resonance spectroscopy (MRS) of ^{31}P for the quantification of ATP, PCr and Pi as well as BOLD fMRI were applied in healthy subjects.

Methods

This section is part of the previous published study (114). It comprised two different protocols (EK 119/2008 and EK166/2009). In the first protocol, 14 healthy male Caucasian subjects were subjected to MR studies (age: 27±7 years, body mass index: 22.4±1.9 kg/m^2). In the second protocol, 9 healthy male subjects (age: 27±8 years, body mass index: 22.2±1.3 kg/m^2) were recruited to assess isometric muscle strength. Both protocols followed a randomized crossover design. After informed consent was obtained, all of the subjects underwent a complete health examination, including a physical examination, ECG and laboratory screening. The inclusion criteria were no history or signs of clinically relevant illness during the two weeks preceding the first day of the study and no contraindications for MR scanning. The subjects were drug-free (including over-the-counter medications) for three weeks prior to the screening and until completion of the study. Four or eight study days with a washout interval of ≥6 days were scheduled for each participant according to a pre-defined protocol. The participants abstained from the consumption of alcohol and stimulating beverages containing xanthine derivatives for 12 hours before each trial period and were studied after an overnight fast. The participants also avoided heavy physical exercise for 3 days prior to the MR and force measurements.

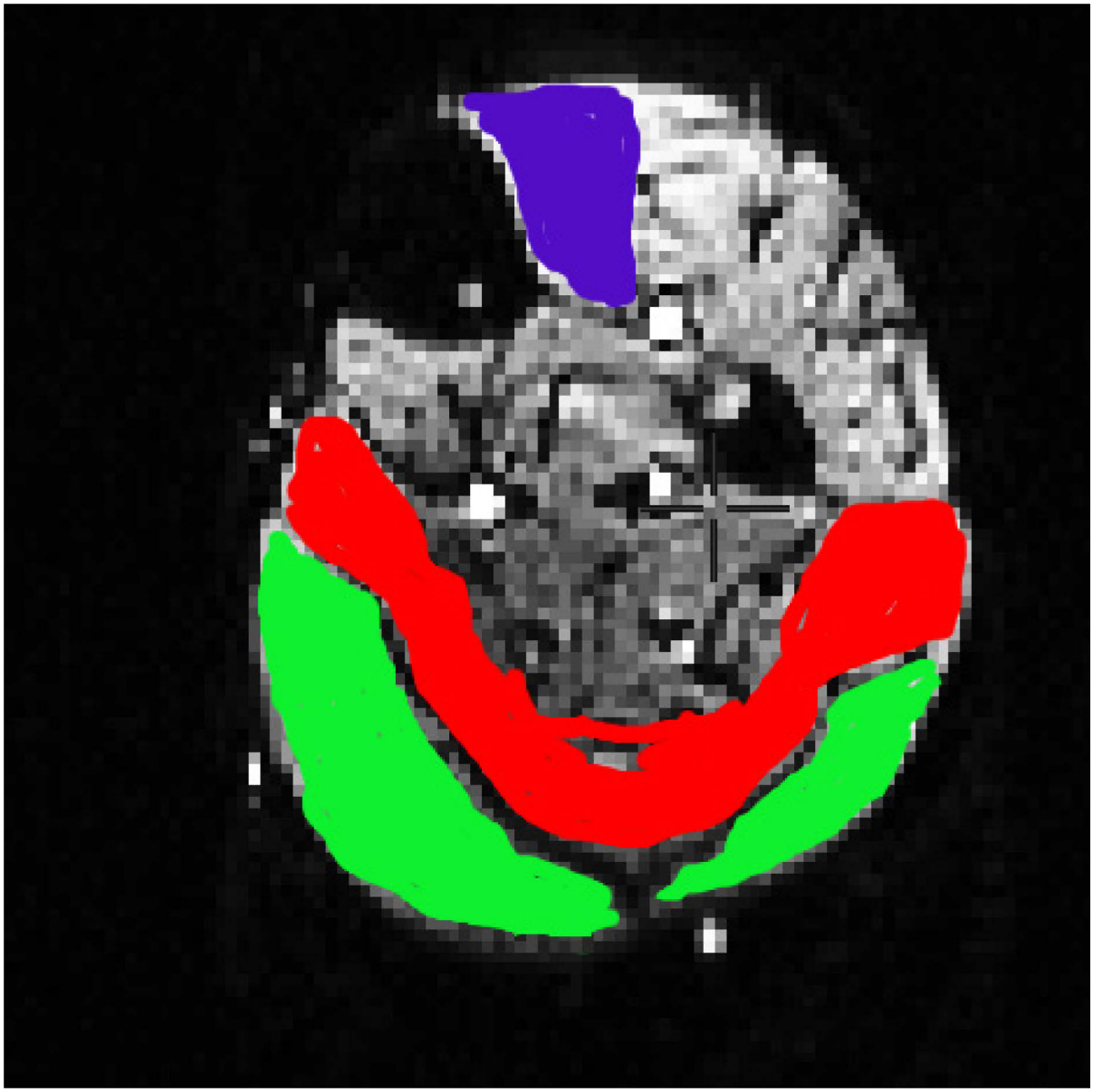
MR examinations for ischemic preconditioning

MR signals were acquired beginning at two minutes prior to ischemia until 30 minutes after release of the cuff. The measurements were performed using a 3T Tim Trio whole-body scanner (Siemens Medical Solutions, Erlangen, Germany). The right leg of the subject was fixed to a wooden exercise rig as described previously (103). Changes in high-energy phosphate levels were recorded using a circular, double-resonant $^{31}P/^{1}H$ surface coil with a diameter of 105/95 mm (RAPID, Germany), which was positioned below the medial head of the right gastrocnemius muscle. A pulse-acquire procedure was used to collect the data. At the beginning of the experiment, the pulse was calibrated to maximize the signal yield. The dynamic scan lasted for approximately 50 minutes, during which a single acquisition was collected every 4 seconds. PCr and Pi resonances were quantified using AMARES (115) in jMRUI (116), pH was calculated from their frequency difference. Post-exercise post-ischemic PCr recovery was fitted to a single exponential equation plus a linear component to account for long-term instabilities in perl/PDL (117) using the Fit-Levmar module.

BOLD signals were calculated from fat-suppressed echo-planar images (EPI), recorded by a flexible coil wrapped around the subjects' calf (TR= 0.5 s, 90 deg. Flip angle, 5400 scans, TE= 44 ms, 128 pixels, 5 slices, 102 phase encodings, reconstruction to 128, 1.4 mm in-plane resolution, 5-mm-thick slices). Dicom images were exported and converted into the minc (http://www.bic.mni.mcgill.ca/ServicesSoftware/MINC) format for further processing. 100 images were blurred and averaged. This served as the reference to which all EPI were registered to correct for motion. They were resampled in the slice direction because the registration was done in three dimensions to correct also for through

plane motion. A full 12 parameter linear registration, the minctracc utility, was used to calculate the transformation. To improve signal to noise ratio (SNR) and to minimize computation time, the parameters were calculated for five averaged scans. After image registration, manually drawn regions of interest (ROI) covering the soleus, gastrocnemius and tibialis anterior muscle were extracted (Figure 3-1). Large vessels were filtered based on the characteristic hyperintense signal, which disappears during ischemia. Afterwards the signal was summed to achieve a time course for each individual muscle. This was then characterized by taking values at predefined points.

Figure 3-1: Regions of interest drawn for BOLD fMRI assessment

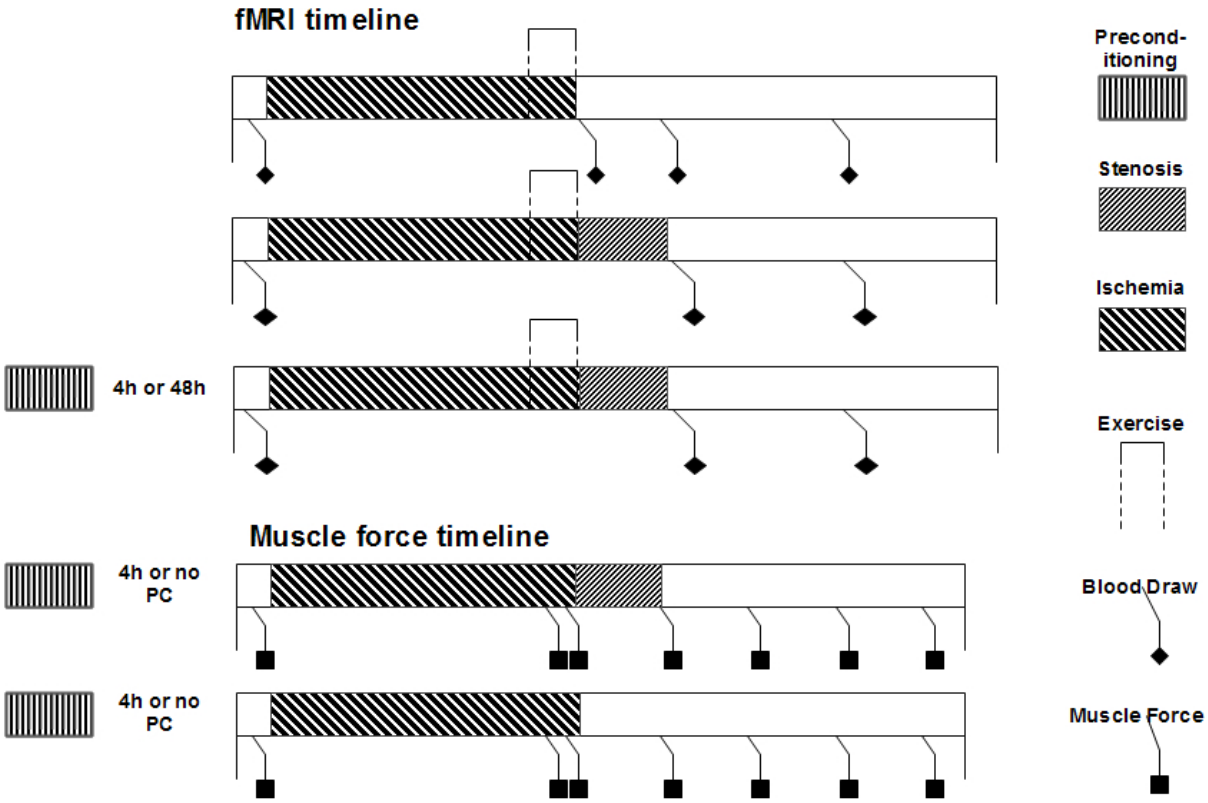


Transversal EPI image of the calf; tibialis anterior muscle (violet), soleus muscle (red) and gastrocnemius muscle (green).

Ischemic preconditioning protocol

IPC was administered on two study days in each MR protocol and on two study days together with the measurement of muscle force (Figure 3-2). In the MR studies, the intervals between mechanical intervention and the initiation of measurements were 4 and 48 hours to detect the short- and long-term effects of IPC, respectively. Because long-term IPC had no effect on the MR measurements in the first experiments, IPC was subsequently performed only 4 hours prior to the measurements obtained in the muscle force trial. IPC consisted of three 5-min periods of ischemia that were separated by two 10-min reperfusion intervals. For ischemia, a cuff that was inflated to 200 mm Hg was placed on the right thigh.

Figure 3-2: Timeline for ischemic preconditioning and measurements



Ischemia protocol

Limb ischemia was administered to the right leg for 20 min using a thigh cuff that was inflated to suprasystolic pressure (200 mmHg). The predetermined cuff pressure was achieved within 4 seconds of inflation using pressurized air. During the last two minutes of ischemia in the MR protocol, the subjects performed plantar flexions at a half-maximal contraction force. These plantar flexions were carried out on an exercise rig every 4 seconds until exhaustion, as described previously (109). Low flow reperfusion according to our stenosis model was induced by deflating the thigh cuff to 30 mmHg below the systolic pressure for a total of 5 minutes following ischemia.

Muscular force assessment

Four trial days were scheduled for each participant. On two days, ischemia-reperfusion was administered both with and without postischemic stenosis. On the other two days, IPC was performed 4 hours prior to ischemia both with and without post-ischemic stenosis. The cuff pressure reduction to 30 mmHg below systolic pressure caused post-ischemic stenosis. Muscular strength was measured prior to ischemia, during the last 2 min of ischemia and every 5 min after reperfusion for a total of 20 minutes. The Biodex 3 dynamometer (Biodex Medical Systems, Shirley, New York, USA) was used to quantify the isometric plantar flexion/dorsiflexion strength of the right leg ankle muscles, according to the specifications of the manufacturer (118-120).

The participants performed three sets of maximal voluntary isometric plantar flexion/dorsiflexion contractions (ankle angle: 15° flexion, knee flexion: 20-30°), which were maintained for 5 seconds each. The maximal isometric force was measured and normalized to body weight. During ischemia, only plantar flexion strength was measured, and the results corresponded to the muscular work performed in the exercise rig.

FACS and laboratory analysis

Venous blood was drawn during MR studies. Blood was collected for measurements of creatine kinase (CK), lactate dehydrogenase (LDH), free haemoglobin (Hb) and high-sensitive C-reactive protein (CRP) and for fluorescence activated cell sorting (FACS) analysis of CD62P and CD42b expression on monocytes before ischemia and at 5 minutes, 15 minutes, and 24 hours after reperfusion. CD62P and CD42b expression reflect monocyte / platelet complex formation and activation (121, 122).

Blood samples were incubated with monoclonal antibodies according to manufacturer's instructions (eBioscience™, San Diego, CA, USA). Fixation of leukocytes and red cell lysis were performed by utilizing OptiLyse®B (BECKMAN COULTER$_{TM}$, Beckman Coulter, Inc., Fullerton, CA, USA). Samples were assembled and analyzed using a FACSCalibur™ system with CellQuest™ software (BD, Franklin Lakes, NJ, USA). Monocytes were gated and a minimum of 20,000 events was recorded for each sample. Monocyte surface markers were expressed as percentage of positive events measured in flow cytometric dot plot.

Statistics

The data sets were analyzed descriptively, and the results are presented as the mean ± standard deviation (SD) or the median (quartiles) for parametric and non-parametric data, respectively. To compare the outcome parameters among the groups, analysis of variance or the Kruskal-Wallis test were used to evaluate parametric and non-parametric data, respectively. P-values less than 0.05 were considered significant. All statistical analyses were performed using SPSS V17 for Macintosh (SPSS Inc., Chicago, Illinois, USA).

Results

This section was previously published (114).

^{31}P MRS - scanning

The PCr signal decreased substantially during ischemia and exercise to 44±13% of the baseline value (Table 3-1, Figure 3-3). Following complete reperfusion, the PCr signal recovered quickly, the average time constants are reported in table 3-1. Normalized levels were observed within 230±102 seconds. In contrast, the PCr signal increased modestly during the 5-min post-ischemic stenosis period from trough values of 40±11% to 46±15% of the baseline value. A rapid recovery of the PCr signal was again observed within 243±83 seconds after full reperfusion. Pi increased during ischemia and exercise (Table 3-2). The effect of reperfusion was similar with or without preceding stenosis. Reperfusion significantly reduced Pi from the baseline NMR signal ($p<0.001$). PCr signals normalized at the end of the observation period. pH values increased slowly during ischemic rest and dropped rapidly during exercise and initial recovery (Table 3-3). They slowly returned to baseline values during further recovery. During stenosis, pH decreased slightly.

IPC 4 hours prior to ischemia/exercise and post-ischemic stenosis significantly increased the maximal PCr reperfusion signal intensity compared to ischemia/exercise and post-ischemic stenosis alone, but had no influence on the speed of recovery. IPC 48 hours before ischemia had no effect on the time course of PCr. IPC had no significant impact on pH or Pi. During the measurements, no changes in ATP were observed.

Table 3-1: PCr MRS signals. Mechanical preconditioning was applied 4h or 48h before ischemia.

PCr (% of baseline)	Ischemia and exercise	Post-ischemic stenosis	Post-ischemic stenosis and 4h IPC	Post-ischemic stenosis and 48h IPC
Number of subjects	*(n=7)*	*(n=8)*	*(n=8)*	*(n=8)*
Ischemia	79±3*	76±8*	79±5	76±3*
Ischemic exercise	44±13*	40±11*	49±17*	39±12*
Post-ischemic stenosis	-	46±15*	55±16*	45±17*
Reperfusion	106±5	104±10	121±20†	109±9
End	105±3	103±9	117±20	108±13
PCr recovery time-constant (s)	46±20	49±17	43±15	47±17

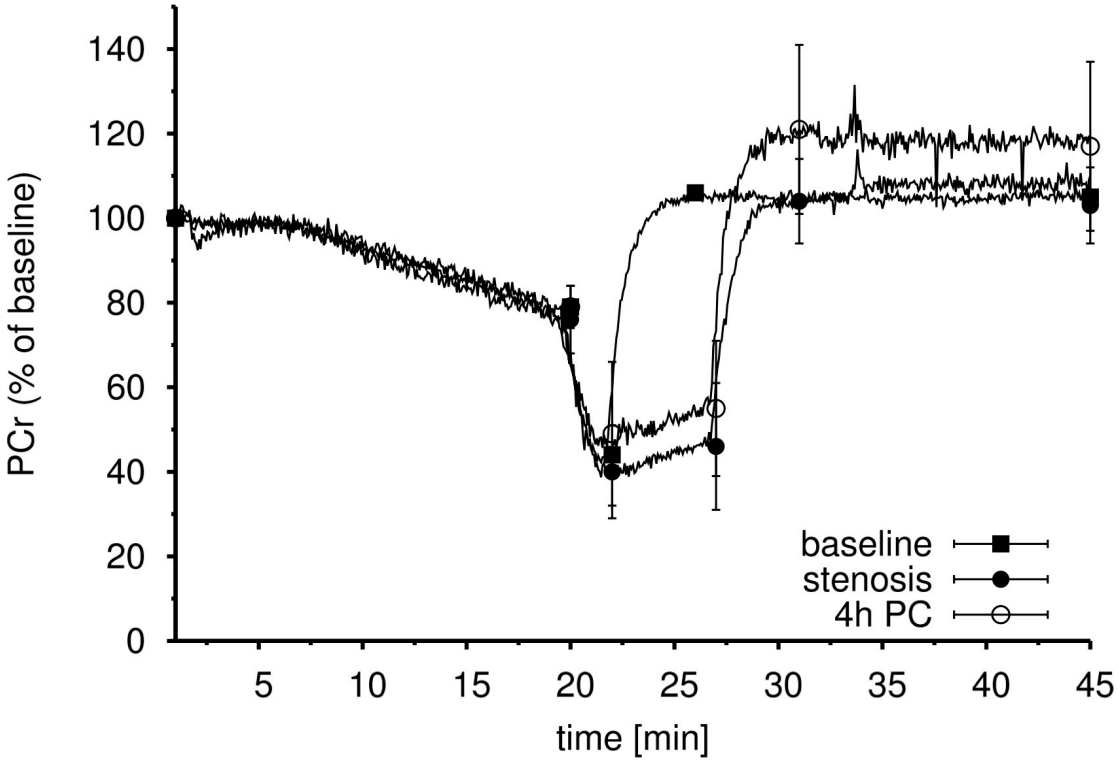
Data represent the means±SD. The baseline is defined as the NMR signal prior to ischemia. PCr: phosphocreatine, IPC: ischemic preconditioning, * $p<0.05$ versus baseline, † $p=0.05$ vs. day of post-ischemic stenosis

Table 3-2: Inorganic phosphate MRS signals. Mechanical preconditioning was applied 4h or 48h before ischemia.

Inorganic phosphate (% of baseline)	Ischemia and exercise	Post-ischemic stenosis	Post-ischemic stenosis and 4h IPC	Post-ischemic stenosis and 48h IPC
Number of subjects	*(n=7)*	*(n=8)*	*(n=8)*	*(n=8)*
Ischemia	334±44*	305±62*	321±89*	310±48
Ischemic exercise	731±183*	657±203*	681±193*	654±281*
Post-ischemic stenosis	-	558±229*	578±165*	532±260*
Reperfusion	51±21	37±15	58±24	55±26
End	110±11	90±84	116±34	92±25

Data represent the means±SD. The baseline is defined as the NMR signal prior to ischemia. IPC: ischemic preconditioning, * $p<0.05$ versus baseline

Figure 3-3: PCr time course



Time course of phosphocreatine (PCr) at baseline, during ischemia and reperfusion, and in the presence of post-ischemic stenosis with or without mechanical preconditioning 4 hours before ischemia. The data represent the means±SEM (*n*=8).

Table 3-3: pH MRS signals. Mechanical preconditioning was applied 4h or 48h before ischemia.

pH	Ischemia and exercise	Post-ischemic stenosis	Post-ischemic stenosis and 4h IPC	Post-ischemic stenosis and 48h IPC
Number of subjects	*(n=7)*	*(n=8)*	*(n=8)*	*(n=8)*
Baseline	7.03±0.02	7.05±0.03	7.05±0.03	7.07±0.05
Ischemia	7.08±0.02*	7.10±0.04*	7.09±0.02*	7.09±0.02
Ischemic exercise	6.93±0.14	6.92±0.14	6.92±0.17	6.98±0.14
Post-ischemic stenosis	-	6.88±0.12	6.91±0.15	6.96±0.14
Reperfusion	6.85±0.18	6.86±0.12*	6.89±0.17	6.90±0.17
End	7.04±0.02	7.10±0.04	7.07±0.08*	7.08±0.02

Data represent the means±SD. The baseline is defined as the NMR signal prior to ischemia. IPC: ischemic preconditioning, * $p<0.05$ versus baseline

BOLD fMRI - scanning

Ischemia rapidly decreased the BOLD signal intensity within the first 100 seconds of cuff occlusion. The BOLD signals were stable at 88±6%, 86±8%, 84±9% and 84±9%

of baseline after 5, 10, 15 and 18 min of ischemia, respectively (Figures 3-4, 3-5, 3-6 and 3-7). Post-ischemic stenosis produced an additional decline of the BOLD signal to 70±17% of the baseline value. During complete reperfusion, the maximum signal intensity was 124±15% and 123±16% of the baseline value with and without post-ischemic stenosis, respectively.

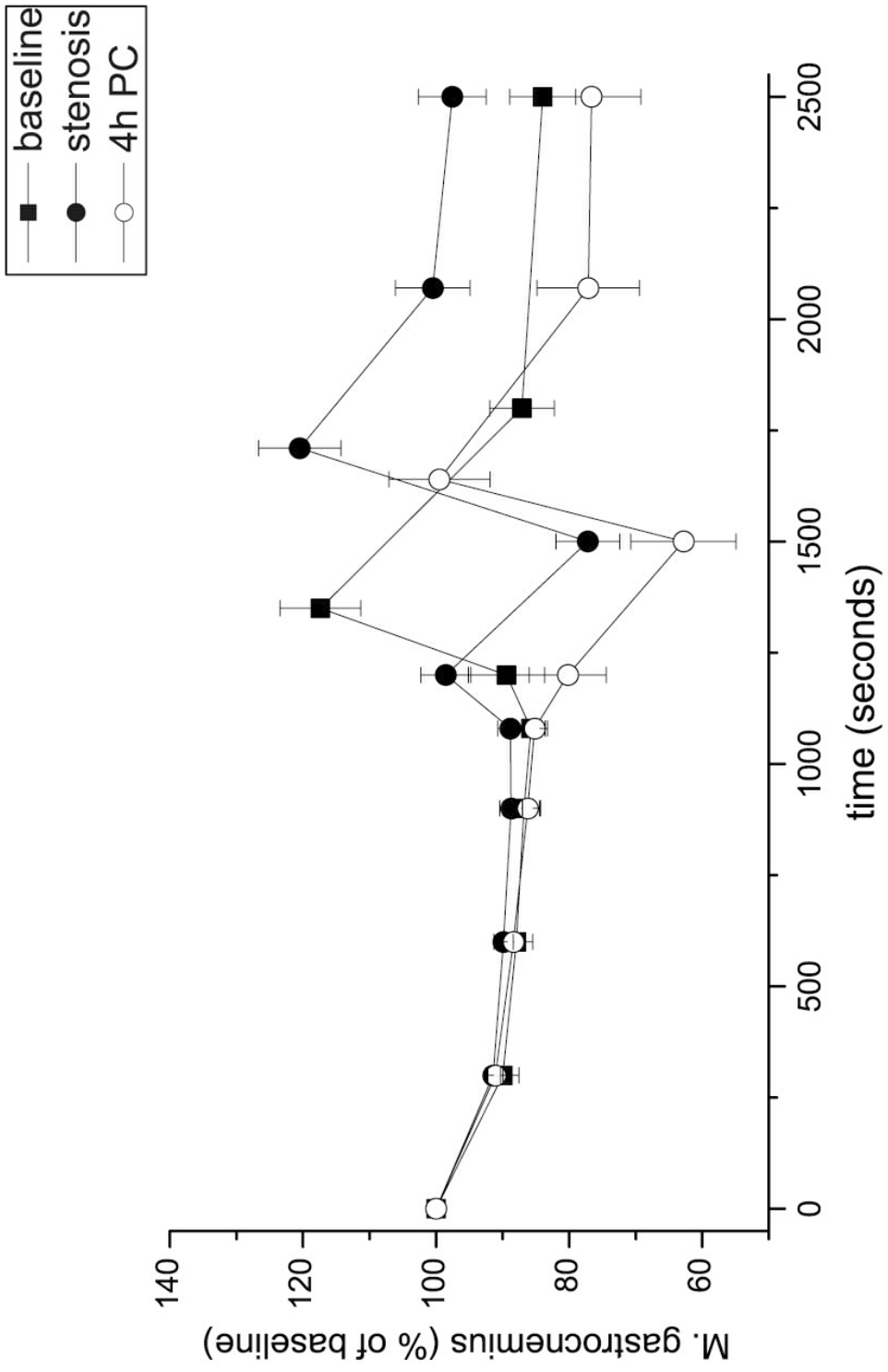
IPC 4 hours prior to ischemia did not affect the BOLD signal decline during ischemia, but it mitigated the peak BOLD signal following post-ischemic stenosis during reperfusion to 108±13% of the baseline value ($p=0.029$ vs. no IPC). This effect of IPC was evident in the gastrocnemius and soleus muscles, which were exhausted by anaerobic exercise, but it was not observed in the tibialis anterior muscle (Table 3-4, Figures 3-4, 3-5 and 3-6). IPC 48 hours prior to ischemia had no effect on the peak BOLD signal after the post-ischemic stenosis.

Table 3-4: Maximum BOLD muscle signal (% of baseline) after IRI and stenosis with or without IPC.

	no IPC (*n*=8)	4h IPC (*n*=8)	48h IPC (*n*=9)
All muscles	124±15	108±13*	123±11
Tibialis anterior muscle	121±12	113±10	128±19
Soleus muscle	130±20	109±14*	129±19
Gastrocnemius muscle	123±18	101±24*	114±8

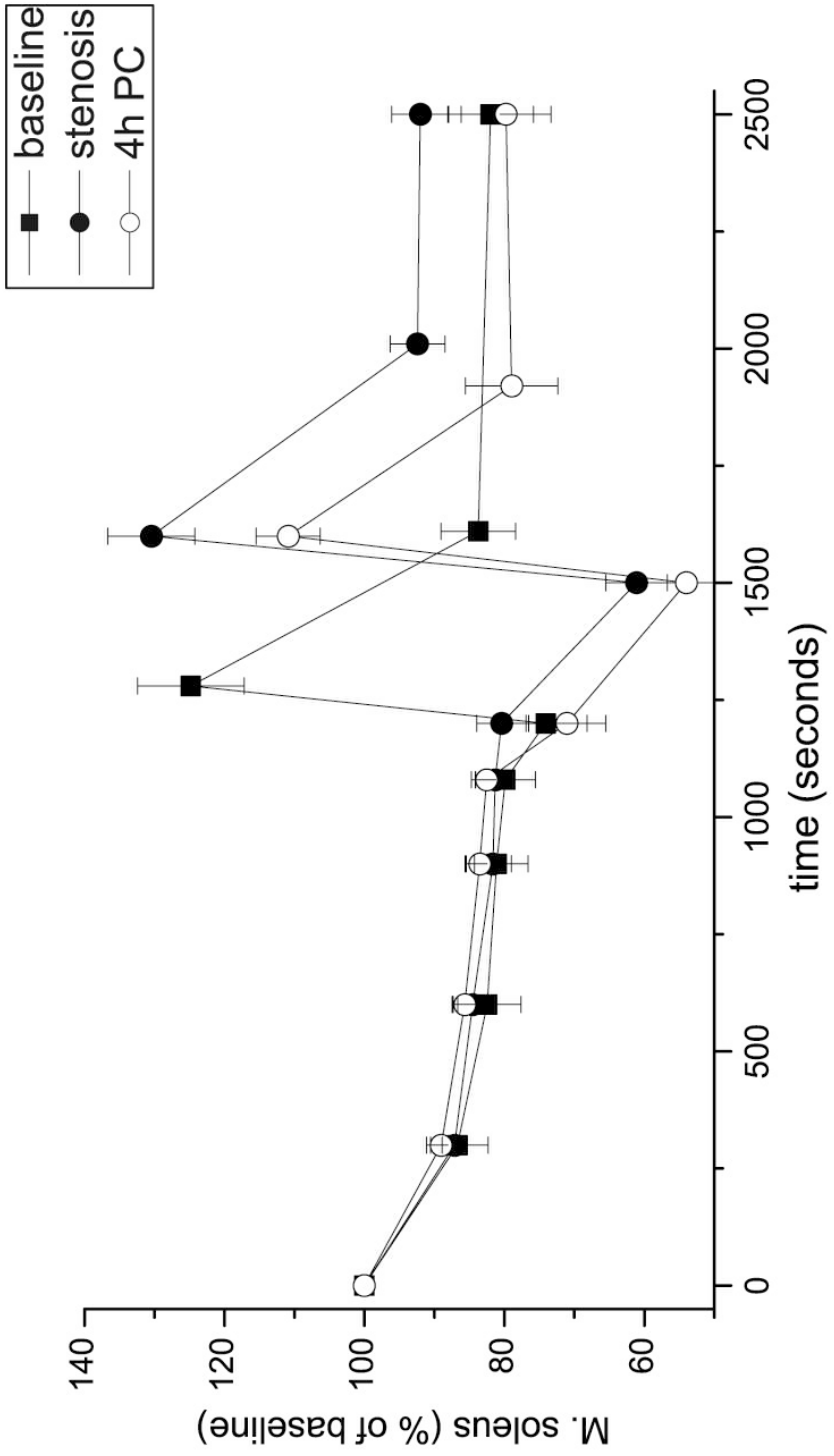
Data represent the means±SD. IPC: ischemic preconditioning, *p<0.05 vs. no IPC

Figure 3-4: BOLD fMRI time course (gastrocnemius)



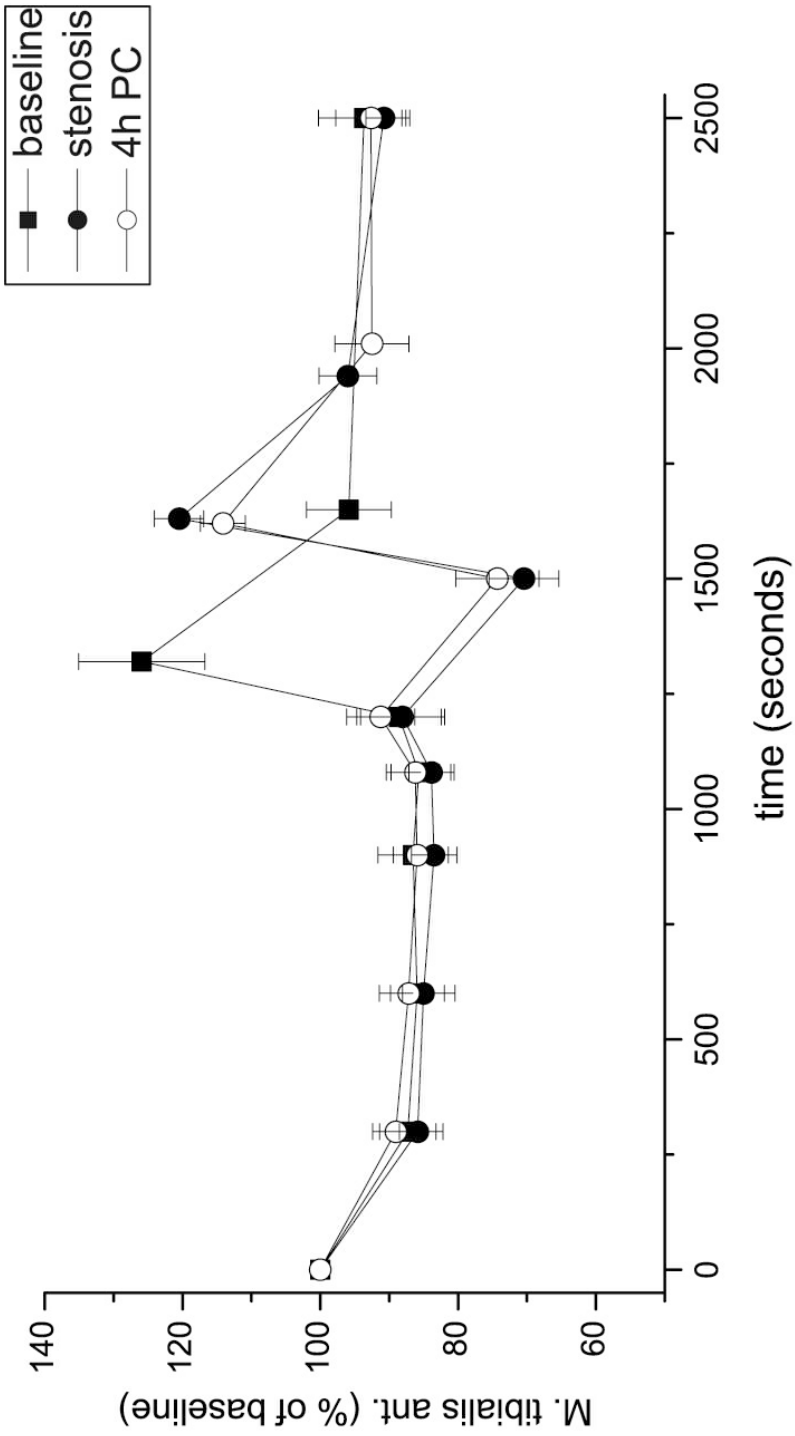
Data represent the means±SEM (*n*=8).

Figure 3-5: BOLD fMRI time course (soleus)



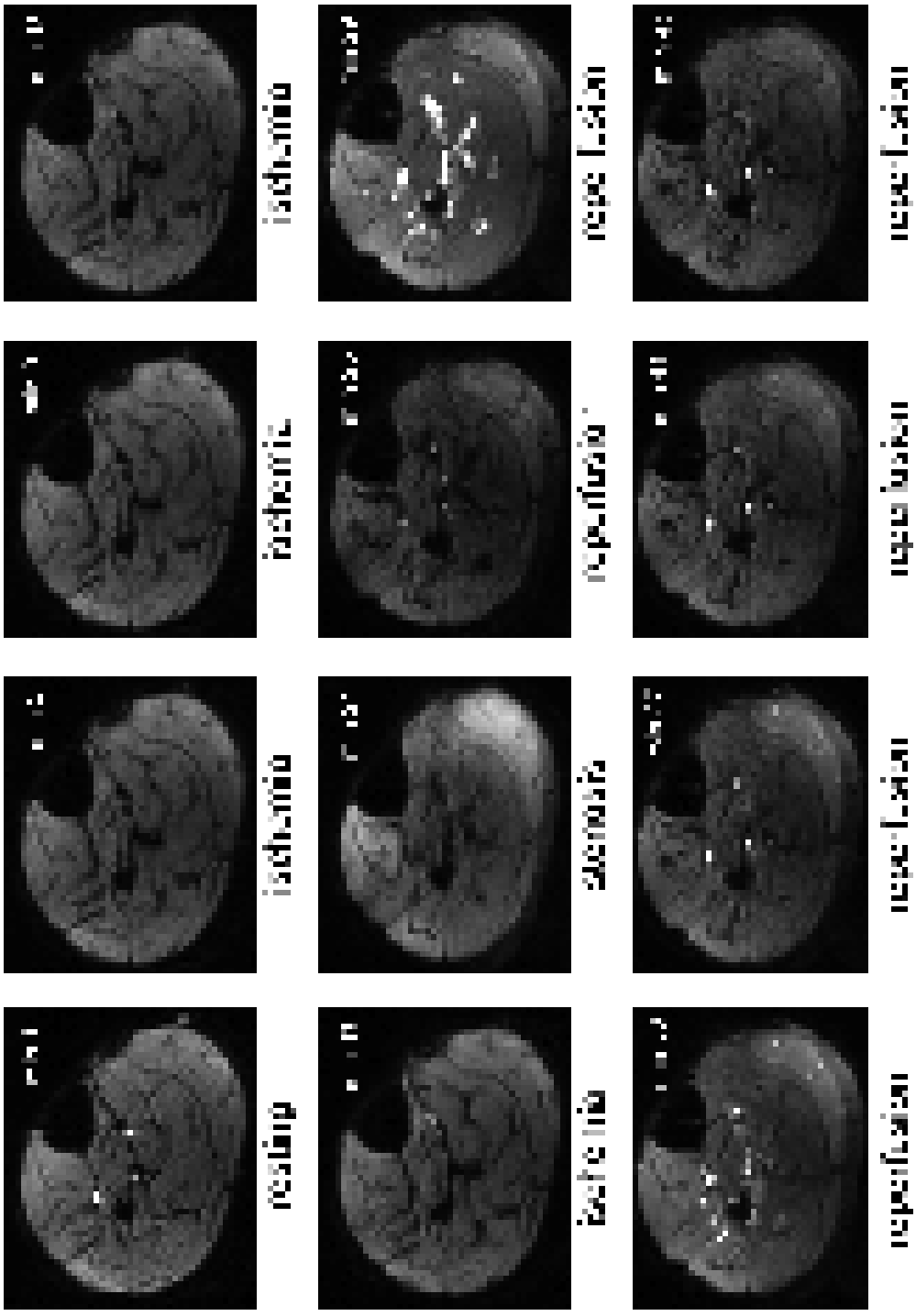
Data represent the means±SEM (*n*=8).

Figure 3-6: BOLD fMRI time course (tibialis anterior)



Data represent the means±SEM (*n*=8).

Figure 3-7: Echoplanar images for the BOLD signal analysis



The images are representative of the BOLD source data. An image for every 4th minute of data collection is shown.

Isometric muscle strength measurement after IPC

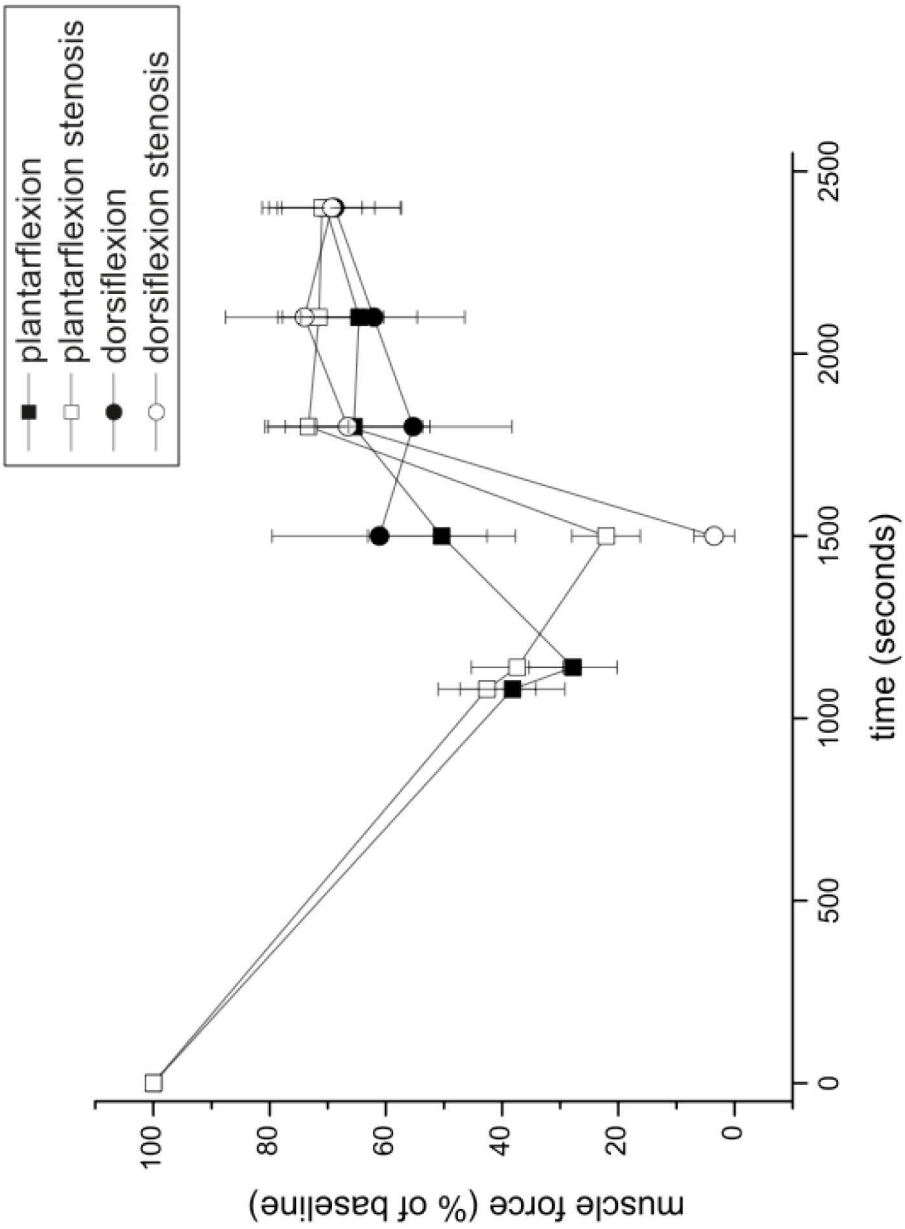
One participant withdrew his consent prior to the first day of the study, and the measurements for two subjects were unavailable for analysis on two study days for technical reasons.

The maximum ankle plantar flexion and dorsiflexion strengths were 129±48 Nm and 48±14 Nm at baseline, respectively. During ischemia, plantar flexion strength was reduced to 28±17% of the baseline value. Post-ischemic stenosis for 5 minutes further decreased plantar flexion strength to 22±16% of baseline (Figure 3-8). After 5, 10 and 15 min of complete cuff release, muscular strength recovered to 66±31%, 65±24% and 70±21% of the baseline value in subjects without post-ischemic stenosis and to 73±18%, 71±19% and 71±18% of the baseline value in subjects with post-ischemic stenosis (p=ns).

Plantar dorsiflexion strength was also reduced in response to ischemia. After the post-ischemic stenosis, muscle strength was 4±9% of the baseline value (p=0.007 vs. complete reperfusion). After 5, 10 and 15 min of complete cuff release, dorsiflexion strength recovered to 55±50%, 62±39% and 69±28% of the baseline value in subjects without post-ischemic stenosis and to 67±38%, 74±36% and 69±32% of the baseline value in subjects with post-ischemic stenosis (p=ns).

IPC 4 hours before ischemia had no effect on the time course of ankle plantar flexion or on dorsiflexion strength recovery.

Figure 3-8: Muscular strength with and without IPC



Ankle plantar flexion and dorsiflexion strength before and during ischemia and after reperfusion with or without post-ischemic stenosis. The data represent the means±SEM (*n*=7).

FACS – Analysis - Systemic laboratory effects of IRI

Data are summarized in Table 3-5. As expected, a substantial variability of CD42b and CD62P expression was noted at baseline before ischemia between subjects and across the different study days. The proportion of CD42b-positive cells increased 5 minutes after reperfusion (p=0.049). CD62P-positive monocytes tended to increase 5 minutes after ischemia, but were reduced compared to baseline after 24 hours (p=0.017).

Post-ischemic stenosis prevented an increase in CD42b-positive monocytes after complete reperfusion. There was no impact of impaired perfusion on CD62P expression.

IPC 4 hours or 48 hours before ischemia had no effect on the course of monocyte CD42b or CD62P expression compared to post-ischemic stenosis condition alone.

Table 3-5: Expression of CD42b and CD62P on monocytes

CD42b	Ischemia and exercise (*n*=11)	Post-ischemic stenosis (*n*=12)	Post-ischemic stenosis and 4h PC (*n*=10)	Post-ischemic stenosis and 48h PC (*n*=14)
Before ischemia	33±13	37±14	41±16	34±15
5 min after ischemia/exercise	50±9*	38±11	46±7	34±11
15 min reperfusion	41±9	35±7	43±12	40±14
24h reperfusion	45±13	38±9	37±11	30±10
CD62P				
Before ischemia	23±13	30±8	34±17	29±17
5 min after ischemia/exercise	32±12	30±11	25±14	24±14
15 min reperfusion	26±12	26±11	29±14	23±15
24h reperfusion	12±7	22±14	22±12	27±20

Data are % of positive cells presented as means±SD. *p<0.05 versus before ischemia

Discussion

Effects of ischemic preconditioning can be described by fMRI

Multi-modal *in vivo* MR in IRI enables the evaluation of mitochondrial oxidative metabolism, muscle metabolic changes and changes in perfusion. The additional measurement of functional skeletal muscle deficits allows for the interpretation of MR data and their relationship with force recovery.

Impaired reperfusion inhibits PCr recovery

Impaired reperfusion had a potent influence on the recovery of PCr in healthy subjects. PCr serves as an energy storage system in muscle; one high-energy phosphate is transferred to ADP via cytosolic creatine kinase for ATP replenishment during periods of increased energy demand. PCr recovery is maintained by oxidative phosphorylation and is catalyzed by mitochondrial creatine kinase (123).

The decline of PCr during ischemia is consistent with previous data (103, 105). However, low-flow reperfusion prevented the recovery of PCr and was paralleled by a further reduction of the BOLD signal and a decrease in muscular strength. The levels of ATP were stable during the entire process. This result may be explained by the increased ATP production via glycolysis in the cytosol, supported by decreasing pH values, which is induced by the preceding ischemia and ischemic contractions (124). Because it is driven by mitochondrial creatine kinase, PCr synthesis depends on mitochondrial ATP generation and is independent of cytosolic ATP production. Therefore, our data indicate that there is an insufficient level of oxidative phosphorylation during low-flow reperfusion and that ATP is stabilized via glycolytic

ATP generation, which is comparable to that observed during hypoxic perfusion (125).

Skeletal muscle PCr was restored far more rapidly after full reperfusion in comparison to muscular strength. In addition, muscular strength further decreased during stenotic reperfusion despite a stable ATP concentration. This seemingly unexpected finding may be readily explained by the data reported by Lanza et al., who state that the reduction of ATP consumption during ischemic contractions is due to increased muscular fatigue (125). Muscular fatigue is related to intracellular pH. Therefore, muscular strength may not be directly affected by PCr or ATP synthesis, thus explaining different recovery times. Unfortunately, post-recovery pH values are hardly measurable due to almost vanishing Pi MR signal. Although the subjects exhibited some variability in muscular strength and in their reactions to the ischemic stimulus, prolonged force impairment in the calf muscle was maintained. Therefore, our data highlight the importance of full and rapid reperfusion (as opposed to low-flow reperfusion) for the rapid recovery of muscle force.

Preconditioning effect on MR signals during reperfusion

Our results also demonstrate the influence of IPC on PCr and the BOLD signal. Whereas IPC was unable to prevent PCr depletion during ischemia or post-ischemic stenosis, peak concentrations of PCr after reperfusion were substantially higher after IPC compared the control group. This effect was only observed when IPC was applied 4 hours prior to ischemia. A mitigation of reperfusion-induced increases in the BOLD signal amplitude was consistently recorded by BOLD imaging in subjects who

were exposed to IPC 4 hours before ischemia. IPC 48 hours before ischemia did not affect the PCr and BOLD signal intensity.

^{31}P MRS signal changes

The ^{31}P spectroscopy was applied to assess muscle metabolism in different populations, conditions and diseases. The simultaneous measurement of ATP, PCr and Pi, and consequently pH, provides insight into the energy status of the tissue and its capacity to undergo oxidative phosphorylation. The effect of IPC on pH failed to gain significance (p=0.071). The PCr time course was the most useful analysis for elucidating the effects of IPC on IRI. This is the first study to demonstrate an effect of IPC using ^{31}P MRS in human skeletal muscle. However, Miyamae et al. have previously described the effects of IPC on ^{31}P spectroscopy in the porcine heart (126), in which, comparable to the present findings, a pronounced PCr overshoot was induced by IPC.

IRI induces cell death primarily via impaired and altered mitochondrial functions. Rapid ATP restoration is crucial for the re-establishment of mitochondrial homeostasis and the prevention of additional cell damage (127). The relatively short period of ischemia employed herein did not result in persistent mitochondrial damage or reduced cellular ATP content. IPC did not have any significant influence on PCr nor pH during resting ischemia, probably due to the fact that there was no damage done. However, previous results have also indicated that the effects of ischemic preconditioning include modifying the early reperfusion period rather than ischemia. This is therefore in line with our results, which report the significant influence of

preconditioning only after the reestablishment of circulation. The pronounced PCr overshoot 4h post IPC indicates a positive tissue response.

BOLD signal changes

The initial rapid decrease in the BOLD signal during ischemia is caused by hemoglobin deoxygenation (128, 129). During reperfusion, the signal increase is attributed to an elevated delivery of oxygenated hemoglobin and vasodilatation (130). In addition, the BOLD signal is influenced by the anatomic and vascular muscle structure and the (de)oxygenation of myoglobin (131-133). The effects of post-ischemic stenosis on venous return may explain the additional decrease in the BOLD signal during impaired reperfusion (130). The venous system fills continuously due to a slow arterial inflow, which increases the amount of deoxygenated hemoglobin and reduces the BOLD signal.

IPC significantly reduced the peak BOLD signal during hyperemia. The protection provided by IPC was most pronounced in the gastrocnemius muscle, which is a fast-twitch glycolytic muscle and is the main contributor to force in repeated plantar flexions (131). Therefore, this finding may be explained by a greater exhaustion of this muscle or by a muscle type-specific signal pattern (128). As previously described, the highest peak BOLD signal during reperfusion is observed in the soleus muscle (134). The soleus muscle is a slow-twitch muscle that is rich in capillaries and myoglobin, which likely account for the pronounced BOLD effect (135).

Flow-mediated hyperemia is a test of forearm resistance and conduit vasculature that is commonly applied to assess endothelial function. IPC has been previously shown to preserve forearm endothelial function after 20 minutes of ischemia (40). IRI leads

to a decrease in hyperemic flow-mediated dilatation that can be overcome by remote ischemic preconditioning. In contrast, the present data showed a less pronounced hyperemic peak in the muscular maximal BOLD signal. Thus, muscular BOLD-based imaging likely reveals far more complex information than solely flow-mediated dilatation because it integrates the effects of the diameter and recruiting of capillaries, the myoglobin saturation status, oxygen consumption and muscle-specific patterns. In this case of muscle exercise on top of ischemia, the interpretation becomes even more difficult. Although this diverse information enables new approaches in research, an accurate interpretation of the findings remains limited due to the lack of data and larger validation studies. For further BOLD signal interpretation in a similar setting, a concurrent monitoring with Doppler, arterial spin labeling or near infrared spectroscopy should be applied.

Multimodal ischemia-reperfusion assessments explain the BOLD signal reduction

An increase in PCr formation was observed during reperfusion 4 hours after IPC, which indicated that energy metabolism was improved during the post-ischemic period. This counter-regulatory PCr production was most likely due to an increase in oxidative phosphorylation in the reperfused tissue. PCr production depends on mitochondrial creatine kinase, consumes energy, and is produced by oxidative phosphorylation. The opposite effect (reduced PCr depletion during the post-ischemic period due to IPC) appears to be very unlikely because PCr levels in the absence of IPC reach pre-ischemic values, and PCr levels in the presence of IPC are higher than the pre-ischemic values. Thus, we suspect that there is a higher rate of oxidative phosphorylation in reperfused tissue after IPC and IRI. This higher rate

increases the demand for oxygen in preconditioned tissue after IRI and the formation of an oxygen gradient. Therefore, a reduced BOLD signal during the reperfusion period 4 hours after IPC may be readily explained by an increased oxygen demand in muscle cells and therefore a reduced ratio of oxygenated versus deoxygenated hemoglobin in comparison to non-IPC tissue. This hypothesis, however, remains to be proven.

Systemic laboratory effects of IRI

IPC 48 hours prior to ischemia decreased the levels of lactate dehydrogenase, as described previously (136). However, a reduction of lactate dehydrogenase was observed before the onset of ischemia. Because the levels of lactate dehydrogenase were within the normal range throughout the observation period, this result may be insignificant.

Platelets and monocytes are key players in IRI (68, 137, 138). Previous clinical trials reported diverging results of leukocyte CD62P or CD42b expression (33, 139). The expression profile in blood taken from a remote vein was also not homogenous in our results. While a transient increase of CD42b positive monocytes shortly after IRI was statistically significant within this group, the wide range of positive cells before ischemia puts this finding into a different perspective. Likewise, CD62P expression was subject to substantial scatter. FACS data therefore have to be interpreted with caution and add to the debate about clinically relevant leukocyte activation after this relatively short period of ischemia.

Chapter 4: Heme arginate for HO-1 induction to prevent ischemia reperfusion injury

Introduction

One of the key mechanisms of IRI is the rise of free oxygen radicals (140). In a second step, neutrophil activation and tissue invasion is part of the deleterious cascade of ischemia-reperfusion injury (IRI) (67).

As a new therapeutic approach in humans, the induction of the enzyme HO-1 may be employed to mitigate IRI (78, 141). HO-1 splits the toxic heme group and produces biliverdin, carbon monoxide (CO) and iron. HO-1 has anti-inflammatory, antioxidant, anti-apoptotic and antiproliferative activities (78, 80). HO-1 thereby may attenuate IRI. It can be induced by HA as described previously in detail (90, 91).

In this therapeutic exploratory study, we aimed to evaluate the effects of HA on skeletal muscle IRI in healthy humans. BOLD fMRI was used to measure alterations in tissue oxygenation with a high spatial and temporal resolution (103, 109). fMRI has previously been used to assess perfusion of the calf muscles in elderly people and patients with peripheral arterial occlusive disease after ischemia reperfusion experiments (101, 134, 142).

Methods

This section was previously published (143).

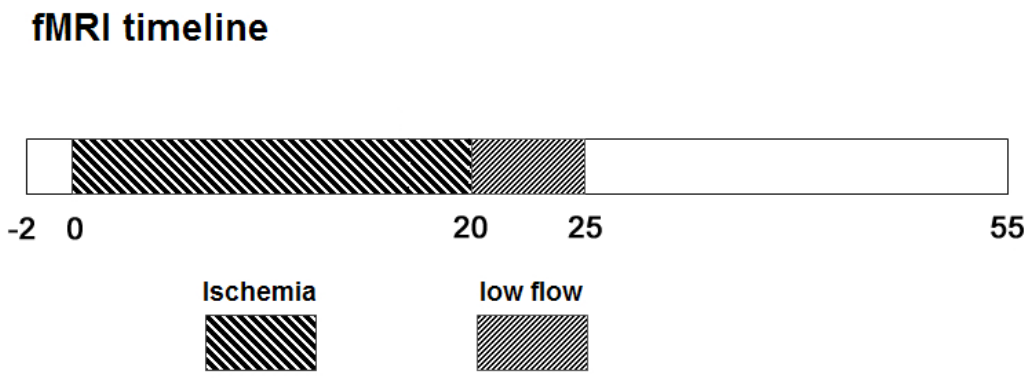
A two period, placebo controlled, observer blinded, randomized crossover trial was performed in 12 healthy male subjects between 18 and 46 years (age [mean ± standard deviation] 28±6 years, body mass index 22.6±2.1 kg/m^2). The Ethics Committee of the Medical University of Vienna approved the study protocol. All participants gave written informed consent prior to inclusion. One screening visit and two study periods with a washout time of at least 10 days in-between were scheduled for each participant. Physical examination, medical history, routine blood works including blood count, clinical chemistry, urine analysis and a 12-lead ECG were performed at the screening visit one week prior to the first study day. Subjects did not take any medication throughout the study and abstained from alcohol and stimulating beverages containing xanthine derivatives (tea, coffee) 48 hours before drug infusion and fMRI. Subjects fasted on study days. After the last treatment a final follow-up examination was performed.

A single dose of 1 mg/kg HA or placebo (sodium chloride) was administered as an intravenous infusion in the alternate study periods. HA was diluted to 110 ml with 0.9% NaCl. The infusion was administered within 15 minutes at an infusion rate of 440 ml/h using an infusomat (IP 85-2, Döring, Munich, Germany). A post-treatment infusion of 250 ml 0.9 % NaCl was administered to rinse perfusion lines.

Each study period consisted of three days. On the first day, placebo or HA were administered. On the second day, the fMRI and ischemia reperfusion protocol was performed. On the third day, a blood draw and a physical examination were done.

Ischemia was administered to the right lower leg for 20 minutes using a cuff inflated to 200 mmHg on the thigh (Figure 4-1). The cuff was deflated to 30 mmHg below systolic pressure for 5 minutes for slow reperfusion, mimicking impaired blood perfusion through the femoral artery. This stenosis model is described in detail in the general methods. A blood draw was performed before ischemia and 24 hours thereafter.

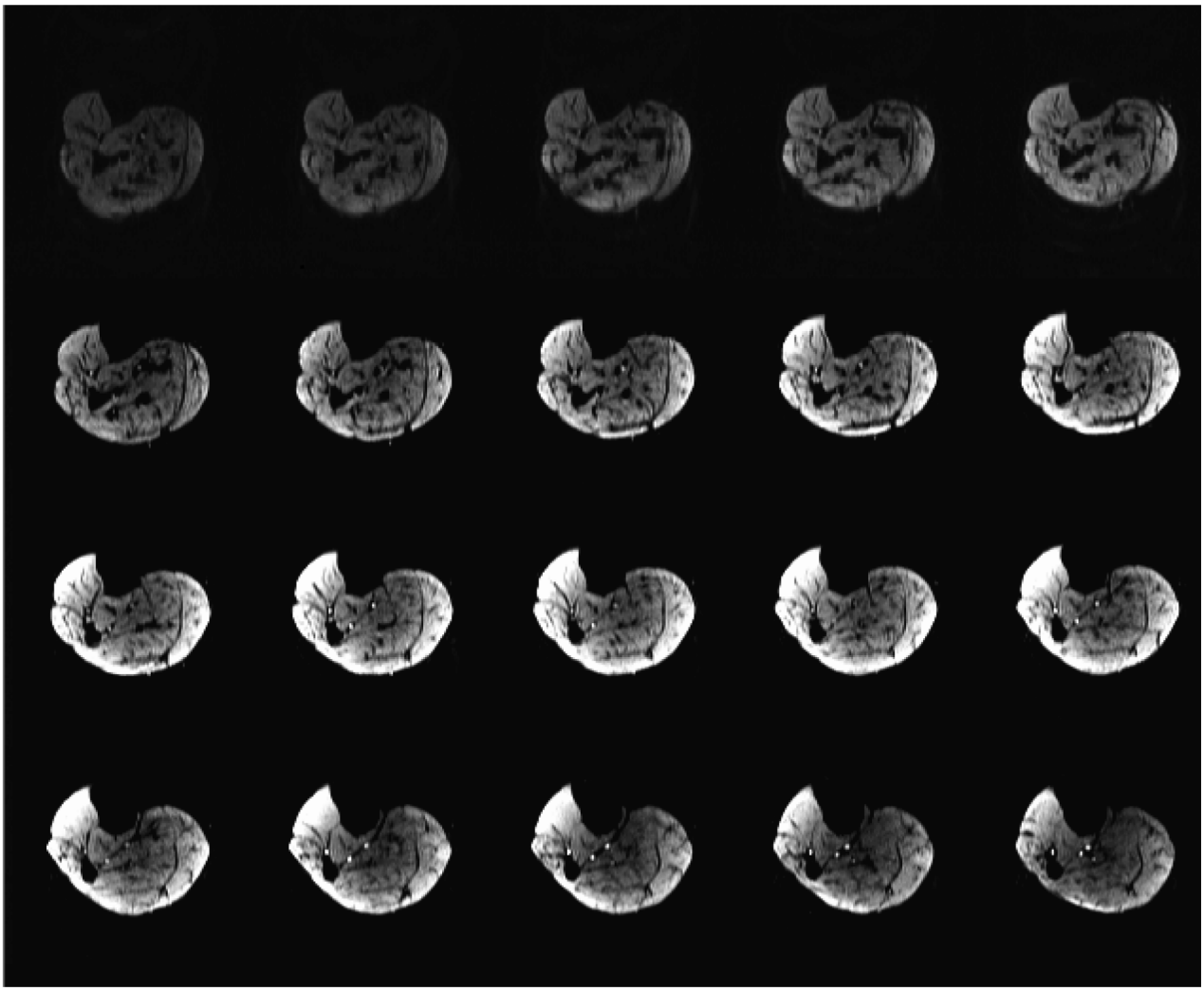
Figure 4-1: fMRI timeline for BOLD measurements



Time course is shown in minutes.

MR operators were blinded to the randomization sequence. A Siemens Tim Trio (Siemens Medical Solutions, Erlangen, Germany) was used for scanning. Anatomical images were acquired at the beginning of the MR examination. BOLD-imaging (EPI) had the following parameters: 128 x 128 matrix, twenty 5 mm axial slices, 1,42 mm in-plane resolution, T_R = 2 s, T_E = 28 ms. Imaging started two minutes before ischemia and lasted until 25 minutes after reperfusion (Figure 4-1 and 4-2). ROIs were drawn around the soleus, gastrocnemius and tibialis anterior muscle for signal analysis. The BOLD signal in the ROIs as well as from all voxels was summed to achieve a single time course for each individual muscle and the complete calf respectively. The starting point of reperfusion was determined manually in all individual data sets.

Figure 4-2: EPI images for every 4th minute during the experiment



To describe the BOLD response in calf muscle during reperfusion, the intensity time courses were fitted against a similar function as is used in dynamic contrast enhanced (DCE) first pass perfusion measurements using Matlab (Mathworks, Natick, MA, USA) using the Curve Fitting Toolbox (144):

$$f(t) = g(t) + s(t) + l(t) \qquad \text{Eq.[1]}$$

f(t) is the sum of gamma variate function *g*(*t*)

$$g(t) = g_0 \cdot (t - t_0)^{g_1} \cdot e^{-g_2 (t-t_0)} \qquad \text{Eq.[2]}$$

and a sigmoid function *s(t)).*

$$s(t) = s_0 \cdot \frac{1}{1 + e^{-s_1 (t - t_0 - t_1)}} \qquad \text{Eq.[3].}$$

A linear function *l(t)* was also added to take into account a possible drift of the signal during the experiment.

$$l(t) = l_0 + l_1 (t - t_0) \qquad \text{Eq.[4].}$$

The values of peak BOLD signal, time to peak and tangent slope were determined numerically from the fitted curves f(t) (Figure 4-3 and 4-4).

Figure 4-3: Graphical description of curve fitting

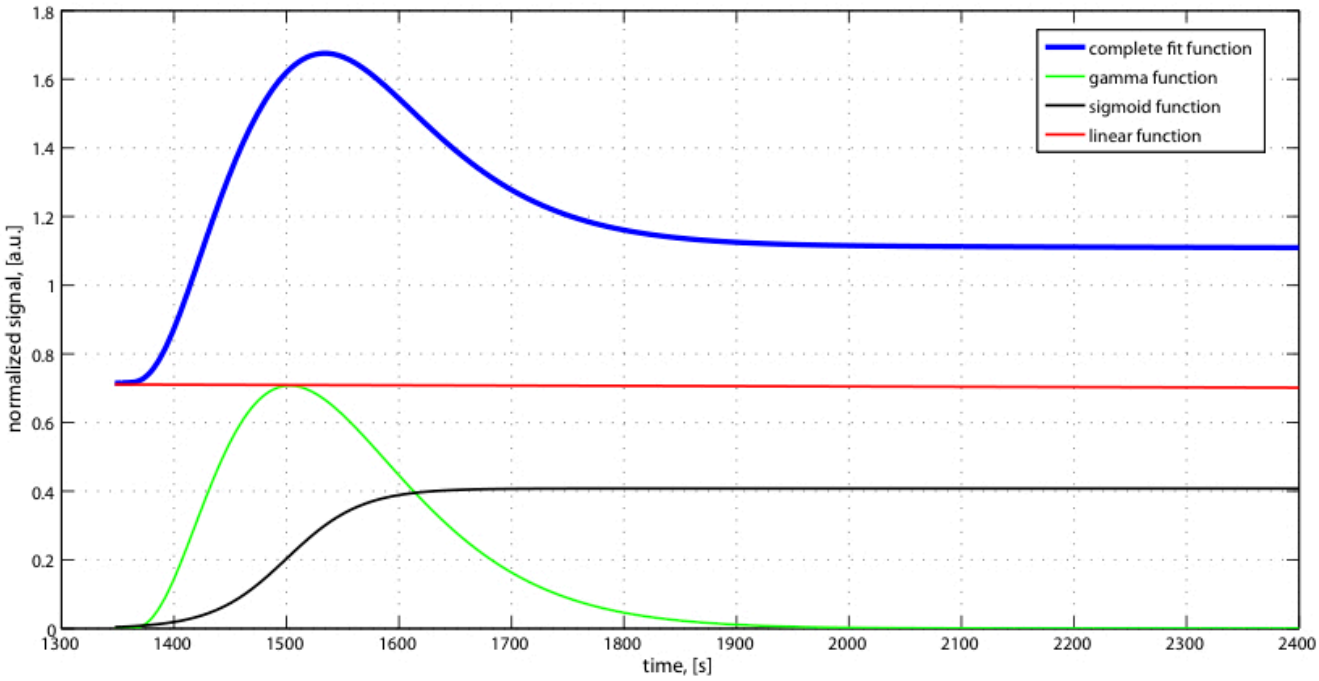
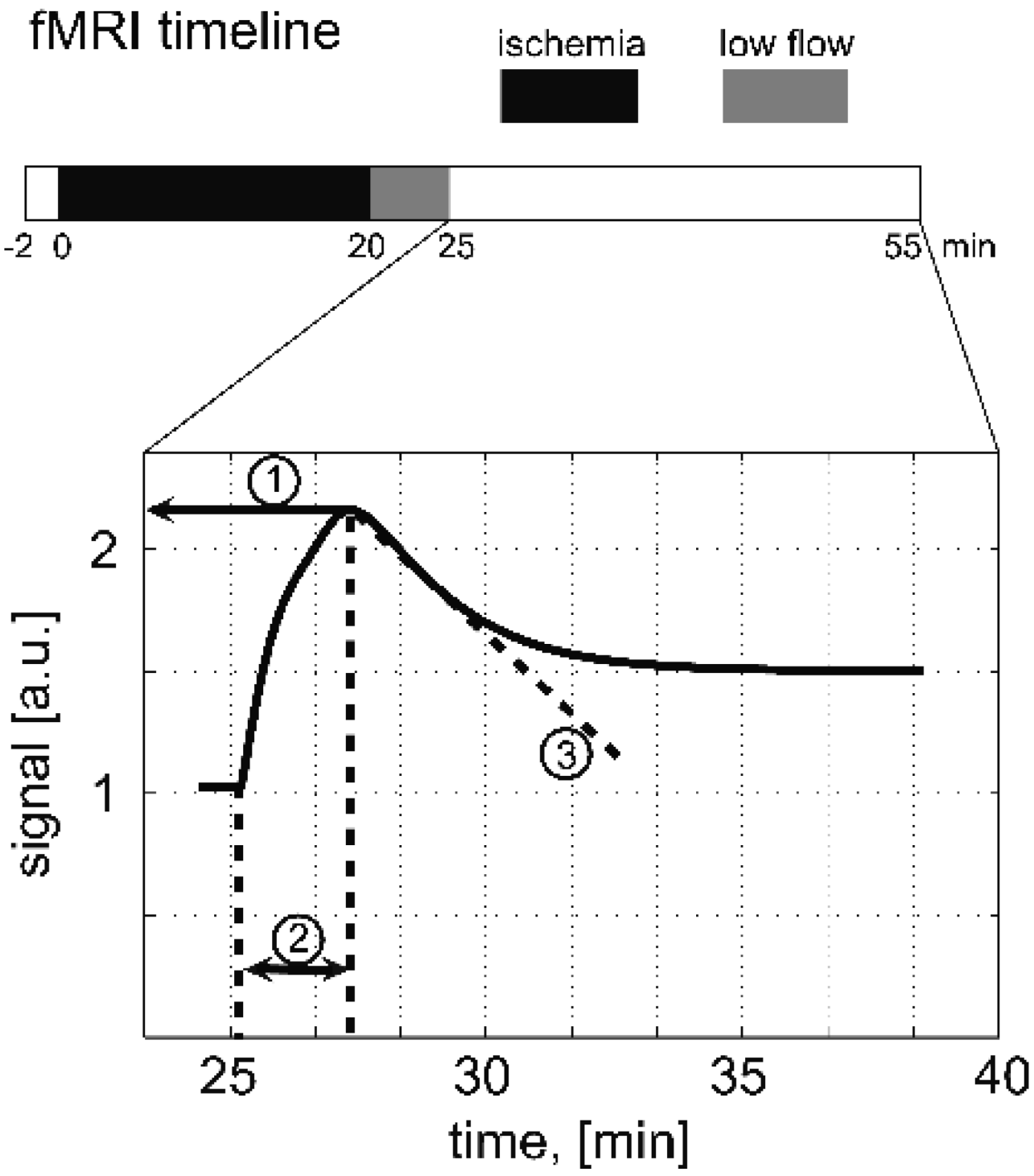


Figure 4-4: Description of parameters derived from fitted curves



Time course is shown in minutes; 1: peak BOLD signal; 2: time to peak; 3: tangent slope; a.u.: arbitrary units; min: minutes.

The effect of HA infusion on nitric oxide synthase was measured by NO_2 and NO_3 plasma concentration prior to HA administration as well as prior to and 15 minutes after ischemia as previously described (145).

Descriptive statistics were used for outcome and safety parameters. The fitted measures time to peak, peak BOLD signal and slope of reperfusion signal decline of each muscle of interest were analyzed as dependent variables using a general linear mixed model, including the fixed factors ‘treatment day’ (placebo or administration of HA) and muscle (soleus, gastrocnemius and tibialis anterior), and the random factor ‘subject’. An interaction of treatment day and muscle was evaluated and dropped from the model if not significant. Pairwise post-hoc comparisons between muscles were corrected for multiple testing using the Fisher’s least significant difference procedure. A two-sided p-value lower than 0.05 was considered to indicate statistical significance. PASW 18.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical computations.

Results

This section was previously published (143).

HA was tolerated well without adverse reaction. fMRI signals could not be analysed in one participant for technical reasons. No significant difference of vital signs or safety blood parameters could be detected between study periods (Table 4-1). An increased creatine kinase serum concentration of up to 490 U/l occurred 24 hours after ischemia in two different subjects (one receiving HA, one receiving placebo) but resolved spontaneously.

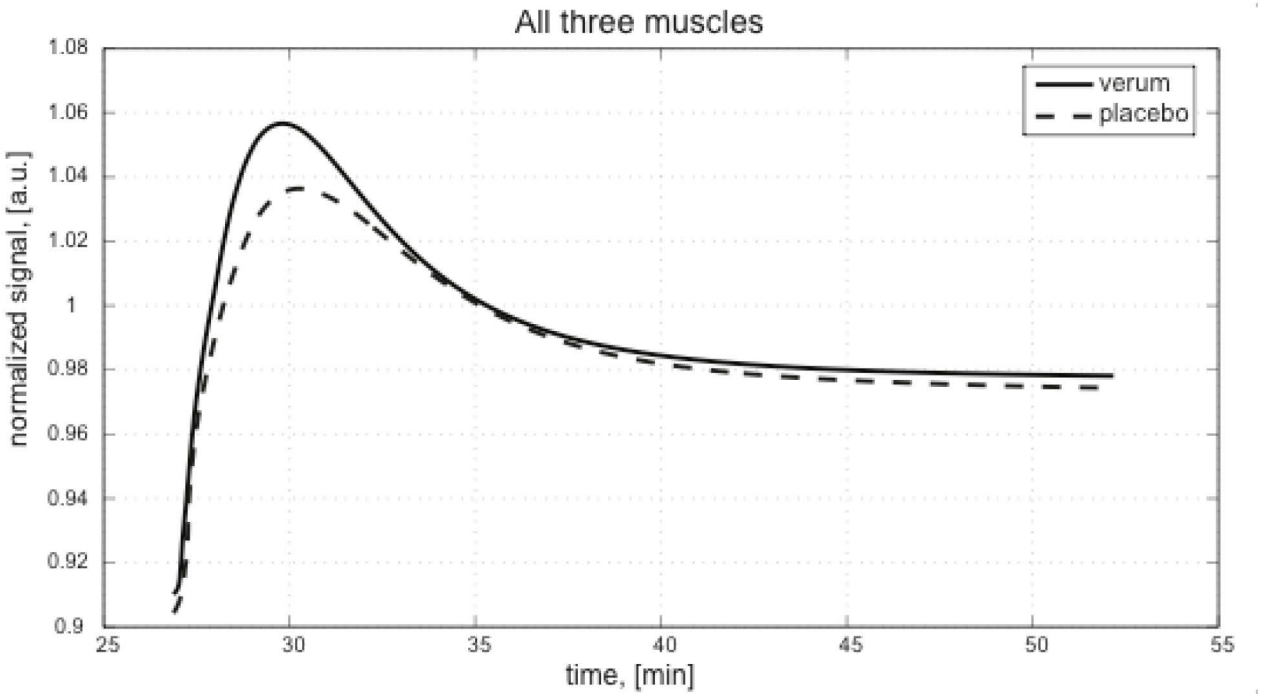
HA administration 24 hours prior to ischemia increased peak BOLD reperfusion signal from 104.5±0.6% to 106.2±0.6% compared to placebo (Figure 4-5; p=0.025). Furthermore, the time to peak reperfusion was reduced from 221±19 seconds to 175±16 seconds versus placebo (p=0.012). The calculated slope of reperfusion signal decline after peak was significantly steeper after heme arginate compared to control condition ($-2.8*10^{-4}\pm0.2*10^{-4}$ vs. $-2.1*10^{-4}\pm0.2*10^{-4}$; p= 0.002).

Table 4-1: Vital signs in treatment periods with placebo or heme arginate

	Placebo	Heme arginate
Systolic blood pressure pre ischemia (mmHg)	115±10	114±8
Diastolic blood pressure pre ischemia (mmHg)	80±8	77±8
Heart rate pre ischemia (bpm)	73±4	72±4
Systolic blood pressure post ischemia (mmHg)	116±10	115±9
Diastolic blood pressure post ischemia (mmHg)	81±7	80±10
Heart rate post ischemia (bpm)	71±2	70±3
Blood iron (μg/dl)	110±41	100±38
Protein (g/l)	76.0±3.8	75.9±2.3
Lactate dehydrogenase (U/l)	152±28	154±28
Creatine kinase (U/l)	148±114	146±87
C – reactive protein (mg/dl)	0.06±0.05	0.08±0.09

Data are presented as mean±SD, n=12.

Figure 4-5: BOLD signal during reperfusion for all three muscles

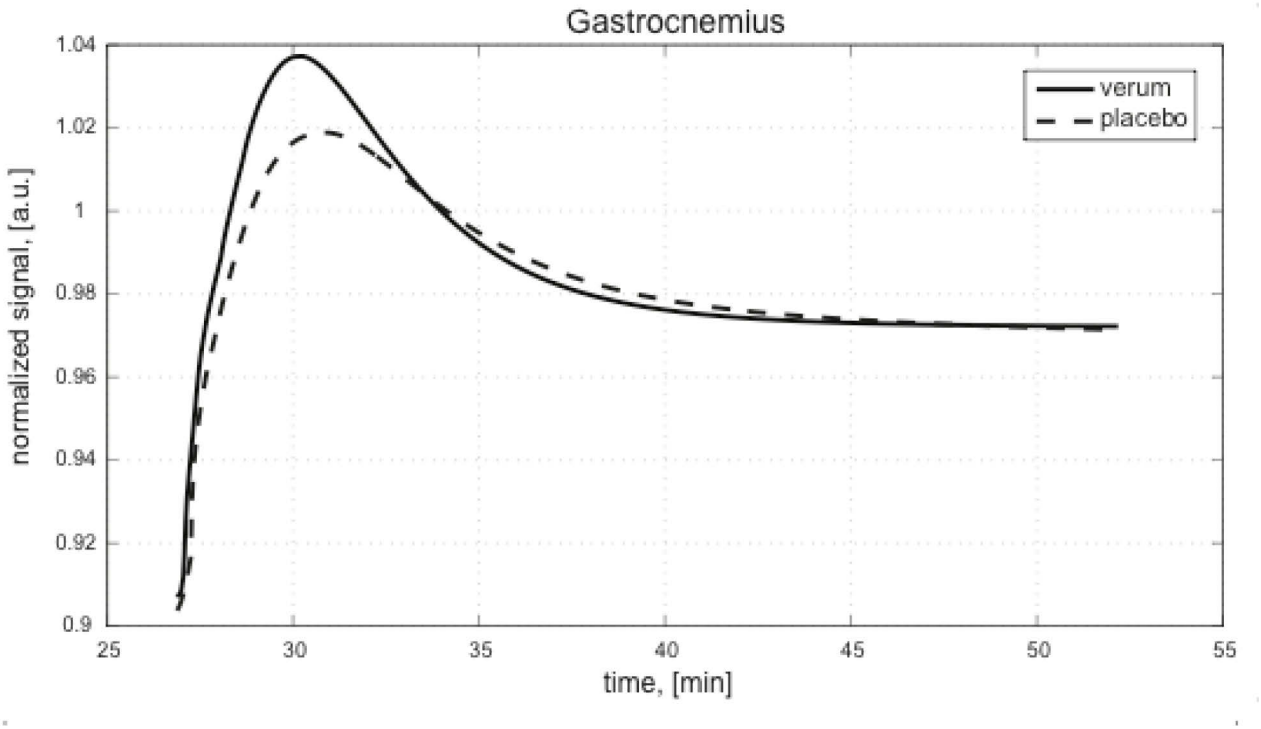


Reperfusion signal was fitted according to the described protocol; a.u.: arbitrary units, normalized to the pre-ischemic signal; min: minutes.

Reperfusion parameters for single muscle groups are depicted in Figure 4-6, 4-7 and 4-8 and summarized in Table 4-2. Peak reperfusion signal after placebo was highest in the soleus muscle and lowest in the gastrocnemius muscle (106.5±0.7% vs. 102.6±0.8%, p=0.019). A similar reaction across calf muscles to HA could be confirmed by statistical analysis.

Plasma concentrations of NO_2 and NO_3 were 2.7±1.4µmol/l and 13.1±8.3µmol/l 24 hours after HA administration compared to 3.5±1.8µmol/l and 12.4±8.3µmol/l after placebo, respectively (p=not significant). Plasma concentrations of NO_2 and NO_3 before HA administration and after ischemia were also unchanged.

Figure 4-6: BOLD signal during reperfusion (gastrocnemius)



Reperfusion signal was fitted according to the described protocol; a.u.: arbitrary units, normalized to the pre-ischemic signal; min: minutes.

Figure 4-7: BOLD signal during reperfusion (soleus)

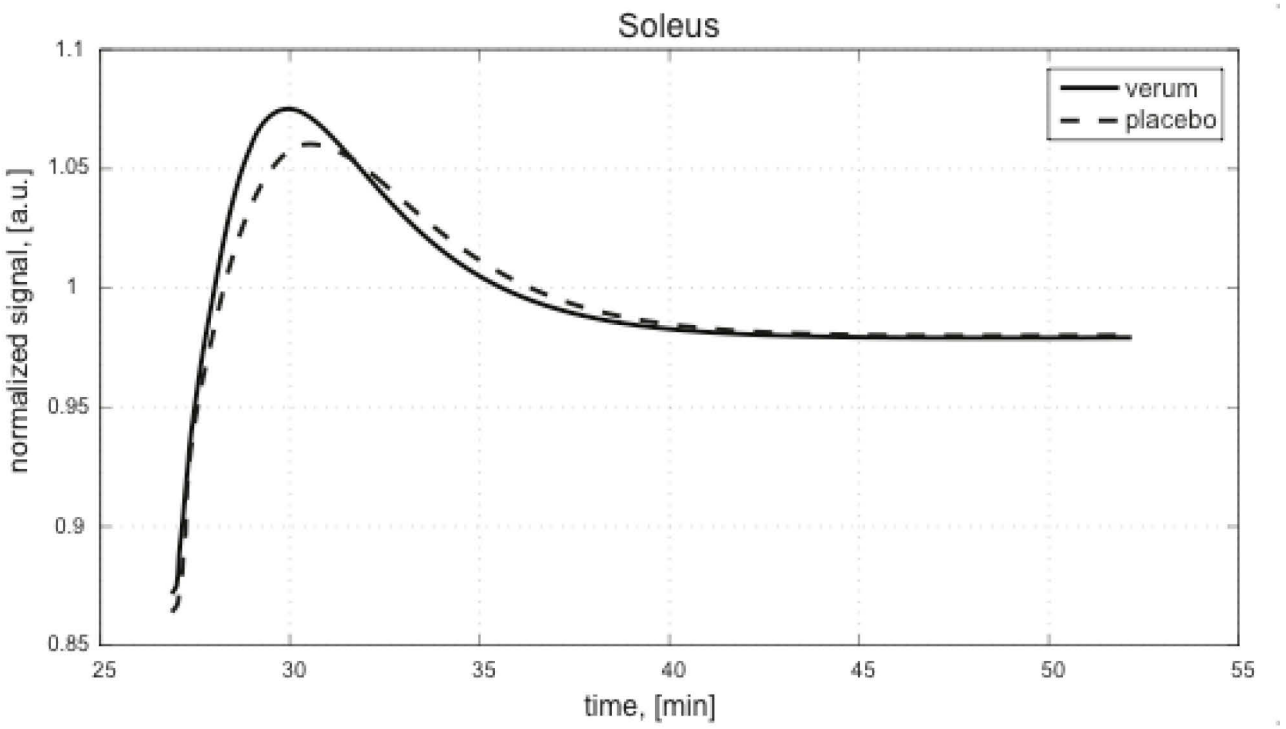


Figure 4-8: BOLD signal during reperfusion (tibialis anterior)

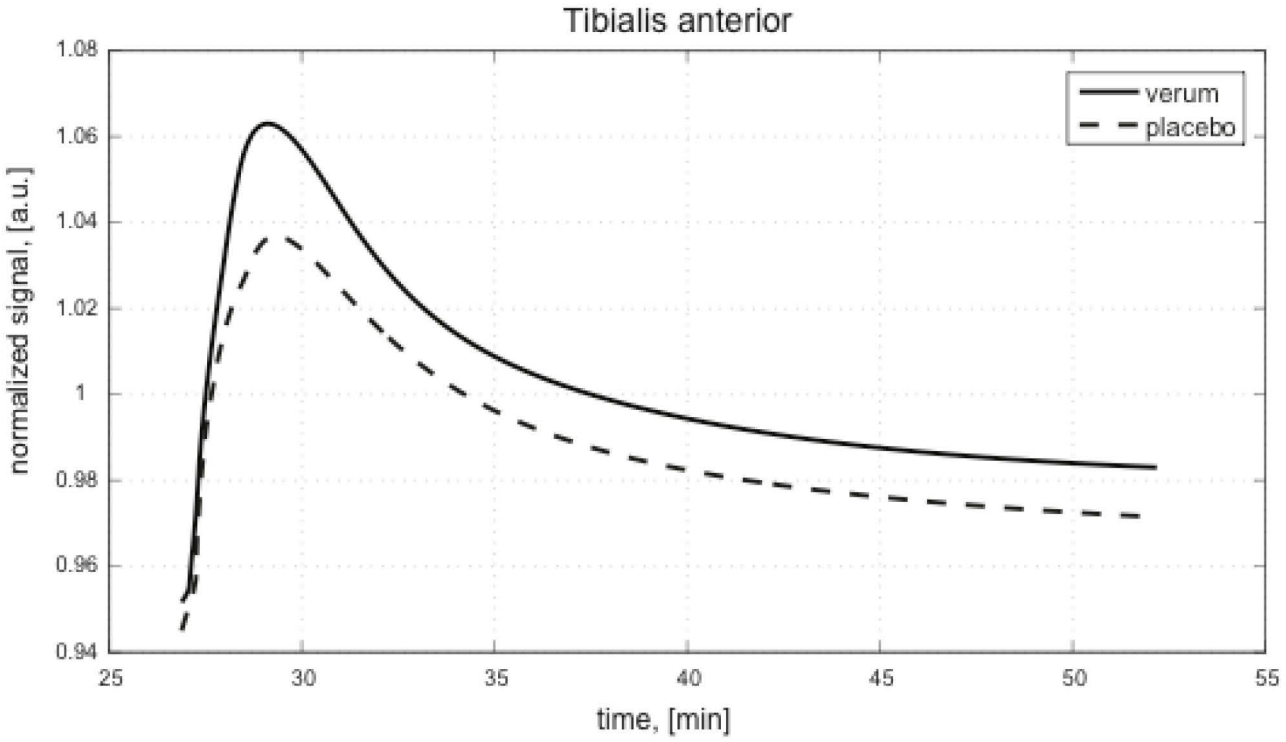


Table 4-2: BOLD signal of calf muscles after ischemia-reperfusion injury

All muscles	**TTP (s)**	**PEAK (%)**	**slope (1/s)**
Placebo	221±19	104.5±0.6	$-2.1*10^{-4}\pm0.2*10^{-4}$
HA	175±16	106.2±0.6	$-2.8*10^{-4}\pm0.2*10^{-4}$
p-value	0.012	0.025	0.002
Gastrocnemius			
Placebo	250±36	102.6±0.8	$-1.6*10^{-4}\pm0.2*10^{-4}$
HA	198±11	104.0±0.6	$-2.2*10^{-4}\pm0.2*10^{-4}$
Soleus			
Placebo	244±31	106.5±0.7	$-2.6*10^{-4}\pm0.4*10^{-4}$
HA	182±8	107.8±0.8	$-3.2*10^{-4}\pm0.4*10^{-4}$
Tibialis ant.			
Placebo	169±28	104.3±1.2	$-2.0*10^{-4}\pm0.3*10^{-4}$
HA	152±13	106.9±1.5	$-3.0*10^{-4}\pm0.4*10^{-4}$

Data are mean±SD, p-value is applicable for all comparisons, n=11. TTP: time to peak; PEAK: peak reperfusion signal; slope: Slope of the tangent at the inflection point of the BOLD signal curve after the peak; HA: heme arginate

Discussion

This study demonstrates beneficial effects of HA pre-treatment on endothelial function and reperfusion patterns after IRI in healthy humans. Full reperfusion signal occurred earlier, was higher and declined faster compared to placebo. We have previously reported that BOLD fMRI is able to characterize reperfusion patterns and the protective effects of ischemic preconditioning in IRI experiments (114). Furthermore, we have shown the ability of HA to induce HO-1 mRNA and protein in humans (91). The induction of HO-1 proved beneficial in several ischemia reperfusion models in animal studies (78, 87, 89). However, the application of HA against IRI in humans has not been reported yet.

We propose a stronger and earlier peak BOLD signal during reperfusion as the healthy response. This hypothesis is supported by two prior trials showing a decreased BOLD fMRI signal in elderly subjects (134, 142). Furthermore, subjects with overt peripheral artery occlusive disease presented with decreased and delayed peak BOLD signal during reperfusion (101). HA increased the peak reperfusion signal by 38% compared to the increase over baseline after placebo administration. This is in line with one report showing a 44% higher peak signal in healthy subjects compared to the BOLD signal increase during reperfusion in elderly subjects (134). We could further demonstrate a faster decline of the reactive hyperemia signal after the peak in the HA period, indicated by a steeper decline of the slope at the inflection point of the reperfusion signal curve.

The BOLD fMRI signal is determined by a combined influence of perfusion, vascular microarchitecture and oxygen saturation of hemoglobin and myoglobin. Unfortunately, the individual contributions of each of these factors to the final observed signal cannot be separated. Regions of interest were drawn around muscles to exclude confounding signal influences from main vessels, fatty tissue and bones. The soleus muscle, a slow-twitch fibre muscle, is known to show a high capillary density (131). It showed the highest peak reperfusion value, as also reported in previous trials (114, 134). Vasodilation of muscular capillaries may therefore be a strong contributor to the BOLD signal. Since this trial had a crossover design and results were analysed with a repeated measurement model, an influence of vessel architecture to the different peak signals is unlikely. Improved perfusion may therefore be the major contributor to the peak BOLD signal. However, muscular metabolism may also influence oxygen extraction and hemoglobin saturation during reperfusion (114). Therefore, the BOLD signal cannot be directly compared to reperfusion patterns measured by flow-mediated dilation or strain gauge plethysmography (146). It seems plausible that a protected endothelial cell function is responsible for faster and increased maximal reperfusion, leading also to more rapid and increased oxygen saturation in the muscles under study. The faster normalization of the reperfusion signal after peak may be an additional sign of protected endothelial cell function and adequate early reperfusion of muscular tissue. This protection may be mediated by decreased heme toxicity and increased antioxidative capacity due to HO-1 induction and the action of its enzymatic products biliverdin/bilirubin and carbon monoxide (78). Ischemia leads to a stasis of red blood cells in the ischemic tissue. Red blood cells are sensitive to reactive oxygen radicals which are increasingly produced during ischemia and early reperfusion (147, 148).

A limitation of this trial is the short period of ischemia. An increased ischemic period may reveal greater tissue injury and alter the protective effects of HA administration, but is not feasible due to ethical concerns. Further, we have not measured HO-1 mRNA and protein levels in this trial. Clinical studies in patients with acute ischemic events are mandatory to validate these experiments accordingly. To draw more general conclusions, a female group and subjects with different underlying diseases should be included in future studies.

Arginine, the substrate of nitric oxide synthase, may exert systemic vasodilator effects mediated by nitric oxide (149). To analyse a possible contribution of increased nitric oxide synthase activity induced by HA, NO_2 and NO_3 levels were measured in plasma. The lack of changes in systemic nitric oxide metabolites argues against major vascular nitric oxide stimulation as a confounder of our results.

Conclusions

In conclusion, application of heme arginate improves reperfusion patterns during IRI and may protect endothelial cells and skeletal muscle against IRI in humans. The potential clinical applications of this intervention have to be addressed in further trials.

Chapter 5: General Considerations and Summary

Comparing BOLD fMRI results after ischemic preconditioning with data after heme arginate administration

Our interpretation of the fMRI results after HA administration has to be discussed with caution in the context of our results after IPC. We reported an increased PCr overshoot and a decreased peak BOLD signal after IPC 4 hours prior to 20 minutes of ischemia. The PCr overshoot indicated an improved mitochondrial function by IPC, which may explain the increased oxygen demand leading to a decreased peak BOLD signal after IPC. Therefore, we suggest that the protective effects of HA are different from the effects of IPC. The induction of HO-1 seems to be downstream in the cascade of IPC (150, 151). Hence, different patterns in fMRI time series of these two protective interventions as demonstrated by fMRI so far are reasonable. As depicted in the introduction, the protection of IPC is effective during two time windows. The administration of HA for the induction of HO-1 is probably more comparable to the second window of protection, which affects the inflammatory response to IRI. Therefore, the short time effects of IPC, which are focused on the mitochondria and are only present during the first hours after preconditioning, may result in significantly different fMRI measurements than the induction of HO-1. However, the late effects of IPC could not be detected by our fMRI protocols. Therefore, we conclude that BOLD fMRI were influenced by mitochondrial metabolism after IPC, whereas HO-1

induction had no influence on mitochondrial performance, but was able to protect endothelial function after IRI. The further investigation of this hypothesis should be performed via experiments which separate perfusion measurement from tissue oxygen saturation. The adequate techniques for this approach are arterial spin labeling and near infrared spectroscopy, which are both planned to be established in the MR Center and are part of ongoing projects. However, the fMRI measurement parameters applied in our two trials were not identical. We increased the number of slices and decreased the slice gap for reduced artefacts from through-plane motion at the cost of reduced temporal resolution and shorter echo time. This had the further consequence of a better signal-to-noise ratio but showed a slightly different contrast. Therefore, a direct, numerical comparison between the trials is not possible (114).

Conclusions

Combined high-field ^{31}P MRS and BOLD fMRI imaging are suitable techniques for the evaluation of ischemia and reperfusion mechanisms in skeletal muscle and can be used to test therapeutic strategies in humans.

IPC had a significant impact on reperfusion energy metabolism and induced a prolonged PCr overshoot. Therefore, a direct action of IPC on mitochondrial function is suggested, which is in line with the current literature. Further, these positive effects of IPC could be reliably measured by ^{31}P MRS. Future trials applying this methodology may be able to optimize IPC protocols.

The beneficial effect of IPC reduced peak reperfusion signal measured by BOLD fMRI. However, BOLD fMRI scanning should be combined with perfusion

measurements in future trials in order to evaluate the exact amount of perfusion effects and metabolic changes during reperfusion.

The infusion of heme arginate improves reperfusion patterns during IRI and may protect endothelial cells and skeletal muscle against IRI in humans. Our data support the hypothesis that the cellular protection conferred by HO-1 induction differs from IPC. The potential clinical applications of this intervention has to be addressed in further trials.

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List of Abbreviations

ADP = adenosine diphosphate

Akt = protein kinase B

ATP = adenosine triphosphate

BOLD = blood oxygen level dependend

cGMP = cyclic guanine monophosphate

CK = creatine kinase

CRP = C-reactive protein

DCE = dynamic contrast enhanced

ECG = electrocardiogram

EPI = echo-planar images

FACS = fluorescence activated cell sorting

fMRI = functional magnetic resonance imaging

GSK-3b = glycogen synthase kinase

H_2O_2 =hydrogen peroxide

HA = heme arginate

Hb = hemoglobin

HO – 1 = Heme oxygenase - 1

IPC = ischemic preconditioning

IRI = ischemia-reperfusion injury

JAK = Janus activated kinase

LAD = left anterior descendent artery

LDH = lactate dehydrogenase

mPTP = mitochondrial permeability transition pore

MR = magnetic resonance

MRS = magnetic resonance spectroscopy

NO = nitric oxide

NOS = nitric oxide synthase

OH = hydroxyl radical

PCr = phosphocreatine

Pi = inorganic phosphate

RISK = reperfusion injury salvage kinases

ROI = region of interest

ROS = reactive oxygen species

SAFE = survivor activating factor enhancement

SNR = signal to noise ratio

STAT3 = signal transducer and activator of transcription 3

SOD = syperoxide dismutase

T = Tesla

List of Tables

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Curriculum Vitae

Martin ANDREAS, MD, MBA

born on the 4th of February, 1983 in Vienna

Department of Cardiac Surgery,

Medical University of Vienna

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Education

Medical University of Vienna

22.03.2010: finished MBA Health Care Management

Master Thesis: *‚Prozessoptimierung und Kapazitätsmanagement im operativen Bereich'*

29.11.2006: start of PhD curriculum 'vascular biology'

thesis in the WWTF sponsored project '*Therapy of Ischemia-Reperfusion-Injury by Heme Oxygenase-1 Induction in Skeletal Muscle and Ischemic Kidney'*

01.10.2001 - 29.11.2006: MD

doctoral theses: *"Pathogens associated with Acute Exacerbation of Chronic Bronchitis"*

Department of Internal Medicine I

received two scholarships for doctoral theses

received scholarships for good academic performance in the 1st and 3rd section

Secondary Education

BRG Maroltingergasse, Vienna, Austria, graduated with distinction on the 12th of June 2001

Absolved postgraduate clinical training for internal medicine

Clinical pharmacology (17 months), Cardiology (5 months), Gastroenterology and Hepatology (5 months)

Absolved postgraduate clinical training for cardiac surgery

Cardiac Surgery 40 months

International Clerkships

Dr. Jeyasekharan Hospital, India, Nagercoil 14.7.2004 – 28.7.2004

Clinical Clerkship at the

Department of Pulmonary and Critical Care Medicine

Mayo Clinic, Rochester, Minnesota 5.7.2005 – 29.7.2005

Research

2004 - 2007

Associate researcher in the laboratory for clinical microbiology,

Department of Internal Medicine I, Medical University of Vienna

2005 - 2011

Associate researcher in the research group of Prof. Dr. Michael Gottsauner-Wolf,

Department of Cardiology, Medical University of Vienna

since 2007

Associate researcher at the cardiovascular research group (Prof. Wolzt),

Department of Clinical Pharmacology, MUV

Current

Research group for valvular heart disease (Prof. Kocher)

Grants

Wiener Bürgermeisterfonds 2008:

Die Bedeutung von VKOR und MGP bei Aortenaneurysmen (23.200 €)

Martin Andreas, Martin Czerny, Heidi Panzenböck, Christine Mannhalter,

Michael Wolzt, Michael Grimm, ***Irene Lang***

Jubiläumsfonds österreichische Nationalbank 2010:

Cardioprotection and Regeneration induced by PICSO (85.000 €)

M. Andreas, C. Khazen, L. Leitner, M. Hülsmann, Ch. Loewe, D. Hutschala, B. Mora, P. Mares, D. Bernhard, P. Wadowski, G.Heinze, ***W. Mohl***

Wiener Bürgermeisterfonds 2011:

Bbeta(15-42) zur Reduktion des Kapillarlecks und des Organschadens als Folge der extrakorporalen Zirkulation an der Herz-Lungenmaschine (42.478,58€)

M. Andreas*, M. Wagner, D. Wiedemann, H. Bergmeister, D. Bernhard, J. Sandkühler, P. Petzelbauer, G. Laufer*

Publications

Heme arginate improves reperfusion patterns after ischemia: a randomized, placebo-controlled trial in healthy male subjects.

Andreas M, Schmid AI, Doberer D, Schewzow K, Weisshaar S, Heinze G, Bilban M, Moser E, Wolzt M.

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Systemic endothelin receptor blockade in ST-segment elevation acute coronary syndrome protects the microvasculature: a randomised pilot study.

Adlbrecht C, **Andreas M**, Redwan B, Distelmaier K, Mascherbauer J, Kaider A, Wolzt M, Tilea IA, Neunteufl T, Delle-Karth G, Maurer G, Lang IM.

EuroIntervention. 2012 Apr;7(12):1386-95. doi: 10.4244/EIJV7I12A218.

Materialization of ghosts: Severe intracardiac masses after pacemaker lead extraction requiring immediate surgical intervention

M. Andreas, D. Wiedemann, A. Kocher, C. Khazen.

Image - Heart Rhythm 2012

Characteristics of TAV- and BAV-associated thoracic aortic aneurysms-Smooth muscle cell biology, expression profiling, and histological analyses.

Blunder S, Messner B, Aschacher T, Zeller I, Türkcan A, Wiedemann D, **Andreas M**, Blüschke G, Laufer G, Schachner T, Bernhard D.

Atherosclerosis. 2012 Feb;220(2):355-61. Epub 2011 Nov 28.

Comparison of measuring energy metabolism by different (31) P-magnetic resonance spectroscopy techniques in resting, ischemic, and exercising muscle.

Schmid AI, Schrauwen-Hinderling VB, **Andreas M**, Wolzt M, Moser E, Roden M.

Magn Reson Med. 2011 Aug 12. doi: 10.1002/mrm.23095. [Epub ahead of print]

Effect of ischemic preconditioning in skeletal muscle measured by functional magnetic resonance imaging and spectroscopy: a randomized crossover trial.

Andreas M, Schmid AI, Keilani M, Doberer D, Bartko J, Crevenna R, Moser E, Wolzt M.

J Cardiovasc Magn Reson. 2011 Jun 30;13(1):32. [Epub ahead of print]

N-terminal-pro-brain natriuretic peptide is decreased in insulin dependent gestational diabetes mellitus: a prospective cohort trial.

Andreas M, Zeisler H, Handisurya A, Franz MB, Gottsauner-Wolf M, Wolzt M, Kautzky-Willer A.

Cardiovasc Diabetol. 2011 Apr 13;10:28

Haem arginate infusion stimulates haem oxygenase-1 expression in healthy subjects.

D Doberer, A Haschemi, **M Andreas**, TC Zapf, B Clive, M Jeitler, H Heinzl, O Wagner, M Wolzt, and M Bilban; Br J Pharmacol. 2010 Dec;161(8):1751-1762.

NT-proBNP is increased in healthy pregnancies compared to non-pregnant controls.

Franz MB, **Andreas M** (shared first), Schiessl B, Zeisler H, Neubauer A, Kastl SP, Hess G, Rhomberg F, Zdunek D, Maurer G, Schlembach D, Heinze G, Szekeres T, Gottsauner-Wolf M.

Acta Obstet Gynecol Scand. 2009;88(2):234-7.

Current positions

Resident at the Department of Cardiac Surgery, Medical University of Vienna

www.muw.ac.at

Vice – President, Alumni-Club, Medical University of Vienna

www.alumni-meduniwien.at

Academic teaching

PGU (physikalische Gesundenuntersuchung)	SS07, SS08, SS09
PKU (physikalische Krankenuntersuchung)	SS08
Ärztliche Gesprächsführung	SS08
Reanimation	WS08
Planung klinischer Studien	SS09
Chirurgische Wundversorgung	WS09, WS10
Training Devices in Cardiac Surgery	SS11, SS12
Applied Research in Cardiac Surgery	WS11/12, SS12

Chairmanships

2nd Chairman of the students representation at the MUV Jul. 03 – Jul. 06

www.uv-medizin.at

Chairman of Medico Oct. 03 – Oct. 06

Medico is a student's platform for medical aid in 3rd world countries.

Chairman of the "Österreichische Mediziner Union" (OEMU) Sep. 03 – Jul. 05

OEMU is a student party at the Medical University of Vienna

www.oemu.at