The role of mitochondrial hexokinase II in ischemia-reperfusion damage
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GENERAL INTRODUCTION

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Chapter 1

Being the world-wide leading cause of death ischemic heart disease equally affects men and women in both middle-to-low-income and high-income countries (72). Major causes are so-called behavioral risk factors, like unhealthy diet and physical inactivity. Ischemic heart diseases manifest as events like acute myocardial infarction, arrhythmias and heart failure. Acute myocardial infarction results from a reduced or completely blocked blood supply to the heart muscle. With proceeding duration of this state a gradually increasing number of cardiomyocytes is irreversibly injured and the degree of tissue loss will incline accordingly. The size of the infarct is the major determinant of further prognosis for patients (5; 56) and therefore needs be reduced as much as possible. The solution to this is not as simple as reducing the length of the anoxic or hypoxic event as much as possible by timely reperfusion, because reperfusion itself also induces cell death. Therefore, ischemia and reperfusion should not be seen as separate targets for therapy. The necessity of a continuous search for new insights and therapeutic possibilities in order to be able to reduce and prevent ischemia-reperfusion damage is obvious. Attenuation of cell death mechanisms initiated during both ischemia and reperfusion is a very promising and effective therapeutic possibility and therefore needs further elucidation.

Ischemia-reperfusion injury

The duration and severity of ischemia, which is the reduction or complete loss of blood flow, and reperfusion, which is the restoration of blood-flow, both have physiological effects on the cellular recovery from the ischemia-reperfusion (I/R) event. The longer tissues are deprived from the necessary oxygen and metabolite supply, the more severe the resulting cellular damage will be. Interestingly, ischemia can also have beneficial effects as very short periods of ischemia (<10 min) actually are protective to tissue (44). However, a longer period of ischemia (10-20 min) results in reversible cellular damage and more persistent ischemia (>20 min) results in severe, irreversible damage (24; 68). Thus, early reperfusion is of utmost importance in order to increase the success of ischemic tissue salvage.

Cellular survival is only possible if cells are still in the reversible damage condition. However, it should be noted that the heart consists of numerous different cell types and
regional differences concerning metabolism and energy needs exist. Throughout the complete heart, cardiac I/R will result in heterogeneous cell death as irreversible cell death develops first in regions with the highest metabolism and energetic needs. Other regions have lower metabolic needs and will suffer significant damage after a more prolonged period of ischemia. This heterogeneity of cell death occurring should be kept in mind when judging the effects of I/R in the complete heart.

Furthermore, besides the loss of flow, the recurrence of blood flow has also been recognized as detrimental as it also induces cell death, called reperfusion injury. It is estimated that approximately up to 50% of I/R injury is caused by reperfusion injury. Ischemia and reperfusion injury are not necessarily separate pathophysiological events due to the physiological events initiated during ischemia, which are continued and/or are of significant influence during reperfusion. Therefore, these pathophysiological terms are therefore referred to as ischemia-reperfusion injury.

**Metabolic features ischemia-reperfusion.** In the normal, normoxic state, cardiomyocytes use fatty acids (~60%) (63) and carbohydrates (glucose, lactate, pyruvate; ~40%) (14; 28) for energy production. However, cardiomyocyte substrate preference depends amongst others on exogenous substrate concentrations, which depend on blood supply. During complete blood flow cessation, as in no-flow ischemia, metabolic residue accumulation and the lack of both oxygen and exogenous substrates supply force the cell to change its metabolic preferences. The first moments of ischemia the rate of glycolysis is increased, but will halt when ischemic conditions sustain. During continuing oxygen deprivation anaerobic metabolism is used as the oxidative metabolism is shut down (3; 32). However, conditions of residual blood flow, referred to as low-flow ischemia, are more usually the case in clinical conditions. The degree of supply cessation determines the extent of metabolic switch to glycolysis (4), which will maintain ATP production. As cellular homeostasis is less disturbed, low-flow ischemia is characterized by less cellular ischemia-reperfusion damage than no-flow ischemia.

**Physiological features ischemia-reperfusion (11; 43) (Figure 1).** Upon the occurrence of no-flow ischemia oxidative phosphorylation and respiration are inactivated due to oxygen deprivation. At the initial moments of ischemia, glycolysis rate increases. However, this
does not result in sufficient ATP production to keep the heart beating. In addition, lactic acid accumulates, as it is not removed by the blood stream, causing acidosis. As a result ATP levels rapidly decrease, which is intensified by reverse operation of the mitochondrial ATP synthase (F$_1$F$_0$-ATPase). During normoxic conditions F$_1$F$_0$-ATPase produces ATP driven by the mitochondrial proton (H$^+$) influx. However, during ischemia it operates in reverse mode and uses ATP in order to maintain the mitochondrial membrane potential ($\Delta\psi_m$).

The intracellular acidosis drives the cytosolic influx of sodium (Na$^+$) through the Na$^+$/H$^+$-exchanger in the plasma membrane. The excess Na$^+$ is transported out of the cell via reverse mode of the Na$^+$/Ca$^{2+}$-exchanger, as a result of depolarized plasma membrane and risen Na$^+$ levels. This step results in reduced Na$^+_i$ but an intracellular calcium (Ca$^{2+}$) overload. ATP lacks for normal operation of the SERCA (sarcoplasmic endoplasmic reticulum Ca$^{2+}$-ATPase) pump, which in normoxic conditions takes up Ca$^{2+}$ into the sarcoplasmic reticulum, and Ca$^{2+}$ is taken up in the mitochondrion via a Ca$^{2+}$ uniporter by using energy from the $\Delta\psi_m$. Mitochondrial Ca$^{2+}$ (Ca$^{2+}_{m}$) is a known possible activator of the mitochondrial permeability transition pore (mPTP). Opening of the pore makes the inner mitochondrial membrane (IMM) permeable, leading to mitochondrial swelling and consequently rupture of the outer mitochondrial membrane (OMM) and release of cytochrome C and triggering of apoptosis (e.g. activation of caspases) and eventually cell death. However, Ca$^{2+}_{m}$ levels alone are not sufficient to open the mPTP as the pore is inhibited by the existing low pH during ischemia.
During reperfusion, the restored oxygen levels cause rapid restoration of pH with still elevated levels of Ca\(^{2+}_m\). This facilitates the activation of mPTP, opening of which activates protein signaling cascades leading to cell death. Furthermore, the mPTP opening leads to cellular ATP depletion at this moment. Whether Ca\(^{2+}_m\) levels will normalize depends on the damage done to SR Ca\(^{2+}_i\) handling proteins during ischemia and whether ATP levels are restored. If Ca\(^{2+}_i\) uptake by SR is disrupted due to damage to the Ca\(^{2+}\) handling proteins, Ca\(^{2+}_i\) levels remain high, which possibly leads to arrhythmias.

The rapid restoration of oxygen during the initial minutes of reperfusion leads to a second possibly damaging consequence as a burst of reactive oxygen species (ROS) arises (64; 81). This is possibly due to damaged electron chain components leading to the generation of superoxide. These detrimental ROS produced during early reperfusion are thought to be an important activator of the mPTP (30) in addition to the, at this time point, elevated Ca\(^{2+}_i\) levels and restored pH.

The described pathophysiological events occurring during reperfusion, if severe enough, can in the end possibly result in membrane damage and rupture, leading to inevitable cell death. In the mean time, before membrane rupture occurs, pro-apoptotic protein signaling cascades are activated by the above described physiological events leading to mPTP opening. The components of both the physiological events and the signaling cascades are possible candidates for I/R damage reduction as they are opposed or inhibited by other cell signaling cascades activated by protective mechanisms.

**Cardioprotective interventions against ischemia-reperfusion injury**

*Ischemic preconditioning.* The cellular processes occurring during I/R actually do provide handles for cardioprotective interventions. The very strong phenomenon of ischemic preconditioning (IPC) was first described in 1986 by Murry *et al.* (44) and uses endogenous physiological protection mechanisms. The infarct size was shown to be reduced with 75% upon applying repetitive brief episodes of I/R followed by a subsequent sustained ischemic period. The protective effect of IPC shows immediately at the first minutes of reperfusion (23; 25), and is thereafter called early preconditioning, and disappears 2-3 hours after the IPC protocol if no subsequent ischemia is applied (18; 45). These short-lived protective IPC effects are mediated by posttranslational modifications and/or
subcellular trafficking. Additionally, a second window of protection exists, called late preconditioning, which becomes apparent at 12-24 hours after the IPC protocol and lasts for 3-4 days (18; 22; 57). This second window is mediated by changed protein expression due to transcriptional effects of IPC.

**Trigger phase.** As a result of an IPC protocol different agonists, such as adenosine, opioids and bradykinin, are released in a pre-ischemic trigger phase (37; 55; 67). These agonists trigger activity of specific G protein-coupled receptors (GPCR) by binding to them (37; 55; 67). The receptors work in parallel as all three GPCR types need to be inhibited in order to completely block the IPC protective effect (15). Other receptors have also appeared to mediate the IPC effect, which are cytokine receptors and receptor tyrosine kinases (12).

**Mediator phase.** A network of intracellular pro-survival signaling pathways is activated at reperfusion by the GPCRs. Two major pathways have been described: the survivor activating factor enhancement (SAFE) pathway (20; 34), which includes JAK and STAT3 protein phosphorylation and tumor necrosis factor (TNF) activation, and the reperfusion injury salvage kinases (RISK) pathway (21). These pathways are not completely separated cascades and both converge on the mitochondrion preventing mPTP formation and opening, thereby making the mitochondrion a key player in cytoprotection against I/R damage.

A group of pro-survival kinases has been denoted as the RISK pathway. The kinase activated first by IPC in this pathway is phosphoinositide 3-kinase (PI3K), which activates phosphatidylinositol 3,4,5-triphosphate (PIP3) leading to activation of amongst others Akt (also named protein kinase B; PKB). Akt is key regulatory component of numerous different cell signaling cascades and thereby regulates numerous different proteins. Very recently, it was shown that solely the Akt1 isoform, and not Akt2, is essential for IPC (33). In cytoprotection against I/R injury, Akt activates endothelial nitric oxide synthase (eNOS), leading to beneficial increased NO production, and inactivates pro-apoptotic proteins p53, BAD and glycogen synthase kinase-3β (GSK-3β). GSK-3β is constitutively active and phosphorylation by Akt inhibits its pro-cell death action. Besides IPC (59; 60), other cardioprotective agents have also been shown to phosphorylate and thereby inactivate GSK-3β (17; 27). Targets of cytosolic GSK-3β are pro-cell death proteins, which
are located in the cytosol as well as at the mitochondrion. However, it is also thought that GSK-3β is partly located at the mitochondrion, where it may regulate adenine nucleotide translocator (ANT) and voltage dependent anion channel (VDAC), both suspected mPTP components, after IPC induced phosphorylation of GSK-3β (10; 46). In this way, mPTP formation is disturbed as ANT and cyclophilin D (CypD), another suspected mPTP component, association is prevented (46). Additionally, GSK-3β inactivation is thought to prevent VDAC phosphorylation and thereby prevent ATP depletion during ischemia through inhibition of ATP transport into the mitochondria (10). Furthermore, GSK-3β has been shown to directly influence different components of the Bcl-2 family. Active GSK-3β phosphorylates the pro-apoptotic Bax, leading to conformational change and translocation to the mitochondrion (36). There, Bax is thought to directly bind to VDAC (48). On the other hand, inactivated GSK-3β increases anti-apoptotic Bcl-2 mitochondrial binding (10). Thus, GSK-3β inactivation leads to direct inhibition of pro-apoptotic signaling and results in decreases mPTP formation and opening.

Conclusively, IPC induced activation of pro-survival pathways, like RISK, ultimately prevent mPTP formation and opening. ATP depletion and release of cytochrome C from the mitochondrion is hereby prevented, resulting in less damaged mitochondria, decreased apoptosis activation and thus increased cell survival chance.

Other conditioning strategies. Besides IPC other protective interventions are known for reduction of I/R injury. The initial minutes of reperfusion appear to be crucial in I/R damage as upon application of brief I/R episodes directly at the onset of reperfusion I/R damage is reduced. This ischemic conditioning strategy was therefore denoted as ischemic postconditioning (IpostC) (31; 74). I/R injury is attenuated to the same extent as IPC, but combining IPC and IpostC does not enhance the degree of protection (19). The clinical relevance is greater for IpostC compared to IPC as the protective intervention takes place after the ischemic insult. Most patient reach the hospital after infarction and medical treatment therefore starts during reperfusion. However, it remains difficult to treat the patient with IpostC as it has only protective effects when applied at the first minutes of reperfusion.
Furthermore, remote preconditioning, which is the application of non-invasive IPC on e.g. a limb results in cellular protection in a distant organ. This clinically interesting application of preconditioning (29) has also been proved protective as remote postconditioning (1). The latter intervention is therefore the most promising in clinical circumstances as it is performed after the ischemic insult.

Pharmacological preconditioning results in similar protective effects as the aforementioned ischemic interventions. Numerous different agents are known which target components of the signaling pathways or detrimental physiological effects involved in I/R injury. Known agents are e.g. cyclosporine, nitric oxide donors, volatile anesthetics and noble gasses. For most agents the exact mechanism of protection is not known, but clinically it would be most beneficial to be able to use each as a postconditioning strategy.

For more efficient use of the aforementioned protective tools, more knowledge concerning the precise mechanisms of action is needed and thus the key components of the interventions should be known for direct pharmacological intervention. In this thesis, we focus on one possible end-effector of the signaling pathways involved in I/R damage, the glycolytic enzyme hexokinase. The role of this protein in both I/R and IPC was studied in heart and skeletal muscle.

**Hexokinase in ischemia-reperfusion injury**

Hexokinases (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) are a group of kinases with a high specificity for glucose as substrate. This means that hexokinases are enzymes which phosphorylate glucose using ATP as the phosphoryl donor resulting in the product glucose-6-phosphate (G-6-P). This is the important first step of the glycolytic pathway as glucose is not able to diffuse out of the cell after phosphorylation, thereby maintaining a glucose gradient for its transport in the cell.

Four different mammalian isoforms exist, hexokinase I-IV (HK I-IV) (70). HKI is expressed throughout the body with particularly high levels in the brain, which is largely dependent on glycolysis. HKII is expressed in insulin-sensitive tissues, like heart, skeletal muscle and
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adipose tissue. HKIII is relatively low-expressed and is found in lung, kidney and liver tissue. Hexokinases I-III are each constituted from two highly homologous 50 kDa halves. In HKI and III only the C-terminal half is catalytically active, while in HKII both C- and N-terminal halves are active in glucose phosphorylation. HK I-III are all prone to direct product inhibition by binding of G-6-P to one or both catalytically active halves. The fourth isoform, hexokinase IV, also known as glucokinase, solely consists of one 50 kDa half, which is not sensitive to G-6-P activity inhibition, and is found in pancreatic B cells and liver tissue. Glucokinase discriminates itself from the other isoforms by its very low affinity for glucose (Km is 100 times higher than that of the other isoforms).

Additionally, both HKI and HKII contain a conserved amino-acid sequence at the N-terminus which enables mitochondrial binding (71). This binding sequence is not apparent in HKIII, which is therefore only found in the cytoplasm, while HKI and HKII are known to be able to translocate between the cytosol and mitochondrion under certain physiological conditions. Under normal conditions, the mitochondrial binding of HK facilitates its access to mitochondrial generated ATP (2). In this manner, an ATP gradient for effective ADP/ATP exchange and ATP production in the mitochondrion is maintained.

Role of HKs in cell death

In addition to their obvious role in glucose metabolism, HKI and II have also been shown to play a role in the regulation of cell survival (47). Firstly, highly malignant tumors are characterized by elevated rates of glycolysis, even in the presence of ample oxygen, also known as the ‘Warburg effect’ (41; 69). This characteristic of rapidly proliferating tumor cells over the surrounding tissue cells is facilitated by corresponding elevated expression and activity of predominantly HKII (52), but also HKI (41). Besides elevated expression, mitochondrial binding of HKI and II in tumor cells further enhances glycolysis rates as their access to ATP is facilitated and makes HKII less sensitive to G-6-P inhibition (50).

Secondly, mitochondrial binding of HKI and II serves another cellular survival mechanism due to their direct influence on anti-apoptotic signaling (51). The exact mechanism of this anti-apoptotic role is not completely clear yet and needs further examination. However,
the dual beneficial role of mitoHK in cell death inhibition leads to the question whether HKs may also have a role in the regulation of cell death due to I/R damage.

**Why could there be a role for mitochondrial HK in cardioprotection?**

Up to now, much basic cardiovascular research is focused on the complete understanding of the mechanisms involved in IPC and thereby I/R. It became apparent that many of the signals induced by IPC converge on the mitochondrion (43). In addition, alterations in glycolysis were also found to be a signature of IPC treated hearts (44). In previous work from our own laboratory this interaction between changes in glycolysis and mitochondrial function was also found using reversible ischemia inducing preconditioning. Additional work, focusing on the activation of mitochondrial oxygen consumption with instantaneous increases in workload (62), demonstrated that glycolysis needs to be active to observe changes in mitochondrial activation after a reversible period of ischemia. This was not observed after irreversible ischemia (77; 79). Thus, alterations in glycolysis induced by reversible ischemia resulted in a specific change in mitochondrial activation that is characteristic of heart tissue in the cardioprotective state.

It has been reported that cardioprotection induced by IPC resulted in decreased anaerobic glycolysis and a decreased build-up of fructose-6-phosphate and lactate, despite normal or even elevated levels of glucose and glucose-6-phosphate during the sustained ischemia (26; 44; 66). Thus, preconditioning induced alterations in glycolysis that are localized just after the first step of glycolysis performed by HK. Previous work showed that this decreased build-up of glycolytic intermediates is not due to an increased channeling of these glycolytic intermediates into the pentose phosphate pathway (75), indicating that the changes described above are probably the result of changes within the control of glycolysis itself.

Regulation of glycolysis by IPC may involve enzyme translocation, with HK being a likely candidate. Especially the known translocation of HK to the mitochondrion could explain many of the phenomena described for IPC. First of all, binding of HK to mitochondria protects against apoptosis induced by oxidative stress (39; 40; 47), which complements the literature on fast growing tumor cells (51) showing that mitoHK provides cell survival benefit. Interestingly, protection by mitoHK was only observed in the presence of glucose,
suggesting that the HK protective effect is not just structural. Thus, translocation of HK to the mitochondrion may constitute part of the cardioprotective phenotype of IPC.

In addition, the aforementioned kinetically advantageous mitochondrial binding of HK brings glycolysis actually under the control of oxidative phosphorylation (70). This may partly explain the often observed increase in aerobic glycolysis of the heart during normoxia following reversible periods of ischemia (80), and the accelerated decrease in anaerobic glycolysis rate during the first period of ischemia in preconditioned hearts (44). This increased coupling of glycolysis with oxidative metabolism may result in a decreased anaerobic glycolytic proton production and consequently attenuated acidosis (38; 71), as is usually observed in preconditioned hearts.

Thus, it seems that increased association of HK with cardiac mitochondria may explain many of the phenomena observed with cardioprotection, offering arguments for a critical evaluation of the role of mitoHK in cardioprotection against I/R damage.

**MitoHK in cardioprotective interventions**

In 2005, the first study was performed that examined whether IPC was associated with HK translocation to the mitochondria within the intact heart (76). It was demonstrated that upon IPC, HK activity in the cytosol decreased, which was accompanied with an increase in the mitochondrial compartment of the IPC treated heart. In addition, two other well-known cardioprotective interventions were examined: morphine and insulin administration. Both morphine and insulin were also able to redistribute HK from the cytosol to the mitochondrion. This was further collaborated by a subsequent study in *in vivo* rat hearts (78). It was shown that well-known cardioprotective anesthetics, such as isoflurane and sevoflurane, are able to keep HK at the mitochondrion during sedation, whereas other non-cardioprotective anesthetics such as ketamine resulted in increases of cytosolic HK as compared to conscious, non-anesthetized, animals. These data clearly demonstrated that IPC induced translocation of HK to the mitochondrion is operative within the intact heart and that this may very well constitute an important end-effector of cardioprotective interventions.
When looking at HK in cell signaling perspective, several cellular studies (39; 40) have demonstrated that activated Akt induces HK translocation to the mitochondrion. Additionally, it has been suggested that activated Akt results in HK translocation to the mitochondria through the phosphorylation and thus inhibition of the pro-cell death GSK-3β (49). Alternatively, it is also possible that activated Akt directly phosphorylates HK in the cytosol and that this facilitates HK association with mitochondria (42). Thus, Akt activation may closely correspond with mitoHK translocation.

In conclusion, cardioprotective signaling in the intact heart appears to be associated with increases of HK at the level of the mitochondrion. Possibly, the anti-apoptotic function of HK translocation may be regulated by the RISK pathway.

Possible mechanisms of mitoHK-mediated cardioprotection against I/R injury

Independently of the above described binding to mitochondria, HK may also offer protection against I/R injury solely by its phosphorylative activity. In a study by Sun et al. (58) it was shown that overexpression of truncated HK proteins lacking the mitochondrial binding domains could indeed partly explain the protective effects of overexpressed full length HK proteins against H₂O₂ treatment in HEK293 cells. Similarly, overexpression of yeast HK, which is not capable of binding to the mitochondrion, protected hypoxic cardiomyocytes against contractile dysfunction (73). However, whether these protective effects of increased HK activity observed in cellular studies, where glucose is usually the sole substrate provided, translate to a more physiological model of I/R in the intact heart, with energy derived from many other substrates, is unclear. Liang et al. (35) have shown that overexpressing yeast HK does not protect against contractile dysfunction following hypoxia in the ex vivo Langendorff-perfused mouse heart. Thus, it is unclear whether increased HK activity will in every case have survival benefit against an I/R insult in the intact heart. This is commensurate with the dual role (detrimental or beneficial) of anaerobic glycolysis in cardiac I/R injury (8).

I/R injury is initiated by Ca²⁺ overload and the generation of ROS, which result in activation of proteases (e.g. caspases, calpains), mitochondrial depolarization and mitochondrial permeability transition pore (mPTP) opening. Although still elusive, the mPTP pore is suggested to be composed of VDAC, ANT, CypD and HK (16). One
mechanism, through which mitoHK could be protective, is by occupying mPTP -VDAC binding sites which otherwise would be occupied by the pro-apoptotic proteins Bax/Bak (48). Whether VDAC is an essential component in this scenario was recently contested by Chiara et al. (6), showing that HK detachment from mitochondria triggers apoptosis independent of VDAC. These authors implied that mitoHK protects by affecting the configuration of the two other components of the mPTP, i.e. ANT and/or CypD, although the precise mechanism was not characterized. One possibility may be that mitoHK activity drains mitochondrial ATP for the phosphorylation of glucose, thereby facilitating ATP to move out of the mitochondria and ADP to move in, and stimulating ATP/ADP exchange. It has been shown by (65) that an early event in apoptosis is a defect in ATP/ADP exchange.

Alternatively, an attractive hypothesis is that mitoHK serves as a specific antioxidant against mitochondrial radical production. Da-Silva et al. (9) have demonstrated that mitoHK reduced mitochondrial membrane potential and ROS production in brain mitochondria. Similar findings have been observed in heart mitochondria (54), and the reduction of oxidative stress has been implicated as a likely candidate for inhibition of the mPTP with IPC (7). According to the abovementioned publications (9; 54), increased mitoHK functionally mimics increased mitochondrial uncoupling, a phenomenon which is currently regarded as one of the mechanisms providing IPC-induced cardioprotection (53). Finally, it has recently been reported that hearts of hypoxia-tolerant fish are characterized by elevated amounts of mitoHK. In this study, it was proposed that the high level of mitoHK serves to create micro-environments with a low ATP/ADP ratio, which could lead to the opening of mitoKATP channels that occur in hypoxic-tolerant fish hearts (61). Such an opening of mitoKATP channels also figures as one of the main IPC triggering mechanism (13).

Overall, many of the purported HK protective mechanisms against cell death are in line with the mechanisms through which IPC and other cardioprotective interventions are currently proposed to operate. This underscores the relevance of studying HK and mitochondrial HK binding in the context of reducing I/R damage of the heart.
Aims of this thesis

In this thesis, we aimed to study the role of HKII in I/R induced cell death and IPC cellular protection in the intact organ, both in vivo and ex vivo. In chapter 2, we studied the influence of HKII on low-flow I/R injury and the influence of standard chow on HK expression in the intact ex vivo mouse heart. Resulting from this, more severe models of no-flow ex vivo and in vivo cardiac I/R and long term recovery were examined in chapter 3. Subsequently, HKII influence on I/R injury was studied in skeletal muscle using in vivo mouse models in mild (chapter 4) and severe I/R injury (chapter 5). In the latter, long-term recovery was also studied allowing examination of the role of HKII in muscle regeneration, angiogenesis and fibrosis.

Spatial and temporal effects of IPC cardioprotection on HK were studied in chapter 6 using an ex vivo rat heart model. Resulting from this study, the role of cellular and mitochondrial bound HKII on IPC cardioprotection and normal cardiac function were studied in ex vivo mouse hearts in chapter 7.

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