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REMOTE ISCHEMIC CONDITIONING AND ITS MOLECULAR MECHANISM IN THE HEART

Ph.D thesis

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2. List of abbreviations

AAR	-	area at risk
AMI	-	acute myocardial infarction
AMPK	-	5' adenosine monophosphate-activated protein kinase
ATG7	-	autophagy-related gene 7
BNIP3	-	Bcl-2/E1B - interacting protein 3
cGMP	-	cyclic guanosine monophosphate
eNOS	-	endothelial nitric oxide synthase
Erk1,2	-	extracellular signal-regulated kinase 1,2
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
GSK3	-	glycogen synthase kinase 3 beta
IL-6	-	interleukin 6
IPostC	-	ischemic postconditioning
IPreC	-	ischemic preconditioning
JAK	-	Janus kinase
LAD	-	left anterior descending coronary artery
LC3	-	microtubule-associated protein 1 light chain 3
LV	-	left ventricle
MEK	-	mitogen-activated protein kinase/ extracellular signal-regulated kinase kinase
mPTP	-	mitochondrial permeability transition pore
MRI	-	magnetic resonance imaging
mTOR	-	mechanistic target of rapamycin
MVO	-	microvascular obstruction
NO	-	nitric oxide
PI3K	-	phosphoinositide 3-kinase
PKG	-	protein kinase G
PTEN	-	phosphatase and tensin homolog
RIC	-	remote ischemic conditioning
RISK	-	reperfusion injury salvage kinase
SAFE	-	survivor activating factor enhancement

- SR - sarcoplasmic reticulum
- STAT3 - signal transducer and activator of transcription 3
- TNF- - tumor necrosis factor alpha
- ULK1 - UNC-51-like kinase 1

3. Introduction

Ischemic heart disease, characterized by limited oxygen and nutrient supply to myocardial cells, and particularly its subgroup of acute coronary syndrome – a clinical syndrome defined as the relatively rapid oxygen and nutrient depletion of the myocardium – has been a leading cause of mortality and morbidity in the developed countries ever since the 1950s [1]. Only in 2012, 7.4 million people died of ischemic heart disease. Although ischemic heart disease mortality is the highest in the developed countries, it will soon become the number one cause of mortality in low-income countries as well [2]. Advanced treatment of ischemic heart disease and acute coronary syndrome has significantly decreased the mortality rates, especially in the high-income countries. The most pioneering improvements to be mentioned involve the establishment of coronary care units with full access to monitors [3], the development of percutaneous coronary catheterization and balloon angioplasty [4], and of the surgical revascularisation techniques [5]. Nevertheless, the effective treatment of established cardiovascular risk factors is also responsible for the reduction in morbidity and mortality [6].

3.1. Ischemia/reperfusion injury

Early restoration of coronary circulation is obligatory in order to prevent the ischemia-triggered myocardial damage. Paradoxically, reperfusion *per se* further exacerbates myocardial injury. Thus, both ischemia and reperfusion are responsible for the overall myocardial damage seen following acute myocardial ischemia and subsequent reperfusion therapy. This is termed ischemia/reperfusion injury (Figure 1) [7].

During ischemia, the nutrient deprivation and decrease in oxygen supply lead to a disturbance of the myocardial homeostasis [8]. After 8-10 seconds hypoxia, all oxygen offered by haemoglobin, myoglobin and creatine-phosphate is extracted and utilized from the cellular proximity [9], cellular oxidative phosphorylation shuts down, and aerobic metabolism shifts to anaerobic one [10]. Once the anaerobic reserve burns out, myocardial cells start to decay. Many factors, such as mitochondrial depolarization and dysfunction, sodium- and calcium overload, intra- and extracellular acidosis and reactive oxygen species production all contribute to the myocardial cell death as coordinated by cardiac myocytes, endothelial and inflammatory cells, *etc.* [11].

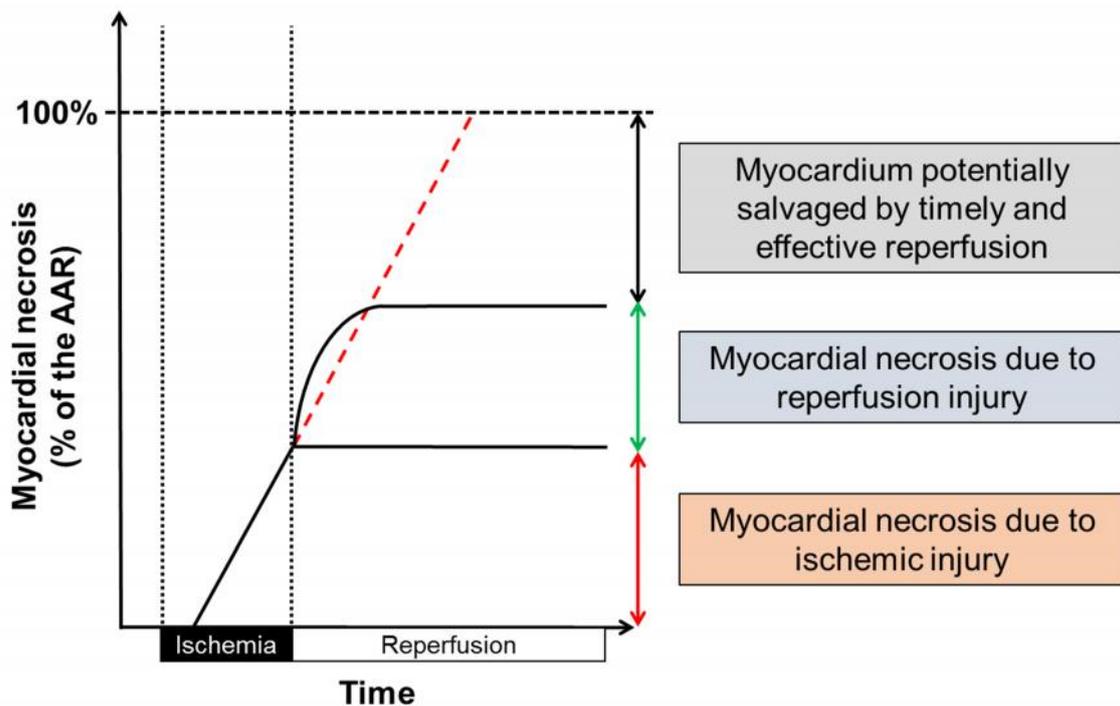


Figure 1. The contribution of ischemia and reperfusion to the final myocardial necrosis. Reperfusion may responsible for 50 % of the total necrosis [7].

The reperfusion injury occurs in the first few minutes of the reperfusion, and similarly to the ischemic injury, is a complex phenomenon [11]. As the myocardial perfusion is restored, tissue oxygen level increases rapidly, metabolites are washed out and different units of the respiratory chain are recruited rapidly. However, the rapid restoration of the downregulated respiratory chain elements results in an increased formation of reactive oxygen species, leading to protein-, lipid- and nucleic acid oxidation [12]. Simultaneously, the already increased cellular calcium concentration [13] further increases via sarcoplasmic calcium release, thereby activating apoptotic pathways [14-16]. Nevertheless, it seems the primary mediator of reperfusion injury is the opening of the mitochondrial permeability transition pore (mPTP) [17].

The (pre)clinical measurement of ischemia/reperfusion injury may be analyzed from three major aspects, which are closely linked together and define the most important endpoint, the mortality: myocardial contractility, electrophysiological function, and irreversible and reversible cellular damage.

Myocardial contractile dysfunction may occur as an acute heart failure during ischemia or reperfusion. Chronic heart failure may develop due to ischemia/reperfusion

injury over years. Importantly, reversibly injured regions of the myocardium may recover completely in a delayed manner termed myocardial stunning, or remain hypoperfused and hibernated [18-20]. The onset of arrhythmias (ranging from isolated premature beats to ventricular fibrillation) during ischemia or upon reperfusion is quite frequent complication of acute myocardial infarction (AMI) [21, 22]. Irreversible and reversible cellular damage can be measured in many ways. It is well-established that the final myocardial necrosis correlates significantly with the mortality [23]. However, the development of the final necrosis is an extremely complex chain of events with many cellular components and players [7]. For instance, it seems that microvascular damage is one of the most important factors contributing to the ultimate damage [7, 24]. Myocardial edema [25], microvascular obstruction (MVO; ischemic area where myocardial perfusion is not restored despite successful revascularization) [26], endothelial dysfunction and microembolisation are major signs of microvascular damage [27].

Many pharmaceutical agents have been proposed to attenuate myocardial ischemia/reperfusion injury. The common feature of these compounds are that they either interfere with well-established detrimental molecular pathways or that they facilitate/mimic the protective endogenous mechanisms activated upon reperfusion (see Section 3.3). The most promising agents identified in preclinical studies have already undergone clinical trials after encouraging preclinical results: adenosine [28], cyclosporin-A [29], erythropoietin [30], statins [31], insulin [32, 33], nitrite/nitrates and soluble guanylate cyclase activators [34, 35], exenatide [36], metoprolol [37] and nicorandil [35]. However, the majority of the studies aiming to modify a single target failed to improve the overall outcome after AMI. Nevertheless, ischemic conditioning enables a more comprehensive way to target multiple pathways orchestrating the effects of the ischemia/reperfusion injury.

3.2. Ischemic conditioning

During ischemic conditioning of the heart, short cycles of ischemia/reperfusion achieve a more resistant state of the myocardium against myocardial ischemia/reperfusion injury due to sustained injury, *i.e.*, index myocardial ischemia (Figure 2). Classification of the ischemic conditioning strategies is based on several parameters including the site of the applied ischemia/reperfusion cycles or based on the timing referenced to the index

ischemia. Ischemic conditioning stimuli can be elicited on the affected coronary artery of the heart or on a coronary artery distant to the index ischemia or even on a remote organ (*e.g.*, kidney, skeletal muscle) termed remote ischemic conditioning (RIC). Temporal variants of ischemic conditioning are ischemic pre-, per- and postconditioning [38], in which the conditioning is applied before, during and after the ischemic event, respectively.

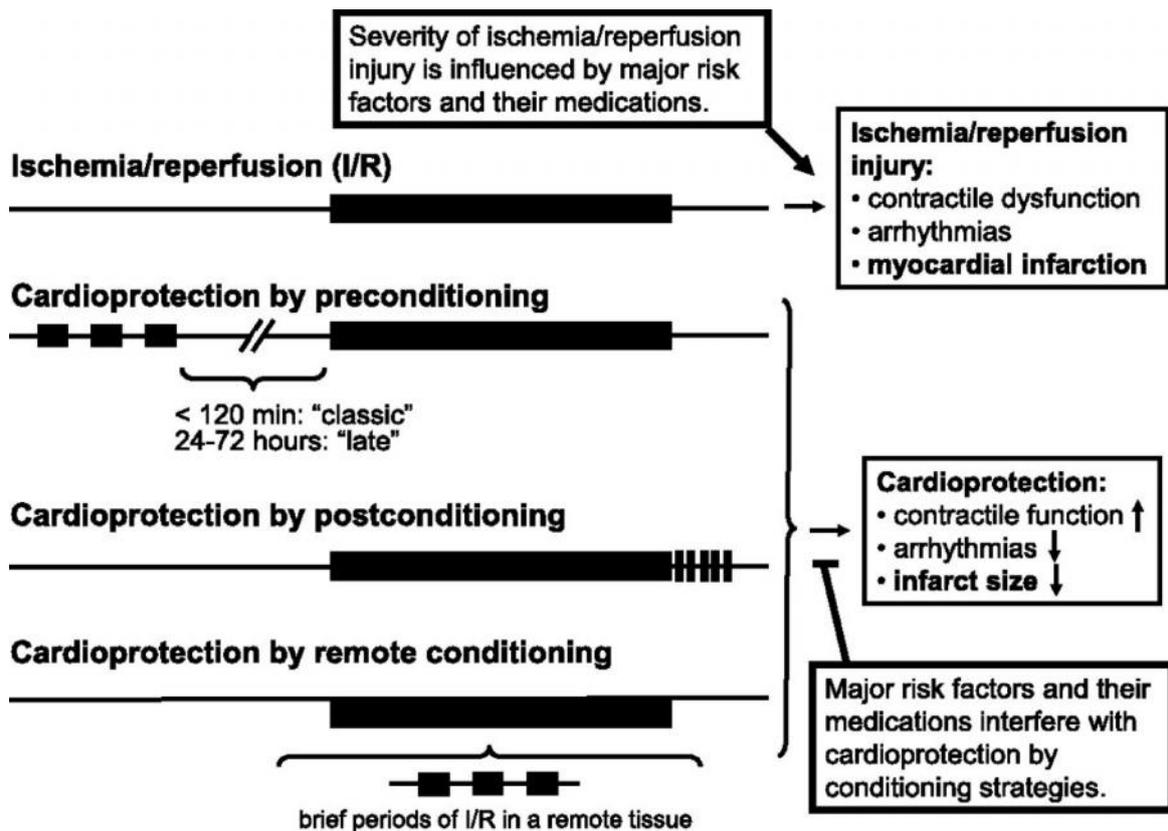


Figure 2. The concept of ischemic pre-, post- and remote conditioning. Black bars represent ischemic periods over time. Figure is adapted from Ferdinandy *et al.* 2014 [39].

3.2.1. Ischemic preconditioning

In 1986, Murry *et al.* showed for the first time that ischemic preconditioning (IPreC; *i.e.*, brief cycles of ischemia and reperfusion of the involved coronary artery before index ischemia) is able to attenuate subsequent myocardial damage [40]. Since then, the cardioprotective effect of IPreC has been confirmed numerous times both in preclinical

models and in clinical trials [41]. It has been demonstrated that IPreC significantly reduces myocardial necrosis [40], enhances postischemic cardiac function [42], improves endothelial dysfunction [43], prevents reperfusion-induced cardiac arrhythmias [44] and increases the resistance against hypoxia [45]. Specially, IPreC might be extremely useful in elective cardiac interventions. It has been reported that IPreC of the human heart results in reduced lactate and/or creatine kinase MB release, attenuated ECG alterations and/or reduced postinterventional complaints of patients after a secondary, elective percutaneous coronary intervention compared to the first intervention [46-48]. Moreover, numerous proof-of-concept studies on coronary artery bypass graft surgery have demonstrated an IPreC-induced cardioprotection proven by a reduction in cardiac biomarkers, such as creatine kinase MB and troponin T or I [41]. It is believed that pre-infarction angina endogenously mimics IPreC with better clinical outcome, nevertheless, this has been challenged by others [41].

3.2.2. *Ischemic postconditioning*

The clinical application of IPreC is limited to elective interventions accompanying myocardial ischemia, as the conditioning stimuli should precede the prolonged ischemia. However, primary physicians, clinicians, *etc.*, diagnose and treat myocardial ischemia after its onset, and IPreC is no longer a therapeutic option. Nonetheless, it has been demonstrated that ischemic conditioning reduces myocardial ischemia/reperfusion injury if applied *after* cardiac ischemia (termed as ischemic postconditioning; IPostC) [49, 50], which has already been postulated in earlier works [51, 52]. Similarly to IPreC, IPostC protects the myocardium from ischemia/reperfusion injury by improving myocardial function, attenuating myocardial oxidative damage, inflammation, edema, *etc.*, after myocardial ischemia, however, the cardioprotection is less pronounced than IPreC [53]. Theoretically, IPostC could be applied either after elective or acute cardiac interventions/cardiothoracic surgeries. Indeed, IPostC has been reported to be cardioprotective both in preclinical [38] and clinical [54, 55] settings. However, to date, the largest clinical trials investigating the efficacy of IPostC did not show any benefit of IPostC on ST-elevation myocardial infarction patients after primary percutaneous intervention (POST and DANAMI 3-iPOST trial) in terms of long-term outcome [56, 57].

3.2.3. Remote ischemic conditioning

IPreC is impossible to apply in acute situations, and IPostC is by definition limited to be effective against the reperfusion-induced myocardial damage only. The discovery of RIC initiated an extensive research activity in the cardiovascular field. Originally, RIC demonstrated that short episodes of ischemia and reperfusion to a canine circumflex artery are able to alleviate the deteriorating effects of prolonged ischemia of the left anterior descending coronary artery (LAD) [58]. Since then, the cardioprotective effect of RIC was demonstrated that not only a distant part of the same organ but applying short ischemia/reperfusion cycles on a distant organ (brain, skin, intestine, kidney, *etc.*) triggers cardioprotection [59]. More importantly, Birnbaum *et al.* discovered that restricted blood flow and electrical pacing of rabbit gastrocnemius muscle protects the myocardium from the subsequent ischemia/reperfusion injury [60]. Noninvasively, Oxman *et al.* described that RIC stimuli could be elicited with a simple tourniquet in rats [61], and thereafter, Günaydin *et al.* [62] and Kharbanda *et al.* [63] showed that this can be demonstrated in humans. This noninvasive approach facilitated the rapid translation into the clinical setting, and it has been justified by many proof-of-concept clinical trials [64-66]. However, the long-term outcome of such interventions is still controversial. Thus far, a completed small-scale clinical trial involving ST-elevation myocardial infarction patients demonstrated long-term efficacy of RIC as assessed by significant improvement of major adverse cardiac and cerebrovascular events [67]. Nevertheless, two large clinical trials reported that RIC did not improve the long-term outcome after cardiac surgery (ERICCA [68] and RIPHEART [69] trials).

3.3. Mechanism of ischemic conditioning

There are thousands of studies investigating the major mechanism of ischemic conditioning. It is clear that ischemic conditioning seems to be a well-conserved, endogenous program of the heart that shares a highly orchestrated spatiotemporal signal transduction with similar elements [53]. Theoretically, the major parts of the signal transduction are the stimulus or trigger, and the intracellular mediators. Since RIC stimulus occurs in a distant organ, it involves a third element as well, namely the signal transport to the target organ.

3.3.1. *Stimuli/triggers*

Cardioprotective triggers are by nature chemical or biological compounds, and many of which are part of the myocardial endogenous adaptation ischemia/reperfusion injury. Ischemia and reperfusion are very complex pathophysiological phenomena. Ischemia and reperfusion induce the release of a mixture of chemical and biological components, and simultaneously trigger endogenous alterations of the cellular homeostasis.

The major chemical stimuli released upon short ischemia/reperfusion are reactive oxygen species, such as nitric oxide. Although reactive oxygen species have a pivotal role in cardioprotection, they behave as a double-edged sword [70]: a small amount of reactive oxygen species recruits protective intracellular signals (*e.g.*, oxidize possibly protective cytosolic kinases) [71-73], while a more pronounced reactive oxygen species formation induces irreversible injury [70]. It has been also indicated that calcium ions [74] and hydrogen sulfide [75] may exert similar cardioprotection.

A significant amount of cardioprotective triggers belongs to the biologically active molecules, which act on specific receptors and throughout regulating intracellular pathways modifies the defense of the myocytes: autocooids (adenosine and bradykinin [76]), neurotransmitters (acetylcholine, catecholamines, endothelin, opioids [77]), peptide hormones (natriuretic peptides [78], adrenomedullin [79]) and cytokines (tumor necrosis factor- and interleukins [80, 81]).

3.3.2. *Signal transport from remote tissue*

RIC is a unique conditioning phenomenon, since the stimulus is applied apart from the site of protection, necessitating a mechanistic pathway conveying the protective information. Two major form of signal transfer have been proposed: neuronal and humoral [38].

It is well-established that an intact afferent neuronal pathway is required for the proper signal transmission [82]. Furthermore, stimulation of the afferent neuronal fibres can mimic RIC-induced cardioprotection [83]. The mechanism of the efferent neuronal pathway is less clear, however, the vagal nerve may have a pivotal role [84, 85].

Humoral signal transfer is based on an early observation where the coronary effluent of a preconditioned heart was transferred to a naïve acceptor heart and exerted a similar

degree of cardioprotection [86]. Since then, many protective factors have been identified, such as stromal-derived factor-1 [87], nitric oxide [88], microRNA-144 [89], hypoxia-inducible factor-1 [90] and apolipoprotein a-I [91].

Nevertheless, the true nature of the protection may be a well-coordinated interplay between neuronal and humoral factors [38, 92].

3.3.3. *Intracellular mediators*

Chemical and biological triggers initiate the temporal and spatial orchestration of intracellular mediators of cardioprotection (Figure 3). The intracellular protective pathway identified was the protein kinase C pathway [93]. Initially, Ytrehus *et al.*, showed that protein kinase C inhibitors abrogated the cardioprotective effect of IPreC subjected to rabbit hearts [93]. Later on, many other pathways have been identified, such as the nitric oxide/cyclic guanosine monophosphate/protein kinase G- [94], the Reperfusion Injury Salvage Kinase (RISK)- [95], the Survivor Activating Factor Enhancement (SAFE)- [96], the hypoxia-induced factor 1 - [97], the mitogen-activated protein kinase- [98] and a microRNA-regulated [99] pathways.

A number of investigations has been conducted to reveal the primary role of the mitochondria and sarcoplasmic reticulum (SR) in the cardioprotection. During short ischemic periods, mPTP opens transiently, preparing or recapitulating the mitochondrial reactive oxygen species- and calcium-homeostasis for the subsequent or elapsed, prolonged ischemia [100, 101]. Conversely, during reperfusion, the mPTP stays closed by the abovementioned mediators, preventing the mitochondrial depolarization [102, 103]. In addition, the activation of the mitochondrial ATP-dependent potassium channels rigorously regulates the calcium-flow and metabolic enzymes [104, 105]. Ion homeostasis is also maintained by the RISK- and SAFE-dependent phosphorylation of connexin-43, a gap junction-related poreforming protein [106]. To preserve the calcium homeostasis, a close functional relationship between mPTP and SR is required [107, 108]. Furthermore, SR facilitates the unfolded, damaged protein degradation [109] and activates autophagy to eliminate injured organelles [110, 111].

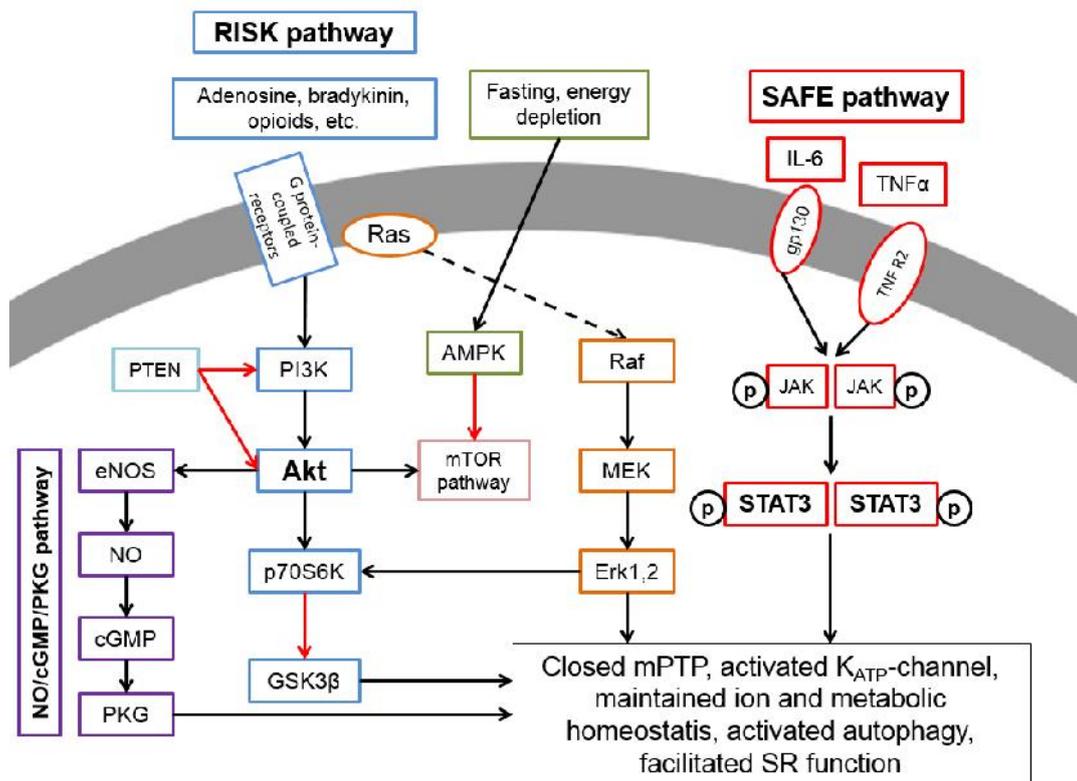


Figure 3. Major intracellular pathways of ischemic/pharmacological conditionings. (Own graph based on Heusch, 2015 [53]). RISK – Reperfusion Injury Salvage Kinase; SAFE – survivor activating factor enhancement; PTEN – phosphatase and tensin homolog; PI3K – phosphoinositide 3-kinase; eNOS – endothelial nitric oxide synthase; NO – nitric oxide; cGMP – cyclic guanosine monophosphate; PKG – protein kinase G; GSK3 – glycogen synthase kinase 3 beta; AMPK – 5’ adenosine monophosphate-activated protein kinase; mTOR – mechanistic target of rapamycin; MEK – mitogen-activated protein kinase/ extracellular signal-regulated kinase kinase; Erk1,2 – extracellular signal-regulated kinase 1,2; IL-6 – interleukin 6; TNF- – tumor necrosis factor alpha; JAK – Janus kinase; STAT3 - signal transducer and activator of transcription 3; mPTP – mitochondrial permeability transition pore; SR – sarcoplasmic reticulum

3.4. Interaction between comorbidities and ischemic conditioning

3.4.1. Interaction with chronic comorbidities

In the clinical routine, the majority of ischemic heart diseases has been preceded by risk factors, such as hyperlipidemia, hypertension, diabetes mellitus, chronic renal failure, ageing, *etc.* [39, 112]. Moreover, to prevent the development or manifestation of ischemic heart diseases, patients are treated with a great amount of medications [39]. Comorbidities, aging and medications significantly influence and alter the structure and intracellular homeostasis of the heart. However, during preclinical research, healthy, juvenile and drug-naïve animals are used to test the efficacy of ischemic conditioning phenomena. Hypercholesterolemia significantly deteriorates the myocardial homeostasis and function without the development of atherosclerosis [113]. It has been shown that hyperlipidemia abolishes the cardioprotective effect of IPreC and IPostC [114-116].

Interestingly, IPreC, but not IPostC, could be elicited in hypertensive preclinical models [117, 118]. Similarly, IPreC [119], IPostC [120] and RIC [121] are ineffective in the presence of diabetes mellitus (type 1 or 2). Mechanisms contributing to the abolished cardioprotective strategies have been attributed to the impaired RISK/SAFE and nitric oxide/cyclic guanosine monophosphate/protein kinase G pathways, to the increased oxidative-nitrative stress, to the altered 5' AMP-activated protein kinase function, *etc.* (see reviews for details [39, 112]). Nevertheless, the cardioprotective effect of IPreC was preserved in chronic renal failure [122].

3.4.2. Interaction with acute comorbidities

It is well-established that chronic comorbidities significantly alter the cardiac homeostasis and metabolism, and therefore the response to the same stimuli, such as ischemic conditioning [39]. However, it is not well-studied how acute pathologies without any chronic comorbidities (*e.g.*, hypertensive crisis, hyperglycemia and acute renal failure) influence IPreC, IPostC or RIC.

Apart from being a major component of chronic metabolic diseases, hyperglycemia may also occur in acute situations for example as seen in sympathetic overactivation

during acute coronary syndrome [123]. Hyperglycemia in non-diabetic patients – a group of patients in which hyperglycemia is unlikely to persist – is generally associated with adverse outcomes after an AMI [124]. It is not known whether this acute hyperglycemia is the cause of adverse outcomes or it only reflects the severity of the AMI [123]. Furthermore, it has been shown that hyperglycemia inhibits cardioprotection conferred by IPreC and cardioprotection by various pharmacological agents [125-127].

4. Aims

- To test the cardioprotective effect of RIC in rat and porcine model of AMI.
- To investigate whether acute hyperglycemia interferes with the cardioprotective effect of RIC.
- To investigate whether RIC signal is transported via extracellular vesicles.

5. Materials and methods

These investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996), to the EU Directive (2010/63/EU) and was approved by the animal ethics committee of the Semmelweis University and by the animal ethics committee of Hungarian National Food Chain Safety Office (SOI/31/26-11/2014).

5.1. Experimental protocol to test remote ischemic conditioning *in vivo* in a rat model of cardiac ischemia/reperfusion injury

Unfasted, 220–280 g male Wistar rats were anesthetized with 60 mg/kg pentobarbital. The absence of pedal reflex was considered as a sign of deep surgical anesthesia. Electric activity of the heart was monitored (AD Instruments, Bella Vista, Australia). Blood pressure was measured in the carotid artery (AD Instruments, Bella Vista, Australia). Body temperature was maintained with a heat pad at physiological temperature (35.8–38.3 °C). Rats were ventilated with 10 mL/kg stroke volume at rate of 80 strokes/min (Ugo-Basile, Gemonio, Italy).

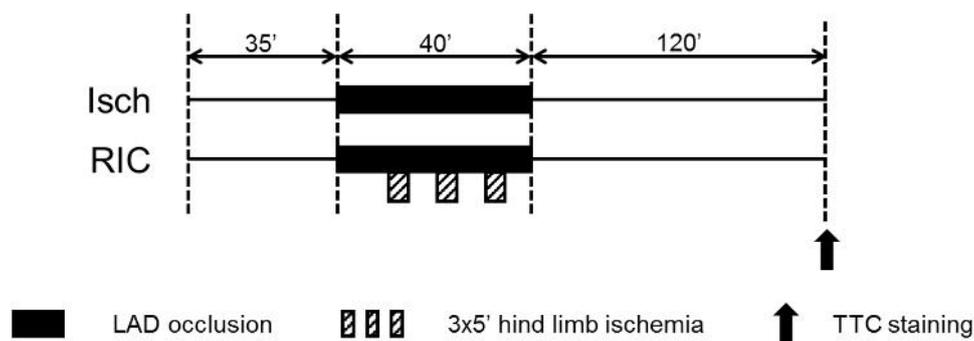


Figure 4. Experimental protocol to test remote ischemic conditioning *in vivo* in a rat model of cardiac ischemia/reperfusion injury. Isch – ischemia only group, RIC – remote ischemic conditioning, LAD – left anterior descending coronary artery, TTC – triphenyltetrazolium chloride.

Rats were randomized into two groups (Figure 4): (1) control ischemic and (2) RIC. At 35 min of the study protocol, LAD was occluded with a 6–0 polypropylene suture via

median thoracotomy for 40 min. Occlusion was confirmed by ST-segment elevation, arrhythmias and paling of the occluded area. RIC was induced by three cycles of 5 min occlusion and 5 min reperfusion of the right femoral vessels starting after 10 min of the LAD occlusion. Both the femoral artery and vein were occluded with a metal vessel clamp after isolation of the vessels from the surrounding connective tissue and femoral nerve. At the end of the 40 min index ischemia, reperfusion was induced by loosening the suture. At the end of the 120 min reperfusion, hearts were harvested in order to evaluate the extent of myocardial necrosis.

5.2. Experimental protocol to test remote ischemic conditioning *in vivo* in a clinically relevant porcine model of cardiac ischemia/reperfusion injury

Domestic female pigs (25-35 kg; genotype: DanBred hybrid) were purchased from the Product Development and Monitoring Research Center of University of Kaposvár, and were kept according to the Big Dutchman principles. Animals were fed with pregnant sow diet with low energy and balanced protein level produced by Dalmand co. Ltd.

Pigs were block randomized [128] into three groups (Figure 5): ischemia only (Isch; n=17), IPreC (n=12) and RIC (n=17). Three animals died during myocardial ischemia (Isch: 1; IPreC: 1; RIC: 1) and 3 during reperfusion due to therapy resistant malignant ventricular rhythm disturbances (Isch: 2; IPreC: 1; RIC: 0). Furthermore, 3 animals were excluded due to procedural technical reasons. The final case numbers were 14, 9 and 14 in Isch, IPreC, and RIC groups, respectively.

One day prior to the experiments, pigs were given 500 mg acetyl salicylic acid and 300 mg clopidogrel. Animals were sedated with 12 mg/kg ketamine hydrochloride, 1 mg/kg xylazine, and 0.04 mg/kg atropine intramuscularly after overnight fasting. Anesthesia was induced by inhalation of isoflurane (2-2.5 vol%). Animals were intubated endotracheally and anesthesia was maintained by isoflurane oxygen mix inhalation (2-2.5 vol% and 3 L/min). Magnesium sulphate (4.06 mEq diluted in 10 mL, in every 60 min) and continuous amiodarone infusion (300 mg diluted in 500 mL saline) were being administered throughout the procedure via an ear vein. Sheaths were inserted into femoral artery and femoral vein to have entry routes for the catheterization, and

5000-5000 IU heparin was administered via each sheath. During preparation, cardiac function was assessed with echocardiography. Selective angiography of the left coronary artery was performed. After the analysis of the baseline angiogram, balloon catheter (2.75 mm diameter, 8 mm length) (Abbott, Abbott Park, IL, US) was placed in the mid part of the LAD after the origin of the second diagonal branch. For induction of AMI, the intracoronary balloon was inflated with 5 atm for 90 min, followed by deflation of the balloon, resulting in reperfusion (3 h or 3 days) confirmed by coronarography.

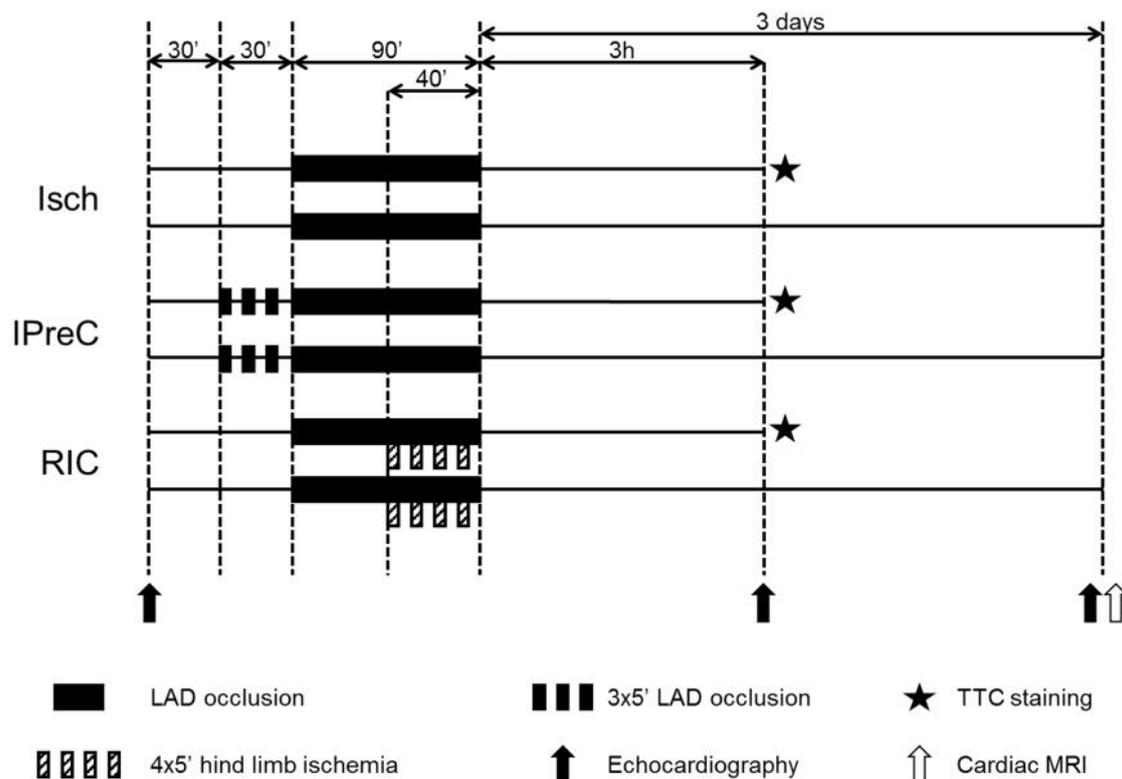


Figure 5. Experimental protocol to test remote ischemic conditioning in an *in vivo* porcine model of acute myocardial infarction. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, LAD – left anterior descending coronary artery, TTC – triphenyltetrazolium chloride, MRI – magnetic resonance imaging.

In IPreC group, LAD was occluded by the inflation of the balloon at 5 atm 3 times for 5 min followed by 5 min of reperfusion, while in other animals the balloon was left deflated for 30 min. Then LAD was occluded by inflating coronary balloon, which was confirmed by coronarography. RIC was performed by four cycles of 5 min occlusion and 5 min reperfusion of the femoral vessels by tightening and releasing a snare around the

right hind limb starting at the 50th min of LAD occlusion. We documented the hind limb ischemia in three ways: 1) apparent lividity during ischemia and pronounced hyperemia during reperfusion were observed, 2) in each animal, a superficial femoral artery was cannulated distal to the occlusion, and blood pressure was measured during the intervention. The minimum of 30/30 mmHg blood pressure with 0 mmHg pulse pressure was achieved while the wire was tightened around the hind limb, and 3) in one particular animal, we performed femoral angiography before and during hind limb ischemia as well. The interventional cardiologist was not blinded during the investigation due to the nature of the procedure: IPreC was achieved by inflating a balloon in the LAD, which require an unblinded interventional cardiologist. Nonetheless, the interventional cardiologist was not aware of the allocation until the initiation of the experimental intervention.

After reperfusion, 5000 IU heparin was initiated by intracoronary administration. Final reperfusion was confirmed with coronarography and the catheters were subsequently removed. Anesthesia was either maintained for 3 h or in the case of 3 days reperfusion, wounds were closed and anesthesia was terminated by the withdrawal of isoflurane. Analgesia was applied by intramuscular injections of 1 g metamizole. An antibiotic cocktail (100 mg benzathine benzylpenicillin, 100 mg procaine benzylpenicillin, 200 mg dihydrostreptomycin-sulphate) was given im. before recovery.

5.3. Experimental protocol to test the effect of hyperglycemia on remote ischemic conditioning in an *in vivo* rat model of ischemia/reperfusion injury

In vivo experiments of RIC in rats has been performed as described in Section 5.1. Rats were randomized into 2 groups (Figure 6): control normoglycemic, and acute hyperglycemic. Acute hyperglycemic animals received 50 % dextrose (vWR, Radnor, PA, US) infusion via the tail vein from the start of the experimental protocol. A blood glucose level of 15–20 mM was reached within 5 min by an infusion rate of 150 μ L/min. Then infusion rate was adjusted to maintain blood glucose levels between 15 and 20 mM throughout the entire protocol (0–60 μ L/min, with an average of 50 μ L/min), which was measured every 15 min (Accu-Check, Roche, Basel, Switzerland). In normoglycemic animals, an equal osmolarity, 46 % mannitol solution was administered (vWR, Radnor,

PA, US, induction rate: 150 $\mu\text{L}/\text{min}$ for 5 min, then 50 $\mu\text{L}/\text{min}$).

After 35 min of *in vivo* perfusion, half of the animals from normoglycemic and acute hyperglycemic groups were sacrificed, and hearts were excised, immersed readily in ice-cold Krebs-Henseleit solution until they were mounted. They were then perfused in Langendorff mode for 1 min with oxygenated (95 % oxygen/5 % CO_2 gas mixture) Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO_4 , 1.25 mM CaCl_2 , 1.2 mM KH_2PO_4 , 25 mM NaHCO_3 and 11 mM glucose) at 37 °C to wash out blood, as described earlier [129]. The hearts were then snap-frozen in liquid nitrogen and stored at -80 °C until further experiments. The other half of the animals were further randomized into four groups: (1) control ischemic with normoglycemia (NG + Isch), (2) RIC with normoglycemia (NG + RIC), (3) ischemic with acute hyperglycemia (AHG + Isch) and (4) RIC with acute hyperglycemia (AHG + RIC). At 35 min of the study protocol, LAD

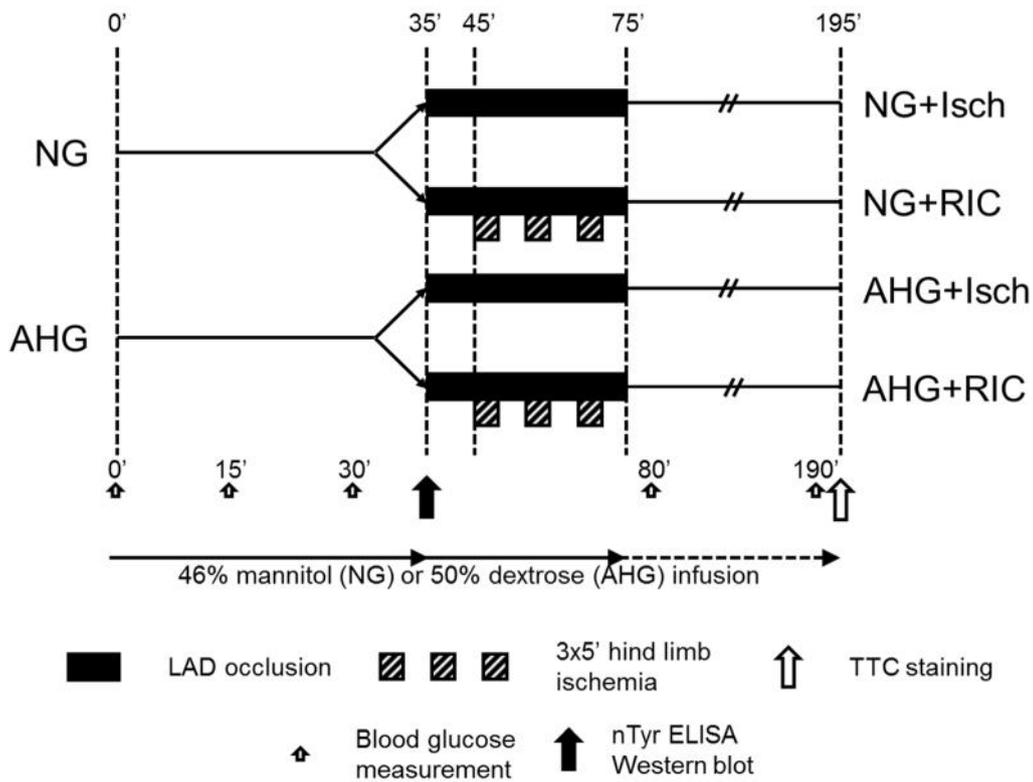


Figure 6. Experimental protocol to test the effect of hyperglycemia on remote ischemic conditioning in an *in vivo* rat model of acute myocardial infarction.

NG – normoglycemia, AHG – acute hyperglycemia, Isch – coronary ischemia, RIC – coronary ischemia and remote ischemic conditioning, nTyr – 3-nitrotyrosine, ELISA – enzyme-linked immunosorbent assay, TTC – triphenyltetrazolium chloride.

was occluded with a 6–0 polypropylene suture via median thoracotomy for 40 min. Occlusion was confirmed by ST-segment elevation, arrhythmias and paling of the occluded area. RIC was induced by three cycles of 5 min occlusion and 5 min reperfusion of the right femoral vessels starting 10 min after the LAD occlusion. Both the femoral artery and vein were occluded with a metal vessel clamp after isolation of the vessels from the surrounding connective tissue and femoral nerve. At the end of the 40 min index ischemia, reperfusion was induced by loosening the suture. At the end of the 120 min reperfusion, hearts were harvested for evaluation of the degree of myocardial necrosis.

5.4. Experimental protocol to test the vesicular nature of remote ischemic conditioning *ex vivo*

Male Wistar rats (250–350 g) were anesthetized by 85 mg/kg ketamine and 10 mg/kg xylazine and heparinized (500 U/kg). Hearts were isolated and perfused in Langendorff mode with 37 °C Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose) for 20 min for stabilization. Hearts were randomized into five groups (Figure 7): 1) 30 min global ischemia (Isch); 2) additional IPreC with 3×5 min global ischemia/reperfusion; 3) perfused with perfusate collected from Isch hearts prior to the 30 min global ischemia starting from the 20th min (Isch perf); 4) perfused with perfusate collected from IPreC hearts prior to the 30 min global ischemia starting from the 20th min (IPreC perf); 5) perfused with vesicle-depleted perfusate collected from IPreC hearts prior to the 30-min global ischemia starting from the 20th min (Depl perf). After 120 min of reperfusion, hearts were sliced into 2 mm slices, and stained with triphenyltetrazolium chloride as in Section 5.5.2.

Extracellular vesicles were isolated from collected coronary perfusates by filtration and differential centrifugation. Freshly prepared coronary perfusates were dialyzed in cellulose dialysis tube (Sigma, St. Louis, MO, US) against 0.45 % saline containing 5 mM EDTA for 4 h at room temperature. CFs were then vacuum-distilled to 40 mL (for approximately 40 min) at room temperature. Concentrated coronary perfusates were filtered through a 800 nm filter (Merck, Darmstadt, Germany) by gravity, and filtrates were centrifuged at 12,200×g for 20 min at 4 °C. Pellets were saved as

microvesicle/microparticle fraction. Supernatants of the previous centrifugation were filtered through a 200 nm filter (Merck, Darmstadt, Germany) by gravity and centrifuged at 100,000×g for 90 min at 4 °C in Beckman MLA-55 rotors. Pellets were saved as exosome-rich pellet and the supernatant was saved as vesicle-depleted perfusate. Vesicle-depleted perfusates were then reconstituted to their original volume measured at the time of coronary perfusate collection with Krebs-Henseleit solution (*e.g.*, in case 200 mL coronary perfusate was collected, 40 mL of depleted perfusate was combined with 160 mL Krebs-Henseleit solution). Reconstituted perfusates were used in heart perfusion experiments on the same day.

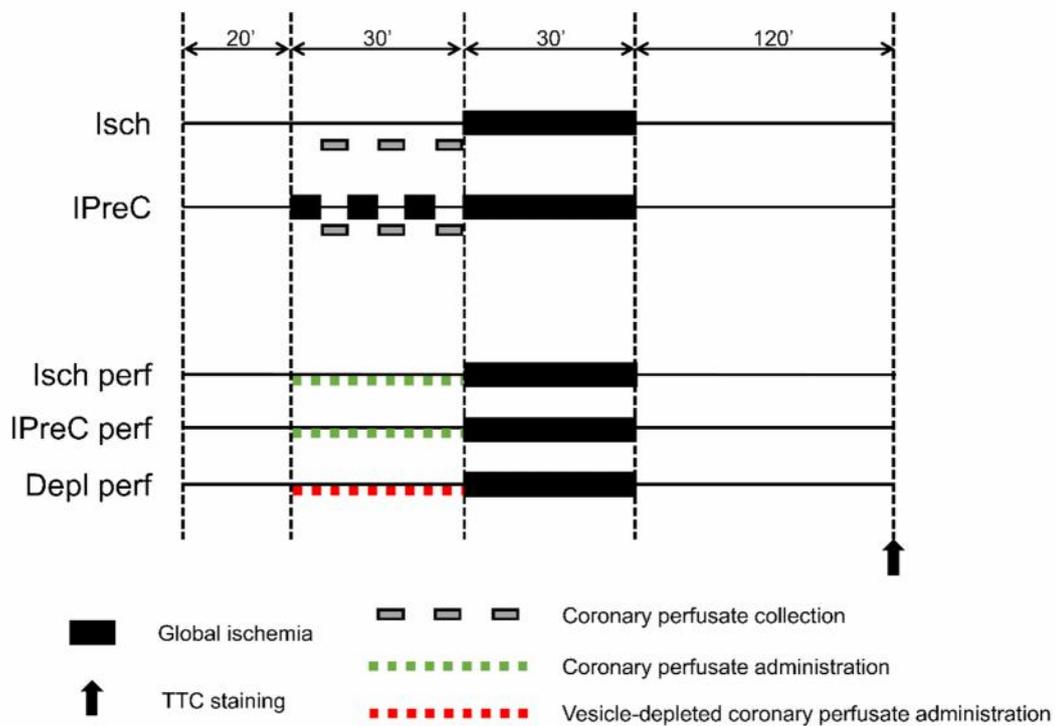


Figure 7. Experimental protocol to test vesicular nature of remote ischemic conditioning *ex vivo*. Isch – ischemia only, IPreC – ischemic preconditioning, perf – perfused with perfusate collected from Isch or IPreC hearts, Depl perf – perfused with perfusate collected from IPreC hearts and depleted from extracellular vesicles, TTC – triphenyltetrazolium chloride.

5.5. Measurement of area at risk, myocardial function, myocardial necrosis, edema and microvascular obstruction

5.5.1. Assessment of area at risk with coronary angiography analysis

All animals underwent coronary angiography according to the protocol established by the catheterization laboratory. Anterograde flow in the artery before and after balloon inflation was characterized using the TIMI (Thrombolysis in Myocardial Infarction) system [130]. TIMI myocardial perfusion grade and myocardial blush grade were assessed visually on the angiogram and made by expert interventional cardiologist, and all data were entered prospectively into a database. Myocardial blush grade has been defined as follows: 0, no myocardial blush or contrast density; 1, minimal myocardial blush or contrast density; 2, moderate myocardial blush or contrast density but less than that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery; and 3, normal myocardial blush or contrast density, comparable with that obtained during angiography of a contralateral or ipsilateral non-infarct-related coronary artery. When myocardial blush persisted (“staining”), this phenomenon suggested leakage of contrast medium into the extravascular space and was graded 0 [131, 132]. No digital techniques were used. The area at risk (AAR) was established by using the modified APPROACH score [133].

5.5.2. Assessment of myocardial necrosis by triphenyltetrazolium chloride staining

Hearts were excised at the end of the indicated reperfusion periods. Rat hearts from *in vivo* experiments were perfused with oxygenated (95 % oxygen/5 % CO₂ gas mixture) 37 °C Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose) in Langendorff mode for 1 min. LAD was reoccluded, and the AAR was negatively stained with Evans blue (Sigma, St. Louis, MO, US) retrogradely. Then 2 mm slices were cut. Rat hearts from *ex vivo* experiments were cut into 2 mm slices without Evans blue (Sigma, St. Louis, MO, US) staining at the end of reperfusion.

Porcine hearts were placed immediately in ice-cold saline. Coronary angiography of

the pig was reviewed, and the occlusion site was identified. LAD was reoccluded *ex vivo* at the same place as *in vivo* with a pean, and Evans blue (Sigma, St. Louis, MO, US) was injected into the coronary arteries through their orifices to negatively stain the AAR. Then 10 mm slices were cut. Heart slices were incubated in 1 % triphenyltetrazolium chloride (Sigma, St. Louis, MO, US) at 37 °C for 15 min to stain viable areas. After overnight fixation with 4 % formalin, slices were weighed and scanned for blind planimetric analysis (InfarctSize 2.4b software [Pharmahungary Group, Szeged, Hungary]). AAR was expressed as the proportion of the left ventricular (LV) mass, and necrosis as the proportion of the AAR mass. In case of hearts receiving *ex vivo* global ischemia, AAR was equal to the LV.

5.5.3. Assessment of myocardial function, necrosis, edema and microvascular obstruction with porcine cardiac magnetic resonance imaging

Three days (70-78 h) after the deflation of the balloon in LAD of pigs, anesthesia was induced by inhalation of isoflurane-oxygen mix. Prior to cardiac magnetic resonance imaging (MRI), atracurium was administered and ventilation was maintained with mechanical ventilation. For the assessment of myocardial necrosis and edema, cardiac MRI was performed using a 1.5 T clinical scanner (Avanto, Siemens, Erlangen, Germany) using a phased array coil and a vector electrocardiogram system. Cine MRI images were acquired using a retrospectively electrocardiography-gated, steady-state free precession cine MRI technique (Cinetruefisp sequence) in short-axis and long-axis views of the heart using 1.2 ms echo time, 40 ms repetition time, 65 degree flip angle, 15 segments, 360 mm field-of-view, 8 mm slice thickness, and 256×256 image matrix. The image resolution was 1.4×1.4*8 mm. Dark blood prepared IR-TSE sequence single slice breath-hold acquisition T2w protocol with IR preparation (technical detail: TI=170 ms) 15 segments using every second trigger pulse was used to detect edema (technical details: TE 74 ms, flip angle 180 degree). The slice position and resolution was identical as cine images. The late gadolinium-enhanced images were acquired to determine the amount of myocardial necrosis and MVO. A 2-dimensional single shot Truefisp sequence with non-selective IR pulse shift acquisition to a diastolic phase of the cardiac cycle by adjusting the TR was

used 12 to 15 min after administration of a gadolinium-based contrast agent (0.13 mmol/kg gadobutrol [Bayer, Whippany, NJ, US]), with slice positions identical to the cine images. Typical in-plane resolution was 1.4×1.4×8 mm (Technical details: echo time 1.2 ms, flip angle 50°, triggering to every other heart beat). The inversion time was set to null the signal of viable myocardium and ranged from 280 to 320 ms. Left and right ventricular end-diastolic and end-systolic volumes, stroke volumes, ejection fractions and masses were quantified using manual planimetry of end-diastolic and end-systolic short-axis SSFP cine images with MASS 7.6 analysis software (Magnetic Resonance Analytical Software System, Medis Medical Imaging Software, Leiden, The Netherlands).

Myocardial necrosis and edema were quantified after manual planimetry both on the delayed contrast enhancement and T2-weighted images by delineation of myocardium with signal intensity 4 SDs above the mean signal obtained in the remote non-infarcted myocardium using MASS 7.6 analysis software. If present, the hypointense zone in the center of the hyperenhancement (MVO) was quantified and added to the infarct volume as previously described [134]. Values were expressed relative to the LV mass.

5.6. Characterisation of extracellular vesicles

5.6.1. Detection of extracellular vesicles with transmission electron microscopy

Extracellular vesicle pellet was prepared by centrifuging samples at 100,000×g for 90 min at 4 °C in Beckman MLA-55 rotors. Formalin (4 %) was layered carefully onto the pellets. After formalin was washed out, pellets were post-fixed with 1 % osmium tetroxide (OsO₄) for 20-30 min, and block stained with 1 % uranyl acetate (in 50 % ethanol) for 20-30 min. Then, pellets were dehydrated by graded ethanol for 5 min in 70 %, 90 %, 96 %, and 3×5 min in 100 % ethanol. Pelleted samples were embedded in Taab 812 (Taab Laboratories, Aldermaston, UK). Then ultrathin sections were prepared. Prepared samples were analyzed under Hitachi 7100 electron microscope equipped by Veleta, a 2k×2k MegaPixel side-mounted TEM CCD camera (Olympus, Tokyo, Japan). Contrast and brightness of electron micrographs were edited in Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA, US).

5.6.2. Size distribution of extracellular vesicles with dynamic light scattering

Size distribution of extracellular vesicles was measured by dynamic light scattering measurements by using an ALV goniometer with a Melles Griot diode-pumped solid-state laser at 457.5 nm wavelength (type: 58 BLD 301). The intensity of the scattered light was measured at 90° and the autocorrelation function was calculated using an IBM PC-based data acquisition system. Particle size distributions were determined by the maximum entropy method. The diameter of the particles was calculated using sphere approximation.

5.7. Arrhythmia analysis in rat model of ischemia/reperfusion injury

An electrocardiogram was recorded throughout the entire experiment. Arrhythmia analysis was performed according to the Lambeth conventions, and arrhythmia incidence and duration scores were calculated [135].

5.8. Protein detection with Western blot in various samples

5.8.1. Sample preparation for Western blot

In order to demonstrate presence or absence of extracellular vesicles in the perfusate of Langendorff perfused hearts, coronary perfusate collected from Isch and IPreC hearts (n=3) were dialyzed and concentrated as described above. After passing concentrated samples through 800 nm filters by gravity, a total extracellular vesicle pellet was prepared by centrifuging samples at 100,000×g for 90 min at 4 °C in Beckman MLA-55 rotors. Supernatants of the centrifugation were kept as extracellular vesicle-depleted perfusates. Total extracellular vesicle pellets were resuspended in 35 µL radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, US) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, US) containing protease inhibitor was added to extracellular vesicle-depleted perfusates as well in 1/10 ratio. Twenty µL of each sample was mixed

with 5× Laemmli-buffer (Bio-Rad, Hercules, CA) and loaded on 4-20 % Tris-glycine SDS-polyacrylamide gels (Bio-Rad, Hercules, CA).

LV tissue was homogenized in radioimmunoprecipitation assay buffer (Cell Signaling, Danvers, MA, US) supplemented with protease inhibitor (Roche, Basel, Switzerland), sodium fluoride (Sigma, St. Louis, MO, US) and phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, US). Protein concentration of the homogenates was measured by Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Waltham, MA, US).

5.8.2. General description of Western blot

Twenty µL of each sample (samples containing extracellular vesicles) or equal amount of protein was mixed with reducing 5× Laemmli buffer, loaded and separated in a 4-20 % precast Tris-glycine SDS polyacrilamide gel (Bio-Rad, Hercules, CA, US) with 90-110 V. Proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, US) at 350 mA for 2 h or 200 mA overnight. Proper transfer was visualized with Ponceau staining (Sigma, St. Louis, MO, US). Membranes were blocked with 5 % bovine serum albumin (Santa Cruz Biotechnology, Dallas, TX, US) or non-fat dry milk (Bio-Rad, Hercules, CA, US) in Tris-buffered saline containing 0.05 % Tween-20 (Sigma, St. Louis, MO, US) at room temperature for 2 h. Membranes were probed with primary antibodies purchased from Cell Signaling (Danvers, MA, US) overnight at 4 °C (HSP60 - #4870; phospho-mTOR [Ser2448] - #2971; mTOR - #2972; phospho - S6 [Ser235/236] - #2211; ribosomal S6 - #2317; phospho-AKT [Ser473] - #9271; AKT - #9272; phospho-AMP-activated protein kinase [AMPK ; Thr172] - #2535; AMPK - #5831; phospho-extracellular signal-regulated kinase 1/2 [Erk1/2; Thr202/Tyr204] - #9106; Erk1/2 - #9107; microtubule-associated protein 1 light chain 3 A/B [LC3A/B] - #4108; beclin-1 - #3495; SQSTM1/p62 - #5114; phospho-UNC-51-like kinase 1 [p-ULK1; Ser555] - #5869; ULK1 - #4773; autophagy-related gene 7 [ATG7] - #8558; Bcl-2/E1B - interacting protein 3 [BNIP3] - #3769; loading control: glyceraldehyde-3-phosphate dehydrogenase [GAPDH] - #5174), and with corresponding horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, Danvers, MA, US) for 2 h at room temperature.

Signals were detected with an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA, US) by Chemidoc XRS+ (Bio-Rad, Hercules, CA, US). Antibodies detecting phosphorylated epitopes were removed with Pierce Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, US) before incubation with antibodies detecting the total protein.

5.8.3. Special Western blot description of Triton X-100-insoluble SQSTM1/p62 detection

LV tissue was homogenized with TissueLyser (Qiagen, Venlo, The Netherlands) in a homogenation buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 10 % glycerol and 2 % Triton X-100 (pH 8.0) supplemented with protease inhibitor (Roche, Basel, Switzerland), sodium fluoride (Sigma, St. Louis, MO, US) and phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, US). Homogenates were centrifuged (10,000×g, 10 min, 4 °C) supernatant was carefully removed and discarded. Pellet was washed with the above mentioned homogenation buffer once again (10,000×g, 10 min, 4 °C). Pellets were then resuspended in sample buffer containing 62.5 mM Tris-HCl, 5 % glycerol and 1.3 % SDS. Protein concentration of the homogenates was measured by Bicinchoninic Acid Assay kit (Thermo Fisher Scientific, Waltham, MA, US). Equal amount of protein (20 µg) was loaded, separated and processed under reducing conditions as described above.

5.9. Myocardial 3-nitrotyrosine measurement for the assessment of the nitrative stress

Free myocardial 3-nitrotyrosine was measured from LV samples harvested at 35 min with 3-nitrotyrosine ELISA (Cayman, Ann Arbor, MI, US) according to the manufacturer's protocol.

5.10. Statistics

Data was expressed as *mean \pm standard error of mean*. *Student's t-test* was performed where two groups including continuous variables were compared. *One-way ANOVA* was performed in case of the comparison of multiple groups including continuous variables. *Repeated measures ANOVA* was used if multiple groups with continuous variables were compared at different time points. To test the effect and interaction of two variables (*i.e.*, AHG and RIC), *two-way ANOVA* was performed. After *ANOVA* studies, either *LSD* or *Dunnett's post hoc test* was applied. To limit the case numbers in the cardiac MRI study, *one-way ANOVA* was performed by using bootstrapping with 1000-sample replacement [136], as frequently used in clinical studies [137-139] and recommended for preclinical studies as well [140]. Groups with discrete variables (*i.e.*, scores) were compared with *Kruskal–Wallis analysis*. Mortality statistics has been developed by using the *Kaplan–Meier estimation*. Statistical significance was accepted if $p < 0.05$. Statistical tests were performed with IBM SPSS Statistics, Version 19.

6. Results

6.1. The cardioprotective effect of remote ischemic conditioning in rat and porcine hearts

6.1.1. The cardioprotective effect of remote ischemic conditioning in an *in vivo* rat model of ischemia/reperfusion injury

To investigate whether RIC attenuates myocardial necrosis, RIC was tested in an *in vivo* rat model of AMI as described in Section 5.1 and Figure 4. RIC significantly attenuated the myocardial necrosis compared to the Control group ($p < 0.05$; $n = 5-6$; Figure 8a). There was no difference between the AAR of groups ($n = 5-6$).

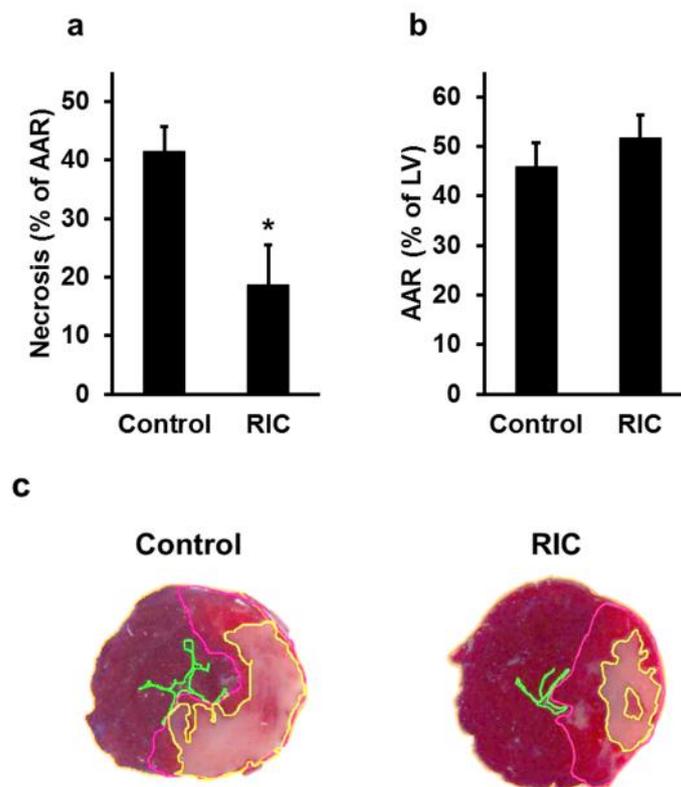


Figure 8. a RIC attenuated myocardial necrosis. b AAR was not different between groups. c Representative images of Evans blue/triphenyltetrazolium chloride-stained heart sections indicating myocardial necrosis and AAR. Orange: slice outline, green: ventricular chamber, purple: AAR, yellow: myocardial necrosis. * $p < 0.05$ vs. Control.

RIC – remote ischemic conditioning, AAR – area at risk, LV – left ventricle.

6.1.2. *The cardioprotective effect of remote ischemic conditioning in an in vivo porcine model of ischemia/reperfusion injury*

To investigate whether RIC attenuates myocardial necrosis, RIC was tested in an *in vivo* porcine model of ischemia/reperfusion injury as described in Section 5.2 and Figure 5.

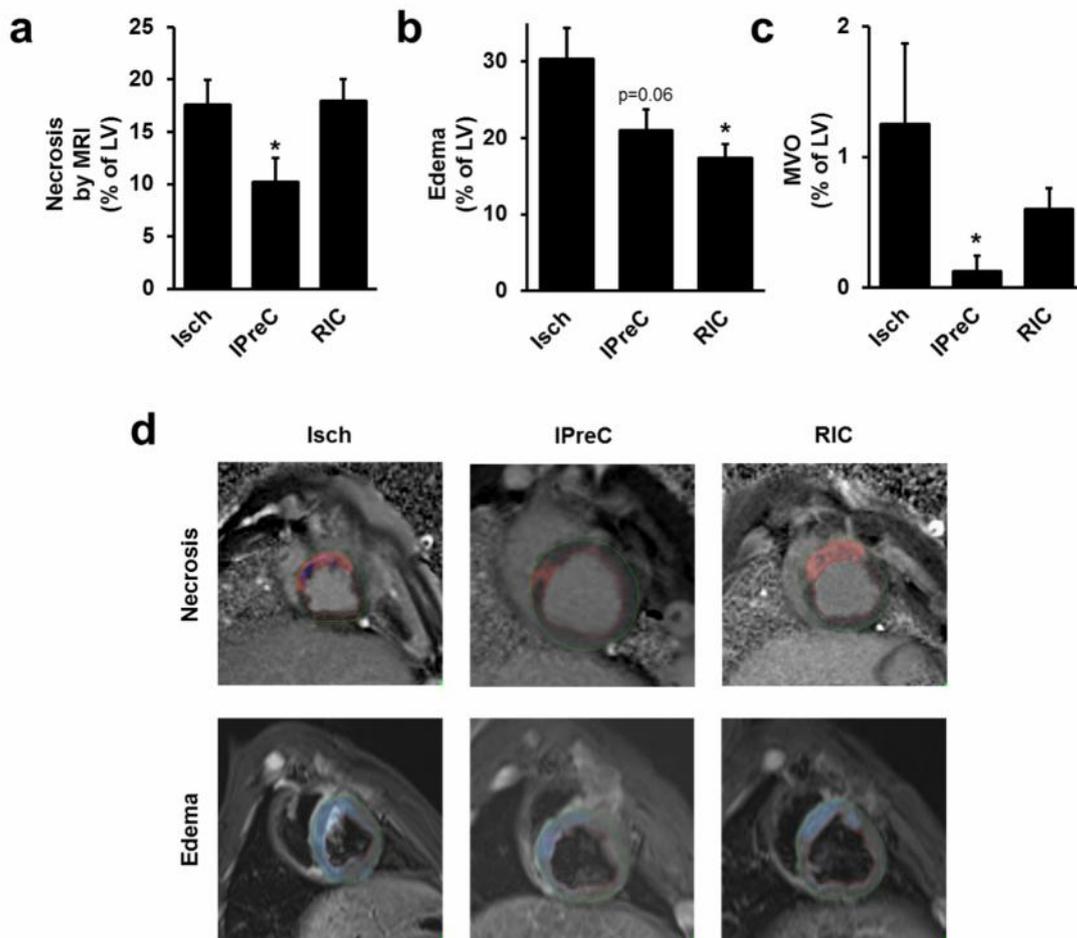


Figure 9. **a** IPreC, but not RIC, reduced myocardial necrosis. **b** IPreC and RIC decreased myocardial edema. **c** IPreC reduced MVO. **d** Representative MRI images of myocardial necrosis and edema. Green line: epicardial outline, red line: endocardial outline, red area: myocardial necrosis, dark blue area: MVO, light blue area: myocardial edema. * $p < 0.05$ vs. Isch. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, MRI – magnetic resonance imaging, LV – left ventricle, MVO – microvascular obstruction.

Table 1. At baseline, hemodynamic parameters were not significantly different between groups, whereas the heart rate was significantly lower in IPreC and RIC groups as compared to the Isch group. *p<0.05 vs. Isch. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, HR – heart rate, SBP – systolic blood pressure, DBP – diastolic blood pressure, MABP – mean arterial blood pressure.

Baseline					
	HR (1/min)	SBP (mmHg)	DBP (mmHg)	MABP (mmHg)	dP/dt (mmHg/s)
Isch	109.5±5.2	112.6±5.9	69.3±4.6	87.4±5.1	1037±81
IPreC	90.6±9.4	102.0±10.8	65.5±8.1	82.6±7.2	838±141
RIC	106.2±6.6	107.5±6.5	67.8±3.8	85.2±5.1	976±91
Reperfusion					
10 min					
Isch	130.2±5.1	93.6±6.4	54.1±4.0	71.1±4.6	800±54
IPreC	92.4±14.4*	82.2±4.4	53.8±4.7	63.4±3.8	560±196
RIC	113.9±4.4*	86.5±4.6	50.0±2.2	65.2±3.0	647±53

Table 2. There was no difference in various angiographic parameters between various groups. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, LV – left ventricle.

	APPROACH score (% of LV)	TIMI score	Myocardial blush grade	TIMI myocardial perfusion grade
Isch	19.2±2.2	2.4±0.3	2.5±0.3	2.3±0.3
IPreC	18.6±2.4	2.2±0.3	2.4±0.3	2.3±0.3
RIC	19.7±2.5	2.3±0.3	2.5±0.3	2.5±0.3

6.1.2.1. Myocardial necrosis, edema and microvascular obstruction by cardiac magnetic resonance imaging

To quantify myocardial necrosis, edema and MVO, cardiac MRI was performed 3 days after coronary occlusion and reperfusion. Myocardial necrosis (% of LV) was not affected by RIC, however, IPreC attenuated it significantly as compared to the Isch group ($p < 0.05$; $n = 4-8$), although only 4 IPreC cases were studied with MRI (Figure 9a). Myocardial edema (% of LV) was significantly decreased by RIC as compared to the Isch group ($p < 0.05$; $n = 7-7$), however, only a tendency of decrease was observed by IPreC ($n = 4$; $p = 0.06$; Figure 9b). At baseline, hemodynamic parameters were not significantly different between groups, whereas the heart rate was significantly lower in IPreC and RIC groups as compared to the Isch group (Table 1). AAR based on angiographic score was not different between groups (Table 2). Furthermore, angiographic measures of reperfusion, *i.e.*, TIMI score, myocardial blush grade, and TIMI myocardial perfusion grade were not affected by conditioning stimuli (Table 2). MVO volume (% of LV) was significantly decreased by IPreC ($p < 0.05$), but not by RIC as compared to the Isch group ($n = 4-8$; Figure 9c).

6.1.2.2. Myocardial necrosis and area at risk evaluated by *ex vivo* staining

To assess myocardial necrosis and AAR by an *ex vivo* histological method, the gold standard triphenyltetrazolium chloride- and Evans blue staining were applied after 3 h of reperfusion. RIC did not decrease myocardial necrosis (% of AAR), however, IPreC significantly decreased it as compared to the Isch group ($n = 5-6$; Figure 10a). There was no difference in AARs between the experimental groups (% of LV) as evaluated by Evans blue staining (Figure 10b).

6.1.2.3. Myocardial function by magnetic resonance imaging and echocardiography

Myocardial function was analyzed by cardiac MRI. Myocardial function was not different between groups after 3 days of reperfusion (Table 3). Similar data was obtained by echocardiographic analysis (Data not shown).

Table 3. There was no difference in myocardial function between groups as assessed by cardiac MRI. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, HR – heart rate, LV – left ventricle, RV – right ventricle, EDVI – end-diastolic volume index, ESVI – end-systolic volume index, SVI – stroke volume index, EF – ejection fraction, COI – cardiac output index.

	Isch	IPreC	RIC
HR (1/min)	96.26±7.76	100.50±5.06	100.13±5.07
LV EDVI (mL/kg)	3.63±0.25	3.10±0.02	3.55±0.09
LV ESVI (mL/kg)	2.07±0.15	1.81±0.14	2.09±0.17
LV SVI (mL/kg)	1.49±0.11	1.29±0.15	1.40±0.10
LV EF (%)	43.47±1.67	41.64±4.75	41.55±2.77
LV COI (L/min/kg)	0.15±0.02	0.11±0.01	0.14±0.02
RV EDVI (mL/kg)	2.86±0.21	2.80±0.18	2.59±0.10
RV ESVI (mL/kg)	1.40±0.09	1.65±0.05	1.32±0.08
RV SVI (mL/kg)	1.46±0.15	1.15±0.18	1.28±0.08
RV EF (%)	43.95±2.43	40.63±3.66	47.42±2.61
RV COI (L/min/kg)	0.14±0.02	0.09±0.01	0.12±0.01

6.2. The cardioprotective effect of remote ischemic conditioning is abolished by acute hyperglycemia

To investigate the effect of acute hyperglycemia on the efficacy of RIC, acute hyperglycemia was induced with a 50 % dextrose infusion during *in vivo* ischemia/reperfusion experiments as described in Section 5.3 and Figure 6. Blood glucose levels were significantly elevated due to dextrose perfusion in both AHG+Isch and AHG+RIC groups compared to the corresponding normoglycemic groups from at least the 15th min of perfusion (Table 4, preliminary results showed that acute hyperglycemia developed in 5 min [17.2±1.8 mM, n=4]). Elevated blood glucose levels did not influence the heart rate and blood pressure of the rats (Table 5).

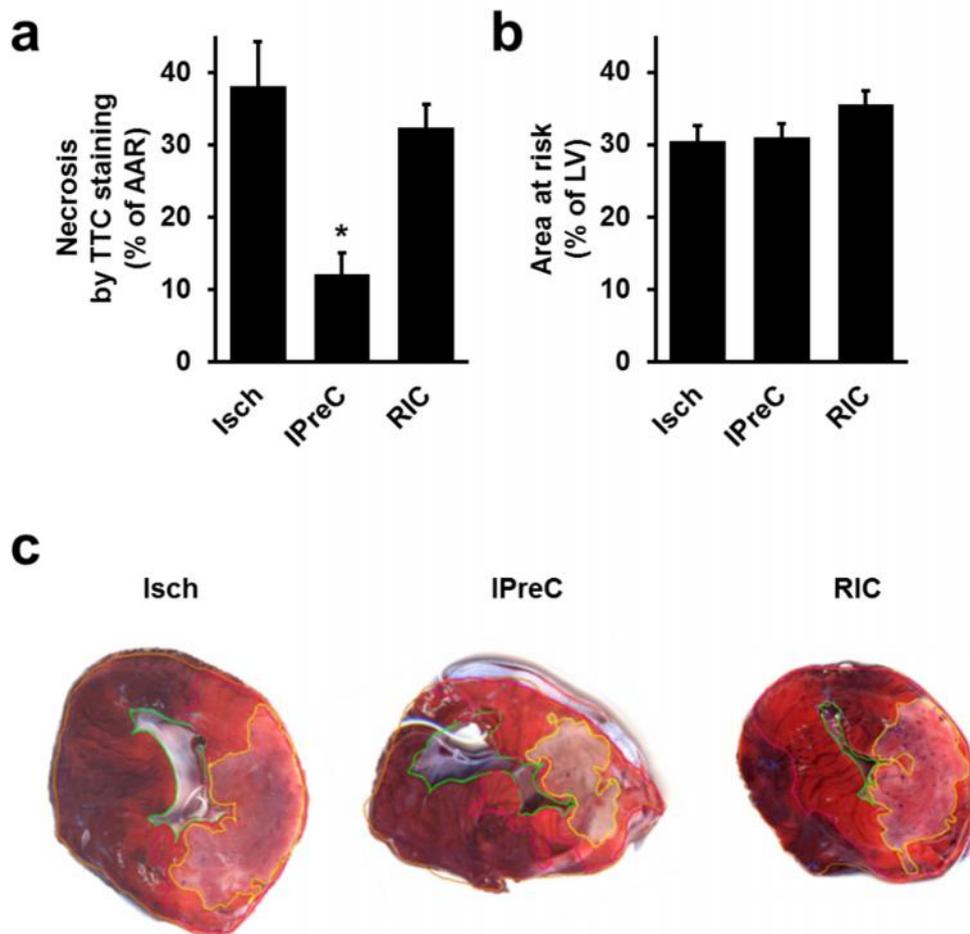


Figure 10. **a** IPreC, but not IPostC and RIC, reduced myocardial necrosis. **b** There was no difference between AARs. **c** Representative images of Evans blue/triphenyltetrazolium chloride-stained heart sections indicating myocardial necrosis and AAR. Orange: slice outline, green: ventricular chamber, purple: AAR, yellow: myocardial necrosis. * $p < 0.05$ vs. Isch. Isch – ischemia only, IPreC – ischemic preconditioning, RIC – remote ischemic conditioning, AAR – area at risk, LV – left ventricle.

6.2.1. Myocardial necrosis is not reduced by remote ischemic conditioning during acute hyperglycemia

Myocardial necrosis was significantly smaller in NG+RIC group compared to NG+Isch ($p < 0.05$; $n = 7-10$), while RIC failed to decrease myocardial necrosis in

AHG+RIC group when compared to AHG+Isch (n=5-6; Figure 11a). Furthermore, acute hyperglycemia *per se* did not aggravate cardiac necrosis (Figure 11a). There was no difference between the AAR of various groups (n=5-10; Figure 11b).

Arrhythmia analyses revealed that acute hyperglycemia compared to normoglycemia significantly increased the incidence and duration of arrhythmias during the whole myocardial ischemic period, but arrhythmia incidence and duration were not different during reperfusion (Table 6). RIC *per se* did not alter arrhythmia scores from the time point it was applied, as compared to Isch group (Table 6). Furthermore, RIC did not decrease arrhythmia scores during acute hyperglycemia compared to AHG+RIC (Table 6).

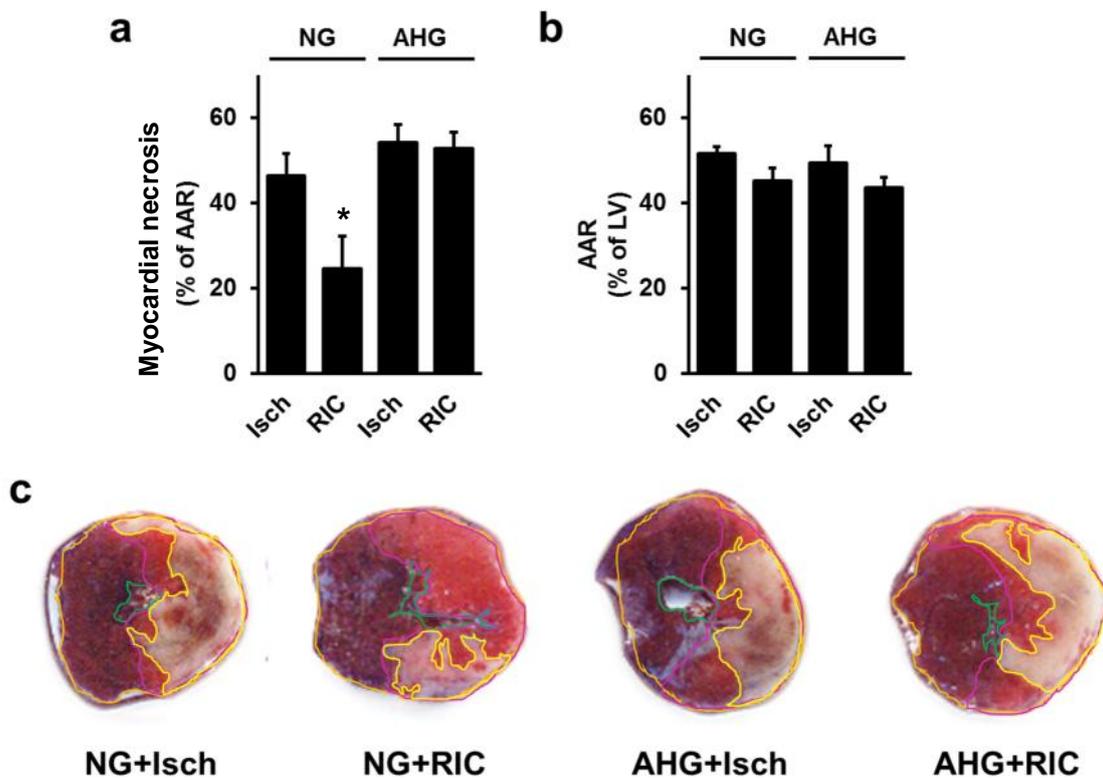


Figure 11. a Acute hyperglycemia abolished cardioprotective effect of RIC. **b** There was no difference between AARs. **c** Representative images of Evans blue/triphenyltetrazolium chloride-stained heart sections indicating myocardial necrosis and AAR. Orange: slice outline, green: ventricular chamber, purple: AAR, yellow: myocardial necrosis. * $p < 0.05$ vs. NG + Isch. # $p < 0.05$ vs. NG + RIC. NG – normoglycemia, AHG – acute hyperglycemia, Isch – ischemia only group, RIC – remote ischemic preconditioning, AAR – area at risk, LV – left ventricle.

6.2.2. Nitrate stress is increased by acute hyperglycemia

Increased oxidative and/or nitrate stress is often implicated in the disruption of cardioprotective interventions. The 3-nitrotyrosine content, a marker of nitrate stress, was therefore measured in the hearts of normoglycemic and acute hyperglycemic rats at 35 min. Cardiac 3-nitrotyrosine was significantly elevated due to acute hyperglycemia ($p < 0.05$; $n = 8-8$; Figure 12).

Table 4. Blood glucose (mM) is elevated in acute hyperglycemia. * $p < 0.05$ vs. corresponding time point of NG+Isch group. # $p < 0.05$ vs. corresponding time point of NG+RIC group. NG – normoglycemia, AHG – acute hyperglycemia, Isch – ischemia only group, RIC – remote ischemic preconditioning.

	0'	15'	30'		70'	190'
NG	8.6±0.3	6.8±0.2	8.4±0.7	NG+Isch	9.2±0.7	9.1±1.1
				NG+RIC	9.2±0.6	8.5±0.5
AHG	8.5±0.3	17.7±1.0*	21.3±2.1*	AHG+Isch	18.0±1.3*	18.6±0.6*
				AHG+RIC	19.9±1.4#	18.4±0.6#

6.2.3. mTOR pathway is overactivated by acute hyperglycemia

Because the oxidative and nitrate stress have been previously shown to interact with mTOR pathway [141], we evaluated the expression and/or phosphorylation of mTOR pathway associated proteins. Phosphorylation levels of mTOR (Ser2448) and S6 (Ser235/236) were significantly elevated ($p < 0.05$; $n = 7-9$; Figure 13a-b), which indicates that the activity of mTOR complex I was increased in acute hyperglycemia. Phosphorylation of AKT at site Ser473 was also significantly elevated in acute hyperglycemia ($p < 0.05$; $n = 7-9$; Figure 13c), however, other mTOR regulators, such as phosphorylated AMPK (Thr172) and Erk1/2 (Thr202/Tyr204) were unchanged in acute hyperglycemia as compared to normoglycemia ($n = 7-9$; Figure 13d-e).

Table 5. Acute hyperglycemia does not influence heart rate (HR) and mean arterial blood pressure (MABP). NG – normoglycemia, AHG – acute hyperglycemia, Isch – ischemia only group, RIC – remote ischemic conditioning.

<u>HR (L/min)</u>	0'	15'	30'	40'	60'	80'	100'	120'	140'	160'	180'
NG	428±10	409±12	415±9	438±15	422±12	430±13	419±10	411±12	411±18	425±20	421±14
				NG+Isch							
AHG	420±12	417±8	403±9	419±15	432±14	418±14	420±8	428±10	416±9	427±9	428±13
				NG+RIC							
				AHG+Isch							
				AHG+RIC							
				410±8	435±10	409±12	414±16	408±11	415±11	406±18	396±18
<u>MABP (Hgmm)</u>	0'	15'	30'	40'	60'	80'	100'	120'	140'	160'	180'
NG	111±5	114±7	114±6	116±13	110±10	109±10	110±9	109±9	107±8	108±7	106±11
				NG+Isch							
AHG	112±7	125±8	119±8	111±6	118±6	109±7	105±7	107±7	100±8	105±7	106±7
				NG+RIC							
				AHG+Isch							
				AHG+RIC							
				98±16	118±13	106±11	116±10	109±13	113±11	106±13	103±12

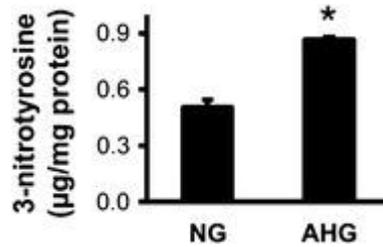


Figure 12. Acute hyperglycemia increased cardiac nitrative stress. 3-nitrotyrosine content of hearts of NG or AHG rats. * $p < 0.05$ vs. NG. NG – normoglycemia, AHG – acute hyperglycemia.

6.2.4. Autophagy is not affected by acute hyperglycemia

Since oxidative/nitrative stress and mTOR pathway have been shown to interact with autophagy, expression and/or phosphorylation of autophagy-related proteins were assessed in normoglycemia and acute hyperglycemia. LC3II/LC3I ratio was significantly decreased due to acute hyperglycemia ($p < 0.05$; $n = 7-9$; Figure 14a), however, other autophagy-related proteins such as Beclin-1, total and Triton X-100-insoluble SQSTM1/p62, phospho-ULK1 (Ser555), ATG7 and BNIP3 were unchanged in acute hyperglycemia ($n = 7-9$; Figure 14b-g).

6.3. Remote ischemic conditioning is mediated by extracellular vesicles

Vesicular transfer of RIC mediators was investigated in an *ex vivo* setup of cardiac ischemia/reperfusion injury as described in Section 5.4 and Figure 7.

6.3.1. Ischemic preconditioning increases the release of cardiac extracellular vesicles

Coronary perfusates from IPreC hearts contained more extracellular vesicles than perfusates isolated from control (Isch) hearts as evidenced by immunoblotting against HSP60, a well-accepted marker of extracellular vesicles (Figure 15a). Electron micrographs revealed that these extracellular vesicles can be classified as

microvesicle/microparticles as defined by a diameter of >100 nm and light vesicular structure (Figure 15b), and exosomes of <100 nm in diameter (Figure 15b). To further demonstrate that size of the particulate matter in coronary perfusate conforms to the range of exosomes and microvesicles, we assessed their size distribution by dynamic light scattering in perfusates from IPreC hearts. As the representative diagram (Figure 15c) shows, three populations of particles could be distinguished in our samples. Besides a small fraction of particles with hypothetical diameter of approximately 10 nm, the main particulate constituents fell into the size range of exosomes (<100 nm) and microvesicles (100–1000 nm).

Table 6. Acute hyperglycemia exacerbates the incidence and duration of arrhythmias during ischemia. *p<0.05 vs. corresponding time point of NG+Isch group. #p<0.05 vs. corresponding time point of NG+RIC group. NG – normoglycemia, AHG – acute hyperglycemia, Isch – ischemia only group, RIC – remote ischemic conditioning.

Arrhythmia incidence scores

	0-35 [°]		35-45 [°]	45-75 [°]	75-195 [°]
NG	4.87±1.28	NG+Isch	1.79±0.73	2.47±0.69	8.10±3.01
		NG+RIC	3.09±1.57	3.03±0.98	6.56±1.12
AHG	1.99±0.45	AHG+Isch	14.06±3.34*	23.17±2.1*	8.79±1.97
		AHG+RIC	10.69±6.68	12.88±6.87#	7.72±3.19

Arrhythmia duration scores

	0-35 [°]		35-45 [°]	45-75 [°]	75-195 [°]
NG	3.65±0.84	NG+Isch	1.59±0.61	2.18±0.57	7.58±2.92
		NG+RIC	1.88±0.73	2.18±0.67	4.98±0.82
AHG	1.99±0.45	AHG+Isch	7.49±1.58*	14.46±2.84*	8.27±1.95
		AHG+RIC	4.08±1.43	8.98±3.62#	7.71±3.18

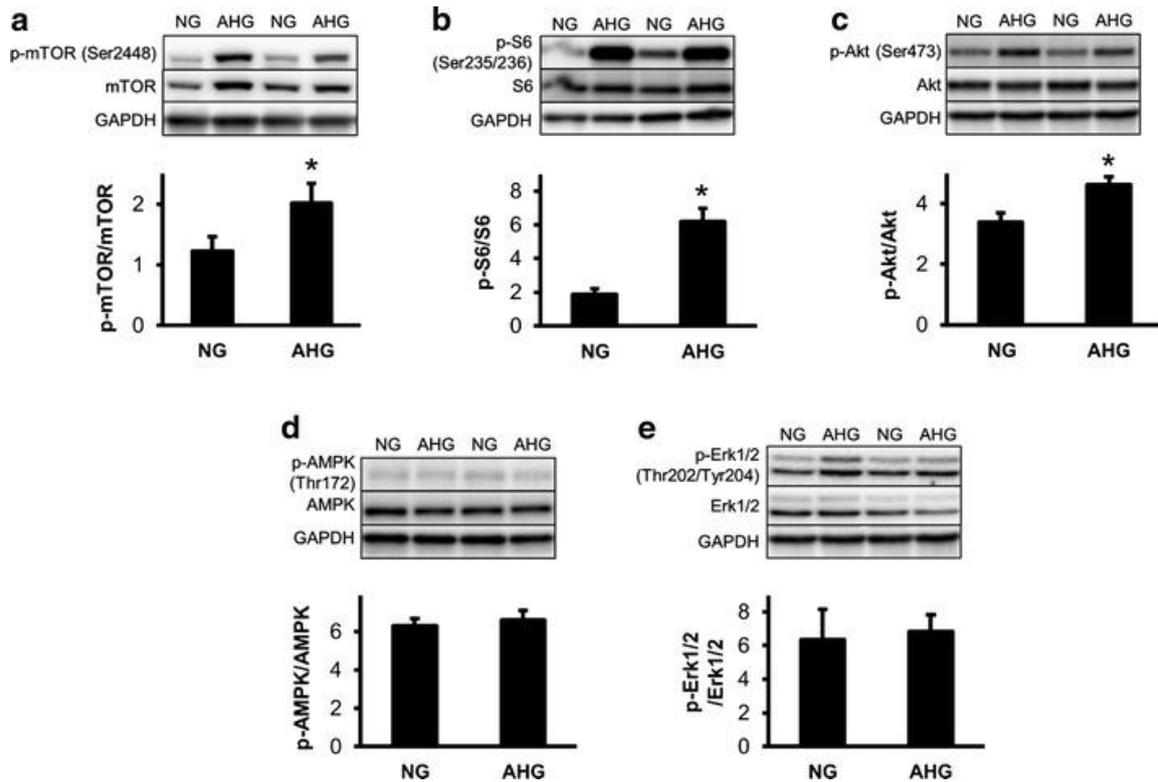


Figure 13. Acute hyperglycemia activated mTOR pathway. **a-e** Protein expression and/or phosphorylation of various mTOR-related proteins in the left ventricle. * $p < 0.05$ vs. NG. NG – normoglycemia, AHG – acute hyperglycemia.

6.3.2. Coronary perfusate depleted from extracellular vesicles did not decrease myocardial necrosis

Isolated hearts that received 3x5-5 min ischemia and reperfusion (IPreC) before 30 min global ischemia demonstrated significantly reduced myocardial necrosis as compared to aerobically perfused hearts (Isch) ($p < 0.05$, $n = 5-8$). In hearts treated with coronary perfusates collected from IPreC hearts (IPreC perf), myocardial necrosis was significantly lower than in hearts treated with coronary perfusate from Isch hearts (Isch perf) ($p < 0.05$, $n = 5-8$). Perfusates from IPreC hearts which had been depleted of extracellular vesicles were also given to recipient hearts (Depl perf). Myocardial necrosis after 30 min ischemia and 2 h reperfusion in Depl perf hearts did not differ significantly from the extent of necrosis observed in Isch perf hearts ($n = 5-8$; Figure 16).

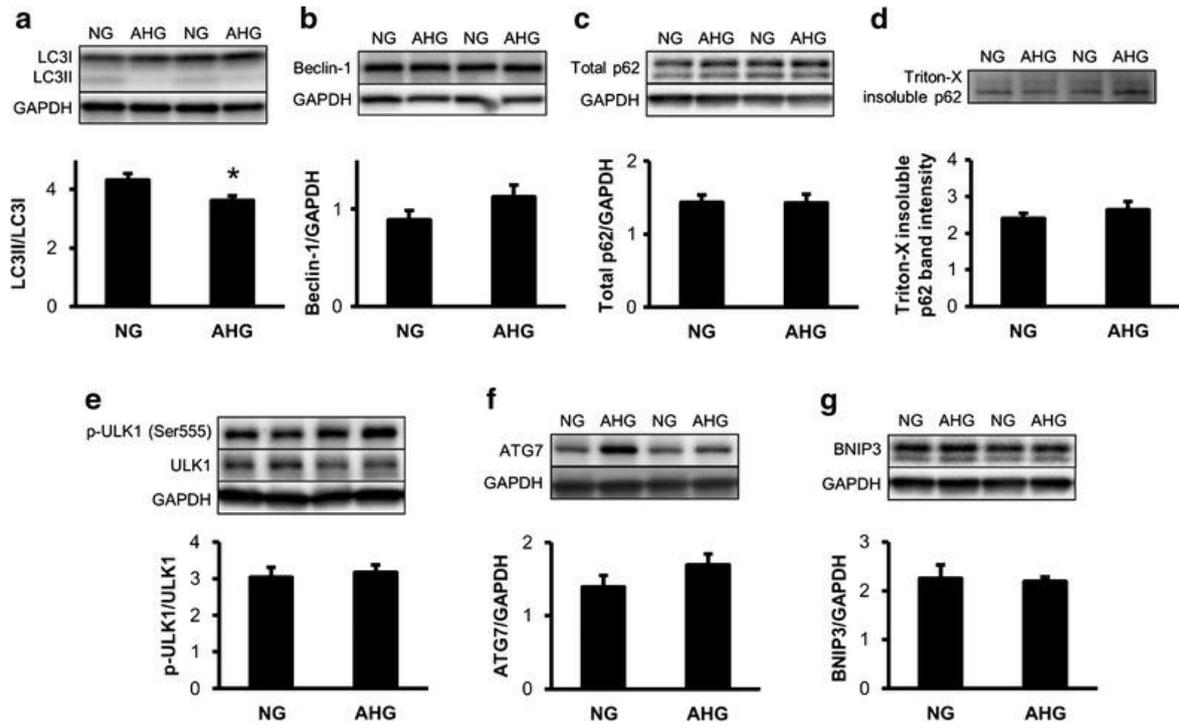


Figure 14. Acute hyperglycemia did not disturb autophagy. **a-g** Protein expression and/or phosphorylation of various autophagy-related proteins in the left ventricle. * $p < 0.05$ vs. NG. NG – normoglycemia, AHG – acute hyperglycemia.

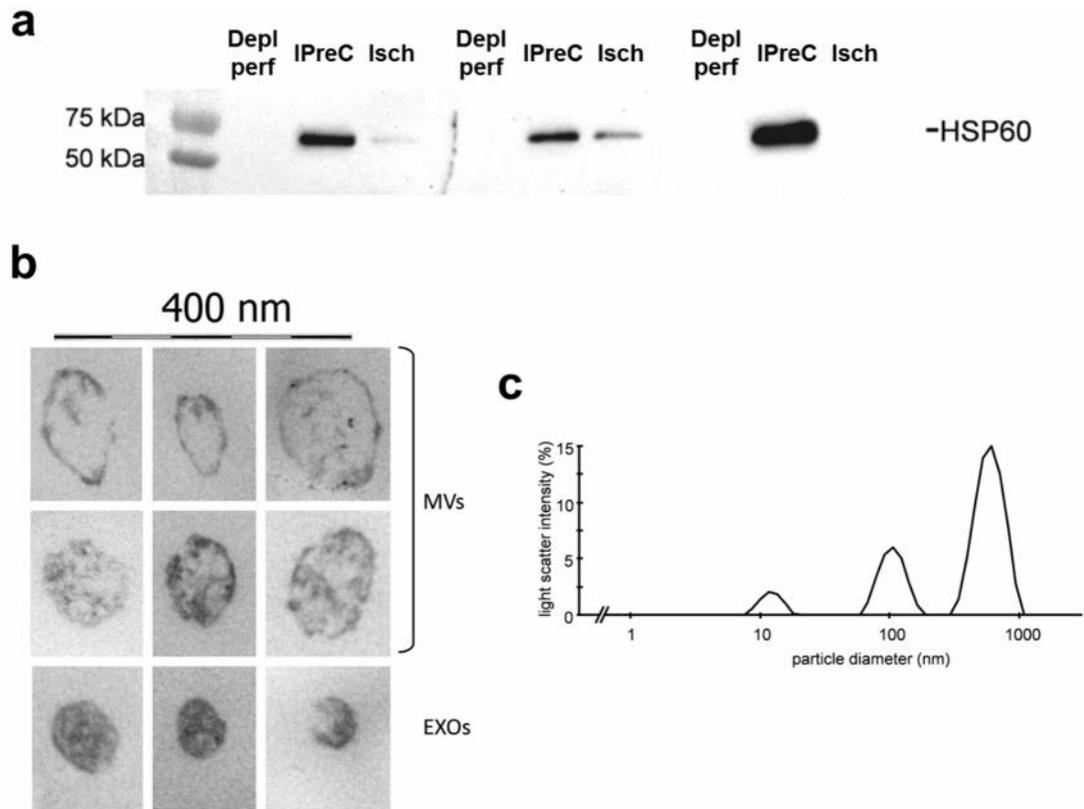


Figure 15. a HSP60 immunoblots of depleted perfusates (Depl Perf), extracellular vesicle pellets of preconditioned (IPreC) and control perfusates (Isch). **b** Representative electron micrographs of extracellular vesicles in the size ranges of microvesicles and exosomes isolated from coronary perfusate of preconditioned hearts. Scale bar represents 400 nm. **c** Dynamic light scattering analysis of perfusates showing distribution of different vesicle populations. MVs – microvesicles, EXOs – exosomes.

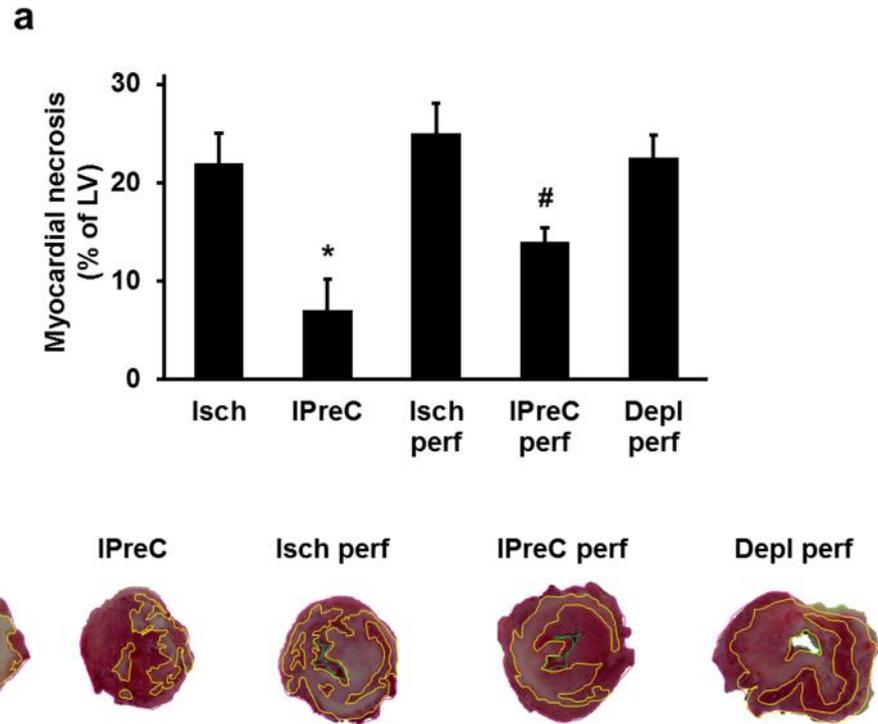


Figure 16. a Perfusate collected from preconditioned hearts significantly decreased myocardial necrosis, while perfusate depleted from extracellular vesicles did not. **b** Representative images of triphenyltetrazolium chloride-stained heart sections indicating myocardial necrosis and AAR. Green: ventricular chamber, purple: area at risk, yellow: myocardial necrosis. * $p < 0.05$ vs. Isch; # $p < 0.05$ vs. Isch Perf. LV – left ventricle, Isch – ischemia only, IPreC – ischemic preconditioning, perf – perfused with perfusate collected from Isch or IPreC hearts, Depl perf - perfused with perfusate collected from IPreC hearts and depleted from extracellular vesicles.

7. Discussion

7.1. The promise of remote ischemic conditioning: the role of microvasculature

The translation of cardioprotective conditioning stimuli into the clinical practice has proven difficult and disappointing despite numerous positive proof-of-concept clinical trials [142, 143]. The neutral results have been attributed to many factors, such as recruitment of inadequate patient populations, different types of revascularisation techniques and liberal inclusion principles (inclusion of late revascularisations, comorbidities and comedications) [142, 143]. Moreover, the strict adherence to certain endpoints, such as myocardial necrosis, and the neglect of the microvasculature might also hinder the successful translation of various cardioprotective strategies [27]. Translational models of AMI play important roles in the development of interventions for the clinical practice [142-145]. For this purpose, pigs are excellent model animals, since their cardiac anatomy and cardiovascular physiology exhibit similarities to the human heart [146]. Although the pig is suitable for closed-chest experimentation, the majority of the studies on the effect of ischemic conditioning on AMI have been performed on open-chest models [102, 147], and only a few studies are available in closed-chest models.

7.1.1. Remote ischemic conditioning did not reduce myocardial necrosis

According to previous studies [38], we demonstrated that RIC reduced myocardial necrosis in an *in vivo* rat model of AMI. However, in our porcine model of AMI, RIC did not reduce myocardial necrosis, but the positive control, IPreC, did. Previously, RIC [63, 148] has been shown to reduce myocardial necrosis in closed-chest swine models of AMI. The discrepancy between our results and those of others might be explained by the significantly different perioperative medication and experimental design. The abovementioned studies applied medications required only for the perioperative procedures (*e.g.*, anesthesia, pain control), however, the therapeutical management of AMI consists of other drugs as well.

We treated animals with acetylsalicylic acid and clopidogrel according to the clinical guidelines [149, 150]. However, it has been shown that cyclooxygenase-2 is an essential

mediator of IPreC [151] and of IPostC [152], and that its blockade neutralizes the cardioprotective effect of late IPreC [153]. Similarly, clopidogrel, a P2Y12 antagonist, has been retrospectively shown to reduce cardiac necrosis and to decrease cardiovascular events after AMI in clinical trials [154, 155], which might be attributed to its antiplatelet activity and a direct cardioprotective effect. Yang *et al.* demonstrated that IPostC did not further reduce myocardial necrosis when the P2Y12 antagonist, cangrelor pretreatment was applied in rabbits [156]. However, cardioprotection could be elicited by an extended, eight cycle-long IPostC in closed-chest pigs pretreated with acetylsalicylic acid and clopidogrel [157]. These data indicate that in order to ensure translational value of animal studies it is essential to apply perioperative medication according to clinical guidelines [39].

In our present study, anesthesia was maintained by isoflurane. It is well-documented that certain anesthetics, including the fluranes, induce cardioprotection [158] and/or interfere with cardioprotective interventions [159], making the assessment of the effect of conditioning stimuli more difficult. In our present study, we observed a decrease in myocardial necrosis by IPreC but not by RIC with isoflurane anesthesia. Although other studies have shown cardioprotective efficacy of remote IPostC with the use of isoflurane in closed-chest porcine model [148]. This discrepancy might be explained by significant differences between experimental protocols. In conclusion, there is a huge body of evidences that indicate the fact that application of antiplatelet drugs and inhalative anesthetics may interfere with various conditioning maneuvers, however, we did not specifically investigate them in our present study.

7.1.2. Remote ischemic conditioning reduced myocardial edema

Here we demonstrated with *ex vivo* Evans blue staining and *in vivo* angiography scoring that AAR was not affected by conditioning stimuli, while myocardial edema was significantly decreased by RIC indicating that the extensive damage of the cardiac microvasculature was prevented. Similarly, a clinical trial showed that myocardial edema is attenuated by RIC [66], however, others reported different results [65, 160]. These results indicate that edema might be independent from AAR in cardioprotection studies, and for this reason, applying myocardial edema as AAR may introduce bias and lead to

false conclusions in clinical trials. To date, no clinical trial has been conducted to reveal the prognostic role of myocardial edema in ST-segment elevation AMI, although it has been shown that non-ST-segment elevation AMI patients with myocardial edema had higher mortality [161].

7.1.3. Remote ischemic conditioning did not reduce microvascular obstruction

It is well-established that the volume or even the presence of MVO, another clinically detectable marker of microvascular injury, correlates with long-term outcome of AMI (for review, see [26]). In our present experimental model, RIC did not reduce MVO. Similarly to our findings, no other clinical trials confirmed the MVO-reducing ability of RIC [66, 160]. Nevertheless, the assessment of myocardial edema and MVO in preclinical and clinical studies may provide additional valuable indices.

7.1.4. Remote ischemic conditioning did not influence myocardial function

In our present study, we observed that myocardial function was not different between groups after reperfusion, although IPreC reduced myocardial necrosis. Our results are in line with a number of ischemic conditioning studies in translational models or in clinical trials. IPostC/RIC has been shown to reduce myocardial necrosis after AMI in porcine models, although myocardial function was not different between groups [157, 162, 163]. Similarly, White *et al.* demonstrated in a clinical trial that RIC did not improve early cardiac function after ST segment elevation AMI, while myocardial necrosis was significantly attenuated by RIC [66]. Furthermore, it seems that early post-AMI cardiac function is not necessarily a good predictor for the future outcome in ischemic conditioning translational studies: For instance, Munk *et al.* showed that RIC does not influence cardiac function at day 1 after AMI, however, cardiac function was improved at day 30 [164]. In summary, acute post-AMI myocardial function may be determined by many other facts than myocardial necrosis, such as, *e.g.*, actual sympathetic tone, regional wall motion [165], collateral flow [166], transmural extent of infarction [166].

7.2. Acute hyperglycemia abolishes the cardioprotective effect of remote ischemic conditioning

We have demonstrated for the first time in the literature that acute hyperglycemia with no preceding diabetes mellitus abolished the myocardial necrosis limiting effect of RIC in an *in vivo* rat model with acute coronary occlusion and reperfusion. Furthermore, we have shown here that acute hyperglycemia did not influence autophagy, but increased oxidative stress in the heart plausibly through the activation of the AKT-mTOR pathway.

7.2.1. Remote ischemic conditioning is influenced by acute hyperglycemia

Our finding that experimentally induced acute hyperglycemia with no preceding diabetes diminishes cardioprotective effect of RIC, supports previous observations showing that other forms of cardioprotection may be affected by acute hyperglycemia. Kersten *et al.* described that cardioprotection by IPreC is absent during acute hyperglycemia in dogs [126]. Similarly, acute hyperglycemia diminished cardioprotection conferred by isoflurane-induced preconditioning, however, it was reversible by increasing the minimum alveolar concentration in dogs [125]. It has been recently shown that hyperglycemia at admission does not deteriorate RIC, however, in this patient cohort, the presence of comorbidities, such as treated or untreated diabetes, have not been reported [167]. Although we showed here that acute hyperglycemia did not influence the extent of myocardial necrosis, a few studies have reported that acute hyperglycemia without any pre-existing pathophysiological conditions aggravate myocardial necrosis [126, 168, 169]. However, the majority of publications concludes that acute hyperglycemia *per se* do not change myocardial necrosis [170-172]. The seemingly contradictory findings may have been the result of applying different glucose concentrations, since reports suggesting harmful effects of acute hyperglycemia applied consistently higher glucose concentrations (*i.e.*, over 30 mM).

7.2.2. *mTOR is overactivated due to acute hyperglycemia*

The underlying mechanism of the loss of RIC-induced cardioprotection by acute hyperglycemia is not fully understood. Increased oxidative and nitrative stresses are implicated in the disruption of cardioprotective interventions by metabolic comorbidities [39, 114, 173-176], while conditioning stimuli such as RIC alleviates nitrative stress [173]. It was shown here that nitrative stress was also increased in acute hyperglycemia in rat heart *in vivo*, and similar results have been shown in isolated rat hearts perfused with hyperglycemic solution [177]. These findings clearly signal the pivotal role of excessive nitrative stress in the loss of cardioprotection in disturbed glucose homeostasis.

Oxidative and nitrative stresses have also been shown to directly disrupt autophagy (for review, see [178]). Therefore, we assessed cardiac autophagy and its regulatory pathways in acute hyperglycemia. However, we found that autophagy was unlikely to be disrupted, as only the LC3II/LC3I ratio was significantly reduced, while other autophagy-related parameters were not. Although cardiac autophagy was not modulated, its most important regulator, the mTOR pathway, was largely activated by acute hyperglycemia. Since it has been shown that the inhibition of mTOR by rapamycin elicits cardioprotective effect *in vivo* [179, 180], and that RIC, while protecting the myocardium against ischemia, downregulates mTOR [181]. We therefore hypothesize that the upregulation of mTOR pathway might be responsible for the observed loss of cardioprotection by RIC in the setting of acute hyperglycemia. Furthermore, it has also been previously shown that under nutrient excess and oxidative stress, such as that seen in hyperglycemia, the mTOR pathway and its upstream modulator AKT are increasingly activated [182-184]. It is well established that activation of AKT upon reperfusion plays a central role in the mediation of cardioprotection conferred by IPreC, IPostC and RIC (for review, see [53]). However, the cardioprotective effect of AKT activation before cardiac ischemia is controversial. Although genetic activation of AKT (24 h or 48 h prior to ischemia) have been found to protect the heart from ischemic insults [185, 186], more acute activation of AKT by SC79 and chronic AKT activation in ob/ob mice prior to ischemia have not conferred protection against ischemia/reperfusion injury [187, 188]. We showed here that acute hyperglycemia-induced AKT activation prior to myocardial ischemia does not alter myocardial necrosis. These discrepancies could be explained by

the fact that genetic activation of AKT induces an overwhelming alteration in cardiac gene expression profile [189] which might have not had time to develop in our acute hyperglycemic experiments. Moreover, we also showed that despite the acute hyperglycemia-induced activation of AKT, the cardioprotective effects of RIC are lost. Similarly, it has previously been reported that AKT activation prior to ischemia significantly interferes with protective stimuli, such as IPreC and IPostC [188, 190]. Therefore, one may conclude that the timing and the method of activation of AKT can profoundly influence its role in cardioprotection. Furthermore, AKT has a central role in the insulin signalling cascade and in the modulation of the mTOR pathway [191]. Here we evidenced an increased phosphorylation of AKT in acute hyperglycemia, however, others found opposing trends in various cellular and *in vivo* models of hyperglycemia [171, 192, 193]. This discrepancy might be attributed to the substantial difference in the activation state of insulin signalling between model systems (*i.e.*, missing insulin in streptozotocin-treated animals or limited supply of insulin in cell cultures). Nevertheless, our current results demonstrate that AKT activation in an *in vivo* model with intact insulin and glucose homeostasis is detrimental on cardioprotection.

7.3. Remote ischemic conditioning is mediated by extracellular vesicles

We have shown here for the first time in the literature that the release of extracellular vesicles from the heart after IPreC stimuli is increased and that extracellular vesicles are responsible for the transmission of RIC signals for cardioprotection.

Several humoral and neuronal transmitter mechanisms have previously been hypothesized to play a role in the propagation of RIC, however, to date none of them are generally accepted [38]. Dickson *et al.* proposed first the involvement of humoral transmission pathways, showing that transfusion of blood from preconditioned rabbits confers cardioprotection in a naïve non-preconditioned animal against ischemia/reperfusion injury [86]. Later, Breivik *et al.* showed that the soluble factor is likely to be hydrophobic [194]. The role of neuronal pathways has also been studied, but results are also still controversial [82-85].

Here we evidence a novel, vesicular mechanism for the transmission of

cardioprotective signals from a preconditioned heart to another heart subjected to coronary occlusion and reperfusion, which might explain how the suspected humoral and/or released neuronal factors of remote conditioning are transmitted. Ischemia-induced release of extracellular vesicles from cultured cardiomyocytes was reported by Malik *et al.* recently, which is in agreement with our current findings that extracellular vesicle-release of isolated hearts increases after brief ischemic episodes [195]. Elsewhere, exosomes derived from mesenchymal stem cell cultures have been shown to exert cardioprotection in mice [196], and microvesicles isolated from cell culture medium of GATA-4-overexpressing bone marrow stem cells protected neonatal cardiomyocytes from ischemic injuries [197]. Since in the latter two reports extracellular vesicles from untreated cells induced pro-survival signals, based on our current findings, we cannot exclude the possibility that extracellular vesicles released from the heart under basal conditions might be also cardioprotective, would their amount be as high as after preconditioning stimuli. Seemingly controversial to our findings, microvesicles derived from blood of animals underwent hind limb ischemia/reperfusion failed to decrease myocardial necrosis in rats [198], which might suggest that exosomes rather than microvesicles are responsible for the propagation of cardioprotective signals.

7.4. Limitations

Although here we assessed the cardioprotective effect of RIC as well as the positive control IPreC in a clinically relevant closed-chest pig model of AMI, our study has some limitations. It has recently been shown that the extent of myocardial edema has two peaks over time in swine [199], but not in humans [200]. Therefore, in this study alteration in myocardial edema at day 3 might not necessarily reflect attenuation of edema by conditioning, but the altered dynamics of edema. A long-term follow up would provide more relevant data to the design of clinical studies. Therefore, here we could only speculate whether attenuation of myocardial edema by RIC could be interpreted as a valid marker of the measure of ischemia/reperfusion injury, or whether improved long-term outcome depends only on the reduction of myocardial necrosis. Furthermore, according to the clinical routine, cardiac MRI was performed at day 3 [66], whereas *ex vivo* histopathological staining at 3 h of reperfusion according to the preclinical standards [201]. Myocardial edema and definite AAR, *i.e.*, Evans blue staining, were evaluated in

separate groups. However, we calculated APPROACH score to estimate AAR in the same cohort, in which cardiac MRI was performed.

We evaluated the effect of acute hyperglycemia on the myocardium without ischemia or RIC. It is well established that ischemia and cardioprotective interventions significantly and dynamically influence autophagy and oxidative stress [110, 173, 202]. Thus, if such parameters were to be assessed after ischemia, *i.e.*, in cardiac tissues with different level of exposure to ischemic insult to corresponding ischemic, border and remote zones, it would be unclear whether a possibly deteriorated autophagy and increased oxidative stress are causes or consequences of ischemia and/or reperfusion injury. Nevertheless, such experiments are still warranted to clarify the role of the mTOR pathway, autophagy and oxidative stress in the loss of RIC in acute hyperglycemia.

We demonstrated that extracellular vesicles are required to RIC in an *ex vivo* model. Since our original publication [203], *in vivo* increase of extracellular vesicles by RIC has been demonstrated [204]. Furthermore, our results are based on indirect evidences. It was recently demonstrated that RIC-induced extracellular vesicles isolated from blood plasma protect the heart from the ischemia/reperfusion injury similarly to RIC [204]. Nevertheless, there are significant technical difficulties to isolate and characterize extracellular vesicles [205], which definitely needs to overcome many technical issues in extracellular vesicle research in general.

8. Conclusions

- **Remote ischemic conditioning attenuates myocardial necrosis in rat, while it protects the microvasculature in porcine model of acute myocardial infarction.**

We demonstrated that myocardial necrosis was attenuated by RIC in a rat model of ischemia/reperfusion injury. In contrast, myocardial necrosis was not affected by RIC in a clinically relevant, closed-chest porcine model of ischemia/reperfusion injury, however, the positive control IPreC reduced myocardial necrosis. The coronary microvascular system was protected by RIC, since it attenuated myocardial edema as assessed with cardiac MRI. Our results indicate that myocardial edema may be an important parameter to be assessed in the context of ischemia/reperfusion injury and these parameters might change independently from that of myocardial necrosis. Furthermore, since the intact microcirculation implies improved long-term outcome, its careful evaluation might help to avoid false negative results in preclinical or clinical studies of cardioprotection.

- **Acute hyperglycemia abolishes the cardioprotection conferred by remote ischemic conditioning.**

We demonstrated that the cardioprotective effect of RIC is lost in acute hyperglycemia. The mechanism of this phenomenon may involve an acute hyperglycemia-induced increase of oxidative stress and activation of the AKT-mTOR pathway, but not the disruption of cardiac autophagy. This data suggests that the efficacy of RIC might be compromised in clinical settings, if acute hyperglycemia is present. Therefore, acute hyperglycemia should be controlled during management.

- **Extracellular vesicles mediate remote ischemic conditioning.**

We showed that perfusate collected from a conditioned heart transfers cardioprotection to a naïve heart. Furthermore, cardioprotection is no longer transferred once extracellular vesicles are removed indicating that extracellular vesicles are required to the manifestation of RIC. However, further molecular and *in vivo* experimentation is warranted to decipher the nature of actual effector factors carried by these vesicles.

9. Summary

Remote ischemic conditioning (RIC; short ischemia/reperfusion cycles of the hindlimb during myocardial ischemia) is a promising strategy to improve the prognosis of acute myocardial infarction (AMI). However, its translation into the clinical practice has been proven difficult, which has been primarily attributed to clinically irrelevant preclinical models and to the unclear underlying mechanism behind it.

Therefore, we aimed to investigate:

- 1) the cardioprotective effect of RIC in a clinically relevant model of AMI,
- 2) the cardioprotective effect of RIC in acute hyperglycemia,
- 3) the mechanism by which RIC is transferred from the remote organ.

RIC was tested *in vivo* in a clinically relevant porcine model of AMI. The cardioprotective effect of RIC was assessed with cardiac magnetic resonance imaging and histological staining. RIC did not decrease myocardial necrosis as compared to the control ischemic group, however, it attenuated myocardial edema which is a measure of microvascular damage.

Acute hyperglycemia was induced in an *in vivo* rat model of AMI. Although RIC reduced myocardial necrosis in normoglycemic rats as evaluated by histological staining, the cardioprotective effect of RIC was abolished in acute hyperglycemia, which, based on our immunoblot analyses, might be attributed to the overactivated AKT-mTOR pathway.

The mechanism of the RIC stimuli was investigated in an *ex vivo* rat heart perfusion system. As assessed by histological staining, there was a decrease in the myocardial necrosis when naïve hearts were pretreated prior to the AMI with perfusate collected from a perfused heart undergoing ischemic conditioning. The mechanism might be related to the increased extracellular vesicle content of the conditioned perfusate. No protection was observed when extracellular vesicles were removed from the perfusate.

In conclusion, our results highlight the importance of suitable translational models to investigate AMI and the strict control of carbohydrate metabolism during AMI. Furthermore, to assess ischemia/reperfusion injury, indicators of microvascular pathology, *i.e.*, myocardial edema should be considered. Lastly, extracellular vesicles mediate RIC, however, the identification of their cargo is still warranted.

10. Összefoglalás

A távoli iszkémiás kondicionálás (RIC; (rövid iszkémia/reperfúziós ciklusok alkalmazása a hátsó végtagon) ígéretes kardioprotektív módszer az akut miokardiális infarktusz (AMI) következményeinek mérséklésére. Azonban a klinikai gyakorlatba való átültetése egyelőre sikertelen, melynek hátterében feltehetően a klinikailag irreleváns preklinikai modellek, illetve a RIC tisztázatlan mechanizmusa áll.

Tanulmányunk során a következőket vizsgáltuk:

- 1) a RIC kardioprotektív hatását egy klinikailag releváns AMI modellben;
- 2) a RIC kardioprotektív hatását akut hiperglikémia során;
- 3) a RIC protektív szignáljának távoli szövetben lévő transzportját.

A RIC hatékonyságát egy *in vivo*, klinikailag releváns sertés AMI modellben vizsgáltuk. Kardioprotektív hatását szív mágneses rezonancia képalkotással és hisztológiai festéssel vizsgáltuk. A kontroll csoporthoz képest a RIC nem csökkentette a miokardiális nekrosis mértékét, azonban a mikrovaszkuláris károsodás egyik markerét, a miokardiális ödémát mérsékelte.

Az akut hiperglikémiát egy *in vivo*, patkány AMI modellben vizsgáltuk. A hisztológiai festéssel meghatározott miokardiális nekrosis mértékét a RIC csökkentette normoglikémia során a kontroll csoporthoz képest, azonban ez a kardioprotektív hatás akut hiperglikémia során elmaradt. Ennek hátterében immunoblot vizsgálataink alapján feltehetően a túlaktiválódott AKT-mTOR útvonal áll.

A RIC mechanizmusát *ex vivo* patkány szív perfúziós rendszerben tanulmányoztuk. Az iszkémiás kondicionálás során gyjtött perfuzátum az AMI következtében kialakult, szövettani festéssel igazolt miokardiális nekrosis mértékét csökkentette a kontroll csoporthoz képest. A kardioprotekció mechanizmusa feltehetően a kondicionált perfuzátumok megnövekedett extracelluláris vezikulatartalmának tulajdonítható. Az extracelluláris vezikulák eltávolításával a kardioprotekció is megszűnt.

Eredményeink kiemelik a megfelelő transzlációs modellek alkalmazásának fontosságát az AMI vizsgálatában, valamint a szénhidrát anyagcsere szerepét. A kardioprotekció mértékének megítélésében fontos a mikrovaszkuláris károsodás (pl.: miokardiális ödéma) meghatározása is. A RIC kardioprotektív hatását extracelluláris vezikulák mediálják, tartalmuk analízise jelentős kutatási területül szolgálhat.

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12. List of own publications

12.1. Own publications involved in the current thesis

Baranyai T, Giricz Z, Varga ZV, Koncsos G, Lukovic D, Makkos A, Sárközy M, Pávó N, Jakab A, Czimbalmos C, Vágó H, Ruzsa Z, Tóth L, Garamvölgyi R, Merkely B, Schulz R, Gyöngyösi G, Ferdinandy P. In vivo MRI and ex vivo histological assessment of the cardioprotection induced by ischemic preconditioning, postconditioning and remote conditioning in a closed-chest porcine model of reperfused acute myocardial infarction: importance of microvasculature. *J Transl Med.* 2017 Apr 1;15(1):67. [IF: 3.694]

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