Age-Dependent Effects of Remote Preconditioning in Hypertensive Rat Hearts are Associated With Activation of RISK Signaling

Veronika FARKAŠOVÁ[1], Lucia KINDERNAY[1], Miroslav FERKO[1], Tomáš RAJTÍK[1,2], Adrian SZOBI[2], Tatiana RAVINGEROVÁ[1]

[1]Centre of Experimental Medicine Slovak Academy of Sciences, Institute for Heart Research, Bratislava, Slovak Republic, [2]Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic

Received September 5, 2022
Accepted November 7, 2022

Summary

Remote ischemic preconditioning (RIPC) represents one of the forms of innate cardioprotection. While being effective in animal models, its application in humans has not been always beneficial, which might be attributed to the presence of various comorbidities, such as hypertension, or being related to the confounding factors, such as patients' sex and age. RIPC has been shown to mediate its cardioprotective effects through the activation of Reperfusion Injury Salvage Kinase (RISK) pathway in healthy animals, however, scarce evidence supports this effect of RIPC in the hearts of spontaneously hypertensive (SHR) rats, in particular, in relationship with aging. The study aimed to investigate the effectiveness of RIPC in male SHR rats of different age and to evaluate the role of RISK pathway in the effect of RIPC on cardiac ischemic tolerance. RIPC was performed using three cycles of inflation/deflation of the pressure cuff placed on the hind limb of anesthetized rats aged three, five and eight months. Subsequently, hearts were excised, Langendorff-perfused and exposed to 30-min global ischemia and 2-h reperfusion. Infarct-sparing and antiarrhythmic effects of RIPC were observed only in three and five months-old animals but not in eight months-old rats. Beneficial effects of RIPC were associated with increased activity of RISK and decreased apoptotic signaling only in three and five months-old animals. In conclusion, RIPC showed cardioprotective effects in SHR rats that were partially age-dependent and might be attributed to the differences in the activation of RISK pathway and various aspects of ischemia/reperfusion injury in aging animals.

Key words

Remote ischemic preconditioning • Hypertension • Ischemia/reperfusion • Protective signaling

Corresponding author

T. Ravingerová, Institute for Heart Research, Centre of Experimental Medicine Slovak Academy of Sciences, Dúbravská cesta 9, 841 04 Bratislava, Slovak Republic. E-mail: usrdravi@savba.sk

Introduction

Ischemic heart disease (IHD) and acute myocardial infarction (AMI) are leading causes of morbidity and mortality worldwide [1,2]. Although reperfusion of ischemic myocardium is a main prerequisite for salvage of the viable tissue, revascularization may paradoxically induce ischemia/reperfusion (I/R) injury and accelerate cardiomyocyte death in terms of greater size of AMI and post-AMI myocardial dysfunction that may later progress into heart failure [3]. Therefore, identification of novel therapeutic strategies to protect the heart during myocardial reperfusion is of great importance [4]. Since 1993, when Przyklenk *et al.* [5] demonstrated that brief episodes of ischemia in the circumflex branch of left coronary artery protected remote virgin myocardium from subsequent sustained ischemia in the region supplied by left anterior descending coronary artery, the application of this method and its modifications defined as remote ischemic preconditioning (RIPC) has been extended to various animal models [6,7,8]. Moreover, a number of clinical investigations has demonstrated a protective effect of RIPC in patients undergoing cardiac bypass and heart valve surgery [9,10], or heart transplantation [11]. RIPC has been described as one of the forms of an endogenous

cardioprotective mechanism when brief episodes of sublethal ischemia or other moderate stress applied to a remote organ protect another organ/tissue including myocardium against the detrimental effects of prolonged severe ischemia and reperfusion injury [12]. Different from classical ischemic preconditioning, protective effect of RIPC could be achieved noninvasively using a pressure cuff placed on the upper/lower limb, which made the intervention more clinically relevant [13]. RIPC has been shown to exert its cardioprotective effect due to a transfer of protective signal from the distant organ through the autonomic nervous system [6,14] directly to the heart and subsequently activating pro-survival signaling cascades such as Reperfusion Injury Salvage Kinase (RISK) pathway including kinases PKB(Akt), ERK1/2 and inactivating GSK-3β, directly in the heart [4,15]. However, many studies of RIPC have been performed in the models underestimating the role of lifestyle-related risk factors in the pathogenesis of cardiovascular diseases (CVD) [16]. Diseases, such as such diabetes, hypertension, and hypercholesterolemia contribute to the development of IHD [13,17,18,19].

Since Szilvassy *et al.* [20] observed the loss of preconditioning protection in hypercholesterolemic model, it has been well documented that cardiovascular risk factors may interfere with cardioprotective interventions [21,22]. The loss of the IS-limiting effect of ischemic preconditioning and postconditioning has been shown in various models of hyperlipidemia [23,24,25,26,27] and in preconditioning against I/R under conditions of hyperglycemia [28].

Moreover, confounding factors, such as age or sex, may play a great role when considering myocardial response to ischemia (even in the period of maturation [29] and the effect of preconditioning [21,30]. Both, ageing and sex contribute to the differences in the heart susceptibility to ischemia and to the outcome of preventive interventions [31,32]. The aims of the present study were: (i) to examine whether RIPC was effective in the hypertensive rats in the *ex vivo* model of AMI; (ii) to determine whether aging can attenuate the response to RIPC in the SHR rats; and (iii) to define the intracellular signaling pathways involved in the cardioprotective mechanisms of RIPC in the hypertensive rats.

Materials and Methods

Ethical approval

All animal experiments were performed in accordance with the State Veterinary Administration of the Slovak Republic, legislation No 289/2003(3548/12-221), as well as the journal's principles and standards for reporting animal experiments checklist. The study protocol was approved by the Animal Research and Care Committee of the Centre of Experimental Medicine Slovak Academy of Sciences v.v.i., Institute for Heart Research, and ethical committee of the CEM SAS, (protocol code 3754/18-221/3, 11.1.2019).

Animals

Seventy-two spontaneously hypertensive male rats (SHR) purchased from the Department of Toxicology and Laboratory Animal Breeding (Dobrá Voda, Slovak Republic) were used. The animals were housed five per cage (according to their age and group), under conditions of standard 12 h light/12 h dark cycle (light: 8:00-20:00), fed standard laboratory chow *ad libitum* with free access to drinking water. All animals were left intact to adapt for 10 days before the initiation of the experiments.

Experimental groups

Rats of age three (juvenile), five (young adults) and eight (mature adults) months were randomly divided into the control group (SHR; 7 rats for each age group) and group preconditioned with remote ischemic preconditioning (SHR-RIPC; 7 rats for each age group for infarct size evaluation and 5 rats for each age group for Western blot analysis). Baseline measurement of mean arterial blood pressure (MBP) by noninvasive tail-cuff plethysmography (ADInstruments, Germany) was performed 48 h before anesthesia. Rats from SHR-RIPC groups underwent the RIPC protocol consisting of three cycles of 5-min non-invasive hind limb occlusion followed by 5-min reperfusion under thiopental anesthesia (50-60 mg/kg i.p.) together with heparin (500 IU). Limb occlusion/reperfusion was induced by inflation/deflation of the pressure cuff (D. E. Hokanson, Inc. USA) placed on the right hind limb. The cuff was inflated to 240-250 mm Hg (31.99-33.33 kPa) according to an increased blood pressure in SHR rats. Control SHR rats were only anesthetized for 30 min.

Perfusion technique

Immediately after RIPC or 30 min anesthesia, the hearts from SHR and SHR-RIPC animals were rapidly excised, placed into cold saline buffer (4 °C; 154 mM NaCl) and subsequently cannulated *via* the aorta and perfused in the Langendorff mode at a constant perfusion pressure of 120 mm Hg (proportionally adjusted to the higher blood pressure in SHR *in vivo*) and

at 37 °C. The perfusion solution was a modified Krebs-Henseleit buffer gassed with 95 % O_2 and 5 % CO_2 (pH 7.4) containing (in mM): glucose 11.0; $CaCl_2$ 1.6; NaCl 118.0; $NaHCO_3$ 25.0; $MgSO_4$ 1.18; KH_2PO_4 1.28; KCl 3.0.

An epicardial electrogram (EG) was registered by means of two stainless steel electrodes attached to the apex of the heart and the aortic cannula and continuously recorded. Heart rate (HR) was calculated from the EG. Left ventricular (LV) pressure was measured by means of a non-elastic water-filled balloon inserted into the left ventricle *via* the left atrium and connected to a pressure transducer (MLP844, ADInstruments, Germany). LV systolic pressure (LVSP), LV diastolic pressure (LVEDP), LV developed pressure (LVDP, systolic minus diastolic pressure), maximal rates of pressure development [$+(dP/dt)_{max}$] and fall [$-(dP/dt)_{max}$] as the indexes of contraction and relaxation, as well as the coronary flow (CF) were used to evaluate cardiac function that was analyzed using PowerLab/8SP Chart 7 software (ADInstruments, Germany).

Protocol of ischemia/reperfusion

After the onset of perfusion and set up of all perfusion parameters, hearts from all experimental animals were left to stabilize for 15 min. Subsequently, global ischemia was induced by clamping of aortic inflow for 30 min, followed by 40 min reperfusion for the evaluation of postischemic recovery of contractile function (expressed in percentage of pre-ischemic values) and further 80 min reperfusion (2 h altogether) for the determination of the size of myocardial infarction as the primary end-point of injury. Non-elastic water-filled balloon inserted into the left ventricle was removed after 40-min of reperfusion. Isolated hearts for tissue samples for Western blot analysis were subjected to 15 min stabilization, followed by 30 min global ischemia and 40 min reperfusion.

Quantification of arrhythmias

Susceptibility to reperfusion-induced ventricular arrhythmias, such as premature ventricular contractions (PVCs), ventricular tachycardia (VT) and fibrillation (VF) was evaluated during 10-min reperfusion [33].

Determination of infarct size

The size of the infarcted area and the area at risk size were delineated by staining with 2,3,5-triphenyl-tetrazolium chloride (TTC). After staining, the hearts were cut perpendicularly to the long axis of the heart, into 1-mm thick slices, and determined by a computerized planimetric method as described previously [13]. The infarct size (IS) was expressed as percentage of the area at risk (AR) size that represented the entire area of left ventricle.

Preparation of tissue protein fractions

In parallel experiments, the tissue samples used for Western blot analysis were obtained from the LV of hearts of all experimental groups (n=5 per group) after 40 min of reperfusion (ischemia/reperfusion samples). The total LV tissue samples (300-350 mg) were wiped in liquid nitrogen, resuspended in ice-cold buffer A containing (in mmol/l): 20 Tris-HCl, 250 sucrose, 1.0 EGTA, 1.0 dithiothreitol (DTT), 1.0 phenylmethyl-sulphonyl fluoride (PMSF), and 0.5 sodium ortho-vanadate (pH 7.4), and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 800× g for 5 min at 4 °C. Pellets after the first centrifugation were discarded and the supernatants were centrifuged again at 16100× g for 30 min. The supernatants after the second centrifugation were used for further analysis. The protein concentrations were measured by the method of Bradford [34].

Electrophoresis and Western blot analysis

Samples of the protein fractions containing equivalent amounts of proteins per lane (70 μg per lane) were separated by 7-12.5 % SDS-PAGE gel electrophoresis. For Western blot assays, proteins were transferred to a nitrocellulose membrane. Specific anti-Akt 1/2/3 (Santa Cruz Biotech. Cat. sc-8312, Lot #E0913, RRID: AB_671717), anti-p-Akt (Cell Signaling Tech. Cat. #4058L, Lot #30, RRID: AB_331168), anti-GSK-3α/β (Santa Cruz Biotech. Cat. sc-7291, Lot #LD0915, RRID: AB_2279451), anti-p-GSK-3β (Santa Cruz Biotech. Cat. sc-11757, Lot #D1913, RRID: AB_2279471), anti-ERK 1/2 (Santa Cruz Biotech. Cat. sc-135900, Lot #L1715, RRID: AB_2141283), anti-pERK 1/2 (Cell Signaling Tech. Cat. 9101S, Lot #30, RRID: AB_331646), anti-Bax (Santa Cruz Biotech. Cat. sc-7480, Lot #J2313, RRID: AB_626729), anti-Bcl (Santa Cruz Biotech. Cat sc-783, Lot #H2914, RRID: AB_2243455), anti-caspase 3 (Merck Millipore, Cat.: AB 1899, Lot 2659167, RRID: AB_91084), antibodies were used for the primary immunodetection. Peroxidase-labelled anti-rabbit (Cell Signaling Tech. Cat. #7074S, Lot #27, RRID: AB_2099233) or anti-mouse (Cell Signaling Tech. Cat. #7076S, Lot #25, RRID: AB_330924) immunoglobulin were used as the

secondary antibody. Bound antibodies were detected by the enhanced chemiluminescence (ECL) method (Pierce Thermofischer Scientific, USA). The optical density of individual bands was analyzed by PCBAS 2.08e software and normalized to GAPDH (anti-GAPDH, Santa Cruz Biotech. Cat. sc-25778, Lot #A0515, RRID: AB_10167668) as an internal control.

Statistical evaluation

The data were expressed as means ± S.E.M. One-way ANOVA and subsequent Student-Newman Keuls test, as well as Mann-Whitney U test (for arrhythmia evaluation) using GraphPad Prism version 6.00 (GraphPad Software, USA) were used. Differences were considered as significant at $P<0.05$.

Results

Biometric parameters and baseline function of isolated hearts

Mean values of body weight and mean blood pressure (MBP) from all experimental groups are shown in Table 1. Preischemic (baseline) functional parameters of HR, LVSP, LVEDP, LVDP (LV systolic minus LV diastolic pressure), $+(dP/dt)_{max}$, $-(dP/dt)_{max}$ in the hearts of three-, five- and eight-month-old (3-m; 5-m; 8-m) SHR and SHR-RIPC animals are summarized in Table 1. Body weight and contractile parameters were significantly higher in both 8-m groups in comparison with those in 3-m and 5-m groups.

Table 1. Biometric data of experimental animals and baseline values of hemodynamic parameters of isolated rat hearts.

Age	**3-m**		**5-m**		**8-m**	
Group	**SHR**	**SHR-RIP**	**SHR**	**SHR-RIP**	**SHR**	**SHR-RIP**
Body weight [g]	250 ± 7.5	242 ± 6.9	266 ± 11.6	256 ± 2.9	319 ± 6.9$^{\#,\&}$	327 ± 5.9$^{\#,\&}$
MBP [mm Hg]	190 ± 5	189 ± 6	201 ± 6	201 ± 4	196 ± 3	198 ± 4
HR [beats/min]	247 ± 10	252 ± 6	237 ± 6	232 ± 7	258 ± 13	242 ± 15
LVSP [mm Hg]	117 ± 6	102 ± 9	112 ± 9	111 ± 5	141 ± 4$^{\#,\&}$	141 ± 5$^{\#,\&}$
LVEDP [mm Hg]	6.3 ± 2.1	4.4 ± 1.8	9.5 ± 0.8	7.0 ± 1.1	4.7 ±0.3$^{\&}$	3.7 ± 0.7$^{\&}$
LVDP [mm Hg]	110 ± 7	98 ± 8	102 ± 10	104 ± 6	136 ± 4$^{\#,\&}$	138 ± 5.5$^{\#,\&}$
$+dP/dt_{max}$ [mm Hg/s]	2885 ± 128	2669 ± 201	2747 ± 161	2704 ± 195	3480 ±227$^{\#,\&}$	4002 ± 127*$^{\#,\&}$
$-dP/dt_{max}$ [mm Hg/s]	1930 ± 184	1796 ± 186	1641 ± 219	1700 ± 145	2232 ± 83$^{\&}$	2495 ± 65*$^{\#,\&}$

HR – heart rate; MBP – mean blood pressure; LVSP – left ventricular systolic pressure; LVEDP – left ventricular end-diastolic pressure; LVDP – left ventricular developed pressure (LV systolic minus LV diastolic pressure; $+(dP/dt)_{max}$, $-(dP/dt)_{max}$ – maximal rates of pressure development and fall; respectively. * $P<0.05$, SHR-RIPC vs. SHR; $^{\#}$ $P<0.05$, 8-m SHR-RIPC/SHR vs. 3-m SHR-RIPC/SHR; $^{\&}$ 8-m SHR-RIPC/SHR vs. 5-m SHR-RIPC/SHR; SHR – spontaneously hypertensive rats; SHR-RIPC – SHR with remote ischemic preconditioning; 3/5/8-m – age of animals in months. Data are means ± S.E.M., n=7 per group.

Age-dependent effect of remote ischemic preconditioning on ischemia-reperfusion injury

The size of myocardial infarction

The increasing age markedly increased the extent of lethal injury (size of infarction) after I/R in control SHR animals and in those exposed to RIPC (Fig. 1A). At the age of eight months, infarct size was significantly larger in comparison with that in 3-m and 5-m control SHR group. Animals subjected to RIPC exhibited positive adaptive response resulting in significantly decreased size of infarction in the hearts of 3-m and 5-m old animals as compared to control SHR of the same age. On the other hand, in 8-m old rats, application of RIPC did not attenuate the size of myocardial infarction in their hearts (Fig. 1A).

Reperfusion arrhythmias

The occurrence of reperfusion arrhythmias in control SHR animals of different age exhibited no significant differences. Markedly decreased sensitivity to reperfusion arrhythmias was observed after RIPC application. As shown in Figure 1B, hearts from 3-m and 5-m SHR-RIPC animals exhibited significantly shorter duration of a severe form of ventricular arrhythmias – ventricular tachycardia (VT) compared to that in the hearts of control SHR animals of the same age groups.

On the contrary, opposite effect of RIPC on arrhythmogenesis during early reperfusion period was observed in 8-m SHR-RIPC group, since total duration of

VT was significantly increased as compared to 8-m control SHR (Fig. 1B) showing rather negative effect of RIPC in this elder group.

In addition, 8-m SHR-RIPC animals showed significantly higher sensitivity to reperfusion arrhythmias, where total duration of VT was significantly increased in comparison to that in 5-m and 3-m SHR-RIPC rats (Fig. 1B).

Age-dependent effect of remote ischemic preconditioning on myocardial stunning

In SHR controls, recovery of contractile function represented by LVDP and LVEDP restoration after I/R did not differ among all age groups. However, different age-related results were observed in the preconditioned groups (Fig. 1C, 1D). RIPC markedly enhanced LVDP and LVEDP recovery in the hearts of 3-m-old SHR (54.8±7.7 vs. 34.5±6.0 % in control SHR, P<0.05) and 5-m-old SHR animals (73.2±4.7 vs. 45.9±9.1 % in control SHR, P<0.05). However, this effect was not seen in 8-m-old SHR (54.6±9.6 vs. 52.3±13.2 in control SHR), where RIPC application caused no effect on the restoration of contractile function in the preconditioned hearts as compared with the non-preconditioned controls (Fig. 1C, D).

Age-dependent activation of RISK pathway after remote ischemic preconditioning

As illustrated in Figures 2A, B and 3A, B, Akt and ERK1/2 phosphorylation at 40 min of reperfusion was significantly enhanced in the hearts from the 3-m and 5-m SHR-RIPC animals as compared to their respective

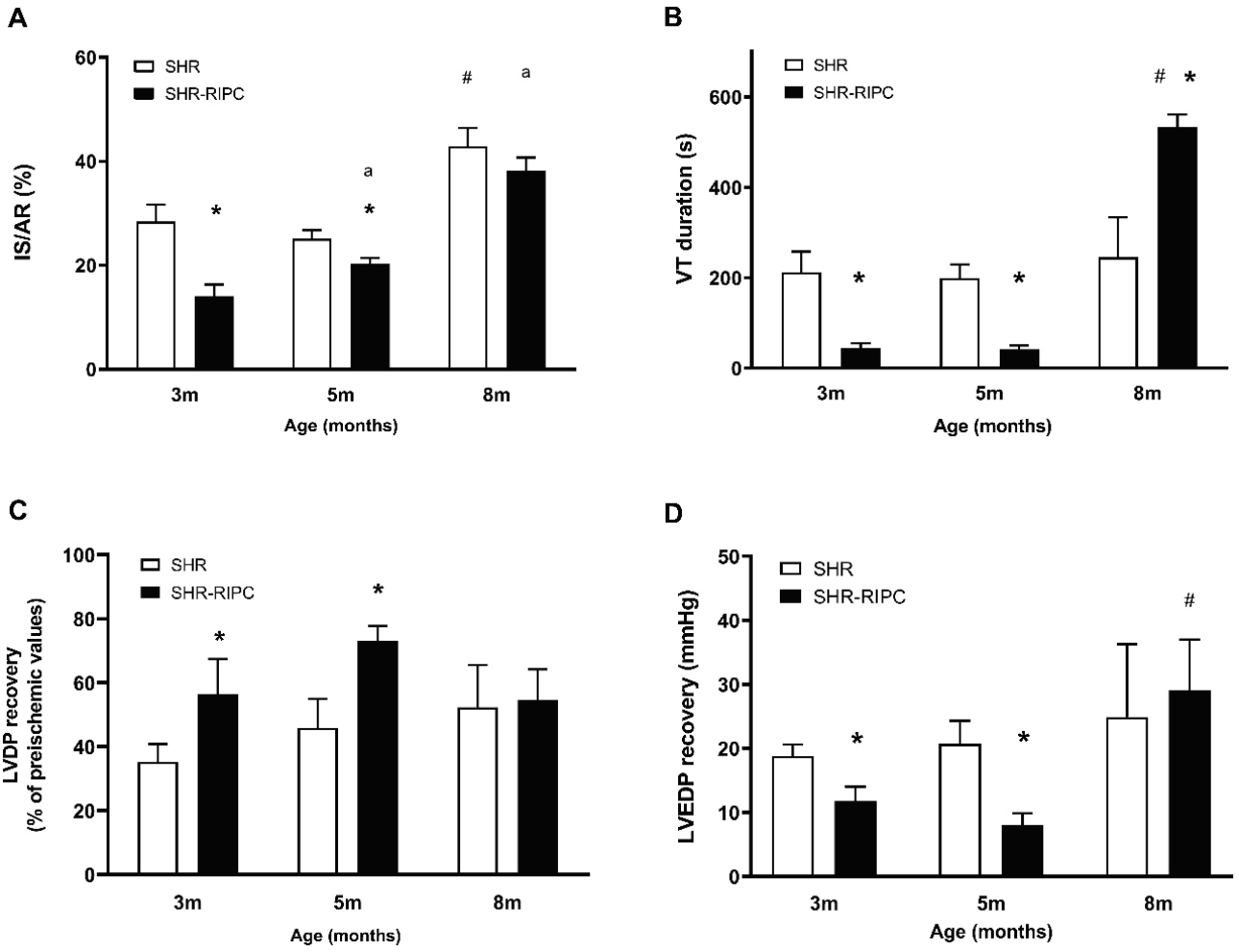

Fig. 1. Age-dependent effect of RIPC on outcomes of I/R injury. (**A**) Age-dependent effect of RIPC on infarct size (IS) after I/R expressed in percentage of area at risk (AR) size. (**B**) Age-dependent effect of RIPC on total duration of reperfusion -induced ventricular tachycardia. (**C**) Age-dependent effect of RIPC on LVDP recovery after I/R. (**D**) Age-dependent effect of RIPC on LVEDP recovery after I/R. SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; LVDP – left ventricle developed pressure; * P<0.05, SHR-RIPC vs. SHR; # P<0.05, 8-m SHR-RIPC/SHR vs. 3-m and 5-m SHR-RIPC/SHR; [a] P<0.05, 5/8-m SHR-RIPC vs. 3-m SHR-RIPC; Data are means ± S.E.M., n=7 per group.

non-preconditioned SHR controls. Accordingly, GSK-3β phosphorylation as a downstream target of Akt and ERK 1/2 was significantly increased in 3-m and 5-m SHR-RIPC groups as compared with control SHR ones (Fig. 4A, B). Positive effect of RIPC on RISK activation was not observed in the 8-m SHR-RIPC group. Moreover, in these hearts from 8-m SHR-RIPC, there was a trend towards a decreased Akt and ERK 1/2 phosphorylation as compared to 8-m control SHR group (Fig. 2C, 3C). Similarly, GSK-3β phosphorylation in 8-m SHR (Fig. 4C) exhibited no significant change after RIPC. These results are suggesting that RISK pathway is not activated in this age group.

Apoptotic signaling after RIPC is influenced by age

RIPC treatment caused age-related changes in apoptotic signaling linked to activation of RISK pathway. Increased Akt and ERK 1/2 phosphorylation (activation) in 3-m and 5-m SHR-RIPC groups was associated with decline of apoptotic signaling, where Bax/Bcl-2 ratio (Fig. 5A, B), as well as caspase-3 activation (Fig. 6A, B) were significantly decreased in comparison to 3-m and 5-m control SHR. However, RIPC treatment in 8-m SHR animals did not reduce apoptotic signaling and its markers (Figs 5C, 6C).

Discussion

Several studies explored effectivity of different forms of conditioning in hypertensive or aged individuals. Some studies reported that ischemic preconditioning and RIPC reduced IS in normotensive as well as in hypertensive rat hearts *ex vivo* [13] and *in vivo* [35]. In the latter study, it was shown that in both, aged normotensive and hypertensive animals, the effects of ischemic preconditioning were maintained. Our present study shows that non-invasive RIPC applied in SHR rats *in vivo* was effective in attenuation of myocardial reperfusion injury in the *ex vivo* model of I/R. Moreover, it revealed age-dependent efficacy of RIPC in limiting the size of myocardial infarction, reducing the duration of severe reperfusion tachyarrhythmias and myocardial stunning in the hearts of younger animals but not in the elder ones. In contrast to our findings, Lu *et al.* [36] demonstrated no protective effect of remote ischemic preconditioning on the *in vivo* I/R injury outcomes in 12 weeks old SHR rats. On the other hand, our results are in agreement with those by Ebrahim *et al.* [37] who demonstrated the loss of preconditioning protection in the aged hypertensive rats. In addition, ischemic postconditioning did not reduce IS in hypertensive rat hearts, different from its effect in normotensives, as has been shown by Wagner *et al.* [38], probably due to a decreased phosphorylation (and activation) of GSK-3β. These discrepancies may be attributed to different forms of protection (pre-, per-, post-conditioning versus RIPC), their timing, distinct type of conditioning protocols used to induce cardioprotection and different I/R injury models.

While the mechanisms of ischemic preconditioning in hypertensive and aged animal models have been relatively well-studied, the effect and mechanisms of RIPC in these conditions are still poorly understood. Activation of the RISK pathway is a commonly accepted mechanism involved in local ischemic pre- and postconditioning in normal animals [4]. Thus, IPC reduced myocardial infarct in SHR rats [39] and in rats with hypertrophy induced by transverse aortic constriction and increased blood pressure [27]. The authors showed that Akt, GSK-3β [39,27] and ERK1/2 [27] phosphorylation was increased in the preconditioned hearts. Moreover, treatment with both GSK-3β inhibitors before and after ischemia exhibited exactly the same cardioprotective action as IPC suggesting that its infarct size-limiting effect is related to GSK-3β-dependent mechanism [39].

Therefore, we assumed that RIPC activated the RISK pathway and conferred protection against I/R injury in the hearts of hypertensive animals as well. Indeed, in our study, cardioprotective effects were associated with the age-dependent changes in the activation of RISK pathway and pro/antiapoptotic mechanisms in the hearts of SHR animals.

Specifically, the RIPC treatment significantly increased phosphorylation (activation) of Akt (Fig. 2) and ERK 1/2 (Fig. 3) kinases and decreased GSK-3β activity (Fig. 4) in the hearts from 3-m and 5-m old SHR rats. Moreover, RIPC suppressed apoptotic signaling manifested by reduction of Bax/Bcl-2 ratio (Fig. 5A, B), and caspase-3 activation (Fig. 6A, B) in the groups of younger animals. However, this protocol of RIPC was not capable to stimulate activation of Akt, ERK 1/2 and inactivate GSK-3β in 8-m-old SHR group, as well as to suppress apoptotic signaling. These findings could explain the failure of RIPC to afford cardioprotection in the group of 8-m-old animals.

A loss of protection by ischemic preconditioning was demonstrated in aged myocardium [40] that could be attributed to a failure to reduce interaction of

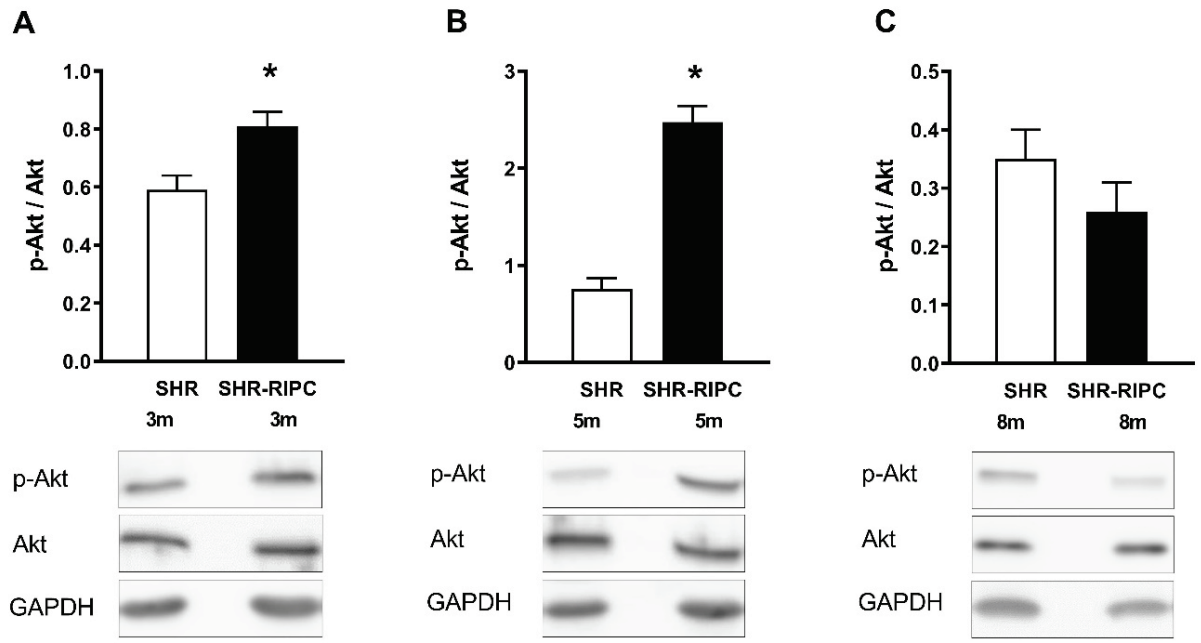

Fig. 2. Western Blot analysis of Akt and p-Akt protein expression. (**A**) p-Akt/Akt ratio – Akt phosphorylation is increased in 3-m SHR-RIPC compared to SHR. (**B**) p-Akt/Akt ratio – Akt phosphorylation is increased in 5-m SHR-RIPC compared to SHR. (**C**) p-Akt/Akt ratio – Akt phosphorylation is not changed in 8-m SHR-RIPC compared to SHR. Lower part of A) B) C): representative blots showing the expression of Akt and p-Akt. SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; *P<0.05, SHR-RIPC vs. SHR; Data are means ± S.E.M., n=5 per group.

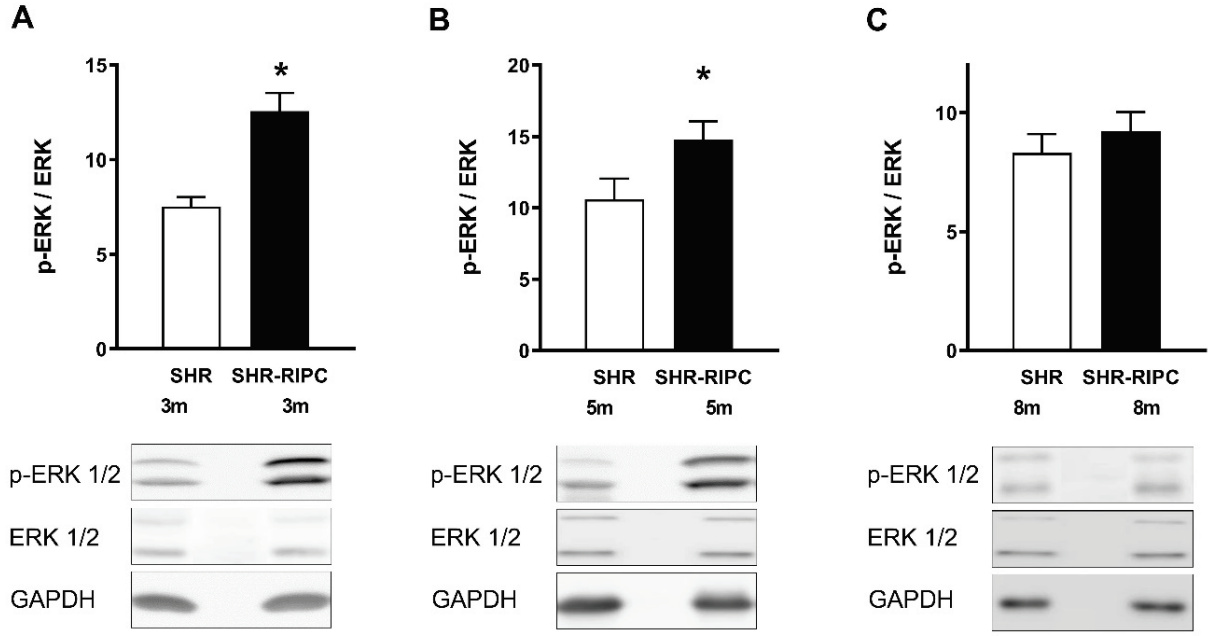

Fig. 3. Western Blot analysis of ERK 1/2 and p-ERK 1/2 protein expression. (**A**) p-ERK 1/2 **/**ERK 1/2 ratio - ERK 1/2 phosphorylation is increased in 3-m SHR-RIPC compared to SHR. (**B**) p-ERK 1/2/ERK 1/2 ratio – ERK 1/2 phosphorylation is increased in 5-m SHR-RIPC compared to SHR. (**C**) p-ERK 1/2/ERK 1/2 ratio – ERK 1/2 phosphorylation is not changed in 8-m SHR-RIPC compared to SHR. Lower part of A) B) C): representative blots showing the expression of ERK 1/2 and p-ERK 1/2. *P<0.05, SHR-RIPC vs. SHR; SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; Data are means ± S.E.M., n=5 per group.

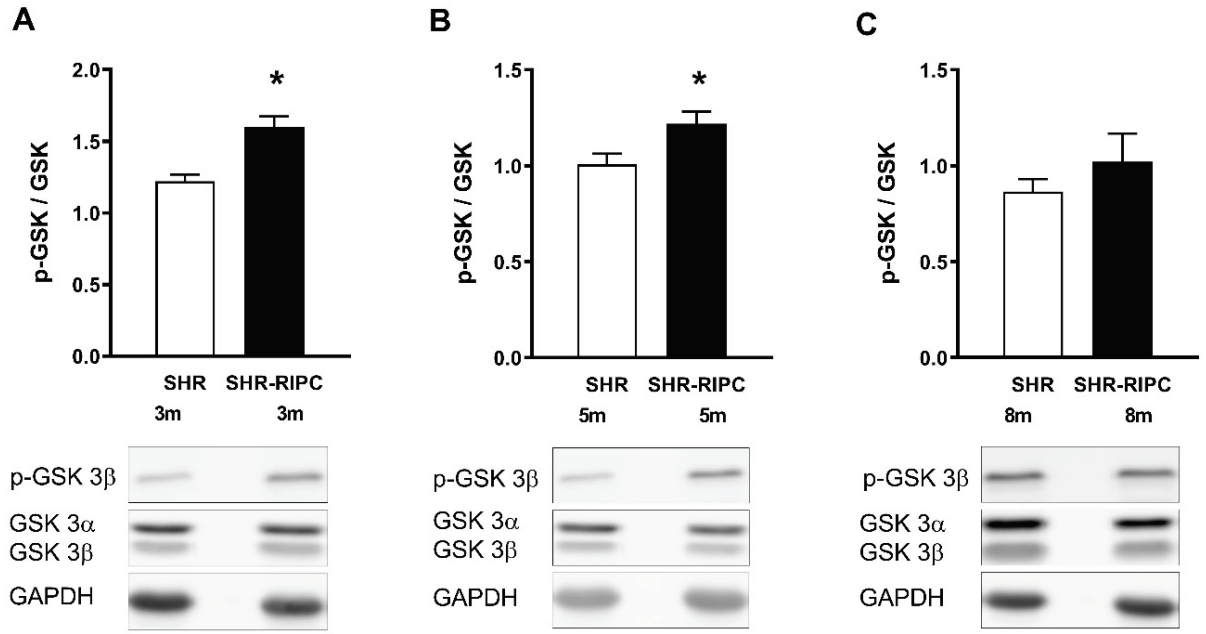

Fig. 4. Western Blot analysis of GSK-3β and p-GSK-3β protein expression. (**A**) p-GSK-3β/GSK-3β ratio – GSK-3β phosphorylation is increased in 3-m SHR-RIPC compared to SHR. (**B**) p-GSK-3β/GSK-3β ratio – GSK-3β phosphorylation is increased in 5-m SHR-RIPC compared to SHR. (**C**) p-GSK-3β/GSK-3β ratio – GSK-3β phosphorylation is not changed in 8-m SHR-RIPC compared to SHR. Lower part of A) B) C): representative blots showing the expression of GSK-3β and p-GSK-3β. * P<0.05, SHR-RIPC vs. SHR; SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; Data are means ± S.E.M., n=5 per group.

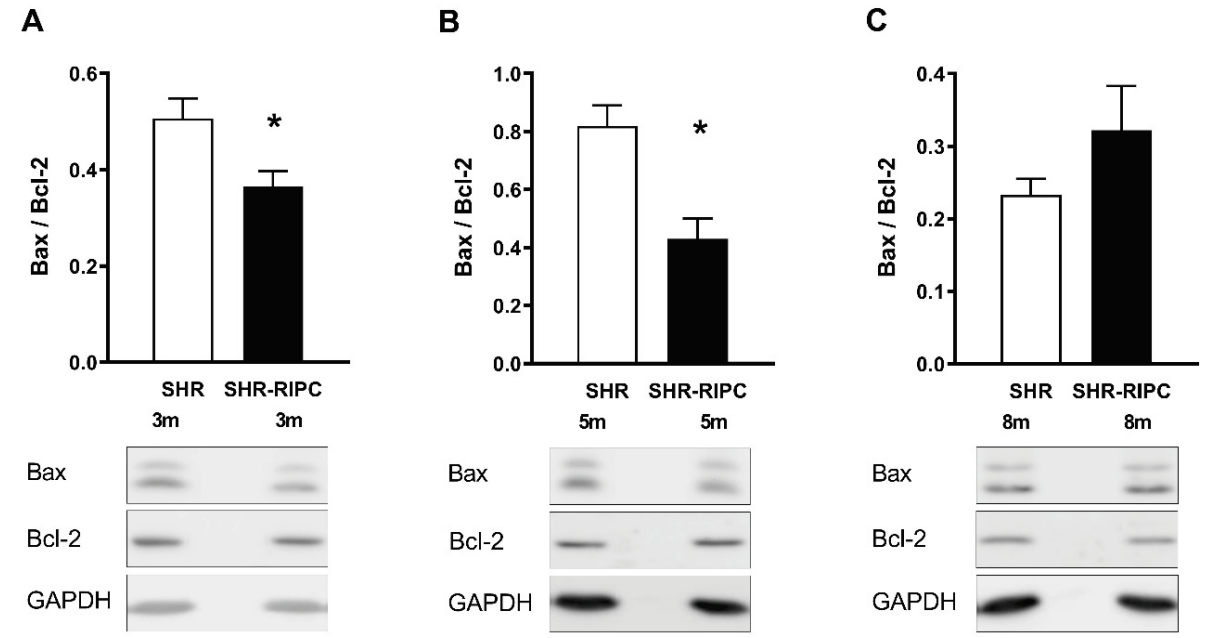

Fig. 5. Western Blot analysis of Bax and Bcl-2 protein expression. (**A**) Bax/Bcl-2 ratio – pro-apoptotic signaling is decreased in 3-m SHR-RIPC compared to SHR. (**B**) Bax/Bcl-2 ratio – pro-apoptotic signaling is decreased in 5-m SHR-RIPC compared to SHR. (**C**) Bax/Bcl-2 ratio – pro-apoptotic signaling is not changed in 8-m SHR-RIPC compared to SHR. Lower part of A) B) C): representative blots showing the expression of Bax and Bcl-2. * P<0.05, SHR-RIPC vs. SHR; SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; Data are means ± S.E.M., n=5 per group.

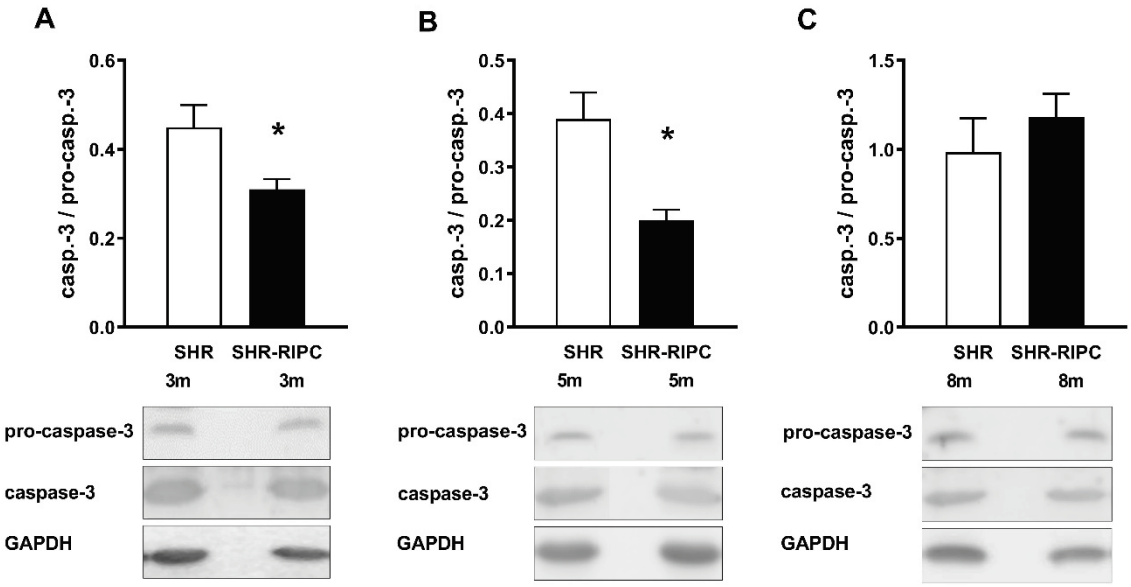

Fig. 6. Western Blot analysis of pro-caspase-3 and caspase-3 protein expression. (**A**) caspase-3/pro-caspase-3 ratio – pro-apoptotic signaling is decreased in 3-m SHR-RIPC compared to SHR. (**B**) caspase-3/pro-caspase-3 ratio – pro-apoptotic signaling is decreased in 5-m SHR-RIPC compared to SHR. (**C**) caspase-3/pro-caspase-3 ratio – pro-apoptotic signaling is not changed in 8-m SHR-RIPC compared to SHR. Lower part of A) B) C): representative blots showing the expression of pro-caspase-3 and caspase-3. * $P<0.05$, SHR-RIPC vs. SHR; SHR – spontaneously hypertensive rats; SHR-RIPC – SHR exposed to remote ischemic preconditioning; 3/5/8-m – SHR rats of age three/five/eight months; Data are means ± S.E.M., n=5 per group.

cyclophilin D, a MPTP modulator, and adenine-nucleotide-translocase [41]. Delayed MPTP opening reduced myocardial IS and time to MPTP opening in young rats but not in old individuals [42].

In accordance, in our study, we observed increased phosphorylation of Akt, ERK and GSK-3β as the main components of RISK pathway in the hearts of 3-m and 5-m SHR rats, as well as an anti-apoptotic effect. However, infarct size-limiting, antiarrhythmic and antistunning effects of RIPC were lost in 8-m-old SHR rats. Moreover, RIPC significantly increased duration of severe ventricular arrhythmias in this age group. The failure of RIPC to precondition the hearts of the elder group of animals might be related to a lower activity of "pro-survival" mechanisms. However, other mechanisms cannot be excluded and require further exploration.

Limitations

In the present study, we tested only one RIPC protocol using three cycles of 5-min ischemia/ 5-min reperfusion applied before myocardial ischemia. Although this RIPC protocol failed to protect hearts from I/R injury in 8-m-old SHR, it cannot be excluded that the threshold for RIPC-induced protection is elevated in the elderly SHR rats. In other words, more than three cycles of 5-min ischemia/5-min reperfusion during RIPC might be necessary to protect the elderly SHR hearts from I/R injury. Since normotensive control rats were not employed in this study, we cannot directly define the impact of hypertension and aging on RIPC effectiveness. Earlier studies have shown that increased ROS production in mitochondria and reduced myocardial perfusion during reperfusion in SHR hearts, could contribute to augmentation of I/R injury in this model of hypertension. Also, our study did not explore the role of the SAFE pathway. Since phosphorylation of RISK kinases typically peaks early in reperfusion, the assessment of RISK proteins at 40 min of reperfusion may not indicate the most relevant changes that may have occurred.

Conclusions

Despite insufficient information on the cardioprotective effects of RIPC in individuals with combined cardiovascular risk factors, specific medications are not fully explored. Moreover, most experimental studies on cardioprotection are performed in young and healthy animal models in the absence of classical risk factors of CVD. Taken together, this study provides new insight into the mechanisms responsible for cardiac protection of RIPC in hypertensive rats.

Extending the knowledge about this form of cardioprotection and its effects in the presence of different comorbidities may contribute to a more successful application of RIPC in clinical situations.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This research was funded by grants of The Slovak Research and Development Agency (APVV-19-0540, APVV-20-0242), Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic (VEGA SR) 2/0104/22, 1/0016/20, 1/0775/21 and IT Monitoring System (ITMS) 26230120009. The authors are thankful to Mrs. I. Formanková and Ing. D. Pancza for their excellent technical assistance.

References

1. Finegold JA, Asaria P, Francis DP. Mortality from ischaemic heart disease by country, region, and age: Statistics from World Health Organiation and United Nations. Int J Cardiol 2013;168:934-945. https://doi.org/10.1016/j.ijcard.2012.10.046
2. Nowbar AN, Gitto M, Howard JP, Francis DP, Al-Lamee R. Mortality from ischemic heart disease. Circ Cardiovasc Qual Outcomes 2019;12:e005375. https://doi.org/10.1161/CIRCOUTCOMES.118.005375
3. Bulluck H, Yellon DM, Hausenloy DJ. Reducing myocardial infarct size: challenges and future opportunities. Heart 2016;102:341-348. https://doi.org/10.1136/heartjnl-2015-307855
4. Hausenloy DJ, Yellon DM. Targeting myocardial reperfusion injury--the search continues. N Engl J Med 2015;373:1073-1075. https://doi.org/10.1056/NEJMe1509718
5. Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. Circulation 1993;87:893-899. https://doi.org/10.1161/01.CIR.87.3.893
6. Abdul-Ghani S, Fleishman AN, Khaliulin I, Meloni M, Angelini GD, Suleiman M-S. Remote ischemic preconditioning triggers changes in autonomic nervous system activity: implications for cardioprotection. Physiol Rep 2017;5:e13085. https://doi.org/10.14814/phy2.13085
7. 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 M, 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;15:67. https://doi.org/10.1186/s12967-017-1166-z
8. Ravingerova T, Farkasova V, Griecsova L, Carnicka S, Murarikova M, Barlaka E, Kolar F, Bartekova M, Lonek L, Slezak J, Lazou A. Remote preconditioning as a novel "conditioning" approach to repair the broken heart: potential mechanisms and clinical applications. Physiol Res 2016;65(Suppl 1):S55-S64. https://doi.org/10.33549/physiolres.933392
9. Candilio L, Malik A, Ariti C, Barnard M, Di Salvo C, Lawrence D, Hayward M, Yap J, Roberts N, Sheikh A, Kolvekar S, Hausenloy DJ, Yellon DM. Effect of remote ischaemic preconditioning on clinical outcomes in patients undergoing cardiac bypass surgery: a randomised controlled clinical trial. Heart 2015;101:185-192. https://doi.org/10.1136/heartjnl-2014-306178
10. Xie JJ, Liao XL, Chen WG, Huang DD, Chang FJ, Chen W, Luo ZL, Wang ZP, Ou JS. Remote ischaemic preconditioning reduces myocardial injury in patients undergoing heart valve surgery: randomised controlled trial. Heart 2012;98:384-388. https://doi.org/10.1136/heartjnl-2011-300860
11. Wang G, Zhang Y, Yang L, Chen Y, Fang Z, Zhou H, Zhang C, Lei G, Shi S, Li J. Cardioprotective effect of remote ischemic preconditioning with postconditioning on donor hearts in patients undergoing heart transplantation: a single-center, double-blind, randomized controlled trial. BMC Anesthesiol 2019;19:48. https://doi.org/10.1186/s12871-019-0720-z
12. Przyklenk K, Darling CE, Dickson EW, Whittaker P. Cardioprotection 'outside the box'--the evolving paradigm of remote preconditioning. Basic Res Cardiol 2003;98:149-157. https://doi.org/10.1007/s00395-003-0406-y

13. Ravingerová T, Farkašová V, Griecsová L, Muráriková M, Carnická S, Lonek L, Ferko M, ET AL. Noninvasive approach to mend the broken heart: Is "remote conditioning" a promising strategy for application in humans? Can J Physiol Pharmacol 2017;95:1204-1212. https://doi.org/10.1139/cjpp-2017-0200
14. Donato M, Goyeneche MA, Garces M, Marchini T, Pérez V, Del Mauro J, Höcht C, Rodríguez M, Evelson P, Gelpi RJ. Myocardial triggers involved in activation of remote ischaemic preconditioning. Exp Physiol 2016;101:708-716. https://doi.org/10.1113/EP085535
15. Tamareille S, Mateus V, Ghaboura N, Jeanneteau J, Croué A, Henrion D, Furber A, Prunier F. RISK and SAFE signaling pathway interactions in remote limb ischemic perconditioning in combination with local ischemic postconditioning. Basic Res Cardiol 2011;106:1329-1339. https://doi.org/10.1007/s00395-011-0210-z
16. Bøtker HE. The future of cardioprotection-pointing toward patients at elevated risk as the target populations. J Cardiovasc Pharmacol Ther 2020;25:487-493. https://doi.org/10.1177/1074248420937871
17. Adameová A, Kuzelová M, Andelová E, Faberová V, Pancza D, Svec P, Ziegelhöffer A, Ravingerová T. Hypercholesterolemia abrogates an increased resistance of diabetic rat hearts to ischemia-reperfusion injury. Mol Cell Biochem 2007;295:129-136. https://doi.org/10.1007/s11010-006-9282-8
18. Ravingerová T, Neckár J, Kolár F. Ischemic tolerance of rat hearts in acute and chronic phases of experimental diabetes. Mol Cell Biochem 2003;249:167-174. https://doi.org/10.1007/978-1-4419-9236-9_21
19. Ferko M, Farkasova V, Jasova M, Kancirova I, Ravingerova T, Duris Adameova A, Andelova N, Waczulikova I. Hypercholesterolemia antagonized heart adaptation and functional remodeling of the mitochondria observed in acute diabetes mellitus subjected to ischemia/reperfusion injury. J Physiol Pharmacol 2018;69:685-697. https://doi.org/10.26402/jpp.2018.5.03
20. Szilvassy Z, Ferdinandy P, Szilvassy J, Nagy I, Karcsu S, Lonovics J, Dux L, Koltai M. The loss of pacing-induced preconditioning in atherosclerotic rabbits: role of hypercholesterolaemia. J Mol Cell Cardiol 1995;27:2559-2569. https://doi.org/10.1006/jmcc.1995.0043
21. Ferdinandy P, Hausenloy DJ, Heusch G, Baxter GF, Schulz R. Interaction of risk factors, comorbidities, and comedications with ischemia/reperfusion injury and cardioprotection by preconditioning, postconditioning, and remote conditioning. Pharmacol Rev 2014;66:1142-1174. https://doi.org/10.1124/pr.113.008300
22. Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, Heusch G, Vinten-Johansen J, Yellon DM, Schulz R. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res 2010;87:406-423. https://doi.org/10.1093/cvr/cvq129
23. Andreadou I, Iliodromitis EK, Lazou A, Görbe A, Giricz Z, Schulz R, Ferdinandy P. Effect of hypercholesterolaemia on myocardial function, ischaemia-reperfusion injury and cardioprotection by preconditioning, postconditioning and remote conditioning. Br J Pharmacol 2017;174:1555-1569. https://doi.org/10.1111/bph.13704
24. Landim MB, Dourado PM, Casella-Filho A, Chagas AC, da-Luz PL. High plasma concentrations of asymmetric dimethylarginine inhibit ischemic cardioprotection in hypercholesterolemic rats. Braz J Med Biol Res 2013;46:454-459. https://doi.org/10.1590/1414-431X20132595
25. Xu Y, Ma LL, Zhou C, Zhang FJ, Kong FJ, Wang WN, Qian LB, Wang CC, Liu XB, Yan M, Wang JA. Hypercholesterolemic myocardium is vulnerable to ischemia-reperfusion injury and refractory to sevoflurane-induced protection. PLoS One 2013;8:e76652. https://doi.org/10.1371/journal.pone.0076652
26. Wu N, Zhang X, Guan Y, Shu W, Jia P, Jia D. Hypercholesterolemia abrogates the cardioprotection of ischemic postconditioning in isolated rat heart: roles of glycogen synthase kinase-3β and the mitochondrial permeability transition pore. Cell Biochem Biophys 2014;69:123-130. https://doi.org/10.1007/s12013-013-9778-2
27. Ma LL, Kong FJ, Guo JJ, Zhu JB, Shi HT, Li Y, Sun RH, Ge JB. Hypercholesterolemia abrogates remote ischemic preconditioning-induced cardioprotection: role of reperfusion injury salvage kinase signals. Shock 2017;47:363-369. https://doi.org/10.1097/SHK.0000000000000737
28. Zálešák M, Blažíček P, Gablovský I, Ledvényiová V, Barteková M, Ziegelhöffer A, Ravingerová T. Impaired PI3K/Akt signaling as a potential cause of failure to precondition rat hearts under conditions of simulated hyperglycemia. Physiol Res 2015;64:633-641. https://doi.org/10.33549/physiolres.932883

29. Griecsová L, Farkašová V, Gáblovský I, Khandelwal VK, Bernátová I, Tatarková Z, Kaplan P, Ravingerová T. Effect of maturation on the resistance of rat hearts against ischemia. Study of potential molecular mechanisms. Physiol Res 2015;64(Suppl 5):S685-S696. https://doi.org/10.33549/physiolres.933222
30. Kindernay L, Farkasova V, Neckar J, Hrdlicka J, Ytrehus K, Ravingerova T. Impact of maturation on myocardial response to ischemia and the effectiveness of remote preconditioning in male rats. Int J Mol Sci 2021;22:11009. https://doi.org/10.3390/ijms222011009
31. Ledvenyiova V, Pancza D, Matejiková J, Ferko M, Bernatova I, Ravingerova T. Impact of age and sex on response to ischemic preconditioning in the rat heart: differential role of the PI3K-AKT pathway. Can J Physiol Pharmacol 2013;91:640-647. https://doi.org/10.1139/cjpp-2012-0414
32. Ruiz-Meana M, Boengler K, Garcia-Dorado D, Hausenloy DJ, Kaambre T, Kararigas G, Perrino C, Schulz R, Ytrehus K. Ageing, sex, and cardioprotection. Br J Pharmacol 2020;177:5270-5286. https://doi.org/10.1111/bph.14951
33. Curtis MJ, Walker MJ. Quantification of arrhythmias using scoring systems: an examination of seven scores in an in vivo model of regional myocardial ischaemia. Cardiovasc Res 1988;22:656-665. https://doi.org/10.1093/cvr/22.9.656
34. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254. https://doi.org/10.1016/0003-2697(76)90527-3
35. Dai W, Simkhovich BZ, Kloner RA. Ischemic preconditioning maintains cardioprotection in aging normotensive and spontaneously hypertensive rats. Exp Gerontol 2009;44:344-349. https://doi.org/10.1016/j.exger.2009.02.005
36. Lu X, Bi YW, Chen KB. Olmesartan restores the protective effect of remote ischemic perconditioning against myocardial ischemia/reperfusion injury in spontaneously hypertensive rats. Clinics (Sao Paulo) 2015;70:500-507. https://doi.org/10.6061/clinics/2015(07)07
37. Ebrahim Z, Yellon DM, Baxter GF. Ischemic preconditioning is lost in aging hypertensive rat heart: independent effects of aging and longstanding hypertension. Exp Gerontol 2007;42:807-814. https://doi.org/10.1016/j.exger.2007.04.005
38. Wagner C, Ebner B, Tillack D, Strasser RH, Weinbrenner C. Cardioprotection by ischemic postconditioning is abrogated in hypertrophied myocardium of spontaneously hypertensive rats. J Cardiovasc Pharmacol 2013;61:35-41. https://doi.org/10.1097/FJC.0b013e3182760c4d
39. González Arbeláez LF, Pérez Núñez IA, Mosca SM. Gsk-3β inhibitors mimic the cardioprotection mediated by ischemic pre- and postconditioning in hypertensive rats. Biomed Res Int 2013;2013:317456. https://doi.org/10.1155/2013/317456
40. van den Munckhof I, Riksen N, Seeger JP, Schreuder TH, Borm GF, Eijsvogels TM, Hopman MT, Rongen GA, Thijssen DH. Aging attenuates the protective effect of ischemic preconditioning against endothelial ischemia-reperfusion injury in humans. Am J Physiol Heart Circ Physiol 2013;304:H1727-H1732. https://doi.org/10.1152/ajpheart.00054.2013
41. Zhu J, Rebecchi MJ, Glass PS, Brink PR, Liu L. Interactions of GSK-3β with mitochondrial permeability transition pore modulators during preconditioning: age-associated differences. J Gerontol A Biol Sci Med Sci 2013;68: 395-403. https://doi.org/10.1093/gerona/gls205
42. Liu L, Zhu J, Brink PR, Glass PS, Rebecchi MJ. Age-associated differences in the inhibition of mitochondrial permeability transition pore opening by cyclosporine A. Acta Anaesthesiol Scand 2011;55:622-630. https://doi.org/10.1111/j.1399-6576.2011.02421.x