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Effect of Maturation on the Resistance of Rat Hearts Against Ischemia. Study of Potential Molecular Mechanisms

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Summary

Reduced tolerance to ischemia/reperfusion (IR) injury has been shown in elder human and animal hearts, however, the onset of this unfavorable phenotype and cellular mechanisms behind remain unknown. Moreover, aging may interfere with the mechanisms of innate cardioprotection (preconditioning, PC) and cause defects in protective cell signaling. We studied the changes in myocardial function and response to ischemia, as well as selected proteins involved in "pro-survival" pathways in the hearts from juvenile (1.5 months), younger adult (3 months) and mature adult (6 months) male Wistar rats. In Langendorff-perfused hearts exposed to 30-min ischemia/2-h reperfusion with or without prior PC (one cycle of 5-min ischemia/5-min reperfusion), we measured occurrence of reperfusion-induced arrhythmias, recovery of contractile function (left ventricular developed pressure, LVDP, in % of pre-ischemic values), and size of infarction (IS, in % of area at risk size, TTC staining and computerized planimetry). In parallel groups, LV tissue was sampled for the detection of protein levels (WB) of Akt kinase (an effector of PI3-kinase), phosphorylated (activated) Akt (p-Akt), its target endothelial NO synthase (eNOS) and protein kinase Cε (PKCε) as components of "pro-survival" cascades. Maturation did not affect heart function, however, it impaired cardiac response to lethal IR injury (increased IS) and promoted arrhythmogenesis. PC reduced the occurrence of malignant arrhythmias, IS and improved LVDP recovery in the younger animals, while its efficacy was attenuated in the mature adults. Loss of PC protection was associated with age-dependent reduced Akt phosphorylation and levels of eNOS and PKCε in the hearts of mature animals compared with the younger ones, as well as with a failure of PC to upregulate these proteins. Aging-related alterations in myocardial response to ischemia may be caused by dysfunction of proteins involved in protective cell signaling that may occur already during the process of maturation.

Key words

Myocardial ischemia • Preconditioning • Aging • Survival cascades

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Introduction

Ischemic heart disease as one of the most widespread cardiovascular diseases can be aggravated by lifestyle risk factors, such as: hyperlipidemia, hypercholesterolemia, hypertension, diabetes mellitus and aging. Moreover, age itself represents an independent risk factor that may cause molecular, structural, or biochemical changes in the cardiovascular system. On the other hand, some stressful stimuli related to risk factors, play a dual role in the pathogenesis of ischemia and besides deleterious effects, may trigger adaptive processes in the heart resulting in its greater ischemic tolerance. The concept of the heart´s own protection is based on the principle, that the heart is able to protect itself by adaptation to various forms of moderate stress (Murry *et al.* 1986, Hearse 2001). One of the most

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studied forms of short-term adaptation is ischemic preconditioning (PC), an adaptive response in which brief exposure to ischemia markedly enhances the ability of the heart to withstand a subsequent sustained ischemic injury (Murry *et al.* 1986). Whether cardioprotection by PC is modified in aging myocardium, has been investigated in animal models, as well as in the human heart. However, the results concerning the effectiveness of PC remain controversial. Most of the studies in animal models proved that increasing age may cause the loss of cardioprotection by PC (Abete *et al.* 2000, 2002, Schulman *et al.* 2001, Boengler *et al.* 2007). On the other hand, some studies demonstrated preservation of cardioprotection even in the elderly (12 months) animals but not in the senescent ones (Schulman *et al.* 2001). The results of clinical studies are very similar to the animal studies. Most of them demonstrated a decreased potency of transient angina (clinical analogue of PC) in elderly humans (Bartling *et al.* 2003, Lee *et al.* 2002), although some of them reported that the presence of previous angina, 48 h before acute myocardial infarction, provided protection in elderly patients – up to 65 years old. However, in patients over 65 years, cardioprotective effect of previous angina has not been shown (Abete *et al.* 1997).

The decrease of ischemic tolerance with increasing age correlates with changes in cellular expression of proteins that are involved in the cardioprotective signaling. This is because in aging heart, several genes are expressed differentially including those that encode proteins participating in signal transduction of PC (Taylor and Starnes 2003). One of the protein kinases involved in PC which is changing with the age is protein kinase C (PKC). Moreover, its isoforms may have distinct effects on the PC (Boengler *et al.* 2007). E.g., increased basal expression of PKCδ was associated with failure of ischemic tolerance with increasing age (Kostyak *et al.* 2006), while decreased expression of PKCε was found in the aged myocardium (Korzick *et al.* 2007).

Cardioprotection induced by PC involves increased phosphorylation of other protein kinases such as Akt, GSK-3β and mitogen-activated protein kinases ERK1/2 and p38 MAPK, and aging heart is characterized by changes in their expression and activation (Boengler *et al.* 2009). Several studies demonstrated age-dependent reduction in the levels of Akt, p-Akt, as well as eNOS and phospho-eNOS levels (Hunter *et al.* 2007, Iemitsu *et al.* 2006). However, to the best of our knowledge, the time of the onset of deleterious modifications has not been explored in details. Only few investigators demonstrated the changes in the efficiency of PC in mice in the relatively early period of life span, between 10 and 18 weeks of age (Turcato *et al.* 2006).

Therefore, our aims were: i) to study the effect of maturation on rat myocardial function and resistance against ischemia; ii) to investigate whether age-dependent changes in the efficiency of adaptative protective mechanisms (preconditioning) may occur within a relatively short life span; iii) to examine the signaling pathways of cardioprotection in association with age (to compare the changes in selected "pro-survival cascades").

Materials and Methods

Animals

In our study, we used male Wistar rats of different age: 1.5 months (juvenile animals), 3 months (young adults) and 6 months old (mature adults). Rats (250-500 g body weight) were fed with a standard pellet diet and had unrestricted access to drinking water *ad libitum*. All experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals (ILAR 1996) and the rules issued by the State Veterinary and Alimentary Administration of the Slovak Republic, based on § 37 (6), legislation No. 488/2002 of the Slovak Parliament. The use and treatment of animals was also approved by the Animal Review Committee at the Institute for Heart Research, SAS, where the experiments were carried out.

Perfusion technique

Rats were anesthetized with thiopental (50-60 $mg.kg^{-1}$) administered intraperitoneally together with heparin (500 IU). The hearts were rapidly excised, placed in ice-cold perfusion buffer, then cannulated *via* the aorta and perfused in the Langendorff mode at a constant perfusion pressure of 73 mm Hg and at 37 °C. The perfusion solution was a modified Krebs-Henseleit buffer gassed with O_2 and 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 was calculated from the EG. Left ventricular 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). Left ventricular 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 heart rate (HR) and coronary flow (CF) were used to assess cardiac function. Heart function was analyzed using PowerLab/8SP Chart 7 software (ADInstruments, Germany).

Induction of ischemia/reperfusion

Global ischemia was induced by clamping of aortic inflow for 30 min and followed by its unclamping for the evaluation of postischemic recovery of contractile function after 40-min reperfusion (expressed in percentage of pre-ischemic values) and determination of the size of myocardial infarction as the primary end-point of injury after 2-h reperfusion.

Quantification of arrhythmias

Susceptibility to reperfusion-induced ventricular tachyarrhythmias, such as premature ventricular contractions (PVCs), ventricular tachycardia (VT) and fibrillation (VF), as well as arrhythmia severity (assessed by a 5-point scoring system according to the most severe form of arrhythmia that occurred in each individual heart) was evaluated during 10-min reperfusion (Curtis and Walker 1988).

Experimental protocols

The hearts of all experimental groups (n=8-12 hearts per group) were assigned to the following protocols. In the protocol of ischemia and reperfusion, after a 20-min stabilization period, the hearts underwent 30-min global ischemia followed by 2-h reperfusion. In the protocol of ischemic preconditioning, after stabilization, the hearts were subjected to one cycle of 5-min ischemia and 5-min reperfusion, prior to sustained (30-min) ischemia/2-h reperfusion.

Determination of infarct size

The size of the infarcted area and the area at risk size were delineated by staining with 2,3,5-triphenyltetrazolium 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 (Ravingerová *et al.* 2009). 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 left ventricles of hearts of all experimental groups (n=4 per group) after stabilization (baseline samples) and after 40 min of reperfusion (ischemia/reperfusion samples) or ischemia/reperfusion with prior preconditioning (PC samples). The tissues 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 phenylmethylsulphonylfluoride (PMSF), and 0.5 sodium orthovanadate (pH 7.4), and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 800g for 5 min at 4 °C. Pellets after the first centrifugation were discarded and the supernatants were centrifuged again at 16100g for 30 min. The supernatants after the second centrifugation, termed as cytosolic fraction, were used for further analysis. The protein concentrations were estimated by the method of Bradford (1976).

Electrophoresis and Western blot analysis

Samples of the protein fractions containing equivalent amounts of proteins per lane (70 μg per lane) were separated by 10 % SDS-PAGE gel electrophoresis. For Western blot assays, proteins were transferred to a nitrocellulose membrane. The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer. Specific anti-Akt 1/2/3 (dilution 1:330), anti-p-Akt 1/2/3 (dilution 1:710), anti-eNOS (dilution 1:200), anti-PKCε (dilution 1:1000) (Santa Cruz Biotechnology) antibodies were used for the primary immunodetection. Peroxidase-labelled anti-rabbit immunoglobulin (Cell Signaling Technology) was used as the secondary antibody (dilution 1:2000). Bound antibodies were detected by the enhanced chemiluminescence (ECL) method. The optical density of individual bands was analyzed by PCBAS 2.08e software and normalized to GAPDH (anti-GAPDH antibody, dilution 1:750) 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 using

GraphPad Prism version 5.00 (GraphPad Software, USA) for Windows (Microsoft Corporation, USA) were used where appropriate. Differences were considered as significant at $P<0.05$.

Results

Effect of age on biometric parameters of animals

Age significantly influenced the biometric parameters of animals. Both, body weight and heart weight were significantly rising with the increasing age. The highest increase in body weight was registered between 3 and 6 months of age, and heart weight increased gradually from 1.5 to 6 months (mo). The data expressed as the ratio of heart weight to body weight are shown in Figure 1.

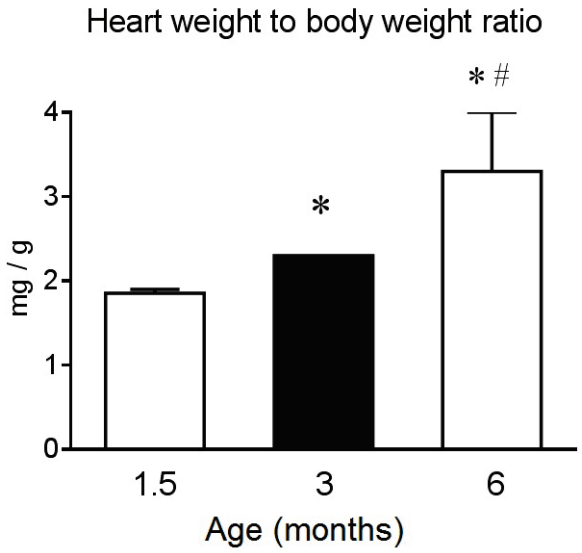

Fig. 1. The effect of age on biometric parameters of the rat heart, body weight and heart weight. Values are means ± S.E.M. from 8-12 hearts per group expressed as a ratio of heart and body weight (in mg / g). * $P<0.05$ vs. 1.5 months, # $P<0.05$ vs. 3 months

Characteristics of isolated hearts

The values of heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular developed pressure (LVDP=LV systolic minus LV diastolic pressure), $+(dP/dt)_{max}$, $-(dP/dt)_{max}$ and coronary flow in the 1.5-, 3- and 6-month-old groups are summarized in Table 1. There were no significant changes in the values of these parameters with age.

The effect of age and preconditioning on ischemia-reperfusion injury

The effect of age and preconditioning on the size of infarction

The increasing age markedly influenced the extent of lethal injury (size of infarction) after ischemia/reperfusion in control (non-adapted) groups. At the age of 3 months, there was a significant increase in infarct size compared with 1.5-month group (3 mo: 33±2.2 vs. 23.6±3 %; $P<0.05$). A further significant increase in the size of infarction was also observed at the age of 6 months compared to the 3-month group (6 mo: IS/AR 39±0.9 vs. 33±2.2 %; $P<0.05$, Fig. 2).

In the groups of animals subjected to PC, there was also a progressive increase in the size of infarction in accordance with the age, similar to unadapted groups (3 mo: IS/AR 16±2.4 vs. 8.6±1.5 %; $P<0.05$, vs. 1.5 mo; 6 mo: 43±7.6 vs. 16±2.4; $P<0.05$, vs. 3 mo).

On the other hand, significant differences were observed when we compared IS/AR in the PC group with that in the control group at the age of 1.5 months (IS/AR 8.6±1.5 vs. 23.6±3 %, $P<0.05$, PC vs. C) and also in the 3-month group (IS/AR 16±2.4 vs. 33±2.2 %, $P<0.05$, PC vs. C) as a manifestation of positive effect of preconditioning. However, there were no significant

Table 1. Preischemic values of hemodynamic parameters of isolated rat hearts.

Group (age)	HR (beats/min)	LVSP (mm Hg)	LVEDP (mm Hg)	LVDP (mm Hg)	$+(dP/dt)_{max}$ (mm Hg/s)	$-(dP/dt)_{max}$ (mm Hg/s)	CF (ml/min)
1.5 months	253 ± 7	86 ± 6	6.1 ± 1	86.1 ± 7.3	2539 ± 316	1467 ± 169	7.4 ± 1.6
3 months	270 ± 7	87.6 ± 4.6	6.8 ± 1.7	87.0 ± 3.5	2339 ± 181	1581 ± 95	6.6 ± 0.7
6 months	261 ± 6	87.7 ± 3.8	5.9 ± 0.8	93.0 ± 3.2	2846 ± 138	1748 ± 95	8.5 ± 0.8

CF – coronary flow, 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, HR – heart rate. Data are means ± S.E.M., n=8-12 per group.

differences between control and PC group at the age of 6 months. Thus, we believe that this finding suggests a reduction in the efficiency of PC in the elder group of rats (Fig. 2).

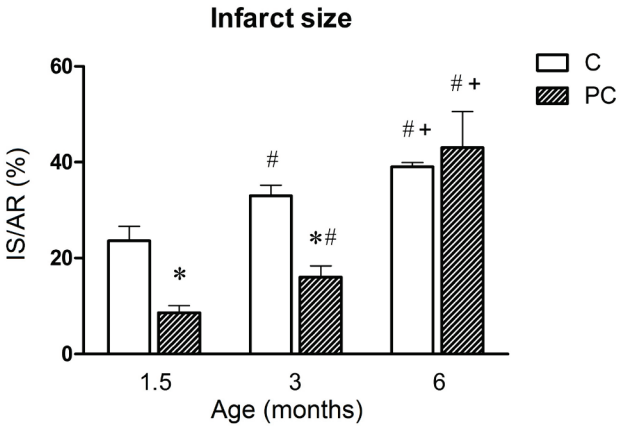

Fig. 2. The effect of age on the size of infarction in non-adapted and adapted (preconditioned) rat hearts. C – control (non-adapted) groups, PC – preconditioned (adapted) groups, IS – infarcted area of left ventricle, expressed in % of risk area (AR). Values are means ± S.E.M. from 8-12 hearts per group. * P<0.05, PC vs. C; #,+ P<0.05-0.01, vs. 1.5 and 3 months

The effect of age and preconditioning on the recovery of left ventricular pressure after ischemia-reperfusion injury

We observed no age-related changes in the recovery of LVDP and of the LVEDP values compared with pre-ischemic values in the control groups (LVDP: 68.9±6.7 at 1.5 mo; 61.8±7.5 at 3 mo; and 74.3±3.8 at 6 mo, in % of pre-ischemic values; LVEDP: 23.9±4.5 at 1.5 mo; 23.7±2.8 at 3 mo; and 21.7±4.7 at 6 mo, in mm Hg).

On the other hand, protective effect of preconditioning on the recovery of LVDP and LVEDP was demonstrated in the groups of younger rats. At the age of 1.5 and 3 months, there was a significant improvement in the recovery of LVDP and also a significant decrease in LVEDP values, in PC groups compared with the control groups (LVDP: 80.8±4.2 vs. 68.9±6.7 at 1.5 mo; 77.1±5.8 vs. 61.8±7.5 at 3 mo; LVEDP: 4.4±2.0 vs. 23.9±4.5 at 1.5 mo, 10.7±1.6 vs. 23.7±2.8 at 3 mo; P<0.05, PC vs. C). In contrast, there were no significant differences in LVDP recovery and LVEDP values between the PC group and the control group, at the age of 6 months. Once again it confirms the loss in the efficiency of PC in the elder group (Table 2).

Table 2. The effect of age and preconditioning on recovery of LVDP and LVEDP after ischemia/reperfusion.

Parameters	Group	1.5 months	3 months	6 months
LVDP	*C*	68.9 ± 6.7	61.8 ± 7.5	74.3 ± 3.8
(% from preischemic values)	*PC*	80.8 ± 4.2*	77.1 ± 5.8*	74.6 ± 8.3
LVEDP (mm Hg)	*C*	23.9 ± 4.5	23.7 ± 2.8	21.7 ± 4.7
	PC	4.4 ± 2*	10.7 ± 1.6*	25.5 ± 12.1

C – control groups, PC – preconditioned groups, LVDP – left ventricular developed pressure (LV systolic minus LV diastolic pressure), LVEDP – left ventricular end-diastolic pressure. Data are means ± S.E.M., n=8-12 per group. * P<0.05, PC vs. C.

The effect of age and preconditioning on the occurrence of reperfusion arrhythmias

We also observed age-related changes in the occurrence of reperfusion arrhythmias. In the control group of 3-month animals, a significant increase of total number of PVCs (Fig. 3A) and a significant increase in the total duration of ventricular tachycardia (VT) (Fig. 3B) were registered, in comparison with 1.5-month experimental group (PVCs: 528±86 vs. 283±56; VT duration: 84±17 s vs. 43.6±5.1 s; P<0.05). In the group of 6-month-old animals, even a greater increase of the total number of PVCs and duration of VT was observed, and this was also significant in comparison with a 3-month experimental group (PVCs: 950±296; VT: 204±73 s; P<0.05) (Fig. 3).

The same age-dependent increase in arrhythmogenesis was observed in the preconditioned groups. There was a progressive increase in the number of PVCs with age, and this increase was also observed when we compared the 6-month group with the 3-month and with 1.5-month group (721±280 vs. 253±56, and vs. 141±23, respectively, P<0.05) (Fig. 3A). Age-related duration of VT was significantly increased in 6-month group compared with 3-month group (170±79 vs. 19±9 s,

P<0.05) and with 1.5-month group (170±79 vs. 18±7 s; P<0.05) (Fig. 3B).

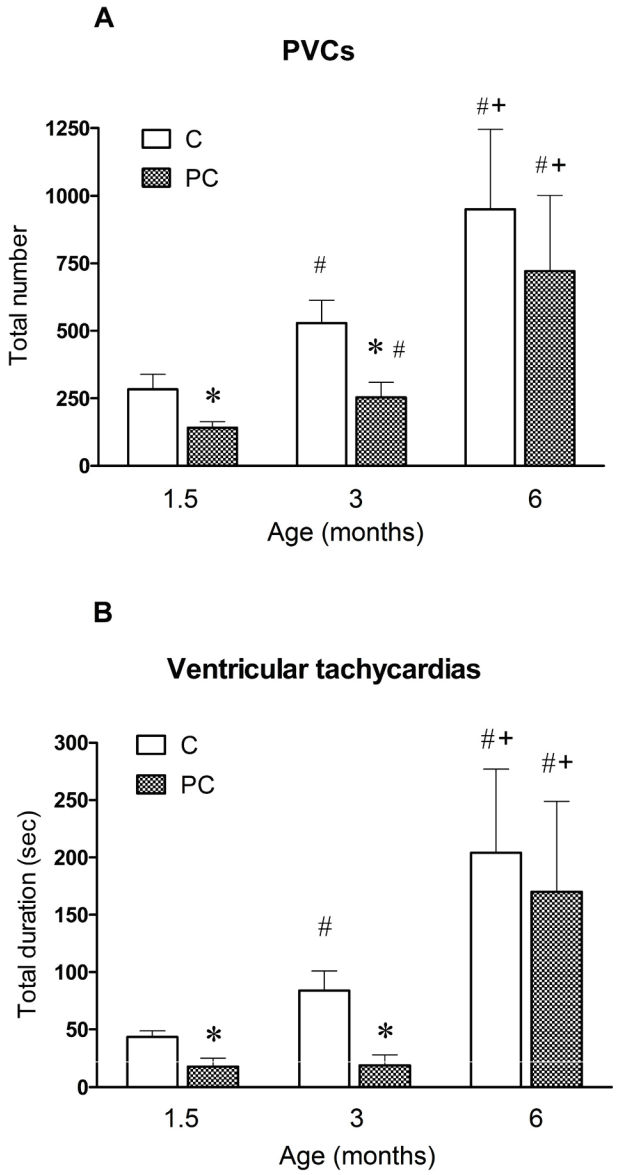

Fig. 3. The effect of age on ventricular arrhythmias in non-adapted and adapted (preconditioned) rat hearts. **A.** The effect of age on total number of PVCs. **B.** The effect of age on total duration of ventricular tachyarrhythmia. PVCs – premature ventricular complexes, C – control (non-adapted) groups, PC – preconditioned (adapted) groups. Values are means ± S.E.M. from 8-12 hearts per group. * P<0.05, PC vs. C; # P<0.05, vs. 1.5 months, + P<0.05, vs. 3 months

Antiarrhythmic effect of PC was manifested by a significant reduction in the number of ventricular extrasystoles and in the total duration of VT when comparing PC groups with the controls at 1.5-month (PVCs 141±23 vs. 283±56; VT: 18±7 s vs. 43.6±5.1 s; P<0.05, PC vs. C) and 3-month groups (PVCs: 253±56 vs. 528±86; VT: 19±9 s vs. 84±17 s; P<0.05, PC vs. C). However, at the age of 6-months, there were no significant differences in the number of PVCs or total duration of ventricular tachycardia between the PC group and the control non-preconditioned group (Fig. 3A and B).

The effect of age on the adaptive mechanisms and changes in the signaling cascade of ischemic preconditioning

The effect of age on Akt phosphorylation in rat myocardium at the baseline conditions

We found the effect of age on the activation (phosphorylation) of protein Akt, which was expressed as the ratio of p-Akt and total Akt and resulted in the progressive reduction of the p-Akt/Akt ratio. Significant changes in p-Akt/Akt occurred at 3 months (decreased by 26 % in comparison with 1.5-month group) and also at 6 months of age in comparison with 1.5-month-old (decreased by 60 %) and 3-month-old animals (decreased by 46 %). The largest decrease in the p-Akt/Akt ratio was observed between 3 and 6 months of age (Fig. 4A).

The effect of age on the protein levels of eNOS and PKCε in rat myocardium at the baseline conditions

With increasing age, a significant decrease in the protein levels of both, eNOS and PKCε, was observed. The levels of eNOS were significantly decreased already at the age of 3 months (by 60 % vs. 1.5 mo), as well as in 6-month group (by 49 % vs. 1.5 mo), in comparison with 1.5-month group of animals. We also observed the reduction in the levels of PKCε already at the age of 3 months in comparison with the 1.5-month group (by 29 % vs. 1.5 mo). At the age of 6 months, the decrease in the levels of PKCε was more pronounced, and it was significant as compared to the 1.5- and also 3-month groups of animals (53 % vs. 1.5 mo; 33 % vs. 3 mo, respectively) (Fig. 4B).

The effect of age and preconditioning on the levels of selected proteins in rat myocardium after ischemia and reperfusion

The effect of age and preconditioning on Akt phosphorylation (activation) in rat myocardium after ischemia and reperfusion

Investigation of impact of age and preconditioning on activation (phosphorylation) of Akt, expressed as the ratio of p-Akt and total Akt was focused on the two main age groups: 3- and 6-month, because the

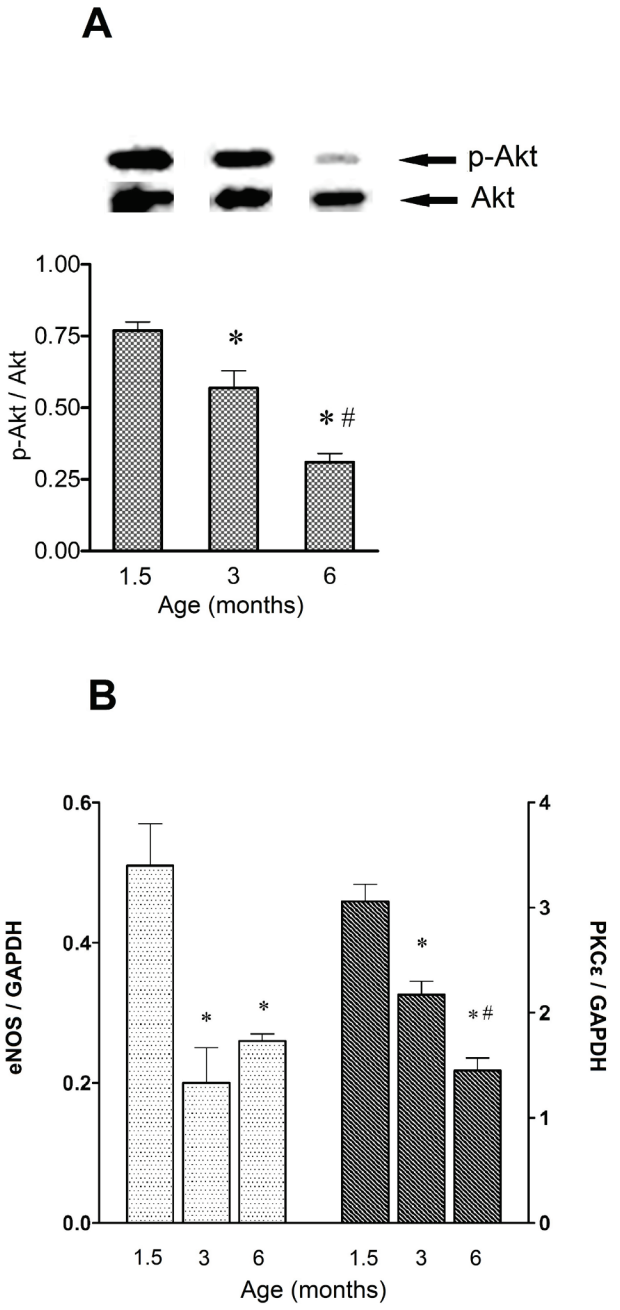

Fig. 4. The effect of age on the changes in the pro-survival proteins in rat hearts at baseline conditions. **A.** The effect of age on Akt phosphorylation (activation). Upper panels – representative blots of p-Akt and Akt. **B.** The effect of age on protein levels of eNOS and PKCε. Akt – protein kinase B, p-Akt – phosphorylated Akt, p-Akt / Akt – ratio of phosphorylated and total Akt, both normalized to GAPDH. PKCε – protein kinase C epsilon, eNOS – endothelial nitric oxide synthase, both expressed in arbitrary units (a.u., normalized to GAPDH). Values are means ± S.E.M. from 4 hearts per group. * P<0.05, vs. 1.5 months; # P<0.05, vs. 3 months

biggest differences were found between these age groups in the previous experiments. The effect of age concerned the reduction of the p-Akt/Akt ratio by 24 % in 6-month group compared with the 3-month control group. On the other hand, no age-dependent changes were observed in the PC groups. However, at the age of 3 months, we observed a significant increase in the p-Akt/Akt ratio in the PC group compared to the controls (by 48 %). In contrast, at the age of 6 months, there were no significant changes in the p-Akt/Akt ratio in PC group compared to the control group (Fig. 5A).

The effect of age and preconditioning on the protein levels of eNOS and PKCε in rat myocardium after ischemia and reperfusion

We also observed the effect of age and preconditioning on the levels of eNOS proteins. The effect of age was manifested by significantly lower levels of eNOS (by 28 %) in 6-month control group compared with 3-month controls, but by no changes in the PC groups.

On the other hand, the effect of PC was manifested by an increase in the protein levels of eNOS by 36 % (compared with the control group) at the age of 3 months, but not in the 6-month groups. This again demonstrates the efficiency of preconditioning at 3 months of age and blunted PC-induced upregulation in the levels of eNOS at 6 months compared with the respective control group (Fig. 5B).

No significant changes in the levels of protein PKCε were recorded in relationship with the age. There was a tendency of an increased PKCε level in the 3-month PC group compared to the control one, which did not reach the level of significance. In the 6-month groups, similar to previous results, no significant differences between the PKCε levels in the PC group and those in the control group were found. These findings further support the hypothesis of failing of preconditioning in the elder animals (Fig. 5C).

Discussion

In the present study we demonstrate for the first time that in the rat hearts, significant changes in their resistance to IR injury, in the effects of myocardial adaptive mechanisms, and also in the protein levels of cardioprotective proteins occurred already during the maturation of rats.

As expected, maturation significantly influenced biometric parameters of rats. With increasing age, there was a gradual increase in heart weight/body weight ratio (Fig. 1), which is in line with the previous studies (Templeton *et al.* 1979, Willems *et al.* 2005, Kostyak *et*

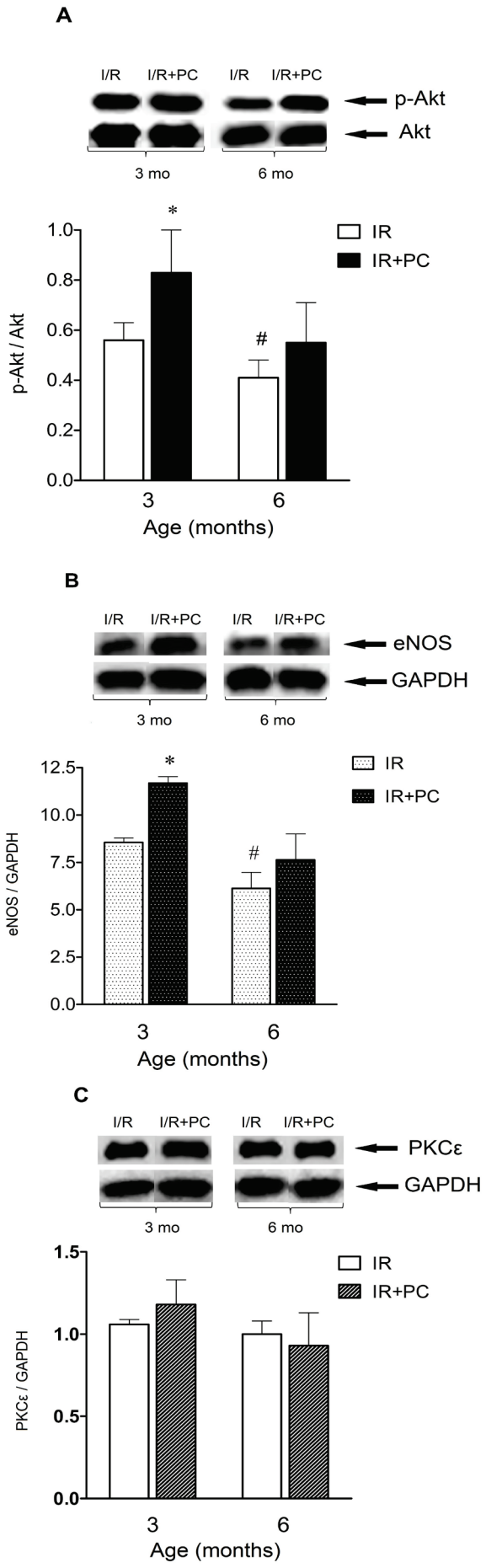

Fig. 5. The effect of age and preconditioning on the changes in the pro-survival proteins in rat hearts after ischemia and reperfusion. **A.** The effect of age and preconditioning on Akt phosphorylation. Upper panels – representative blots of p-Akt and Akt. **B.** The effect of age and preconditioning on the protein levels of eNOS. Upper panels – representative blots of eNOS and GAPDH. **C.** The effect of age and preconditioning on the protein levels of PKCε. Upper panels – representative blots of PKCε and GAPDH. Akt – protein kinase B, p-Akt – phosphorylated Akt, p-Akt / Akt – ratio of phosphorylated and total Akt, both normalized to GAPDH, eNOS – endothelial nitric oxide synthase, expressed in arbitrary units (a.u., normalized to GAPDH). PKCε – protein kinase C epsilon, expressed in arbitrary units (a.u., normalized to GAPDH). IR – samples after ischemia and reperfusion, IR+PC – samples after ischemia and reperfusion with prior preconditioning. Values are means ± S.E.M. from 4 hearts per group. * P<0.05 PC vs. IR; # P<0.05 vs. 3 months

al. 2006, Korzick *et al.* 2007). As for hemodynamic parameters, we did not observe age-dependent changes (Table 1), which is in agreement with several other studies (Anversa *et al.* 1986, Isoyama *et al.* 1987, Delp *et al*. 1998, Pacher *et al.* 2004). However, some studies demonstrated a significant decrease in the heart rate with age, and in even younger rats (Fujita *et al.* 2009).

Our major aim was to investigate the effect of maturation on the resistance of rat heart to ischemia and to explore the age-dependent changes in the efficiency of PC. We focused on the lethal injury (IS), arrhythmias and functional recovery of isolated rat hearts exposed to IR.

We demonstrated that increasing age caused a gradual increase in the size of infarction (Fig. 2) that is in accordance with some studies (Schulman *et al.* 2001, Kostyak *et al.* 2006). Age-related changes in the occurrence of arrhythmias were also evident. An increase in the total number of PVCs and in the duration of ventricular tachycardia with age was observed (Fig. 3). These results are consistent with a study, in which even a 6-week age difference led to a significant increase in ectopic activity and episodes of ventricular tachycardia in comparison with arrhythmogenesis in the 3-month-old male rats (Ledvényiová *et al.* 2013).

However, we did not find age-dependent alterations in postischemic recovery of systolic and diastolic function (Table 2), which is not in line with several studies that show decreased recovery of LVDP and higher values of LVEDP after ischemia and reperfusion in older age (Kostyak *et al.* 2006, Korzick *et al*. 2007, Willems *et al.* 2005). Nevertheless, at least partially, our results may be explained by no differences in SERCA 2a and phospholamban protein levels that were observed in 6-month group of rats in comparison with the younger ones (Babusikova *et al.* 2012). That might be one of the reasons of the unchanged contractile

recovery in relationship with maturation, however, we believe that many other factors (e.g., oxidative state of the myocardium) could be involved in the maintenance of postischemic myocardial function (Matejíková *et al*. 2009). Moreover, the absence of age dependency in the functional recovery during maturation could be related to the differences in pathophysiological mechanisms of various manifestations of I/R injury (reversible myocardial stunning versus irreversible lethal injury).

The present study also demonstrated that in the younger animals (1.5- and 3-month-old), PC reduced the size of infarction in comparison with that in non-adapted controls (Fig. 2) and decreased the number of ectopic beats and duration of ventricular tachycardia (Fig. 3). The latter is also in agreement with antiarrhythmic effect of PC shown in open-chest 3-month-old *in vivo* rats (Humphreys *et al.* 1999). Reduced incidence of reperfusion arrhythmias in animals subjected to PC at younger age has been also demonstrated in other studies (Ledvényiová *et al.* 2013, Takeshima *et al.* 1997). In addition, in the preconditioned hearts of younger animals, a significant increase of LVDP and a decrease in the values of LVEDP post-IR indicate an improved restoration of both, systolic and diastolic function (Table 2), which is in agreement with the data presented in studies by Tani *et al.* (1997) and by Matejíková *et al.* (2009) reporting signaling role of oxyradicals and mitochondrial K_{ATP} channels activity.

On the other hand, at the age of 6 months, no significant differences between the control and PC group with respect to all studied end-points of injury were observed (Fig. 2, Fig. 3, Table 2) suggesting that PC was no longer effective. These findings are in accordance with the results of other studies that demonstrated the efficiency of PC only in younger (3-month-old) animals (Boengler *et al.* 2007, Schulman *et al.* 2001). Less potent antiarrhythmic protection by PC in the hearts of 18-week-old rats than in 12-week-old ones has been also demonstrated in the study by Ledvényiová *et al.* (2013). Furthermore, our data are in line with reported loss of PC protection against postischemic contractile dysfunction in elder male mice as compared with younger animals (Turcato *et al.* 2006).

Based on these results, we propose that preconditioning is starting to lose its effectiveness already at the age of adulthood, at least with the protocol used in this study. However, we believe that the potency of PC could be eventually restored, e.g., by employing a more intensive protocol using multiple episodes of I/R, as can be seen in the study by Schulman *et al.* (2001), in which an increased number of PC stimuli helped to preserve the efficiency of cardioprotection also in 12-month-old rats.

The final goal of this study was to examine the signaling pathways of cardioprotection in association with age and to compare the changes in selected "pro-survival cascades", with a focus on specific proteins: Akt, p-Akt, eNOS and PKCε.

The effect of age was manifested by changes in the ratio of phosphorylated and total Akt at baseline conditions. With increasing age, there was a decrease in the ratio of these proteins (Fig. 4A). Protein levels of eNOS and PKCε showed similar age-related changes (Fig. 4B). The decrease in the levels of these proteins was observed already in the hearts of 3-month-old rats. These results are consistent with those reported by Korzick *et al.* (2007), who demonstrated a decreased expression of PKCε in the aged myocardium. In addition, they also support the importance of eNOS in the myocardium (Ping *et al*. 1999), as well as the fact that its decline may have a negative impact on ischemic tolerance in animals exposed to ischemic insult at the age of 6 months. Our findings are also in line with the study of Iemitsu *et al.* (2006), in which the authors showed a significant age-dependent decrease in the levels of p-Akt, in the p-Akt / Akt ratio, and phospho-eNOS and eNOS ratio.

The effect of PC on the cardioprotective protein levels was examined in the age groups in which we found major differences in the myocardial sensitivity to ischemic injury and in cardioprotective effects of PC, 3- and 6-month-old groups. Since a significant increase in the p-Akt / Akt ratio was observed in PC group post-I/R compared to controls in 3-month-old animals (Fig. 5A), we believe that Akt and p-Akt proteins are involved in cardioprotective effects of PC in this age. This is in line with a study by Hausenloy *et al.* (2005), in which the authors demonstrated that PC led to an enhanced Akt phosphorylation detected in isolated rat hearts post-I/R, and confirmed thus an essential role of this kinase in the PC-induced protection in younger animals. In contrast, we observed no changes in the p-Akt / Akt ratio in PC group compared to the controls in older age (6 months). Similar PC-induced increase in the protein levels of eNOS was observed only in the younger group (Fig. 5B), while in 6-month-old group, no differences in the levels of this protein between the PC and the control group were observed. Thus, we assume that failure of PC to activate Akt/eNOS pathway may account for the loss of cardioprotection in this age group. There was only

a tendency of PC to increase protein levels of PKCε in the 3-month-old group, however, similar to other findings, we did not observe any significant differences in the PKCε levels between the preconditioned and control groups at the age of 6 months (Fig. 5C). Thus, it is conceivable that the results concerning the levels of PKCε and eNOS might copy those concerning the levels of p-Akt, due to mutual interactions of these proteins within the PC cascade (Monti *et al.* 2010, Yang *et al.* 2010, Zhang *et al.* 2005, Rask-Madsen and King 2008).

In conclusion, maturation impaired rat heart response to lethal I/R injury (increased IS) and promoted arrhythmogenesis, while functional recovery was not affected by aging. PC suppressed the occurrence of malignant arrhythmias, reduced the IS and improved functional recovery in the younger animals, while its efficacy was attenuated in the adult mature ones. Loss of cardioprotective effects of PC was associated with reduced Akt phosphorylation and protein levels of eNOS and PKCε in the elder group both, at baseline and post-IR. Alterations in myocardial response to ischemia may be caused by suppression of the mechanisms of innate cardioprotection due to dysfunction of proteins involved in protective cell signaling.

Conflict of Interest

There is no conflict of interest.

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