Severity of Lethal Ischemia/Reperfusion Injury in Rat Hearts Subjected to Ischemic Preconditioning Is Increased Under Conditions of Simulated Hyperglycemia

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Summary

The aim of our study was to characterize resistance to ischemia/reperfusion (I/R) injury in Langendorff-perfused rat hearts and effectivity of ischemic preconditioning (PC) under condition of simulated acute hyperglycemia (SAHG) by perfusion of the hearts with Krebs-Henseleit (KH) solution with elevated glucose concentration (22 mmol/l). I/R injury was induced by 30-min coronary occlusion followed by 120-min reperfusion and PC by two cycles of 5-min occlusion/5-min reperfusion, prior to I/R. The severity of I/R injury was characterized by determination of the size of infarction (IS, expressed in % of area at risk size) and the amount of heart-type fatty acid binding protein (h-FABP, a marker of cell injury) released from the hearts to the effluent. Significantly smaller IS (8.8±1 %) and lower total amount of released h-FABP (1808±660 pmol) in PC group compared with IS 17.1±1.2 % (p<0.01) and amount of h-FABP (8803±2415 pmol, p<0.05) in the non-PC control hearts perfused with standard KH solution (glucose 11 mmol/l) confirmed protective effects of PC. In contrast, in SAHG groups, PC enhanced IS (21.4±2.2 vs. 14.3±1.3 %, p<0.05) and increased total amount of h-FABP (5541±229 vs. 3458±283 pmol, p<0.05) compared with respective non-PC controls. Results suggest that PC has negative effect on resistance of the hearts to I/R injury under conditions of elevated glucose *in vitro*.

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Introduction

Many clinical studies describe protective effects of unstable angina pectoris (UAP) considered as clinical analogue of ischemic preconditioning (PC) (Kloner 1995, Kloner *et al.* 1998, Nakagawa *et al.* 1995, Švorc *et al.* 2003). In contrast, other authors observed higher mortality in UAP patients than in non-UAP patients after acute myocardial infarction (AMI) (Behar *et al.* 1992). Distinct effects of UAP can be attributed to different personal history (e.g., presence of comorbidities, such as diabetes mellitus, hypertension). Clinical and epidemiological studies demonstrated a higher risk of cardiovascular disorders in diabetic patients and a loss of protective effect of UAP (Ishihara *et al.* 2001). On the other hand, experimental data are not unequivocal and suggest that besides higher myocardial vulnerability, diabetes mellitus may be associated with induction of adaptive processes leading to lower susceptibility to ischemia similar to PC (Ravingerová *et al.* 2011, Balakumar and Sharma 2012). The first paradoxical effects of diabetes have been observed in diabetic rats subjected to ischemia/reperfusion (I/R) injury reflected in better recovery of contractile function and reduced Ca^{2+}

overload upon I/R (Tani and Neely 1988). The probable cause of attenuated Ca^{2+} overload in the diabetic myocardium is decreased accumulation of glycolytic products and inhibition of Na^+/H^+ exchanger activity (Feuvray and Lopaschuk 1997, Karmazyn 1995). Likewise, Liu *et al.* (1993) observed antiinfarct effect in the acute phase of streptozotocin (STZ)-induced diabetes mellitus. Duration of diabetic state significantly influences myocardial response to ischemia. Experimental studies have shown that increased resistance to I/R injury in the acute phase of disease can be reversed in the chronic phase of diabetes (Balakumar and Sharma 2012). Experiments with isolated Langendorff-perfused rat hearts demonstrated decreased antiarrhytmic effect (Ravingerová *et al.* 2000) and reduction of improved postischemic contractile recovery associated with prevention of ion shifts (Tosaki *et al.* 1996) in the chronic (8-9 weeks) phase in comparison with acute (1 or 2 week) phase of STZ-induced diabetes. In addition, loss of antiinfarct protection (reduction of infarct size) in the diabetic rats was observed in the chronic phase of the disease in the open-chest *in vivo* animals (Ravingerová *et al.* 2003).

Experimental studies showed that diabetes can also modulate effectivity of PC. In normal physiological conditions, insulin can regulate glucose metabolism through phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway (component of PC cascade) and phosphorylation (and inactivation) of glycogen synthase kinase 3ß (GSK3ß). The latter is known to promote glucose utilization and mitochondrial permeability transition (mPTP) pores closing (Yin *et al.* 2012). Active GSK3ß plays a role in necrosis and apoptosis of cardiomyocytes due to mPTP pores opening triggered by tumor suppressor protein 53 (p53) phosphorylation (Miura *et al.* 2009) and regulation of glycogen synthesis (Montori-Grau 2013). In accordance, the antiinfarct effect of PC in hearts isolated from rats with STZ-induced diabetes was evident only in the case of treatment with GSK3ß inhibitor (Yadav *et al.* 2010). However, hyperglycemia can abrogate PC effect independently of insulin level, too (Clodfelder-Miller *et al.* 2005), e.g., by increasing the threshold for antiinfarct protection that requires a higher number of PC cycles (Tsang *et al.* 2005).

Clinical studies demonstrated that heart-type fatty acid binding protein (h-FABP) has a high potential as an early marker of AMI. It is a low-molecular weight cytoplasmic protein (about 14-15 kDa with 126-134 amino acids in molecule) found in abundance in the heart and released from cardiomyocytes early after the onset ischemia (Chan *et al.* 2004, Veerkamp *et al.* 1999). h-FABP test could be considered as highly specific in the diagnosis of AMI (Alhadi and Fox 2004) with more than 80 % sensitivity of this cardiomarker maintained within first three hours after an ischemic attack (Kleine *et al.* 1992). However, clinical studies revealed increased levels of h-FABP in the serum of UAP patients than were lower than in AMI patients (Tsuji *et al.* 1993). Therefore, h-FABP migh be also regarded as an indicator of reversible damage of myocardial integrity occurring without necrosis.

Aims of our study were to find differences in the size of myocardial infarction and in the release of h-FABP between the hearts of control non-preconditioned and ischemically preconditioned groups, both under standard conditions and under conditions of simulated hyperglycemia. Our second goal was to reveal mutual correlation between the total amount of released h-FABP and the extent of lethal injury (infarct size).

Material and Methods

Animals and experimental protocols

Adult male Wistar rats (250-300 g body weight) fed a standard diet and tap water *ad libidum* were used in the experiments. Animals were anesthetized by sodium pentobarbitone in dose 50-60 mg/kg combined with heparin. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication No 85-23, revised 1996) and approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic. Rats were randomly divided into four groups, and further experiments were performed in Langendorff-perfused hearts.

Perfusion technique

After rapid excision, the hearts were placed in ice-cold perfusion buffer, cannulated *via* the aorta and then perfused in the Langendorff mode with Krebs-Henseleit (KH) solution gassed with 95 % O_2 and 5 % CO_2 (pH 7.4) at constant perfusion pressure of 73 mm Hg and 37 °C. KH solution contained (in mmol/l): NaCl 118.0; KCl 3.2; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; KH_2PO_4 1.18; $CaCl_2$ 2.5; glucose 11.1 or 22.2. Left ventricular (LV) pressure was measured by a water-filled balloon

(inflated to obtain end-diastolic pressure of 5-7 mm Hg) inserted into LV and connected to a pressure transducer (MLP physiological pressure transducer, ADInstruments, Germany). LV systolic and diastolic pressure, LV developed pressure (LVDP; systolic minus diastolic pressure), maximal rates of pressure development and fall, $+dP/dt_{max}$ and $-dP/dt_{max}$, as indexes of contraction and relaxation, heart rate (HR, derived from electrogram) and coronary flow were used to assess heart function using Power-Lab/8SP Chart 7 software (ADInstruments, Germany). The hearts were allowed to stabilize (15 min) before further interventions.

Protocols of ischemia/reperfusion
1. In the control normoglycemic (C/NG) and hyperglycemic (C/HG) groups, the hearts were exposed to 30-min occlusion of left anterior descending (LAD) coronary artery followed by a release of clamping and 120-min reperfusion. During the whole protocol, the hearts were perfused either with standard KH solution (concentration of glucose 11 mmol/l) or with KH solution containing 22 mmol/l glucose. Numbers of animals per group were 8 (C/NG) and 6 (C/HG).
2. In the preconditioned normoglycemic (PC/NG) and hyperglycemic (PC/HG) groups, the hearts were subjected to two cycles of ischemic preconditioning (PC) induced by 5-min LAD occlusion followed by 5-min reperfusion, prior to sustained I/R as in 1. Numbers of animals per group were 9 (PC/NG) and 4 (PC/HG).

Determination of infarct size
Infarct size (IS) and area at risk (AR) size were delineated by double-staining with $KMnO_4$ and 2,3,5-triphenyltetrasolium chloride (TTC), as described elsewhere (Ravingerová *et al.* 2003). IS and AR were measured in 4-5 slices per heart (1 mm thick) and determined by noncommercial planimetric software. IS was expressed in % of AR. In one experiment in PC/NG group, the staining failed to provide the required information.

Analysis of h-FABP released from the hearts
Heart effluent was collected during stabilization, long-term occlusion and reperfusion period. Collecting during reperfusion was divided into six 20 min separated portions. Samples (2 ml) from effluent portions were dialyzed overnight against redistilled water at 4 °C using dialysis tubing cellulose membrane, 1-ml samples obtained from dialyzed samples were frozen at −20 °C, completely lyophilized and subsequently diluted with 110 µl of sample diluent. Diluted samples (100 µl) were analyzed by rat h-FABP ELISA test kit (sandwich ELISA) according to the manual from the manufacturer to determine h-FABP concentration. Concentration values were multiplied by values of collected volumes to determine the amount of h-FABP substance (in pmol) in every portion. With respect to the sample processing, values of h-FABP amounts were multiplied by 0.09. This constant represents a simplified numeric expression of $^{100}/_{1000}$ x $^{100}/_{110}$. Summation of amounts from all previous portions and the amount of actual collected portion was considered as a real value in a respective stage of experiment. Requirement for concentration analysis of h-FABP was the value of coronary flow within the calibrated range during the whole experiment. For these reasons, the data from two experiments in PC/NG and in C/HG groups (one per group), as well as from two experiments in C/NG group were excluded from the analysis. The criterion for subsequent calculation of h-FABP amounts was the concentration value no lower than the lowest standard concentration (16 pmol/ml). In three experiments in C/NG group, where detected concentration values were below this level in all samples, the results of the test were considered as insensitive or false negative. Data related to h-FABP in these experiments were not included in statistical analysis.

Statistical evaluation
All data are expressed as means ± SEM. Comparison of individual groups was performed by Student´s *t*-test. Criterion for significant difference between the groups was value $p < 0.05$. To verify the possible statistical correlation of total amount of h-FABP in the effluent from the hearts with the infarct size, the calculation of the Pearson´s correlation coefficient (r) was used. Computed modified Z-scores (Iglewicz and Hoaglin 1993) were used for testing for outliers. Due to exclusion of extreme values of released total h-FABP in PC/NG and in PC/HG groups (one experiment per group) data associated with released h-FABP in these experiments were not included in statistical analysis.

Results

Characteristic of isolated hearts
The value of heart rate, LVDP, $+dP/dt_{max}$,

−dP/dt$_{max}$, and coronary flow in the NG and HG control groups, as well as in the preconditioned NG and HG groups are summarized in Table 1. There were no significant differences in the values of these parameters between the groups prior to ischemia.

Table 1. Preischemic values of parameters of myocardial function in controls and ischemically preconditioned rat hearts.

Group	C/NG	PC/NG	C/HG	PC/HG
HR (beats/min)	263±21	228±23	269±12	221±30
CF (ml/min)	8.2±0.7	8.0±1.1	12.6±1.7	10.8±1.0
LVDP (mm Hg)	81.6±4.7	79.8±7.8	87.5±5.8	69.3±9.5
+(dP/dt)$_{max}$ (mm Hg/s)	2125±242	2061±353	2309±258	2077±198
−(dP/dt)$_{max}$ (mm Hg/s)	1158±138	1178±193	1490±95	1156±100

C − controls, PC − ischemic preconditioning. HR − heart rate, CF − coronary flow, LVDP − left ventricular developed pressure, +(dp/dt)$_{max}$ − maximal rate of pressure development, −(dP/dt)$_{max}$ − maximal rate of pressure fall. Data are expressed as means ± SEM, n=4-9 hearts per group.
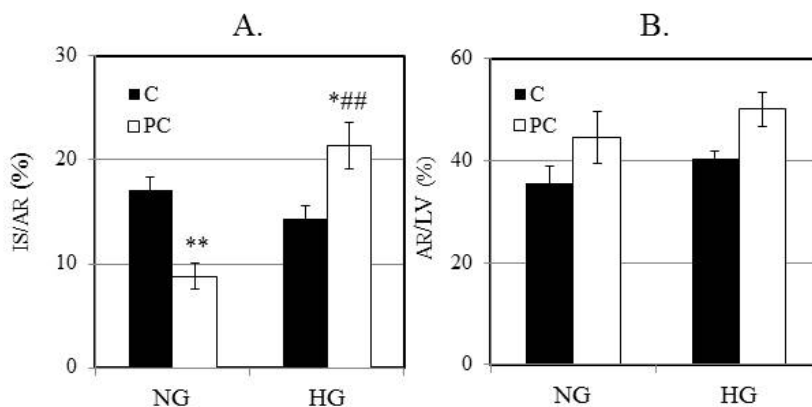

Fig. 1. The effect of hyperglycemia on size of myocardial infarction and area at risk size in the preconditioned rat hearts and in non-preconditioned control hearts. **A**: Infarct size/area at risk (IS/AR) ratio. **B**: Area at risk/left ventricle size (AR/LV) ratio. IS − infarct size, AR − area at risk, LV − left ventricle, NG − groups perfused with standard KH solution, HG − groups exposed to simulated hyperglycemia, C − non-PC control groups, PC − preconditioned groups. Results are means ± SEM, n=4-8 hearts per group. * p<0.05, ** p<0.01, PC vs. C, ## p<0.01, PC/HG vs. PC/NG

Infarct size in non-preconditioned controls and preconditioned groups under NG and HG conditions

Under NG conditions, significantly smaller infarct size was observed in the PC group than in the control non-PC group (8.8±1.0 % vs. 17.1±1.2 %, p<0.01). Under HG conditions, in the PC group, infarct size was significantly larger than in the control non-PC group (21.4±2.2 % vs. 14.3±1.3 %, p<0.05). No significant difference in IS/AR ratio between the control non-preconditioned groups was observed. On the contrary, in the preconditioned hearts, significantly higher IS/AR ratio was found in the HG group as compared with preconditioned NG group (p<0.01) (Fig. 1A). No significant differences in the size of area at risk (AR/LV ratio) among all the groups were observed (Fig. 1B).

Cumulative curves of amount of H-FABP substance released from the hearts in the non-preconditioned controls and preconditioned hearts under NG and HG conditions

Under NG conditions, amount of h-FABP substance released from the hearts was significantly lower in the 100th and the 120th min of long-term reperfusion in the PC group than in the non-PC control group (1677±626 and 1808±660 vs. 6189±1765 and 8803±2415 pmol, p<0.05, respectively) (Fig. 2A). In contrast, in HG conditions, during long-term reperfusion, h-FABP was higher in the PC group than in the control non-PC group starting from the 60th min of reperfusion (3183±415, 3936±396, 4521±442, 5541±229 vs. 1345±558, 1812±532, 2651±415, 3458±283 pmol, respectively, p<0.05) (Fig. 2B). In both conditions, the most significant difference was observed at the end of reperfusion (p<0.01). When comparing the control groups, significantly lower amount of released h-FABP was observed in the HG group from the 100th min of the reperfusion. Comparison of PC groups revealed that

release of h-FABP in the HG conditions was significantly higher than in the preconditioned NG group with the onset at the 80th min and was even more pronounced at the end of experiment (p<0.01).

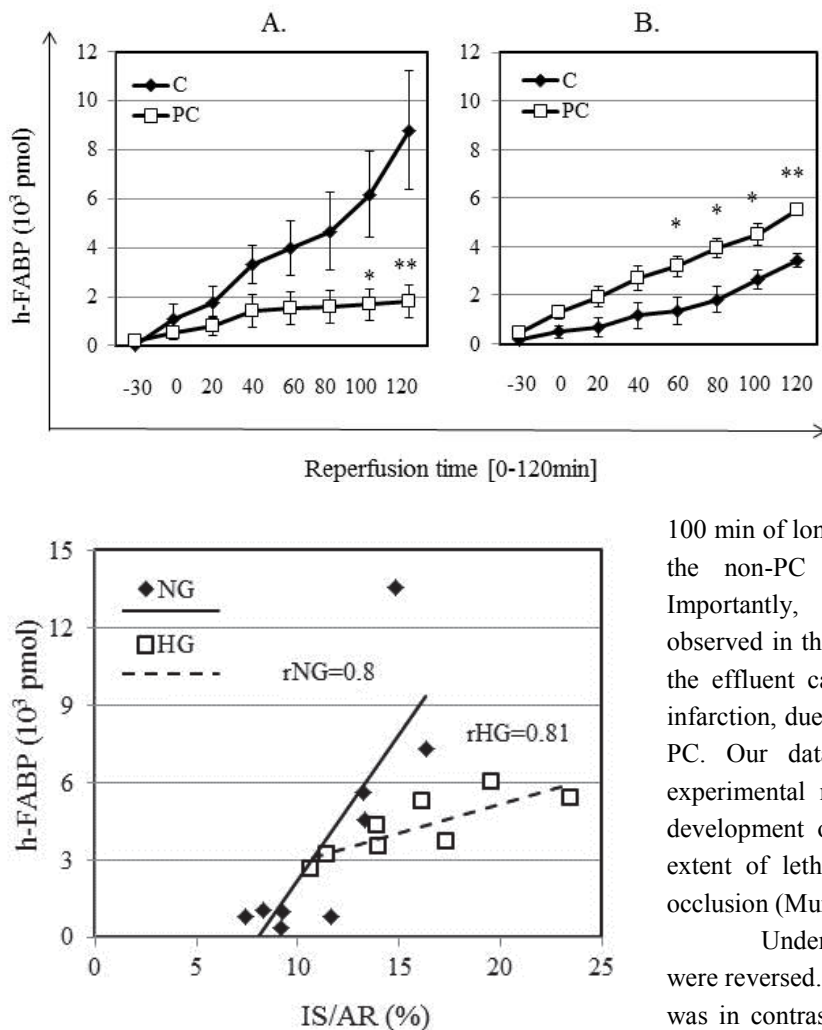**Fig. 2.** Cumulative curves of h-FABP amount released from the hearts of non-PC control groups and PC groups. **A**: Cumulative curves under NG conditions. **B**: Cumulative curves under HG conditions. h-FABP − amount of h-FABP released to effluent from hearts expressed in pmol. Results are means ± SEM, n=3-7 hearts per group. * p<0.05, ** p<0.01, PC vs. respective non-PC group (C)

Fig. 3. Correlation of infarct size and amount of h-FABP in the effluent from the hearts at the end of long term reperfusion. IS/AR − infarct size/area at risk ratio, IS − infarct size, AR − area at risk, h-FABP − amount of h-FABP substance in effluent from hearts (in pmol) at the end of long-term reperfusion

Correlations of total h-FABP amount released from the hearts and the size of myocardial infarction

Dependence of released h-FABP on the size of infarcted area in the non-preconditioned and preconditioned hearts was assessed based on the calculation of the Pearson´s correlation coefficient. Fig. 3 shows high correlation of IS/AR with h-FABP in both NG (rNG=0.8) and HG (rHG=0.81) conditions.

Discussion

Our study demonstrated that significantly lower amounts of h-FABP were released from the hearts after 100 min of long-term reperfusion in the PC-group than in the non-PC control group under NG conditions. Importantly, significantly smaller IS/AR was also observed in the PC group. Lower increase of h-FABP in the effluent can point out to a slower development of infarction, due to increased resistance to I/R conferred by PC. Our data are in agreement with the Murry´s experimental results demonstrating that PC delays the development of irreversible changes and decreases the extent of lethal injury, evoked by long-term coronary occlusion (Murry *et al.* 1991).

Under conditions of simulated HG, the results were reversed. PC significantly increased IS/AR ratio that was in contrast with antiinfarct effect of PC in the NG group (Fig. 1A). Likewise, significantly higher amount of h-FABP was observed in the effluent from the preconditioned hearts than in the effluent from the non-PC group of hearts from 60 min of reperfusion (Fig. 2). It seems that PC under conditions of simulated hyperglycemia has negative effects on cardiac resistance to I/R injury. Comparison of IS/AR and total h-FABP amount in the effluent from the preconditioned and non-preconditioned hearts under NG and HG condition indicates that hyperglycemia influences the development of infarction only in combination with PC. Thus, significantly higher IS/AR ratio and amount of h-FABP in the effluent from 80 to 120 min of reperfusion was observed in the PC/HG group than in the PC/NG group.

On the other hand, no significant differences in IS/AR were found between the NG and HG non-PC control groups (Fig. 1), and the amount of h-FABP released from the hearts of HG group was even lower in the final stages of reperfusion than in the NG group

(Fig. 2). This finding indicates that the hearts perfused with high-glucose medium may be more resistant to ischemic injury. This is in agreement with the results of Chen *et al.* (2006) who have demonstrated that STZ-induced severe acute hyperglycemia was associated with lower release of creatinine phosphokinase from the tissue exposed to I/R injury and with increased heat shock protein 90 and catalase in Langendorff-perfused hearts.

Prognosis of AMI depends on its size (Sobel *et al.* 1972). Determined high association between the IS and total released amount of h-FABP in NG and HG conditions (Fig. 3) implies that h-FABP might be used as a prognosis predictor in AMI patients with a history of UAP. The utility of h-FABP as a determinant of infarct size is confirmed by the study with open-chest dogs (Sohmiya *et al.* 1993). Lower release of h-FABP from the hearts in the control group in HG than in NG conditions with no difference in the size of infarction between these groups demonstrated in our study might refer to protective effect of simulated acute HG that prevents severe myocardial injury in non-infarcted risk area and release of low molecular substances. This is in accordance with the study of Tani and Neely (1988) who found that acute diabetes prevents Ca^{2+} overload in rat hearts exposed to I/R injury and with the Schaffer's *et al.* (2000) study showing that high-glucose medium upregulates apoptotic repressor protein Bcl-2.

In all the groups, release of h-FABP during long-term coronary occlusion was also observed. Its potential cause could be basal release from the normal myocardium, which is evident at the end of stabilization period, and coronary collateral circulation developing in AR which becomes functional between 15 and 30 min of coronary occlusion according to a morphological study in rats (Jia and Sato 1997).

Comparison of PC effectivity under NG and HG conditions was performed in the *in vivo* conditions in open-chest rabbits as well. PC was induced by a single 5 min coronary occlusion followed by a 15 min reperfusion (Vladic *et al.* 2011). In this study, PC in HG conditions had no negative effect on the IS size after I/R evoked by coronary artery occlusion. HG only abolished its beneficial effect. Similarly, attenuated but no negative effect of PC was observed in the *in vivo* experiments with mongrel dogs in HG group (Kersten *et al.* 1998). In these experiments, PC was induced by four short-term coronary artery occlusions and permanent hyperglycemia by continual intravenous infusion of glucose or dextrose. Therefore, it seems unprobable that the differences

between the results of *in vivo* experiments and our *ex vivo* experiments are attributed to the differences between animal species, models or a number of PC episodes. Potential cause of negative effect of PC in our *ex vivo* experiments can be associated with elimination of beneficial action of insulin, PI3K/Akt pathway and inhibition of GSK3β (Yin *et al.* 2012). Deleterious effect of HG without the influence of insulin could be caused by lowering tetrahydrobiopterin (BH_4) cofactor of NO synthase confirmed by cell culture study (Prabhakar 2001). However, beneficial action of insulin is not obvious. Other cell culture studies showed that insulin promotes proapoptotic activity reflected in an increased Bcl-2 like protein 4 (BAX)/B-cell lymphoma 2-protein (Bcl-2) ratio (Ricci *et al.* 2008), caspase-3, cytochrome-c and Bcl-2-associated death promoter (Rajamani *et al.* 2011).

Studies with cultured cardiomyocytes also pointed to deleterious effect of simulated hyperglycemia alone, i.e., in the absence of insulin, due to induction of nuclear factor (NF)-κB (Nizamutdinova *et al.* 2013), activation of protein p53 (Fiordaliso *et al.* 2001), mitochondrial cytochrome-C-mediated caspase-3 pathway (Cai *et al.* 2002) and downregulation of Na^+/K^+-ATPase (Li *et al.* 2009).

In addition, simulated hyperglycemia attenuated protective effect of pharmacological preconditioning associated with Akt pathway in cardiomyocyte cultures (Kim *et al.* 2012).

A component of signal transduction of preconditioning in rat hearts is protein kinase C-δ (PKC-δ), too (Schulz *et al.* 2001), which can be involved in the apoptotic processes (Zhao *et al.* 2012). Both, mannitol and glucose treatment of rat ventricular myocytes *in vitro* led to activation of PKC-δ and its translocation to cell membrane (Shizukuda *et al.* 2002). Impaired cardioprotective signaling pathways of preconditioning and activation of PKC-δ and other proapoptotic factors under conditions of hyperglycemia can explain negative effect of PC in our experiments.

Conclusion

Our results indicate that measurement of perspective cardiomarker h-FABP released from the injured hearts after the onset of ischemia can be used as a determinant of the lethal injury and the effectivity of PC, under certain conditions. Higher release of h-FABP from the hearts in the control group perfused under standard

NG conditions than under conditions of simulated hyperglycemia indicates that acute hyperglycemia may exert cardioprotective effects against ischemic cardiac injury. Based on the evaluation of infarct size and release of h-FABP, deleterious effect of PC under simulated acute hyperglycemia *in vitro* conditions may be suggested. It can be speculated that a probable cause of this negative effect could be the absence of protective influence of insulin and/or impairment of cardioprotective pathways of preconditioning.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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