

Impaired PI3K/Akt signaling as a potential cause of failure to precondition rat hearts under conditions of simulated hyperglycemia

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Short title: High glucose modulates PI3K/Akt in preconditioned hearts

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List of abbreviations

Akt	Protein kinase B
AMI	Acute myocardial infarction
BAX	Bcl-2 like protein
Bcl-2	B-cell lymphoma protein
CF	Coronary flow
+dp/dt _{max}	Maximal rate of pressure development
-dp/dt _{max}	Maximal rate of pressure fall
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h-FABP	Heart fatty acid binding protein
HG	High glucose, simulated hyperglycemia
HR	Heart rate
IP	Ischemic preconditioning
I/R	Ischemia/reperfusion
LV	Left ventricle
LVDP	Left ventricular developed pressure
NG	Normal glucose, normoglycemia
P-Akt	Phosphorylated protein kinase B
PI3K	Phosphatidylinositol-3 kinase
PIP3	phosphatidylinositol-3 phosphate
PKC	Protein kinase C
UAP	Unstable angina pectoris

Summary:

The aim of the study was to evaluate the impact of simulated acute hyperglycemia (HG) on PI3K/Akt signaling in preconditioned and non-preconditioned isolated rat hearts perfused with Krebs-Henseleit solution containing normal (11 mmol/l) or elevated (22 mmol/l) glucose subjected to ischemia-reperfusion. Ischemic preconditioning (IP) was induced by two 5-min cycles of coronary occlusion followed by 5-min reperfusion. Protein levels of Akt, phosphorylated (activated) Akt (P-Akt), as well as contents of BAX protein were assayed (Western blotting) in cytosolic fraction of myocardial tissue samples taken prior to and after 30-min global ischemia and 40-min reperfusion. In „normoglycemic“ conditions (NG), IP significantly increased P-Akt at the end of long-term ischemia, while reperfusion led to its decrease together with the decline of BAX levels as compared to non-preconditioned hearts. On the contrary, under HG conditions, P-Akt tended to decline in IP-hearts after long-term ischemia, and it was significantly higher after reperfusion than in non-preconditioned controls. No significant influence of IP on BAX levels at the end of I/R was observed under HG conditions. It seems that high glucose may influence IP-induced activation of Akt and its downstream targets, as well as maintain persistent Akt activity that may be detrimental for the heart under above conditions.

Key words:

Phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), ischemic preconditioning, simulated hyperglycemia, bcl-2-like protein 4 (BAX)

Introduction:

In 1986 it was first time described by Murry *et al.* that series of short-time coronary occlusions intercepted with reperfusion, termed as ischemic preconditioning (IP), delays cardiomyocyte death. Preconditioning results in activation of G-protein-coupled receptors by ligands, such as adenosine, alpha adrenergic agonists, delta-opioids and bradykinin (Tyagi and Tayal 2002). It was found that protection afforded by all of the trigger substances could be blocked by protein kinase C (PKC) inhibitors, which indicates that PKC could be a common target of these ligands (Downey *et al.* 2007). The mechanism of PKC activation may involve stimulation of phospholipase C via GPCR (G proteins-coupled receptors) that subsequently cleaves phosphatidylinositol-3 phosphate (PIP3) to inositol-1, 4, 5-triphosphate and diacylglycerol, which binds to PKC and activates it (Simkhovich *et al.* 2013). Isolated rat heart experiments revealed that activation of PKC leads to its translocation to particular fraction associated with improvement of cardiac function (Kawamura *et al.* 1998). A basic property of PKC is considered to be modulation of mitochondrial K^+ _{ATP} channels (Wang *et al.* 2001). Their opening causes a mitochondrial swelling. Expansion of the mitochondrial matrix volume improves fatty acid oxidation, respiration and energy production. Thus, this mechanism has become considered as one of key hypotheses to explain protection by IP (O'Rourke 2004). Experiments with isolated rat hearts have shown that phosphatidylinositol-3 kinase (PI3K)/protein kinase B (Akt) stimulation occurs upstream of PKC activation (Tong *et al.* 2000). Akt kinase is stimulated by a number of receptor tyrosine kinases and this is mediated by the action of PI3K (Hemmings and Restuccia 2012). It was proposed that PI3K may be also downstream of PKC (Walser *et al.* 2013). PI3K/Akt promotes physiological heart growth and inhibits pathological myocardial hypertrophy (Walsh 2006). Akt also phosphorylates Bcl-2-associated death promoter, decreases caspase-3 activity (Hussain *et al.* 2014), upregulates antiapoptotic B-cell lymphoma protein (Bcl-2) (Pugazhenti *et al.* 2000)

and prevents conformational changes of bcl-2-like protein 4 (BAX), as well as its incorporation into a mitochondrial membrane. Importantly, Rajtík *et al.* (2012) demonstrated that ischemia-reperfusion (I/R) injury leads to a decrease of Bcl-2/BAX ratio associated with myocardial dysfunction and apoptosis. On the other hand, increased Bcl-2/BAX indicates activation of antiapoptotic (or suppression of apoptotic) processes leading to antiinfarct protection (Hausenloy and Yellon 2004). This effect of PI3K/Akt pathway was confirmed in the experiments with Langendorff-perfused rat hearts and in *in vivo* settings of adaptation to intermittent hypobaric hypoxia (Ravingerová *et al.* 2007).

Many clinical studies demonstrated that unstable angina pectoris (UAP) decreases mortality in patients with acute myocardial infarction (AMI), the most severe form of ischemic heart disease (Kloner 1995, Kloner *et al.* 1998, Nakagawa *et al.* 1995) and, therefore, is considered as a clinical analogue of IP (Švorc *et al.* 2003). In contrast, other authors observed higher mortality in UAP patients than in non-UAP patients after 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, systemic arterial hypertension, hyperlipidemia) and aging. Ischemic heart disease is often combined with these risk factors (Ferdinandy *et al.* 2007). Risk factors may play a dual role in pathogenesis of I/R and besides deleterious effects, some factors may trigger adaptive processes in the heart resulting in its greater ischemic tolerance (Ravingerová *et al.* 2012). However, other experimental studies in open-chest animals have demonstrated that in pathologically altered hearts (e.g., in acute hyperglycemia), protective IP-effect was blunted (Vladic *et al.* 2011, Kersten *et al.* 1998). Tsang *et al.* (2005) have reported that under conditions of experimental diabetes mellitus II type, the heart requires more IP stimuli to reach PI3K/Akt activation. Moreover, under conditions of hypercholesterolemia, IP even impaired heart resistance to ischemia (Juhász *et al.* 2004). In our previous study (Zálešák *et al.* 2014), we have also observed negative effect

of IP under conditions of simulated hyperglycemia, where preconditioned rat hearts had larger size of infarction than non-preconditioned controls. The aims of our present study were to disclose some molecular mechanisms that might underlie this negative effect of IP under conditions of hyperglycemia, with particular regards to the changes in PI3K/Akt signaling.

Material and methods

Animals and experimental protocols

Adult male Wistar rats (250-300g body weight) fed a standard diet and tap water *ad libitum* were used in the experiments. Animals were anesthetised by sodium pentobarbitone in a dose of 50-60 mg/kg (i. p.) 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% O₂ and 5% CO₂ (pH 7.4) at constant perfusion pressure of 73 mmHg and 37 °C. KH solution contained (in mmol/l): NaCl 118.0; KCl 3.2; MgSO₄ 1.2; NaHCO₃ 25.0; KH₂PO₄ 1.18; CaCl₂ 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 mmHg) 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 (20 min) before further interventions (Fig. 1).

Protocols of ischemia/reperfusion

1. In the control normoglycemic (C/NG) and hyperglycemic (C/HG) groups, after stabilization (20 min) and additional 20-min perfusion to match the protocol of IP, the hearts were exposed to 30-min global zero-flow ischemia followed by 40-min recovery of perfusion – I/R injury. During the whole protocol, the hearts were perfused either with a standard KH solution (concentration of glucose 11 mmol/l) or with KH solution containing 22 mmol/l glucose in the case of simulated hyperglycemia. Number of animals per group was 4.
2. In the preconditioned normoglycemic (IP/NG) and hyperglycemic (IP/HG) group, after stabilization (20 min), the hearts were subjected to two cycles of ischemic preconditioning (IP) induced by 5-min stop of aortic inflow and 5-min recovery of perfusion, prior to sustained I/R as in 1. Numbers of animals per group was 4. The schemes of both protocols are shown in Fig. 1.

Preparation of tissue protein fractions

Sampling of tissue for Western blot analysis was performed as described previously (Barlaka *et al.* 2013; Ravingerová *et al.* 2009, 2013). In non-preconditioned and preconditioned hearts, small amounts of left ventricular myocardial tissue (equal size) were taken prior to ischemia, after 30-min global ischemia and after 40-min reperfusion and stored in liquid nitrogen until

usage. Timing of sampling is shown in Fig. 1. The samples were resuspended in ice-cold buffer containing (in mmol/l): Tris-HCl 20, sucrose 250, ethylene-glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid 1.0, dithiotreitol 1.0, phenylmethylsulphonylfluoride and 0.5 sodium orthovanadate (pH 7.4) and homogenized with a Teflon homogenizer. The homogenates were centrifuged at 3000xg for 5 min at 4°C, pellets after centrifugation were discarded and the supernatants were centrifuged again at 10000xg for 30 min. The supernatants after the second centrifugation, termed as cytosolic fraction were used for further analysis. Protein concentration was estimated by the method of Bradford (1976).

Electrophoresis and Western blot analysis

Samples of protein fraction containing equal amounts of protein per lane (40 µg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Western blot analysis, separated proteins were transferred from gel to a nitrocellulose membrane. The quality of the transfer was controlled by Ponceau S staining of nitrocellulose membranes after the transfer and protein loading by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeper. Specific anti-Akt kinase, anti-Bcl-2 and anti-BAX (from Santa Cruz Biotechnology) and anti-phospho (P)-Akt kinase (from Cell Signaling Technology) antibodies were used for the primary immunodetection. Peroxidase-labelled anti-rabbit immunoglobulin (Cell Signaling Technology) was used as the secondary antibody. Bound antibodies were detected by the enhanced chemiluminescence detection method using Carestream PC program. Protein levels of Akt, P-Akt and BAX were normalized to GAPDH. PI3K/Akt activity was expressed as a ratio of P-Akt and Akt. Color burst marker (Sigma Aldrich) was used for quantitative molecular mass determination of transferred proteins.

Statistical evaluation

All data are expressed as means \pm SEM. Comparison of individual groups was performed by multifactorial analysis of variances (ANOVA) and subsequently by Student's *t*-test. Differences between the groups were considered significant at $p < 0.05$.

Results:

Characteristics 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 sustained ischemia.

Akt phosphorylation prior to and after long-term ischemia and after reperfusion

Under conditions of NG, preconditioning tended to increase myocardial P-Akt contents, although the difference between non-preconditioned and preconditioned groups did not reach the level of significance (Fig. 2). Neither did IP under HG conditions influenced Akt phosphorylation (Fig. 2). No differences in the levels of P-Akt between the control NG and HG groups were observed (Fig. 2).

On the other hand, in the IP group, P-Akt was significantly increased at the end of index ischemia as compared with the non-preconditioned controls under NG conditions (0.32 ± 0.004 vs. 0.27 ± 0.01 , $p < 0.05$) (Fig. 3). However, under HG conditions, no significant changes in P-Akt/Akt between the groups of preconditioned hearts and non-preconditioned controls were observed, moreover, P-Akt/Akt in IP group tended to be reduced but the difference between the IP and C groups was not significant (Fig. 3). In contrast, in non-IP

controls, HG significantly increased P-Akt/Akt as compared with NG controls (0.46 ± 0.04 vs. 0.27 ± 0.01 , $p < 0.05$) (Fig. 3).

After reperfusion, P-Akt/Akt in the IP group was significantly lower than in the C group under NG conditions (1.57 ± 0.07 vs. 2.92 ± 0.1 , $p < 0.05$) (Fig. 4), whereas under HG conditions, it was higher than in the C group (1.82 ± 0.17 vs. 1.15 ± 0.12 , $p < 0.05$). In non-IP controls, HG significantly decreased P-Akt/Akt as compared to its value in the NG control group (1.15 ± 0.11 vs. 2.92 ± 0.1 , $p < 0.5$) (Fig. 4).

Influence of preconditioning on the levels of Bcl-2 and BAX proteins at the end of I/R protocol

Under NG conditions, IP decreased BAX protein value against controls (0.302 ± 0.01 vs. 0.37 ± 0.01 , $p < 0.05$) (Fig. 5A). However, under HG conditions, no significant decline of BAX was observed in the preconditioned group (0.39 ± 0.02 vs. 0.43 ± 0.03 , $p = 0.2$) (Fig. 5B). Under both conditions the levels of Bcl-2 were not changed (data not shown).

Discussion:

Our study showed that following two cycles of ischemic preconditioning, PI3K/Akt signaling may start to become up-regulated (Fig. 2), but more apparently, Akt activation occurs during the period of long-term zero-flow ischemia in isolated rat hearts under NG conditions (Fig. 3) that subsequently may lead to a decrease in the level of proapoptotic BAX protein after I/R (Fig. 5A). As no changes in the levels of Bcl-2 were observed, antiapoptotic effect was not presented as the ratio of Bcl-2 and BAX since it would not bring any important additional information. It seems that Akt activation by IP becomes more evident after longer period of time, e.g., like after providing of three IP-cycles when the P-Akt was elevated significantly (Yang *et al.* 2013). Thus, we confirmed that activation of Akt plays one of the

crucial roles in the mechanisms of IP (Hausenloy and Yellon 2006). Antiapoptotic effect of downregulated BAX in the rat hearts caused by IP (documented by decreased DNA fragmentation) has been also demonstrated in the study of Piot *et al.* (1997). However, at the end of long-term reperfusion in the preconditioned hearts, lower Akt activity than in the control group perfused with normal glucose medium was observed in our experiments (Fig. 4). This finding is in accordance with the study of Zu *et al.* (2011) who showed depressed levels of P-Akt and elevated lipid phosphatase and tensin homologue deleted on chromosome ten (PTEN) in sarcoplasm of the preconditioned hearts compared with non-preconditioned controls at the end of I/R. These findings were associated with attenuated PTEN and BAX localization in mitochondria, reactive oxygen species production and depressed infarct size in the IP group. PTEN dephosphorylates the lipid second messenger (PIP3) that prevents activation of the Akt kinase (Newton and Trotman 2014). Therefore, PTEN could be considered one of the negative regulators of PI3K/Akt signaling, as well as protein phosphatases 1 and 2A (Fan *et al.* 2010), pleckstrin homology domain leucine-rich repeat protein phosphatase-1 (Miyamoto *et al.* 2010), and also protein kinase D1, likewise in intestinal epithelial cells (Ni *et al.* 2014). PTEN probably plays a pivotal role in the effectivity of cardioprotection provided by IP. It is supported by the failure of IP in PTEN-knockout mice leading to an enhanced size of infarction as compared with that in the non-IP controls (Zheng *et al.* 2014).

Elevated glucose in medium reversed IP-induced modulations of sarcoplasmic P-Akt levels. After long-term zero-flow ischemia, P-Akt in preconditioned hearts did not differ from that in the non-IP controls (Fig. 3), and at the end of I/R it was significantly higher than in respective controls (Fig. 4). These changes in Akt phosphorylation were associated with attenuation of IP-mediated lowering of BAX value (Fig. 5B). In our previous study, HG also reversed IP-induced effect on heart resistance to I/R injury (Zálešák *et al.* 2014). In that study,

decreased resistance of preconditioned hearts to I/R was manifested by significantly elevated infarct size (normalized to the size of area at risk, AR) against that in the non-IP control group (IS/AR: $21.4 \pm 2.2\%$ vs. $14.3 \pm 1.3\%$), while under NG conditions, antiinfarct IP-induced protection was confirmed (IS/AR: $8.8 \pm 1\%$ vs $17.1 \pm 1.2\%$). It is in agreement with numerous studies that documented negative impact of lifestyle-related comorbidities, such as diabetes, on the mechanisms of innate cardioprotection (Ferdinandy *et al.* 2014, Ferdinandy *et al.* 2007, Ravingerová *et al.* 2012). Similar switching of IP-induced effect on heart resistance against development of infarction was achieved by insulin applied during the entire perfusion protocol of I/R via Akt-dependent mechanisms (Fullmer *et al.* 2013). Persistent cardiac specific Akt activation in transgenic mice also depressed cardiac resistance against infarction (Nagoshi *et al.* 2005). Moreover, Akt hyperactivation suppressed expression of antioxidant proteins due to inhibition of forkhead family transcription factors, which normally up-regulate antioxidant proteins (Los *et al.* 2009, Nogueira *et al.* 2008). Alterations in PI3K/Akt signaling due to Akt hyperactivation may be a cause of these detrimental effects of IP on the heart.

Similar modulation of P-Akt as by IP under NG conditions (P-Akt/Akt elevation after zero-flow ischemia and its depression after reperfusion), was achieved in the non-IP controls with elevated glucose in medium (Figs. 3, 4). PI3K/Akt modulation by elevated glucose was previously observed in the cell culture experiments (Yeshao *et al.* 2005). Moreover, high glucose or hyperosmosis provided by perfusion with mannitol in isolated rat hearts elevated the levels of cardioprotective heat shock protein 90 (Chen *et al.* 2006). In addition, lower total amount of heart fatty acid binding protein (h-FABP, a marker of cell injury) released from the impaired non-IP hearts under HG than under NG conditions suggests that high glucose medium may exert rather protective effects against severe I/R injury under certain conditions (Zálešák *et al.* 2014). Experiments with isolated rat hearts also showed enhanced

resistance of hearts with acute streptozotocin-induced diabetes to myocardial I/R by demonstrating lower release of creatine phosphokinase during I/R (Chen *et al.* 2006).

Experimental results confirm that PI3K/Akt signaling pathway plays a pivotal role in the cardioprotection. However, it seems that long-term-I/R-related regulation of PI3K/Akt signaling proved by elevated P-Akt in sarcoplasm at early stage after IP and by its decrease at the end of experiment is essential for effective execution of cardioprotection.

Study limitations

In this study, normoglycemic and hyperglycemic solutions had different osmotic concentration that might affect the results. It is very probable that influence of high concentration of glucose on intracellular signaling mechanism may involve relative hyperosmosis. Influence of induced hyperosmosis on cardioprotection mediated by IP would be the subject of our further research to differentiate metabolic and non-metabolic effects.

Conclusions:

Simulated acute hyperglycemia alters long-term-I/R-related PI3K/Akt signaling triggered by ischemic preconditioning. A lack of PI3K/Akt up-regulation at early stage of I/R protocol and negative PI3K/Akt regulation at the end of reperfusion, under high glucose conditions, probably plays a role in the failure of preconditioning to confer cardioprotection. PI3K/Akt regulation by high glucose in non-preconditioned hearts similar to that in the preconditioned ones under normal glucose conditions may serve as one of potential explanations of their better resistance against severe I/R injury under certain conditions.

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Figure legends

Figure 1: Experimental protocol of zero-flow ischemia and ischemic preconditioning.

IP- ischemic preconditioning, C - non-IP control protocol.

Figure 2: Cytosolic protein levels of Akt, P-Akt, GAPDH directly after stabilisation period or IP. Effect of normoglycemia and simulated hyperglycemia.

Upper panel: Representative blots of P-Akt, Akt and GAPDH as a loading control. Bottom panel: Quantification of blots by scanning densitometry. Results are shown as a ratio of P-Akt and Akt (normalized to GAPDH) and expressed as means \pm SEM, n=4 hearts per group. IP- ischemic preconditioning, C - non-IP controls, NG - normal glucose, HG – simulated hyperglycemia

Figure 3: Cytosolic protein levels of Akt, P-Akt, GAPDH, after long-term zero-flow ischemia. Effect of normoglycemia and simulated hyperglycemia.

Upper panel: Representative blots of P-Akt, Akt and GAPDH as a loading control. Bottom panel: Quantification of blots by scanning densitometry. Results are shown as a ratio of P-Akt and Akt (normalized to GAPDH) and expressed as means \pm SEM, n=4 hearts per group. * - p<0.05, IP/NG vs. C/NG, + - p<0.05, C/NG vs. C/HG, IP-ischemic preconditioning, C - non-IP controls, NG - normal glucose, HG – simulated hyperglycemia

Figure 4: Cytosolic protein levels of Akt, P-Akt, GAPDH after long-term reperfusion. Effect of normoglycemia and simulated hyperglycemia.

Upper panel: Representative blots of P-Akt, Akt and GAPDH as a loading control. Bottom panel: Quantification of blots by scanning densitometry. Results are shown as a ratio of P-Akt and Akt (normalized to GAPDH) and expressed as means \pm SEM, n=4 hearts per group. * - p<0.05, IP/NG vs. C/NG, # - p<0.05, IP/HG vs. C/HG, + - p<0.05, C/NG vs. C/HG, IP-

ischemic preconditioning, C - non-IP controls, NG - normal glucose, HG – simulated hyperglycemia

Figure 5. Cytosolic protein levels of BAX and GAPDH at the end of long-term I/R under normoglycemia (A) and under simulated hyperglycemia (B).

Upper panel: Representative blots of BAX and GAPDH as a loading control. Bottom panel: Quantification of blots by scanning densitometry. Results are shown as a ratio of BAX and GAPDH. Results are expressed as means \pm SEM, n=4 hearts per group. * - p<0.05, ischemic preconditioning (IP) group vs. respective non-IP control group (C), NG normal glucose, HG – simulated hyperglycemia

Table 1: Preischemic values of parameters of myocardial function in controls and ischemically preconditioned rat hearts under conditions of normoglycemia (NG) and hyperglycemia (HG).

Group	C/NG	IP/NG	C/HG	IP/HG
HR (beats/min)	246 \pm 10.9	248 \pm 12.1	207 \pm 10.5	239 \pm 5.2
CF (mL/min)	9.1 \pm 1.4	12 \pm 0.9	8.8 \pm 0.5	10.3 \pm 0.4
LVDP (mmHg)	67.8 \pm 15.0	73.8 \pm 11.4	84.2 \pm 8.1	76.7 \pm 8.8

$+(dP/dt)_{max}$ (mmHg/s)	1793±337	1927±345	2013±258	2046±228
$-(dP/dt)_{max}$ (mmHg/s)	1119±274	1149±233	1327±150	1242±125

C – controls, IP – 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 a means ± SEM, n=4 hearts per group.

Figure 1

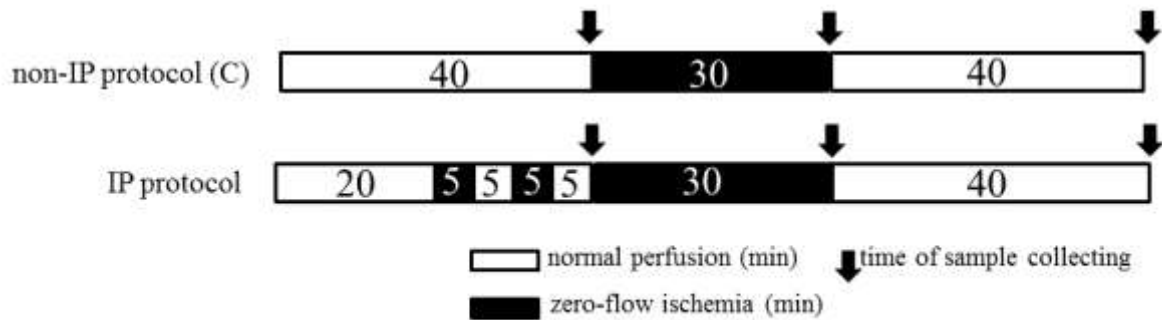


Figure 2

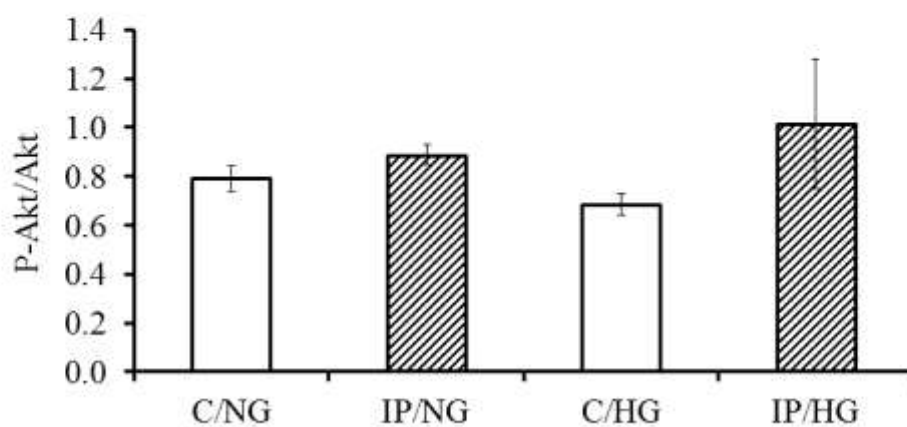
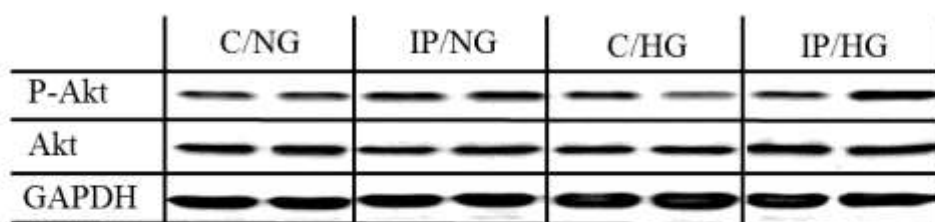


Figure 3

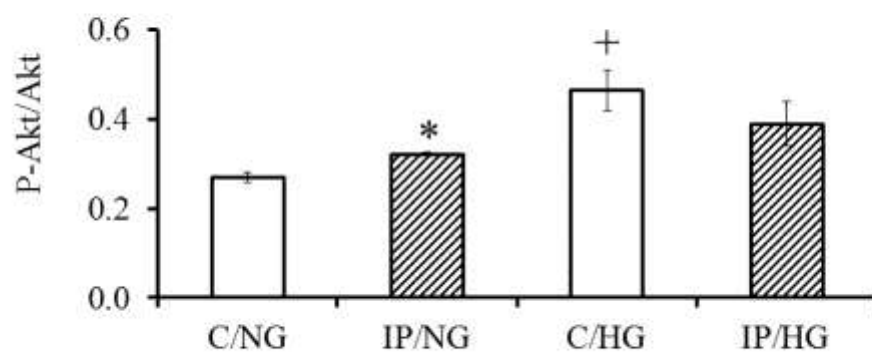
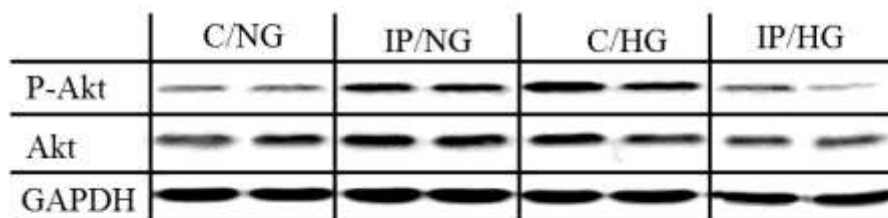


Figure 4

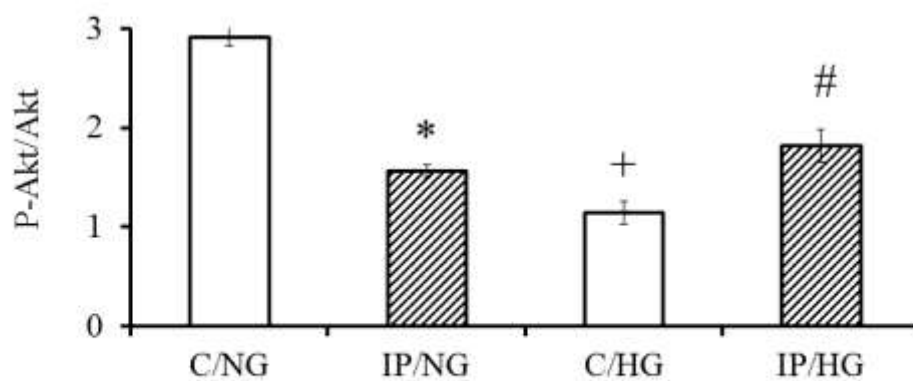
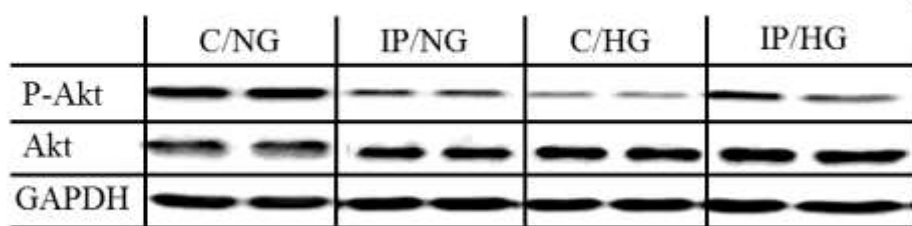


Figure 5

