Protection against ischaemia-induced ventricular arrhythmias and myocardial dysfunction conferred by preconditioning in the rat heart: Involvement of mitochondrial K_{ATP} channels and reactive oxygen species

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Summary

Ischaemic preconditioning (I-PC) induced by brief episodes of ischaemia and reperfusion (I/R) protects the heart against sustained I/R. Although activation of mitochondrial K_{ATP} channels (mitoK_{ATP}) interacting with reactive oxygen species (ROS) has been proposed as a key event in this process, their role in the antiarrhythmic effect is not clear. This study was designed: i., to investigate the involvement of mito K_{ATP} opening in the effect of I-PC (1 cycle of I/R, 5-min each) on ventricular arrhythmias during test ischaemia (TI, 30-min LAD coronary artery occlusion) in Langendorff-perfused rat hearts and subsequent postischaemic contractile dysfunction; ii., to characterize potential mechanisms of protection conferred by I-PC and pharmacological PC induced by mito K_{ATP} opener diazoxide (DZX), with particular regards to the modulation of ROS generation. Lipid peroxidation (an indicator of increased ROS production) was determined by measurement of myocardial concentration of conjugated dienes (CD) and thiobarbituric acid reactive substances (TBARS) in nonischaemic controls, non-preconditioned and preconditioned hearts exposed to TI, I-PC alone, as well as after pretreatment with DZX, mito K_{ATP} blocker 5-hydroxydecanoate (5-HD) and antioxidant N-acetylcysteine (NAC). Total numbers of ventricular premature beats (VPB) that occurred in the control hearts (518 ± 71) was significantly ($P<0.05$) reduced by I-PC (195 ± 40), NAC (290 ± 56) and DZX (168 ± 22). I-PC and NAC suppressed an increase in CD and TBARS caused by ischaemia indicating lower production of ROS. On the other hand, I-PC and DZX themselves moderately enhanced ROS generation, prior to TI. Bracketing of I-PC with 5-HD suppressed both, ROS production during PC and its cardioprotective effect. Conclusions: Potential mechanisms of protection conferred by mito K_{ATP} opening in the rat heart might involve a temporal increase in ROS production in the preconditioning phase triggering changes in the pro/antioxidant balance in the myocardium and attenuating ROS production during subsequent prolonged ischaemia.

Key words Myocardial ischaemia • Arrhythmias • Preconditioning • mito K_{ATP} channels • Rat heart

Introduction

Severe ventricular arrhythmias represent a major challenge for therapeutic intervention due to complexity of pathophysiological mechanisms initiating arrhythmias in ischaemic heart disease (Bril 1996), and the development of a new approach to management of arrhythmias is urgently needed. Imbalance between the formation of reactive oxygen species (ROS) and the availability of endogenous antioxidants caused by ischaemia and reperfusion (I/R) plays an important role in the genesis of myocardial injury (McCord 1985; Kloner *et al.* 1989; Dhalla *et al.* 2000) and might lead to occurrence of malignant ischaemia- and reperfusion-induced arrhythmias (Yang *et al.* 1995; Ravingerová *et al.* 1999). Negative effect of an oxidative load has been further supported by the ability of exogenously administrated free radical scavengers to improve functional recovery of the postischaemic-reperfused heart (Downey *et al.* 1991; Tang and Tang 1991; Qiu *et al.* 1992).

Ischaemic preconditioning (I-PC) is a concept of cardioprotection against lethal ischaemia based on the exploitation of endogenous protective mechanisms activated by prior periods of brief ischaemia (Murry 1986; Ravingerová *et al.* 2002; Das and Sarkar 2005). Recent studies demonstrated that preconditioning (PC) is involved in the attenuation of altered cellular redox state and deleterious effects of ROS (Das and Maulik 2003). Although we do not yet understand completely molecular mechanisms responsible for cardioprotection afforded by PC, compelling evidence suggests that ATP-sensitive K^+ (K_{ATP}) channels localized in different cell compartments are central players in this process (Gross and Fryer 1999; Végh and Parratt 2002; Das and Sarkar 2005). Opening of sarcolemmal K_{ATP} channels has been initially suggested as an end-effector

mechanism in the preconditioning cascade since their blockade with K_{ATP} blocker glibenclamide abolished I-PC in dogs (Gross and Auchampach 1992), strengthened by the observations that K_{ATP} openers could mimic the I-PC-induced protection (Grover *et al.* 1994; Gross and Fryer 1999). However, K_{ATP} modulations may exert both, anti- and proarrhythmic effects (Tosaki *et al.* 1992). Moreover, it has been also revealed that cardioprotection occurs independently from the changes in action potential duration, which is the main determinant of arrhythmogenesis and a target of sarcolemmal K_{ATP} openers (Hamada *et al.* 1998). In line, our previous study demonstrated that K_{ATP} blockade with glibenclamide did not reverse antiarrhythmic protection conferred by I-PC (Ravingerová *et al.* 2002). Furthermore, a selective inhibitor of K_{ATP} channels localized in mitochondria (mito K_{ATP}) 5-hydroxydecanoate (5-HD) abolished protection against contractile dysfunction in guinea pig papillary muscle conferred by hypoxic PC (Ravingerová *et al.* 1998) and cardioprotection afforded by I-PC (Sato *et al.* 2000) including anti-infarct protection in rabbits and rats, without affecting ischaemia-induced shortening of action potential duration (Munch-Ellingsen *et al.* 2000). Finally, Garlid *et al.* (2003) proposed that mito K_{ATP} channel is 2000-fold more sensitive than the sarcolemmal one to K_{ATP} opener diazoxide, which has been shown to mimic the I-PC-induced cardioprotection, and that it is the most likely end-effector involved with I-PC. On the other hand, mito K_{ATP} opening could play not only the end-effector role in the preconditioning cascade, but it can also trigger this chain of events and act as an *upstream* mechanism of protein kinases activation mediated by an increased production of ROS and nitric oxide (Yue *et al.* 2002).

Although application of diazoxide has been shown to exert cardioprotective effects (Asemu *et al.* 1999), the mechanism of antiarrhythmic protection induced by mito K_{ATP} opening remains less elucidated. In the present study, we tested the hypothesis that I-PC elicits its antiarrhythmic effects through the opening of mito K_{ATP} channels and that pharmacologically

induced PC by exogenously applied mito K_{ATP} activator can afford similarly effective antiarrhythmic protection followed by an improvement of postischaemic contactile recovery in isolated rat heart. Our further goal was to elucidate the mechanisms of cardioprotective effect of mito K_{ATP} opening with particular regards to the modulation of ROS production in the preconditioned myocardium.

Materials and methods

Animals

Male Wistar rats (250-300g body weight), fed a standard diet and tap water *ad libitum*, were employed. 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 Care and Use Committee of the Slovak Republic.

Perfusion technique

Rats were anesthetized (sodium pentobarbitone, 60mg/kg, i.p.) and given heparin (500 IU, i.p.). Hearts were rapidly excised, placed in ice-cold perfusion buffer, cannulated via the aorta and perfused in the Langendorff mode at a constant perfusion pressure of 70 mm Hg and at 37 ºC. The perfusion solution was a modified Krebs-Henseleit buffer gassed with 95% O_2 and 5% CO_2 (pH 7,4) containing (in mM): NaCl 118.0; KCl 3.0; $MgSO_4$ 1.2; $NaHCO_3$ 25.0; NaH_2PO_4 1.18; $CaCL_2$ 2.5; glucose 11.1. Reduced potassium and enhanced calcium concentrations in the above buffer were used to promote arrhythmogenesis during ischaemia. Solution was filtered throught a 5 μm porosity filter (Millipore) to remove contaminants. An epicardial electrogram

was registered by means of two stainless steel electrodes attached to the apex of the heart and the aortic cannula.

Left ventricular pressure was measured by means of a non-elastic water-filled balloon inserted into the left ventricle via the left atrium (adjusted to obtain end-diastolic pressure of 5-7 mm Hg) and connected to a pressure transducer (MLP844 Physiological Pressure Transducer, ADInstruments). Left ventricular developed pressure (LVDP, systolic minus diastolic pressure), maximal rates of pressure development and fall, $+dP/dt_{max}$ and $-dP/dt_{max}$, as the indexes of contraction and relaxation, as well as the heart rate (derived from electrogram) and coronary flow were monitored during stabilization, pre-ischaemia period (for the evaluation of the effect of pharmacological interventions on haemodynamic parameters), and were continuously recorded until the end of experiment. Its recovery after ischaemia/reperfusion was expressed as percentage of preischemic baseline values. Heart function and arrhythmias were analyzed using PowerLab/8SP Chart 5 software (ADInstruments). The hearts were allowed to stabilize (15 min) before further interventions.

Induction of ischaemia

At the onset of experiment a ligature was placed loosely around the left anterior descending (LAD) coronary artery close to its origin. Both ends of the suture were threaded through a traction-type plastic occluder. Regional ischaemia (LAD artery occlusion) was induced by traction of the suture against the plastic cannula and clamping the suture. After 30-min ischaemia, the ligature was released to permit reperfusion. A fall in coronary flow by approximately 40% at the onset of ischaemia and its increase upon reperfusion verified the efficacy of LAD occlusion. For the evaluation of postischaemic functional recovery, in the

additional subset of experiments the hearts underwent 25-min global ischaemia (induced by closing of the aortic inflow) followed by 40-min reperfusion.

Quantification of arrhythmias

Susceptibility to ischaemia-induced ventricular arrhythmias was analyzed from the electrogram recording following the guidelines for the study of ischaemia and reperfusion arrhythmias known as The Lambeth Conventions (Walker *et al.* 1988). We focused on the measurement of the total number of ventricular premature beats (VPB) over the whole period of ischaemia, as well as on the incidence and duration of ventricular tachycardia (VT), which was defined as a run of four or more consecutive ectopic beats.

Perfusion protocols

1. Control test ischaemia (TI)

After 15 min equilibration and additional 15 min perfusion, the hearts were subjected either to regional ischaemia (occlusion of LAD) lasting 30 min followed by 10-min reperfusion or to 25-min global ischaemia and 40-min reperfusion (n= 8 per group).

2. Perfusion with antioxidant N- acetylcysteine (NAC+I)

After equilibration, NAC (4mmol/L) was administered for 15 min, prior to TI induced by regional (n= 9) or global (n= 6) ischaemia/reperfusion.

3. Ischaemic preconditioning (I-PC+I)

After equilibration, the hearts were subjected to one cycle of ischaemic preconditioning consisting of 5-min ischaemia and 5-min reperfusion, prior to TI induced by regional (n= 9) or global (n= 6) ischaemia/reperfusion.

4. Pharmacological preconditioning (DZX+I)

Selective mito K_{ATP} opener diazoxide (DZX, 50μM) dissolved in dimethyl sulfoxide (DMSO) was used to mimic I-PC and was administered 15 min prior to TI induced by regional (n= 8) or global (n= 7) ischaemia/reperfusion.

5. Perfusion with selective inhibitor of mito K_{ATP} channels 5-HD in non-preconditioned hearts (5-HD+I)

5-HD (200 μM) was administered 15 min prior to TI induced by regional (n= 6) or global (n= 8) ischaemia/reperfusion.

6. Perfusion with selective inhibitor of mito K_{ATP} channels 5-HD in preconditioned hearts (I-PC + 5-HD+I)

5-HD (200 μM) was administered throughout the preconditioning protocol 15 min prior to TI induced by regional (n= 14) or global (n= 6) ischaemia/reperfusion.

Measurement of lipid peroxidation

In parallel subsets of experiments, concentration of conjugated dienes (CD, a marker of lipid peroxidation due to increased ROS production) was measured in lipid extracts of the left ventricular tissue according to Kogure *et al.* (1982). Briefly, after chloroform evaporation under the inert atmosphere of nitrogen and after the addition of 3 ml cyclohexane, concentration of CD was determined spectrophotometrically (λ=223 nm, ε= 29000 $l.mol^{-1}.cm^{-1}$., SECOMAN). In addition, lipid peroxidation was also evaluated by measuring the formation of thiobarbituric acid reactants (TBARS) according to Ohkawa *et al.* (1979). The sampling for TBARS and CD (n= 6-8 in each group) was performed immediately after equilibration of the hearts (control samples, C) and following TI in the non-preconditioned (TI) and preconditioned hearts (I-PC+I). In addition, concentration of CD was also measured immediately after I-PC or after 15-min pretreatment with

DZX, as well as after I-PC and simultaneous pretreatment with 5-HD (I-PC+5-HD) or NAC (I-PC+NAC).

Chemicals

Diazoxide was obtained from Alexis Biochemicals. 5-hydroxydecanoate and N-acetylcysteine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Diazoxide was dissolved in dimethyl sulfoxide (Lachema, Czech Republic) before being added into experimental solutions. The final concentration of dimethyl sulfoxide was < 0.01%. All other chemicals were from Centralchem (Bratislava, Slovak Republic).

Statistical evaluation

The data were expressed as means ± S.E.M. One-way ANOVA and subsequent Student-Newman-Keuls test were used for comparison of differences in variables with normal distribution between the groups. Variables with non-parametric distribution were compared by using Mann-Whitney (differences in the numbers of VPB, the numbers of episodes of VT and its duration) or Fisher's exact test (incidence of VT). Differences were considered as significant at $P<0.05$.

Results

Characteristics of isolated hearts

The values of heart rate, LVDP, LVEDP, $+dP/dt_{max}$, $-dP/dt_{max}$ and coronary flow in the control non-preconditioned and preconditioned groups, as well as in drug-treated control and preconditioned hearts are summarized in Table 1. There were no significant differences in the values of these parameters between the groups at baseline and before TI.

Effect of NAC, ischaemic preconditioning, diazoxide and 5-HD on susceptibility to ventricular arrhythmias

Occlusion of LAD coronary artery caused an immediate fall in coronary flow that was similar in all experimental groups. Myocardial ischaemia resulted in a high ectopic activity in the control non-preconditioned group with a characteristic bell-shaped pattern of its temporal distribution with maximum between 10 and 20 min of ischaemia (Fig. 1). VT was the most severe form of arrhythmia that occurred in all hearts. Marked attenuation of ectopic activity was observed in the preconditioned hearts (both, I-PC and pharmacologically induced PC), while pretreatment with 5-HD had no effect on the incidence of arrhythmias (Fig. 1, Fig. 3).

Administration of NAC significantly reduced a total number of VPB to 290 ± 56 from 518 ± 71 in the non-treated control hearts, decreased the mean number of the episodes of VT from 12.1 ± 2.4 to 5.3 ± 1.8 and shortened total duration of VT (18.3 ± 4.7 s vs. 43.6 ± 8.6 s in the controls; $P<0.05$; Fig. 2).

I-PC significantly suppressed the total number of VPB to 194.6 ± 39.6 ($P<0.05$; Fig. 2A). The number of episodes of VT (Fig. 2B) and its total duration (Fig. 2C) were also significantly reduced to 0.2 ± 0.1 and to 0.7 ± 0.3 s, respectively, as compared with these parameters in non-preconditioned controls ($P<0.05$).

An antiarrhythmic protection similar to that induced by I-PC was achieved by pharmacologically induced PC following application of diazoxide. Thus, there were only few VPB (167.8 ± 21.9; Fig. 2A) and episodes of VT in the DZX-treated hearts (2.3 ± 0.6; Fig. 2B). Total duration of VT was also significantly reduced in these hearts as compared with the controls (5.3 ± 2.2 s; $P<0.05$; Fig. 2C). This effect of I-PC and DZX was maximal between 5 and 25 min of ischaemia (Fig. 1).

Although bracketing of preconditioning with 5-HD did not significantly increase the total number of VPB (not shown), blockade of mito K_{ATP} channels exacerbated arrhythmias and partially attenuated the effect of I-PC documented by an increased incidence of VT in these hearts (71%) as compared with its incidence in the non-treated preconditioned hearts (22%; $P<0.05$) and 100% in both non-treated and 5-HD-treated ischemic controls (Fig. 3).

Postischaemic recovery of contractile function

Pretreatment with NAC significantly improved recovery of LVDP after TI as compared with non-treated control hearts (50 ± 6 and 29 ± 3% of preischaemic values, respectively; $P<0.05$; Table 2). Both, I-PC and DZX also markedly attenuated postischaemic contractile dysfunction and increased LVDP recovery to 68 ± 5 and 70 ± 5%, respectively ($P<0.05$ vs. controls). Pretreatment with 5-HD did not affect LVDP recovery in non-preconditioned hearts and reversed cardioprotective effect of I-PC (LVDP 21 ± 6%; $P<0.05$ vs. I-PC group; Table 2).

Evaluation of myocardial oxidative state

The levels of TBARS at the end of ischaemia were significantly higher in ischaemic hearts than those detected in non-ischaemic myocardium (50.9 ± 6.4 and 37.8 ± 2.3 nmol/g; $P<0.05$). I-PC decreased the levels of TBARS during subsequent prolonged ischaemia to their value in the non-ischaemic hearts (37.9 ± 2.7 nmol/g; Fig. 4A). A more pronounced effect of ischaemia and I-PC on production of ROS was demonstrated by measurement of concentration of CD. The concentration of CD in non-ischaemic controls (103.8 ± 13 nmol/g) was increased by TI more than two-fold (241.9 ± 34.4 nmol/g; $P<0.05$ vs. baseline pre-ischaemic values) and this effect was reversed in the preconditioned hearts (104.2 ± 12.8 nmol/g; $P<0.05$ vs. non-preconditioned ischaemic hearts; Fig. 4B). As expected, pretreatment with antioxidant NAC also

significantly reduced ROS generation during TI and decreased concentration of CD in the ischaemic myocardium to 110.8 ± 8.5 nmol/g ($P<0.05$ vs. ischaemic controls).

On the other hand, I-PC itself tended to enhance ROS generation prior to TI (CD 137.4 ± 20.6 nmol/g; Fig. 5). Similarly to I-PC, opening of mito K_{ATP} channels with diazoxide led to an elevation in ROS production before TI (CD 148.9 ± 9.1 nmol/g; $P<0.05$ vs. non-ischaemic controls), whereas administration of K_{ATP} channels blocker 5-HD during preconditioning phase markedly suppressed production of ROS before subsequent prolonged ischaemia (CD 85 ± 5.3 nmol/g; $P<0.05$ vs. non-treated preconditioned hearts). This effect was comparable with the reduction of CD following application of NAC in combination with I-PC (Fig. 5).

Discussion

The main objective of this study was to demonstrate whether opening of mito K_{ATP} channels, either endogenous or by exogenously administered mito K_{ATP} opener diazoxide, is involved in production of ROS and in the antiarrhythmic effect of PC during sustained ischaemic challenge.

Occurrence of arrhythmias and myocardial infarction might be the direct consequences of increased production of ROS during myocardial ischaemia. Free radicals have been implicated in the mechanisms of reversible postischemic contractile dysfunction (myocardial stunning), cardiac cell death, electrophysiological derangements and they are also involved in the pathogenesis of chronic cardiovascular diseases (Kevin *et al.* 2005). Myocardial ischaemia-induced arrhythmias may be mitigated by various antiradical interventions, such as inhibition of radicals formation or by scavenging of free radicals (Bernier *et al.* 1989b; Tosaki *et al.* 1993; Sobey *et al.* 1993). In

accordance, in the present study, 15-min pretreatment of the hearts with antioxidant NAC significantly decreased arrhythmias during subsequent ischaemia (Figs 1, 2) and improved postischaemic recovery of contractile function (Table 2). In other studies, NAC administered throughout the experiment has been also shown to reduce myocardial stunning, however, it failed to limit infarct size in the *in vivo* canine model (Forman *et al.* 1988). In addition, in the study of Chen *et al.* (1995), postischemic functional recovery in NAC-treated hearts was not improved as compared with that in the untreated hearts indicating certain controversies in the results concerning the efficiency of antiradical treatment.

In our study, one cycle of I-PC did not change the temporal profile of arrhythmias, but significantly reduced the total number of VPB, number of episodes of VT, as well as the total duration of VT. Administration of diazoxide induced similar antiarrhythmic protection as classical I-PC (Fig. 2). One of the major determinants of arrhythmogenesis is the size of the occluded zone (Curtis 1998). Another factor that might influence the occurrence of arrhythmias is the changes in the heart rate (Bernier *et al.* 1989a). However, we can rule out both factors since similar technique of LAD coronary artery occlusion has been applied in all groups, and there were no differences in the size of ischaemic area between the groups correlating with the reduction of coronary flow (Ravingerová *et al.* 1995), or in the heart rate. These results are in agreement with the data provided by Das and Sarkar (2005) and Végh and Parratt (2002), who demonstrated a reduction of arrhythmias in the preconditioned dogs and rabbits by a mechanism that involved opening of mito K_{ATP} channels. Although electrophysiological mechanisms underlying antiarrhythmic protection conferred by one cycle of ischaemic preconditioning have been shown to involve attenuation of spatial dispersion of repolarization between the epi- and endocardial layers of the myocardium as a substrate for reentry arrhythmias induced by ischaemia (Botsford and Lukas 1998), the role of K_{ATP} channels in antiarrhythmic effects is still a matter of

debate. In the present study, the role of mito K_{ATP} channels activation in the protection against ischaemia-induced arrhythmias was further supported by the finding that application of their inhibitor 5-HD during the preconditioning phase partially abolished antiarrhythmic effect of I-PC and significantly increased the incidence of ventricular tachycardia (Fig. 3). Moreover, the role of mito K_{ATP} channels opening in cardioprotective mechanisms was confirmed by the effects od I-PC and diazoxide on functional recovery upon ischaemia/reperfusion and its reversal by 5-HD (Table 2). Our results are consistent with those of Munch-Ellingsen *et al.* (2000), Végh and Parratt (2002) who found that 5-HD blunted cardioprotection in the preconditioned rats and dogs.

K_{ATP} channel activation may be related to a release of ROS during the preconditioning ischaemia/reperfusion (Cohen *et al.* 2001). $O_2^{\cdot-}$ is a potent activator of myocardial mito K_{ATP} channels. Therefore, ROS, such as $O_2^{\cdot-}$ generated during IPC, may activate mito K_{ATP} channels by their direct action on the sulfhydryl groups of the channel protein leading subsequently to a cardioprotective effect due to K_{ATP} opening (Zhang *et al.* 2001).

On the other hand, experimental evidence suggests that the opening of mito K_{ATP} channels generates ROS and that these radicals represent a part of the cascade leading to cardioprotection (Pain *et al.* 2000; Forbes *et al.* 2001; Patel and Gross 2001). Cardioprotective effect of I-PC and diazoxide may be manifested during the phase of sustained ischaemia by attenuation of ROS production and mobilization (activation) of antioxidant reserves (Maczewski *et al.* 2004; Glantz *et al.* 2005; Morihira *et al.* 2006), and in this respect, the role of mitochondria has been proposed. Moreover, pharmacological preconditioning by diazoxide has been found to be associated with an improved mitochondrial recovery after I/R injury (Honda *et al.* 2005). We demonstrated by two methods suitable for detection of early and advanced changes in the oxidative state that enhanced ROS production in the ischaemic myocardium was effectively suppressed in the preconditioned myocardium and hence, their proarrhythmic effects (Ravingerová *et al.* 1999) might be reduced

as well. Measurement of conjugated dienes as primary products of lipid peroxidation was, however, more sensitive and using this method we were able to detect even less evident changes in oxidative state, such as those that occurred during early preconditioning ischaemia/reperfusion and/or application of mito K_{ATP} channels diazoxide. Our data show that both, I-PC and pretreatment with diazoxide caused an increase in ROS production (documented by an enhanced concentration of CD) prior to sustained ischaemia, which was blocked by coadministration of 5-HD and/or NAC (Fig. 4). This is in agreement with the study of Obata and Yamanaka (2000) who demonstrated that the administration of K_{ATP} channels openers cromacalim and nicorandil increased production of ROS that was blocked by 5-HD. Opening of the mito K_{ATP} channel occurs upstream of the mitochondrial ROS generation in the protective pathway since this protection is abrogated by 2- mercaptopropionylglycine and NAC, a free radical scavenger (Tang and Tang 1991; Forbes *et al.* 2001; Zhang *et al.* 2001;Yue *et al.* 2002).

The role of K_{ATP} channels activation as a final step in cardioprotective signaling mechanisms has been supported by the findings that activated protein kinase C (PKC) phosphorylates sarcolemmal K_{ATP} channels (Hu *et al.* 1996), and that NO, PKC and MAPK-mediated mechanisms facilitate the opening of K_{ATP} channels localized in mitochondria as well (Sato *et al.* 1998; Murphy 2004). A study by Pain *et al.* (2000) demonstrated that diazoxide is protective even if it is only present before, rather than during, the sustained period of ischaemia, suggesting that diazoxide acts as a trigger of preconditioning. It is possible that opening of a K_{ATP} channels may be involved as both a trigger and a mediator of preconditioning (Fryer *et al.* 2000). Exact mechanism by which opening of mito K_{ATP} results in cardioprotection is not completely elucidated so far, although depolarization of the mitochondrial inner membrane and dissipation of membrane potential, in conjunction with limitation of calcium uptake by mitochondria (Holmuhamedov *et al.* 1999), regulation of mitochondrial volume and rate of respiration (Lim *et*

al. 2002), as well as modulation of ROS production (Pain *et al.* 2000) could underlie protective effects. Another potential mechanism of attenuation of cell death by mito K_{ATP} openers is related to the regulation of mitochondrial antiapoptotic proteins (bcl-2) involved in the limitation of the mitochondrial permeability and release of apoptosis-inducing cytochrome c (Shimizu *et al.* 1999).

However, further studies are required to get more insight into the mechanisms by which mito K_{ATP} channels activation might modify electrophysiological processes underlying antiarhythmic protection afforded by PC in the rat heart.

Conclusions

Our results indicate that pharmacological preconditioning of the heart by pretreatment with a selective mito K_{ATP} channel opener diazoxide confers an effective protection against severe ischaemia-induced ventricular arrhythmias (comparable with the effect of classical I-PC) and subsequently attenuates postischaemic contractile dysfunction in the rat heart. Both forms of preconditioning were associated with a temporal moderate increase in generation of ROS prior to TI, and this effect was blocked by both, K_{ATP} channel blockade and antiradical intervention and was followed by an attenuated ROS production by the end of sustained ischaemia. Potential mechanisms of antiarrhythmic protection induced by PC might be related to the changes in the pro/antioxidant state of the myocardium and attenuation of their deleterious effects on the myocardium.

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Figure legend

Figure 1. Temporal pattern of the distribution of ectopic activity in 5-min intervals during 30-min ischaemia in the rat heart.

VPB - ventricular premature beats. Values are means ± S.E.M. of 6 - 9 hearts per group.

* P<0.05 vs. control ischaemic group (TI).

Figure 2. Effects of I-PC, K_{ATP} channels modulations and NAC on ischaemia-induced ventricular arrhythmias in the rat heart.

A: Number of ventricular premature beats. **B**: Number of episodes of VT. **C**: Duration of VT. VPB - ventricular premature beats, VT- ventricular tachycardia. Values are means ± S.E.M. of 6-9 hearts per group. *P<0.05 vs. control ischaemic group (TI).

Figure 3. Effect of K_{ATP} inhibition on the incidence of ventricular tachycardia (VT) in the non-preconditioned and preconditioned rat hearts subjected to test ischaemia (TI).

Values are expressed as % of incidence, n=6-14 in each group.

*P<0.05 vs. control ischaemic group, $^{\#}$ - P<0.05 vs. I-PC.

Figure 4. Concentration of TBARS (A) and conjugated dienes (B) in the rat myocardium subjected to test ischaemia (TI): effect of I-PC. C- non-ischaemic controls. Values are means ± S.E.M, n=6-8 in each group. *- P<0.05 vs. C, $^{\#}$ - P<0.05 vs. TI.

Figure 5. Concentration of conjugated dienes in the rat myocardium before test ischaemia: effects of I-PC, K_{ATP} channels modulations and NAC.

C- non-ischaemic controls, I-PC- ischaemic preconditioning alone, DZX – treatment with diazoxide alone, I-PC+5-HD- 5-hydroxydecanoate applied during ischaemic preconditioning, I-PC+NAC - N-acetylcysteine applied during ischaemic preconditioning. Values are means ± S.E.M, n= 8-9 in each group. * - P<0.05 vs. C, $^{\#}$ - P<0.05 vs. I-PC.

Table 1 Pre-ischaemic values of the haemodynamic parameters in the non-preconditioned and preconditioned hearts. Effects of mito K_{ATP} channels modulations and NAC in isolated rat hearts.

Group	**n**	**CF** *ml/min*	**LVDP** *mmHg*	**LVEDP** *mmHg*	**+dP/dt$_{max}$** *mmHg/s*	**-dP/dt$_{max}$** *mmHg/s*	**HR** *beats/s*
TI	16	13 ± 1	94 ± 3	6 ± 1	2641 ± 185	1947 ± 177	254 ± 7
I-PC+I	15	14 ± 1	93 ± 2	6 ± 1	2878 ± 285	1836 ± 212	272 ± 5
DZX +I	15	15 ± 1	98 ± 1	6 ± 1	2607 ± 221	1877 ± 139	260 ± 7
NAC +I	13	12 ± 1	75 ± 6	5 ± 1	2019 ± 201	1529 ± 111	261 ± 10
5-HD+I	14	13 ± 2	78 ± 6	4 ± 1	2908 ± 251	1750 ± 150	250 ± 7
I-PC + 5-HD+I	20	13 ± 1	81 ± 2	5 ± 1	2456 ± 221	1690 ± 203	253 ± 4

Data are means ± S.E.M., n= 13-20 in each group. CF- coronary flow (ml/min), LVDP- left venricular developed pressure (LV systolic minus LV diastolic pressure; mm Hg), +dP/dt$_{max}$, -dP/dt$_{max}$ - maximum rates of pressure development and fall, respectively (mm Hg/s), LVEDP - left venricular end-diastolic pressure (mm Hg); HR- heart rate (beats/min).

Table 2 Postischaemic recovery of LVDP after global ischaemia/reperfusion. Effects of I-PC, mito K_{ATP} channels modulations and NAC in isolated rat hearts.

Group	**TI**	**I-PC+I**	**DZX +I**	**NAC +I**	**5-HD+I**	**I-PC + 5-HD+I**
%	29 ± 3	68 ± 5*	70 ± 5*	50 ± 6*	26 ± 7	21 ± 6
n	8	6	7	6	8	6

Data are means ± S.E.M. expressed in % of pre-ischaemic values. N= 6-8 in each group.

*- P< 0.05 vs. control ischaemic group (TI).

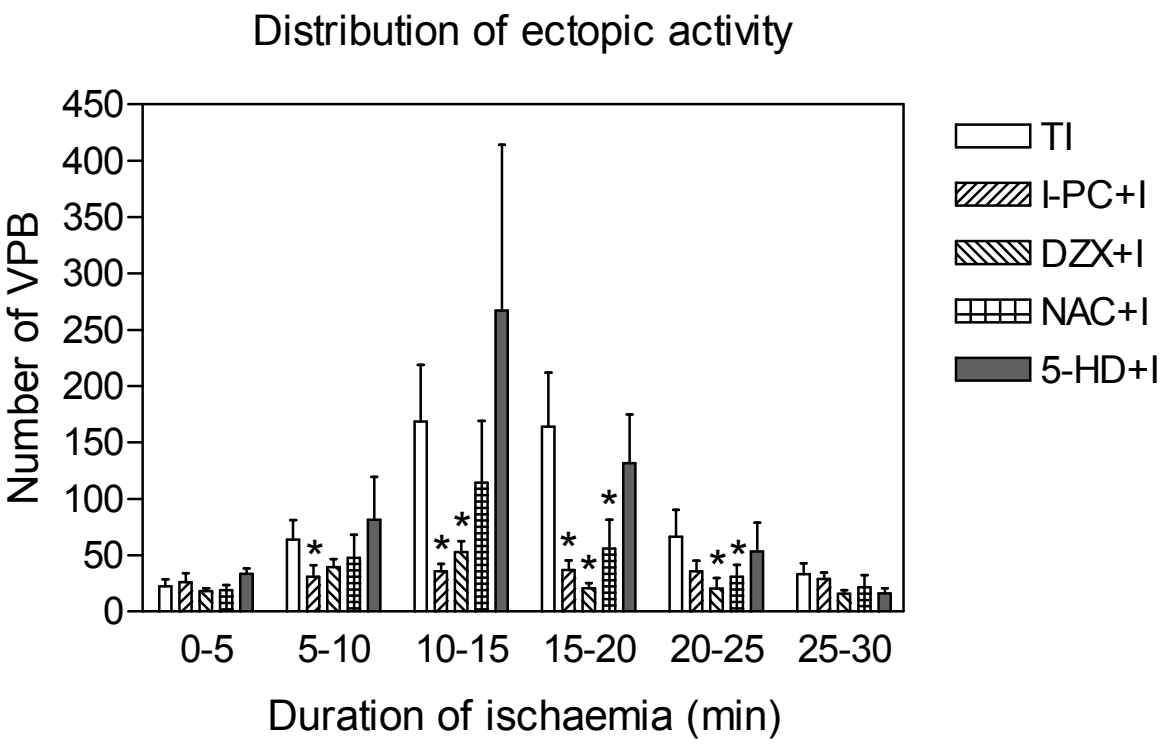
Distribution of ectopic activity
Number of VPB
450
400
350
300
250
200
150
100
50
0
0-5
5-10
10-15
15-20
20-25
25-30
Duration of ischaemia (min)
TI
I-PC+I
DZX+I
NAC+I
5-HD+I

Figure 1.

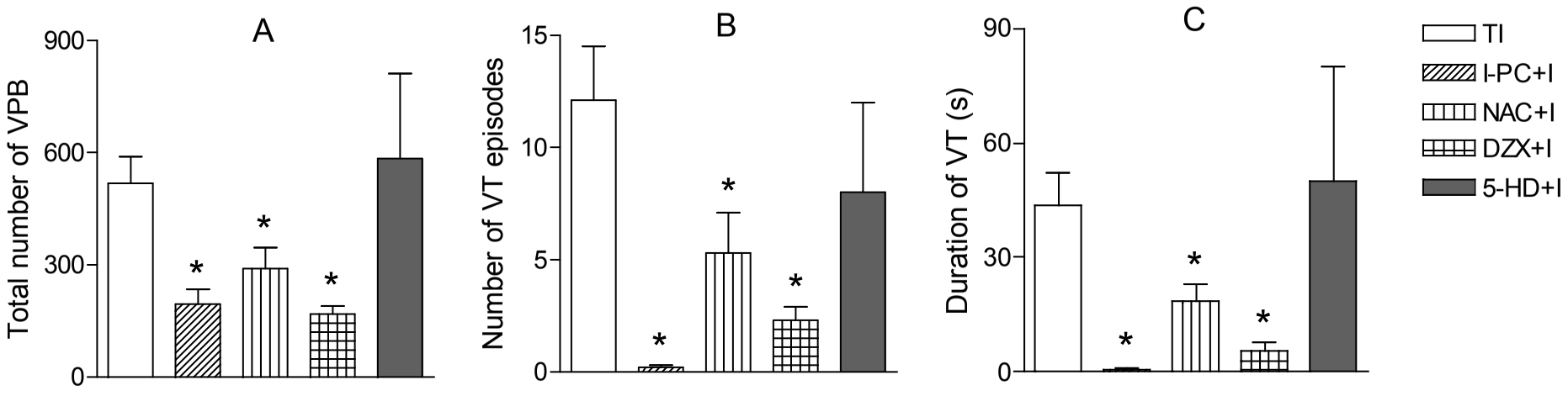
A
Total number of VPB
900
600
300
0
B
Number of VT episodes
15
10
5
0
C
Duration of VT (s)
90
60
30
0
*
TI
I-PC+I
NAC+I
DZX+I
5-HD+I

Figure 2.

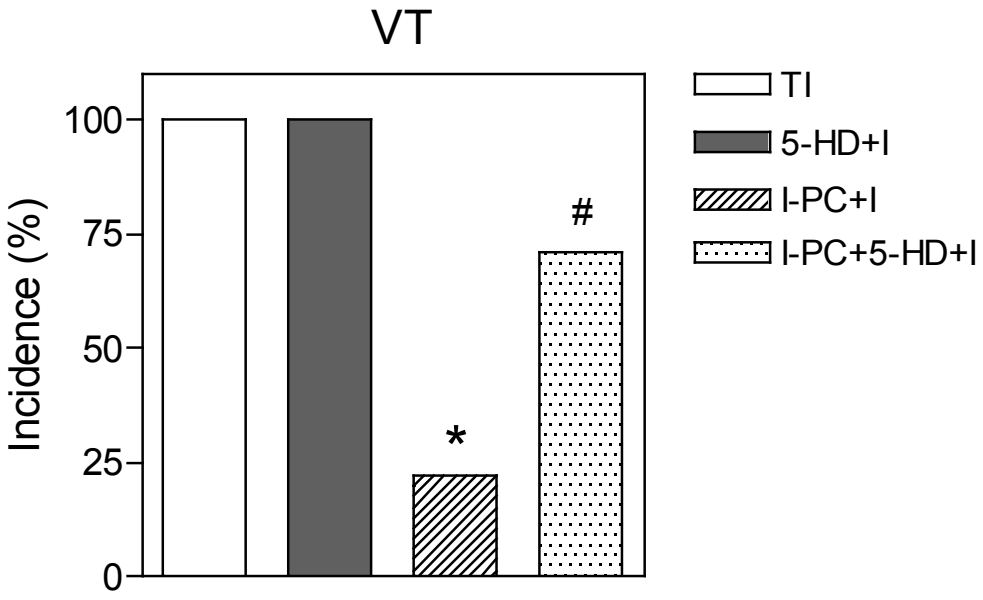

Figure 3

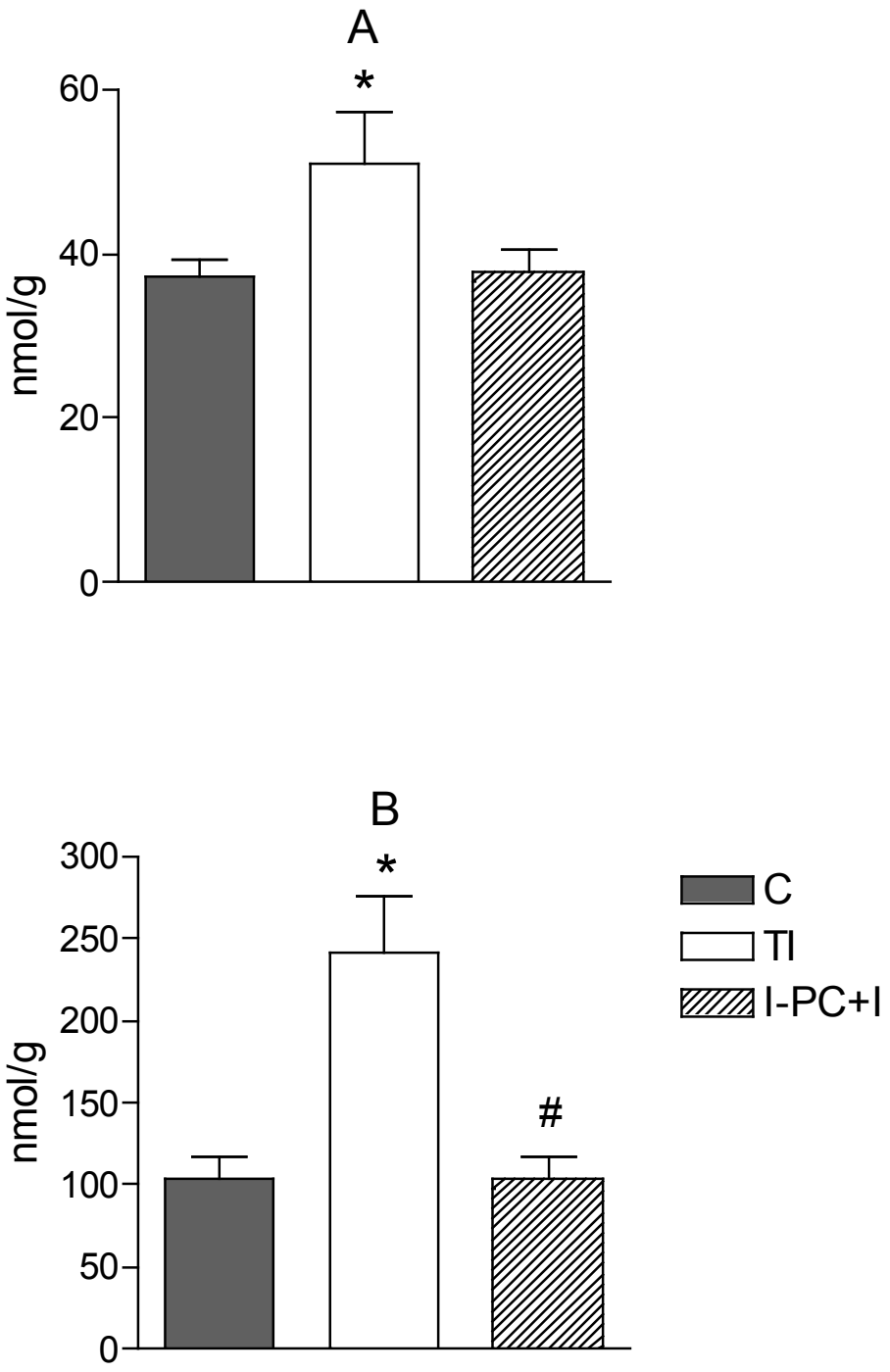

Figure 4.

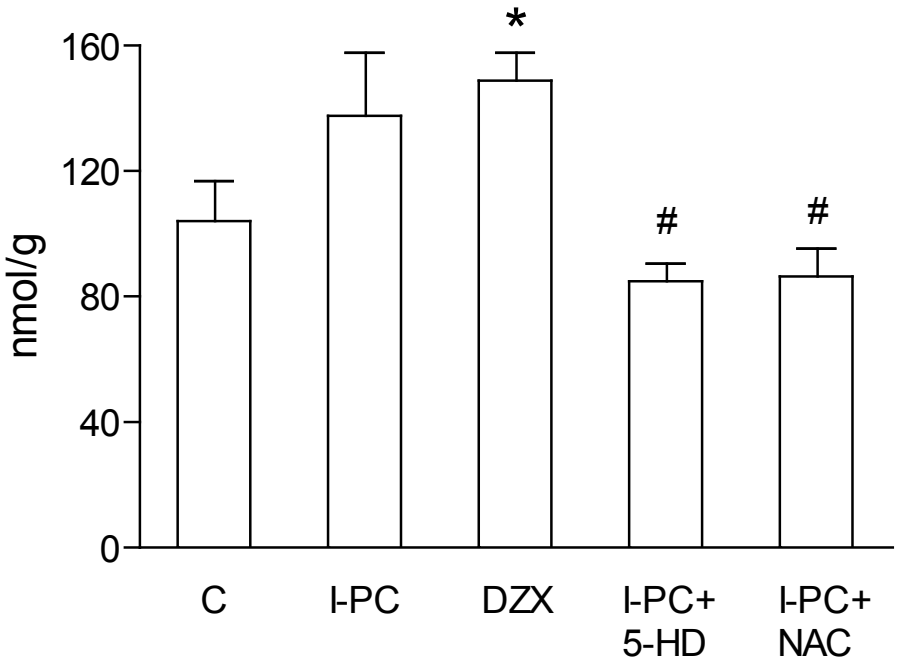

Figure 5.