JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476

Calcium activated potassium channel triggers cardioprotection of ischemic preconditioning

Chun-Mei Cao, Qiang Xia, Qin Gao, Mai Chen and Tak-Ming Wong

Department of Physiology, The University of Hong Kong, Hong Kong SAR, China (C.-M.C., M.C., T.-M.W.); and Department of Physiology, Zhejiang University School of Medicine, Hangzhou, China (Q.X, Q.G)

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: calcium-activated potassium channel in ischemic preconditioning

Corresponding author: Prof. Tak Ming Wong, Department of Physiology, 4/F, Laboratory Block, Faculty of Medicine Building, The University of Hong Kong, 21 Sassoon Road, Hong Kong SAR, China. Tel: 00852-28199194; Fax: 00852-28559730; E-mail: wongtakm@hkucc.hku.hk

The number of text pages: 23

The number of tables: 0

The number of figures: 7

The number of references: 26

The number of words in the *Abstract*: 244

The number of words in the *Introduction*: 337

The number of words in the *Discussion*: 679

ABBREVIATIONS: K_{Ca}, calcium activated potassium; IPC, ischemic preconditioning; mitoK_{ATP}, mitochondrial ATP-sensitive potassium; mPTP, mitochondrial permeability transition pore; LVEDP, left ventricular end diastolic pressure; LVDP, left ventricular developed pressure; ±dp/dtmax, the velocity of contraction and relaxation; MI/A, metabolic inhibition and anoxia; NS, 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2Hbenzimidazol-2-one (NS1619); Pax, paxilline; Dz, diazoxide; 5-HD, 5-hydroxydecanoate; Atr, atractyloside, CsA, cyclosporine A; 2-DOG, 2-deoxy-D-glucose; $Na_2S_2O_4$, sodium dithionite; TTC, 2, 3, 5-triphenyl-tetrazolium chloride; LDH, lactate dehydrogenase

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

We tested the hypothesis that the high-conductance calcium-activated potassium (K_{Ca}) channel is involved in the cardioprotection of preconditioning with ischemic insults. In the isolated perfused rat heart subjected to ischemia/reperfusion, effects of ischemic preconditioning (IPC) on infarct size and lactate dehydrogenase (LDH) release were abolished by 1 μM paxilline (Pax), an inhibitor of the K_{Ca} channel, administered 30 min before, but not during, ischemia. In isolated ventricular myocytes subjected to metabolic inhibition and anoxia (MI/A), preconditioning with MI/A increased their viability, and the effect was abolished by administering Pax before MI/A. Like IPC, 10 μM NS1619 (NS), an opener of K_{Ca} channels, reduced infarct size and LDH release, effects attenuated by Pax. The harmful and protective effects of blockade and activation of the K_{Ca} channel were accompanied by impaired and improved left ventricular contractile functions, respectively. In addition, the effect of NS was not altered by 100 μM 5-HD, an inhibitor of the K_{ATP} channel. Neither was the effect of 100 μM diazoxide (DZ), an activator of the K_{ATP} channel, altered by Pax. Furthermore, opening of the mitochondrial permeability transition pore (mPTP) with 20 μM atractyloside (Atr) abolished the beneficial effects of IPC or NS in the isolated rat heart and myocyte. Inhibition of mPTP opening with 0.2 μM cyclosporin A (CsA) decreased the infarct size and LDH release, and improved the contractile function, effects not attenuated by Pax. In conclusion, the study provides evidence that the K_{Ca} channel triggers cardioprotection of IPC, which involves mPTP.

Ischemic preconditioning (IPC) confers cardioprotection (Murry et al., 1986). Although extensive studies have been performed, the mechanisms by which IPC protects the myocardium against ischemia/reperfusion-induced injury are still not fully understood. Recently, a high-conductance Ca^{2+}-activated potassium (K_{Ca}) channel, located in the inner membrane of the mitochondrion, has been shown to mediate cardioprotection against ischemia/reperfusion (Xu et al., 2002), as does the mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channel (Garlid et al., 1997). Opening of both channels increases mitochondrial uptake of potassium, which is required for optimal functioning of oxidative phosphorylation (Kowaltowski et al., 2001). Since activation of the mitoK_{ATP} channel participates in the cardioprotection of IPC, we hypothesized that activation of the K_{Ca} channel also participates in cardioprotection of IPC.

The mitochondrial permeability transition pore (mPTP) is a multiprotein complex formed at contact sites between the inner and outer mitochondrial membranes (Hunter and Haworth, 1979), opening of which triggers apoptosis/necrosis (Crompton, 1999;Kroemer et al., 1998). The mPTP opens during the first few minutes of reperfusion (Griffiths and Halestrap, 1995), and this results in myocardial injury (Griffiths and Halestrap, 1995;Halestrap et al., 1998). It has been demonstrated that blockade of mPTP opening confers cardioprotection similar to that provided by IPC (Hausenloy et al., 2002;Javadov et al., 2003). It is therefore hypothesized that if IPC confers cardioprotection by activation of K_{Ca}, the mPTP may be involved.

To determine the role of K_{Ca} channel in cardioprotection of IPC, we first determined the effects of preconditioning with ischemic insults on injury induced by ischemia/reperfusion upon blockade of the high conductance K_{Ca} channel with an blocker of the channel, paxilline both the isolated perfused rat heart and isolated myocytes preparations. To delineate the role of the mPTP in the cardioprotection by activation of the K_{Ca} channel, we determined the effect of blockade of

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

mPTP on cardioprotection effects of ischemic insults or activation of the K_{Ca} channel in the isolated perfused rat heart and isolated ventricular myocyte. The results showed that the K_{Ca} channel triggers the cardioprotection of IPC, and the mPTP is involved.

Materials and methods

Isolated perfused heart preparation

The protocols of this study were approved by the Committee on the Use of Experimental Animals for Teaching and Research, The University of Hong Kong.

Male Sprague-Dawley rats of 250-300 g body weight were anesthetized with 60 mg/kg sodium pentobarbitone intraperitoneally (i.p.) and given 200 IU heparin intravenously (i.v.). Hearts were excised rapidly and placed in ice-cold Krebs-Henseleit (K-H) perfusion buffer before being mounted on a Langendorff apparatus for perfusion at 37°C with K-H buffer at a constant pressure (100 cm H_2O) and equilibrated with 95% O_2/5% CO_2. The buffer contained (mM): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.0. For hearts subjected to regional ischemia, a silk suture was placed around the left coronary artery to form a snare. The coronary artery was occluded by pulling the snare to produce ischemia. Reperfusion was achieved by releasing it. In the present study, the isolated heart was subjected to 30 min ischemia followed by 120 min reperfusion, that induced myocardial injury. IPC was achieved by two cycles of 5 min of ischemia followed by 5 min of reperfusion prior to the more sustained ischemia/reperfusion that caused myocardial infarction.

For the study of left ventricular contractile function, a balloon was inserted through the left atrium into the left ventricle and the left ventricular end diastolic pressure (LVEDP) was

adjusted between 4 and 8 mmHg. Cardiac parameters, namely, heart rate, left ventricular developed pressure (LVDP: difference between left ventricular end systolic pressure and end diastolic pressure) and velocity of contraction and relaxation (±dp/dtmax), were monitored continuously. Coronary flow, expressed in ml/min, was measured by timed collection of effluent at regular intervals, using a calibrated tube.

Measurement of the area of risk

For determination of infarct size, the coronary artery was re-occluded at the end of reperfusion and a solution with 2.5% Evans blue was perfused to delineate the area of risk. Hearts were then frozen and cut into slices, which were then incubated in a sodium phosphate buffer containing 1% w/v 2,3,5-triphenyl-tetrazolium chloride (TTC) for 15 min to visualize the unstained infarcted region. Infarct and risk zone areas were determined by planimetry with the software Image/J from NIH. The infarct size was expressed as a percentage of the risk zone.

Determination of myocardial injury by lactate dehydrogenase (LDH) efflux

The effluent from the isolated perfused heart was collected at 5 min of reperfusion and lactate dehydrogenase (LDH) was spectrophotometrically assayed using a kit purchased from Sigma Chemical Co. LDH activity was expressed as units per liter.

Preparation of isolated ventricular myocytes

Single ventricular myocytes were prepared from the heart of male Sprague-Dawley rats by enzymatic dissociation. Immediately after decapitation, the heart was rapidly removed and rinsed

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

in ice-cold Ca^{2+}-free Tyrode's solution. The heart was perfused via a Langendorff apparatus with a 100% oxygenated, non-recirculating Ca^{2+}-free Tyrode's solution. Then the perfusion solution was switched to a 100% oxygenated recirculated, low Ca^{2+} (50 μM) Tyrode's solution containing 0.03% collagenase and 1% bovine serum albumin for 10 min. The ventricles were cut, minced, and gently triturated with a pipette in the low Ca^{2+} Tyrode's solution containing bovine serum albumin at 37°C for 10 min. The cells were filtered through 200-μm nylon mesh, re-suspended in the Tyrode's solution, in which the Ca^{2+} concentration was gradually increased to 1.25 mM in 40 min. Only rod-shaped cells with clear cross-striations were used. For ischemic insults, myocytes were incubated for 5 min with a solution containing 10 mM 2-deoxy-D-glucose (2-DOG) and 10 mM sodium dithionite ($Na_2S_2O_4$), that induce metabolic inhibition and anoxia (MI/A) (Ho et al., 2002;Wu et al., 1999), two consequences of ischemia. This was followed by perfusion with normal K-H solution for 10 min - reperfusion. For preconditioning with MI/A, cells were subjected to three cycles of 1 min superfusion with the MI/A solution, separated by 3 min of superfusion with normal Krebs solution (Ho et al., 2002).

Cell viability

Trypan blue exclusion was used as an index of the viability of the myocyte (Zhou et al., 1996;Hiebert and Ping, 1997). After cells were incubated with 0.4% trypan blue dye for 3 minutes, they were counted in a hemocytometer chamber under a light microscope. Dead cells are not able to exclude trypan blue and thus appear blue. The cell morphology was determined by microscopic examination (Zhou et al., 1996). Only rod-shaped (length/width ratio, >3:1) cells were used for data collection.

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Experiment protocol

Three series of experiments were performed in the isolated perfused heart. The first series was to determine whether blockade of the high conductance K_{Ca} channel abolished cardioprotection of IPC. 1 μM paxilline (Pax) was administered either 30 min before ischemia or throughout the ischemia and 15 min into reperfusion (Fig 1). In the second series of experiments, which determined whether opening of the high conductance K_{Ca} channel reduced injury induced by ischemia/reperfusion, 10 μM NS1619 (NS) was perfused 15 min before ischemia for 10 min in the absence or presence of Pax, which was administered 30 min before ischemia (Fig 2). The protocol for administration of NS was according to a previous study (Xu et al., 2002). In order to determine whether K_{Ca} channel was located up-stream of mPTP, the effect of NS was determined with atractyloside (Atr) administered from 25 min into ischemia to 15 min into reperfusion according to previous study (Fig 3) (Hausenloy et al., 2002). The effect of 0.2 μM cyclosporin A (CsA), an inhibitor of mPTP opening, administered from 25 min into ischemia to 15 min into reperfusion in the presence of Pax was also determined. The third series of experiments determined the relationship between the K_{Ca} and mitoK_{ATP} channels (Fig 4). The effects of activation of one potassium channel were determined in the presence of the blocker of another channel. 100 μM diazoxide (Dz), activator of K_{ATP} channels or 10 μM NS were administered 15 min before ischemia for 10 min (Fig 4). 100 μM 5-HD, an inhibitor of K_{ATP} channels, or 1 μM Pax was administered 30 min before ischemia (Fig 4).

Infarct size and LDH, determined at the end and 5 min into reperfusion, respectively, were used as parameters of injury. LDH was measured at 5 min into reperfusion when its release reaches its peak (Cao et al., 2004).

In the isolated ventricular myocyte, two series of experiments were performed. The first was

aimed at determining whether blockade of the K_{Ca} channel abolished cardioprotection of preconditioning with ischemic insults. Myocytes were incubated with Pax for 30 min before MI/A with and without preconditioning with MI/A or NS (Fig 5). The second series of experiments addressed the relationship between the K_{Ca} channel and mPTP. The effects of activation of the K_{Ca} channel upon opening of mPTP and inhibition of mPTP upon blockade of the K_{Ca} channel were determined (Fig 6). NS and CsA were added 15 min before MI/A for 10 min while Pax and Atr were added 30 min before MI/A.

Trypan blue exclusion was used as a parameter of cell viability.

Chemicals and solutions

Collagenase (type I), bovine serum albumin, NS, 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifluoromethyl-2Hbenzimidazol-2-one (NS1619, NS), paxilline (Pax), diazoxide (Dz), 5-hydroxydecanoate (5-HD), atractyloside (Atr), cyclosporin A (CsA), 2-deoxy-D-glucose (2-DOG), sodium dithionite ($Na_2S_2O_4$), fura-2/AM, trypan blue, and 2, 3, 5-triphenyl-tetrazolium chloride (TTC) were purchased from Sigma Chemical Co. FK506 was purchased from Merck.

The concentrations of Pax, NS (Xu et al., 2002), CsA, Atr (Hausenloy et al., 2002), DZ (Miura et al., 2000), 5-HD (Auchampach et al., 1992), used in this study were based on previous studies.

Statistical analysis

Values are presented as means ± standard error of means (SEM). Statistical comparisons were performed by One-way analysis of variance and the Newman-Keuls test. Differences of $P<0.05$ were regarded as significant.

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Studies in the isolated perfused heart subjected to ischemia/reperfusion

Effect of IPC on injury upon blockade of the K_{Ca} channel by paxilline

IPC significantly reduced the infarct size and LDH release in the isolated perfused rat heart subjected to 30 min ischemia and 120 min reperfusion compared with untreated control hearts (Fig 1), in agreement with the well established cardioprotection by IPC.

When 1 μM Pax, an inhibitor of the K_{Ca} channel, was administered 30 min before ischemia, both the infarct size and LDH release after ischemia were the same as those in the control group i.e. without IPC, indicating that cardioprotection of IPC had been abolished. On the other hand, when the inhibitor was administered only during ischemia and 15 min into reperfusion, both the infarct size and LDH release remained the same as those of the group subjected to IPC, indicating that cardioprotection of IPC was still present.

Effects of activation of K_{Ca} channel with NS on injury

Pretreatment with 10 μM NS, known to activate K_{Ca} channel (Xu et al., 2002), significantly reduced the infarct size and LDH release in isolated perfused rat heart subjected to ischemia/reperfusion, an effect similar to that of IPC (Fig 2). These results are in agreement with a recent study (Wang et al., 2004).

We next determined the relationship between the K_{Ca} channel and the mPTP, which is known to be involved in cardioprotection/injury (Hausenloy et al., 2002). 20 μM Atr, an opener of the mPTP, abolished the effects of IPC on myocardial infarct and LDH release (Fig 3). Consistent with the role of the mPTP in ischemia/reperfusion injury, administration of 0.2 μM CsA, an

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

inhibitor of the mPTP, during reperfusion significantly reduced the infarct size and LDH release in the isolated perfused rat heart subjected to ischemia/reperfusion (Fig 3). 5 μM FK506, which inhibits calcineurin, also an effect of CsA, but does not inhibit mPTP opening (Connern and Halestrap, 1994;Griffiths and Halestrap, 1993), did not affect the infarct size and LDH release when administered during reperfusion, indicating that the cardioprotective effect of CsA was due to its action on the mPTP.

In order to determine whether closure of the mPTP mediated cardioprotection upon activation of K_{Ca} channels by NS (10 μM), cardioprotection was assessed in the presence of Atr (20 μM). As shown in Fig 2, the effects of NS on infarct size and LDH release were attenuated in the presence of Atr, indicating that activation of K_{Ca} channels confers cardioprotection via mPTP at least partly. On the other hand, the effect of inhibiting the mPTP opening with CsA was not altered by pretreatment with Pax, indicating that the mPTP is located downstream from the K_{Ca} channel.

Administration of Atr (20 μM) for 30 min did not alter the infarct size and LDH release (Fig 2).

Effects of DZ on injury

In agreement with the previous observation (Miura et al., 2000), pretreatment with 100 μM DZ, which is known to open the mitoK_{ATP} channel and confer cardioprotection, significantly reduced the infarct size and LDH release in the isolated perfused rat heart subjected to ischemia/reperfusion (Fig 4). Inhibition of the mitoK_{ATP} channels with 5-HD (100 μM) abolished the protective effect both of DZ and IPC (Fig 4). On the other hand the protective effect of DZ was not altered by Pax, nor was the effect of NS affected by 5-HD (Fig 4), indicating no interaction between the two potassium channels. 5-HD alone had no effect on infarct size or LDH release.

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

In agreement with the previous finding (Hausenloy et al., 2002), Atr (20 μM), abolished the protective effects of DZ. On the other hand, the protective effect of CsA (0.2 μM) was not altered by 5-HD (10 μM) (data not shown).

Effects of K_{Ca} blockade on ventricular functions and coronary flow

In all groups, occlusion of the left anterior descending artery resulted in a marked decrease in LVDP, ±dP/dtmax and coronary flow and an elevation in LVEDP at the end of reperfusion (Fig 5). The reductions in LVDP, ±dP/dtmax and the elevation in LVEDP in the group treated with IPC were significantly attenuated, effects that were reduced by either 1 μM Pax or 20 μM Atr. On the other hand, in the group treated with 10 μM NS, reductions in LVDP and ±dP/dtmax during reperfusion were significantly attenuated, suggesting that NS improved the left ventricular contraction and relaxation following ischemic injury. The effects of NS were attenuated by Pax and Atr.

Inhibition of mPTP opening with CsA attenuated the reductions in LVDP and ±dP/dtmax and the elevation in LVEDP during reperfusion, which were not altered by Pax, indicating again that inhibition of the mPTP is downstream from the K_{Ca} channel.

Heart rate and coronary flow during ischemia and reperfusion were the same in these groups (Fig 5).

Studies in isolated ventricular myocytes subjected to metabolic inhibition and anoxia (MI/AP)

Effect of preconditioning with MI/AP on viability upon blockade of of K_{Ca} channel with Pax

In order to confirm the observation from the isolated perfused heart preparation that blockade of K_{Ca} channel abolished cardioprotection of IPC by direct action on the myocardium, we determined the effects of preconditioning with ischemic insults, metabolic inhibition and anoxia (MI/AP), on viability of the myocytes subjected to MI/A upon blockade of the channel. The percentage of rod-shaped unstained cells 10 min into reperfusion was significantly decreased in ventricular myocytes subjected to MI/A compared with that in control group (Fig 6). In the cells subjected to MI/AP, the percentage of rod shaped unstained cells subjected to MI/A was significantly greater than in MI/AR only cells, in agreement with our previous finding (Liu et al., 2004). This increase was attenuated by pretreatment with Pax (1 μM) before MI/A.

10 μM NS (Fig 6) or 0.2 μM CsA (Fig 7) restored the percentage of unstained cells of the MI/AP cells to the level of the control group. 20 μM Atr attenuated the effects of MI/AP or NS (Fig 7). 1 μM Pax abolished the effect of NS, but not the effect of CsA (Fig 7). Atr or Pax alone had no effect at all.

Discussion

There are two important observations in the present study. First, cardioprotection conferred by IPC was abolished by blockade of the high-conductance K_{Ca} channel with Pax administered before ischemia and the effect of Pax was not accompanied by alterations in coronary flow in the isolated perfused rat heart. Secondly, the beneficial effect of preconditioning with brief periods of metabolic inhibition and anoxia, two consequences of myocardial ischemia, on viability of isolated ventricular myocytes subsequently subjected to MI/A, was also abolished by Pax administered before MI/A. So blockade of the K_{Ca} channel abolished cardioprotection of ischemic insult by direct action on myocardium. In agreement with a previous study (Wang et al.,

2004), in the present study, it was found that activation of the channel with its opener, NS, conferred cardioprotection as did IPC or opening of the mitoK_{ATP} channel with diazoxide. These observations provide unequivocal evidence that the high-conductance K_{Ca} channel is involved in cardioprotection of preconditioning with ischemic insults. It is important to note that when the K_{Ca} channel was blocked with Pax 30 min before ischemia, the effect of IPC was abolished in the isolated perfused heart. On the other hand, when Pax was added during ischemia and reperfusion, the effect of IPC remained. This finding indicates that the K_{Ca} channel is a trigger, rather than a mediator of cardioprotection of preconditioning.

Another important finding is the causal relationship between the high-conductance K_{Ca} channel and the mPTP, a crucial step in the cardioprotection of preconditioning (Hausenloy et al., 2002;Javadov et al., 2003;Hausenloy et al., 2004). We discovered that opening the mPTP with Atr, which abolished the beneficial effect of IPC on the heart in terms of protection against injury and contractile function, also abolished the beneficial effects of activating the high-conductance K_{Ca} channel with NS. This is the first demonstration that mPTP plays a permissive role for cardioprotection due to activation of the K_{Ca} channel. The K_{Ca} channels involved are most likely those in the mitochondria, as the channel has been shown to be mainly, if not exclusively, located on the membrane of this organelle (Xu et al., 2002).

We also found that the cardioprotection conferred by activation of mitoK_{ATP} channels with diazoxide was not altered by blockade of K_{Ca} channels nor vice-versa, indicating that these channels act independently. It should be noted that activation of mitoK_{ATP} channels also confers cardioprotection by inhibiting mPTP opening (Hausenloy et al., 2002). So, both potassium

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

channels located in mitochondria confer cardioprotection via the same machinery, the mPTP.

One of the common features of these two channels is influx of K^+ into mitochondria upon activation. That opening of these channels results in cardioprotection suggests that K^+ influx may play a crucial role in cardioprotection. There is evidence that K^+ influx into mitochondria may be important for oxidative phosphorylation, regulation of mitochondrial functions such as reactive oxygen species production and mitochondrial volume regulation (Garlid, 2000;Garlid and Paucek, 2003). It is also interesting to note that the K_{Ca} channel acts as a trigger as shown in the present study whereas mitochondrial K_{ATP} channel, acts as a trigger as well as a mediator (Gross and Peart, 2003). So although both channels share a common feature, namely increased K^+ influx into mitochondria upon activation, they may have unique properties other than influx of K^+, which are responsible for different roles each of them plays. Further studies are warranted.

In the present study we also observed that blockade of the K_{Ca} channel abolished the protective effects of IPC accompanied by impairment of contraction while activation of the channel conferred cardioprotection accompanied by improved contractile functions. Further study is needed to determine whether improved/impaired contractile function is secondary to cardioprotection/injury upon activation/inactivation of the channel.

In conclusion, the study has provided first evidence that activation of K_{Ca} channels triggers cardioprotection of preconditioning with ischemic insults. Cardioprotection of preconditioning is accompanied by improved left ventricular contractile function. The study has also provided first evidence that mPTP plays a permissive role for cardioprotection due to activation of K_{Ca}

channels.

Acknowledgements

We thank Dr. I. Bruce for advice on the use of English, Dr. GH Borchert for advice on viability study and Mr. C. P. Mok for technical assistance.

References

Auchampach JA, Grover GJ, and Gross GJ (1992) Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc.Res.* **26**:1054-1062.

Cao CM, Xia Q, Tu J, Chen M, Wu S, and Wong TM (2004) Cardioprotection of Interleukin-2 Is Mediated via {kappa}-Opioid Receptors. *J.Pharmacol.Exp.Ther.* **309**:560-567.

Connern CP and Halestrap AP (1994) Recruitment of mitochondrial cyclophilin to the mitochondrial inner membrane under conditions of oxidative stress that enhance the opening of a calcium-sensitive non-specific channel. *Biochem.J.* **302 (Pt 2)**:321-324.

Crompton M (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem.J.* **341 (Pt 2)**:233-249.

Garlid KD (2000) Opening mitochondrial K(ATP) in the heart--what happens, and what does not happen. *Basic Res.Cardiol.* **95**:275-279.

Garlid KD and Paucek P (2003) Mitochondrial potassium transport: the K(+) cycle. *Biochim.Biophys.Acta* **1606**:23-41.

Garlid KD, Paucek P, Yarov-Yarovoy V, Murray HN, Darbenzio RB, D'Alonzo AJ, Lodge NJ, Smith MA, and Grover GJ (1997) Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K+ channels. Possible mechanism of cardioprotection. *Circ.Res.* **81**:1072-1082.

Griffiths EJ and Halestrap AP (1993) Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. *J.Mol.Cell Cardiol.* **25**:1461-1469.

Griffiths EJ and Halestrap AP (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem.J.* **307 (Pt 1)**:93-98.

Gross GJ and Peart JN (2003) KATP channels and myocardial preconditioning: an update. *Am.J.Physiol Heart Circ.Physiol* **285**:H921-H930.

Halestrap AP, Kerr PM, Javadov S, and Woodfield KY (1998) Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart. *Biochim.Biophys.Acta* **1366**:79-94.

Hausenloy D, Wynne A, Duchen M, and Yellon D (2004) Transient mitochondrial permeability transition pore opening mediates preconditioning-induced protection. *Circulation* **109**:1714-1717.

Hausenloy DJ, Maddock HL, Baxter GF, and Yellon DM (2002) Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc.Res.* **55**:534-543.

Hiebert L and Ping T (1997) Protective effect of dextran sulfate and heparin on adult rat cardiomyocytes damaged by free radicals. *J.Mol.Cell Cardiol.* **29**:229-235.

Ho JC, Wu S, Kam KW, Sham JS, and Wong TM (2002) Effects of pharmacological preconditioning with U50488H on calcium homeostasis in rat ventricular myocytes subjected to metabolic inhibition and anoxia. *Br.J.Pharmacol.* **137**:739-748.

Hunter DR and Haworth RA (1979) The Ca2+-induced membrane transition in mitochondria. I.

The protective mechanisms. *Arch.Biochem.Biophys.* **195**:453-459.

Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, and Halestrap AP (2003) Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. *J.Physiol* **549**:513-524.

Kowaltowski AJ, Seetharaman S, Paucek P, and Garlid KD (2001) Bioenergetic consequences of opening the ATP-sensitive K(+) channel of heart mitochondria. *Am.J.Physiol Heart Circ.Physiol* **280**:H649-H657.

Kroemer G, Dallaporta B, and Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. *Annu.Rev.Physiol* **60**:619-642.

Liu J, Kam KW, Zhou JJ, Yan WY, Chen M, Wu S, and Wong TM (2004) Effects of HSP70 activation by metabolic inhibition preconditioning or {kappa}-OR stimulation on Ca2+ homeostasis in rat ventricular myocytes subjected to ischemic insults. *J.Pharmacol.Exp.Ther.*

Miura T, Liu Y, Kita H, Ogawa T, and Shimamoto K (2000) Roles of mitochondrial ATP-sensitive K channels and PKC in anti-infarct tolerance afforded by adenosine A1 receptor activation. *J.Am.Coll.Cardiol.* **35**:238-245.

Murry CE, Jennings RB, and Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**:1124-1136.

Wang X, Yin C, Xi L, and Kukreja RC (2004) Opening of Ca2+-Activated K+ Channel Triggers Early and Delayed Preconditioning against Ischemia/Reperfusion Injury Independent of Nitric Oxide Synthases in Mice. *Am.J.Physiol Heart Circ.Physiol.*

Wu S, Li HY, and Wong TM (1999) Cardioprotection of preconditioning by metabolic inhibition in the rat ventricular myocyte. Involvement of kappa-opioid receptor. *Circ.Res.* **84**:1388-1395.

Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, and O'Rourke B (2002) Cytoprotective role of Ca2+- activated K+ channels in the cardiac inner mitochondrial membrane. *Science* **298**:1029-1033.

Zhou X, Zhai X, and Ashraf M (1996) Direct evidence that initial oxidative stress triggered by preconditioning contributes to second window of protection by endogenous antioxidant enzyme in myocytes. *Circulation* **93**:1177-1184.

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

* The study was supported by a Cardiovascular Physiology Research Fund donated by L.C.S.T. (Holdings).

Send reprint requests to: Prof. Tak Ming Wong, Department of Physiology, 4/F, Laboratory Block, Faculty of Medicine Building, The University of Hong Kong, 21 Sassoon Road, Hong Kong SAR, China. E-mail: wongtakm@hkucc.hku.hk

Legends for figures

Figure 1. Effects of ischemic preconditioning (IPC) in the presence of paxilline (Pax) on myocardial infarct (A) or LDH release (B) in the isolated perfused rat heart subject to ischemia of 30 min and reperfusion of 120 min (IR). Experimental protocol was shown in the upper panel. Hearts were preconditioned with two cycles of 5 min of ischemia interspersed with 5 min reperfusion (IPC). 1 μM Pax was either administered for 30 min before ischemia (PaxT) or throughout the ischemia and 15 min into reperfusion (PaxM). Data are expressed as individual values and mean±SEM; n=10 in each group.

** P<0.01 vs IR group

Figure 2. Effects of NS1619 (NS) in the presence of paxilline (Pax) or atractyloside (Atr) on myocardial infarct (A) and LDH release (B) in isolated perfused rat heart subject to 30 min ischemia and 120 min reperfusion (IR). Experimental protocol was shown on the upper panel. 10 μM NS was administered 15 min before ischemia for 10 min, 1 μM Pax for 30 min before ischemia and 20 μM Atr for the last 5 min of ischemia to 15 min into reperfusion.

Data are expressed as individual values and mean±SEM; n=10 in each group

** P<0.01 vs IR group

Figure 3. Effects of IPC or cyclosporin A (CsA) in the presence of atractyloside (Atr) or paxilline (Pax), or FK506 on myocardial infarct (A) and LDH release (B) in the isolated perfused rat heart subject to 30 min ischemia and 120 min reperfusion (IR). Experimental protocol was shown on the upper panel. 1 μM Pax was administered for 30 min before ischemia. 0.2 μM CsA, 5 μmol/L

FK506 and 20 μM Atr were administered from last 5 min of ischemia to 15 min into reperfusion. Data are expressed as individual values and mean±SEM; *n*=10 in each group.

** *P*<0.01 compared with IR group

Figure 4. Effects of diazoxide (DZ), IPC or NS 1619 (NS) on myocardial infarct (A) and LDH release (B) in isolated perfused rat heart subject to 30 min ischemia and 120 min reperfusion (IR). Experimental protocol was shown on the upper panel. 100 μM DZ and 10 μM NS were administered 15 min before ischemia for 10 min. 1 μM Pax and 100 μM 5-HD were administered for 30 min before ischemia. Data are expressed as individual values and mean±SEM; *n*=10 in each group.

** *P*<0.01 compared with IR group

Figure 5. Hemodynamic parameters, heart rate and coronary flow in isolated rat heart.

The experimental protocol and abbreviations are shown in Figs 1-4. LVDP: left ventricular developed pressure; LVEDP: left ventricular end diastolic pressure; +dP/dtmax: the velocity of contraction; -dP/dtmax: the velocity of relaxation. Only the values at the end of reperfusion are presented here. Results on time course changes are available in the supplementary file. Data are expressed as mean±SEM; *n*=8 in each group. * *P*<0.05 *vs* ischemia/reperfusion (IR) group

Figure 6. Effects of preconditioning with metabolic inhibition and anoxia (MI/AP) or NS 1691 (NS) in the presence of paxilline (Pax) on trypan blue exclusion of rat ventricular myocytes subjected to metabolic inhibition and anoxia (MI/A).

Experimental protocol was shown on the upper panel. 10 μM NS was administered 15 min before MI/A for 10 min. 1 μM Pax was administered for 30 min before MI/A.

JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Data are expressed as mean±SEM; *n*=10 in each group.

** *P*<0.01 compared with control group; †† *P*<0.01 compared with MI/AR group.

Figure 7. Effects of preconditioning with metabolic inhibition and anoxia (MI/AP), NS 1691 (NS) or cyclosporin A (CsA) on trypan blue exclusion of rat ventricular myocytes subjected to metabolic inhibition and anoxia (MI/A).

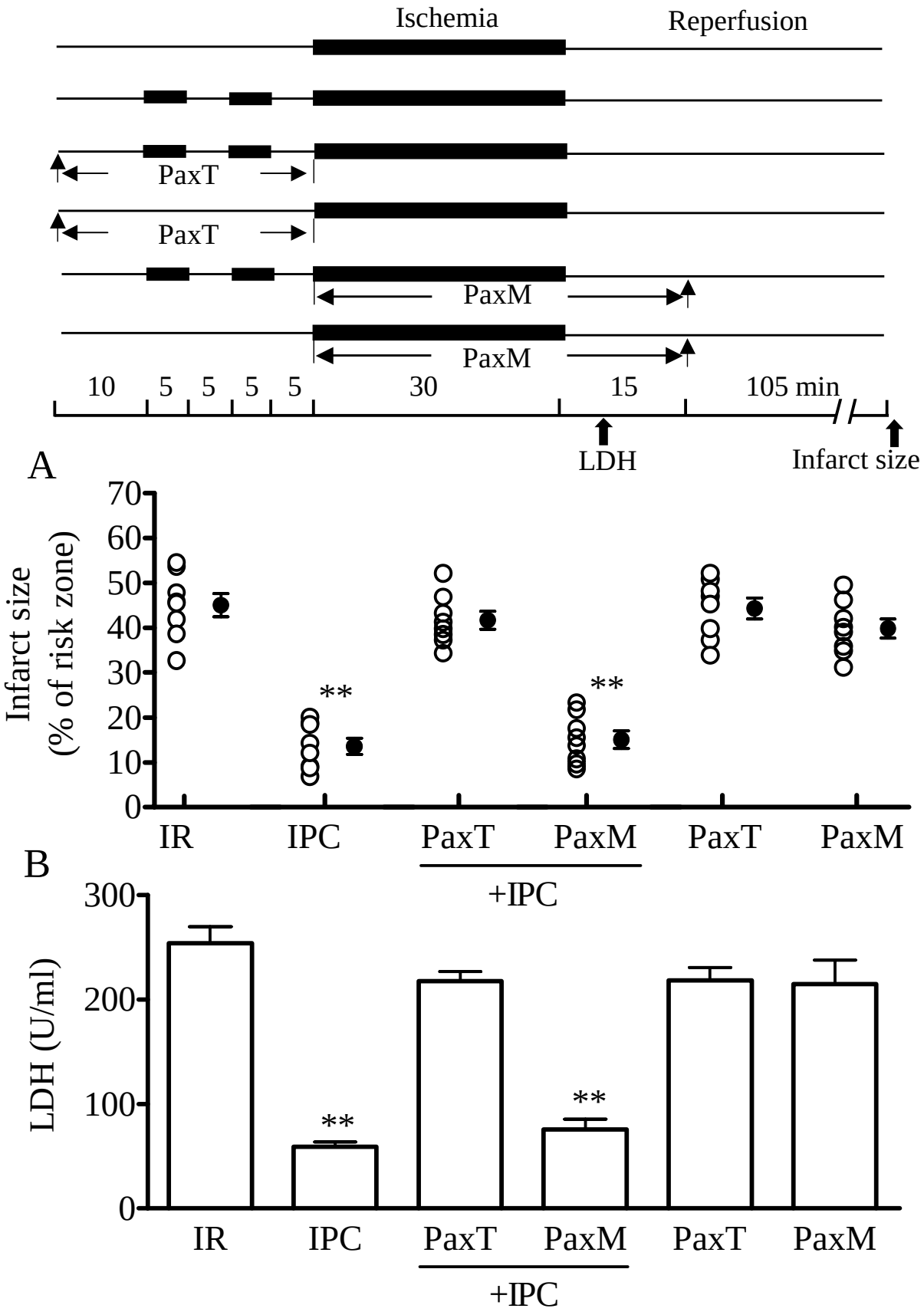
Experimental protocol was shown on the upper panel. 10 μM NS and 0.2 μmol/L CsA were administered 15 min before MI/A for 10 min. 20 μM Atr and 1 μM Pax were administered for 30 min before MI/A.

Data are expressed as mean±SEM; *n*=10 in each group.

** *P*<0.01 compared with control group; †† *P*<0.01 compared with MI/AR group.

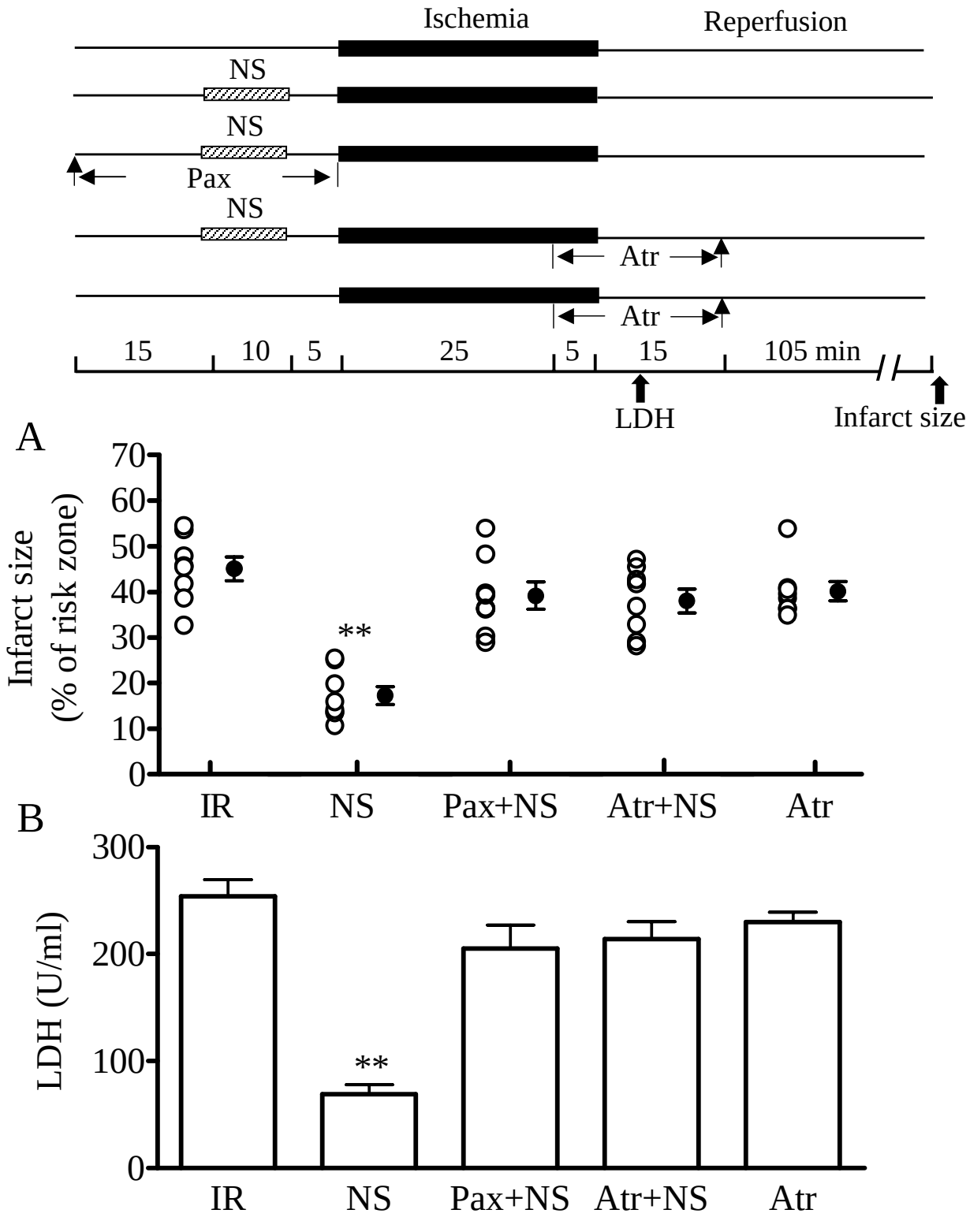
JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



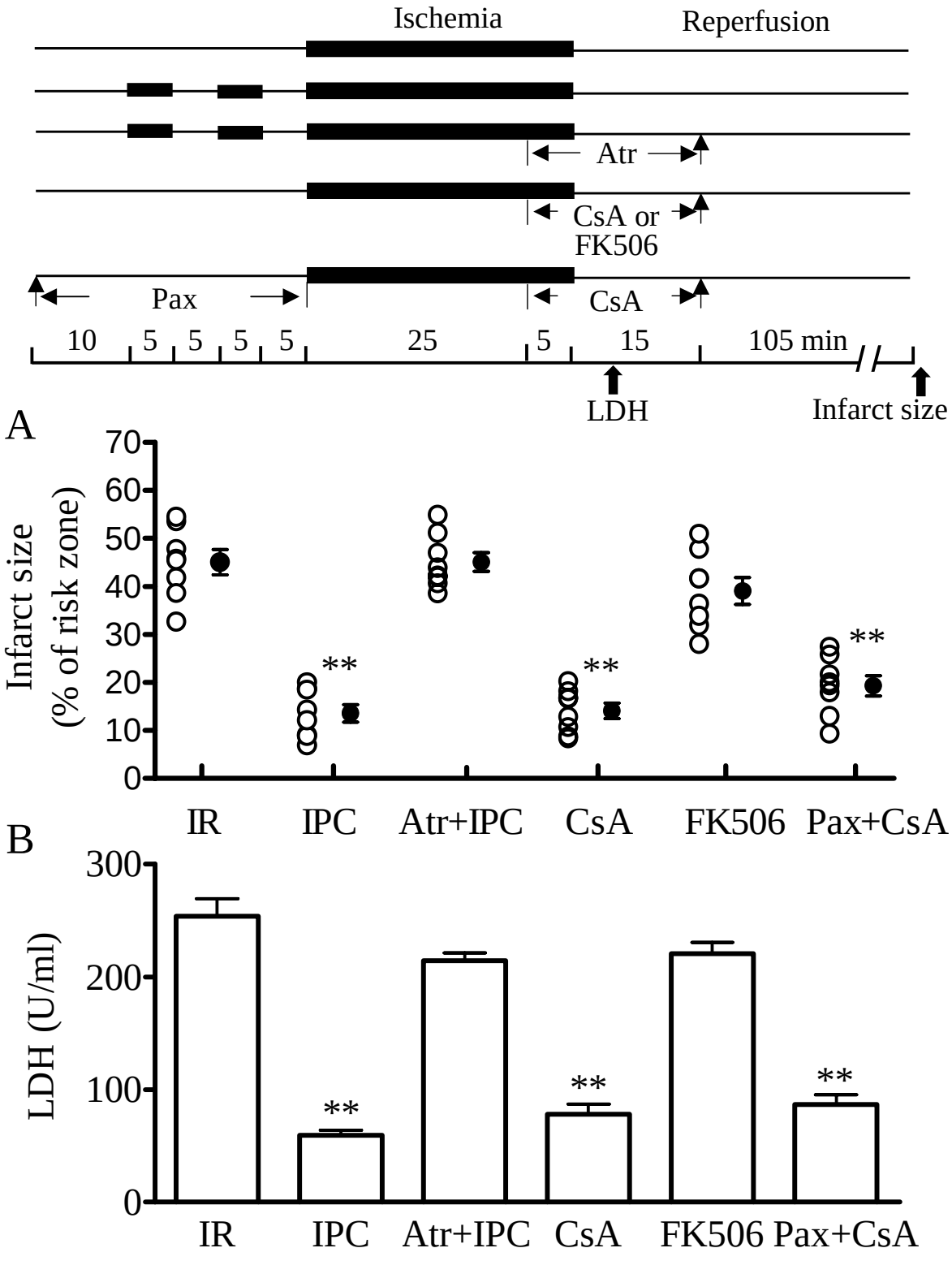
JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



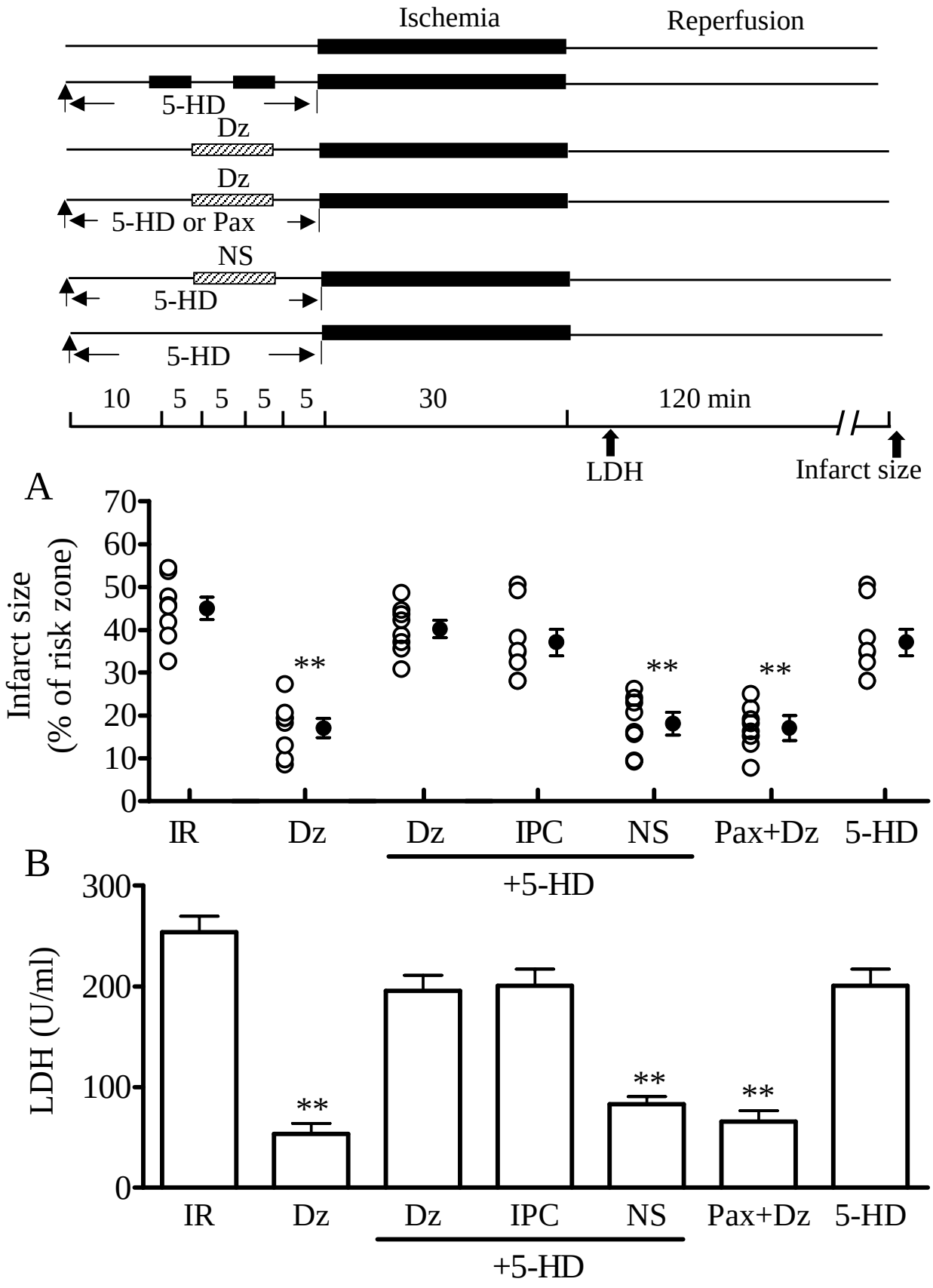
JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



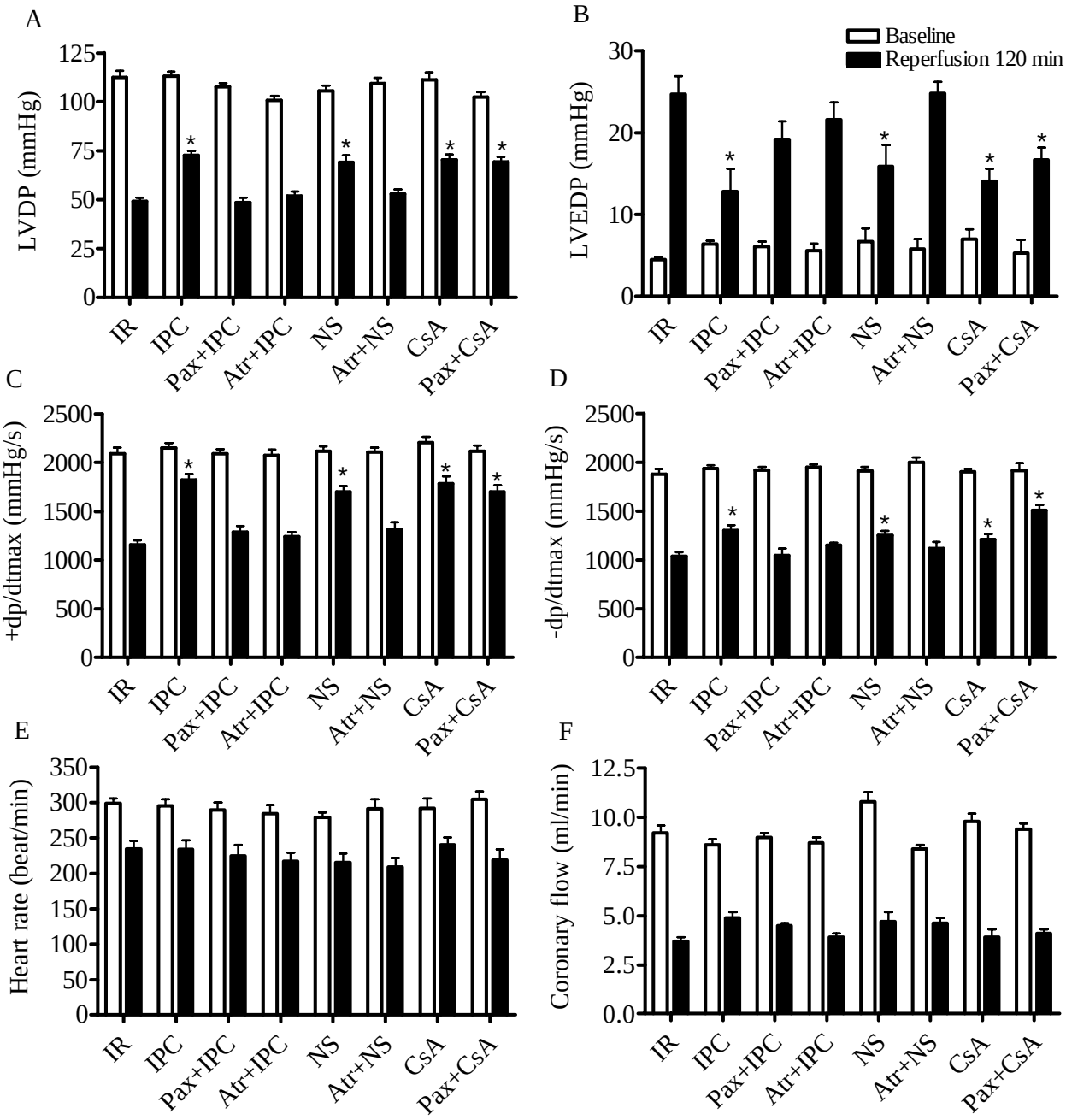
JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



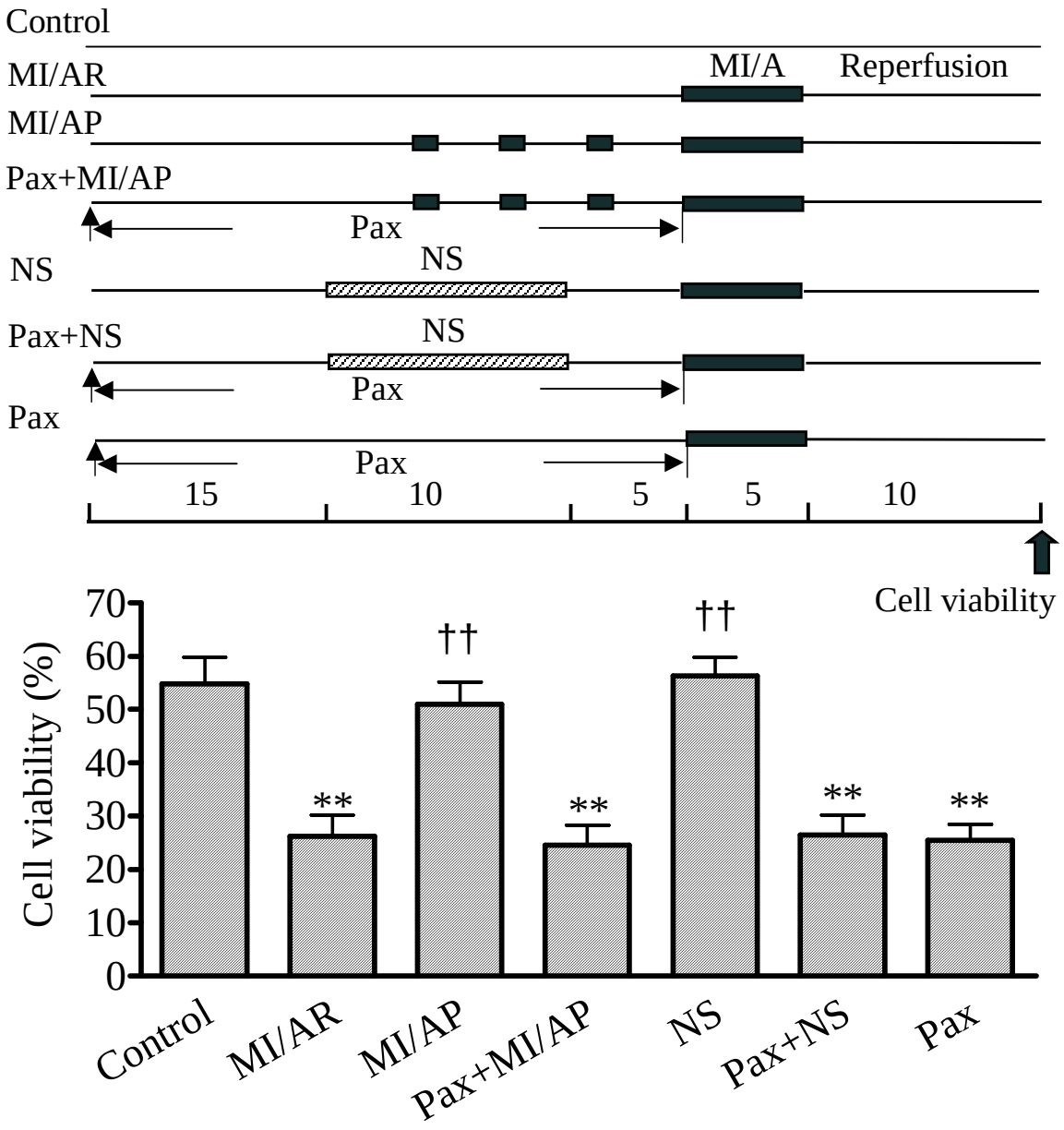
JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



JPET Fast Forward. Published on September 2, 2004 as DOI: 10.1124/jpet.104.074476
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7