Postconditioning attenuates myocardial ischemia–reperfusion injury by inhibiting events in the early minutes of reperfusion

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Abstract

Objective: We previously showed that brief intermittent ischemia applied during the onset of reperfusion (i.e., postconditioning) is cardioprotective in a canine model of ischemia–reperfusion. This study tested the hypothesis that the early minutes of reperfusion (R) during which postconditioning (Post-con) is applied are critical to its cardioprotection. **Methods:** In anesthetized open-chest rats, the left coronary artery (LCA) was occluded for 30 min and reperfused for 3 h. All rats were randomly divided into six groups: Control ($n = 8$): no intervention at R; Ischemic preconditioning (IPC) ($n = 8$): the LCA was occluded for 5 min followed by 10 min of R before the index occlusion; Post-con 1 ($n = 8$): after LCA occlusion, three cycles of 10 s R followed by 10 s LCA re-occlusion were applied during the first minute of R; Post-con 2 ($n = 8$): Six cycles of 10 s R and 10 s re-occlusion were applied during the first 2 min of R; Delayed Post-con ($n = 8$): the ligature was loosened for full reflow for the first minute of R, after which the three-cycle Post-con algorithm was applied; Sham ($n = 6$): the surgical procedure was identical to other groups, but the LCA ligature was not ligated. **Results:** Infarct size (TTC staining) was 23% smaller in Post-con 1 ($40 \pm 2\%$*) than in Control ($52 \pm 3\%$), confirmed by plasma creatine kinase activity (18 ± 2* vs. 46 ± 6 IU/g protein). There was no further reduction in infarct size with 6 cycles of Post-con ($40 \pm 2.9\%$, $p > 0.05$ vs. Post-con 1). Meanwhile, infarct size reduction was significantly greater in the IPC group ($17 \pm 3\%$) than in Post-con1 ($p < 0.01$). The plasma lipid peroxidation product malondialdehyde (MDA, μM/ml) was less after R in IPC and Post-con 1 (0.8 ± 0.07* and 0.8 ± 0.06*) vs. Control (1.21 ± 0.08), consistent with a visual decrease in superoxide anion generation (dihydroethidium staining) in the AAR myocardium after 3 h of reperfusion. Neutrophil accumulation (myeloperoxidase activity, MPO, U/100 g tissue) in the AAR was less in IPC (1.4 ± 0.3*) and Post-con 1 (2.5 ± 0.3*) vs. Control (5.5 ± 0.6). The reductions in infarct size, creatine kinase, MDA and DHE staining were lost with delayed Post-con, while MPO activity remained lower than in Control (3.2 ± 0.4*). **Conclusions:** (1) Post-con at onset of R reduces myocardial injury; (2) cardioprotection may be mediated, in part, by inhibiting oxidant generation and oxidant mediated injury; (3) the first minute of R in the rat model is critical to cardioprotection by Post-con; and (4) cardioprotection by Post-con may be independent of neutrophil accumulation in AAR. *$p < 0.05$ Post-con vs. Control.
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This article is referred to in the Editorial by R.J. Diaz and G.J. Wilson (pages 4–6) in this issue.

1. Introduction

Ischemic preconditioning (IPC) was first introduced by Murry and associates [1] as a potent endogenous form of cardioprotection against ischemic–reperfusion injury. IPC attenuates the incidence and severity of post-ischemic

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arrhythmias [2–6] enhances the recovery of cardiac function after global ischemia, and reduces infarct size and the appearance of apoptosis in hearts subjected to ischemia–reperfusion injury [7,8]. Recently, Zhao et al. [9,10] recently reported another endogenous form of cardioprotection that exerted cardioprotection similar to that observed with IPC. In this study, [10] a short series of repetitive cycles of brief reperfusion and re-occlusion of the coronary artery applied *immediately* at the onset of reperfusion, termed "postconditioning", was also cardioprotective by reducing infarct size, coronary artery endothelial dysfunction, and neutrophil accumulation in the area at risk. This protection was similar in extent to ischemic preconditioning. This study [10] suggested that endogenous mechanisms are put into action within the first few minutes of reperfusion that attenuate reperfusion injury specifically. Earlier studies reporting cardioprotection when mechanical interventions were applied immediately upon reperfusion are consistent with the concept that endogenous mechanisms are engaged during reperfusion which reduce post-ischemic injury. For example, control over the hydrodynamic conditions (intracoronary blood flow and pressure) immediately after the onset of reperfusion to gradually restore reperfusion to the area at risk reduced infarct size, post-ischemic contractile dysfunction, microvascular injury and tissue edema [11–14] in a canine model of myocardial infarction. In addition, gradual reperfusion reduced contractile "stunning" in a model of non-lethal ischemia–reperfusion injury [15]. Furthermore, Peng et al. [16] reported that constraining blood flow at reperfusion to avoid reactive hyperemia attenuated tissue calcium accumulation and preserved high energy phosphate levels in reperfused myocardium. Therefore, endogenous mechanisms can be engaged to attenuate ischemic injury (preconditioning) as well as reperfusion injury (postconditioning).

The early reperfusion period is characterized by a burst of inflammatory-like reactions. Zweier et al. [17], Duilio et al. [18] and others [19,20] have reported a robust respiratory burst of oxygen radical species which peaks during the early moments of reperfusion, but which continues at an elevated level for hours thereafter. In addition, Tsao et al. have reported that neutrophil-endothelial cell interactions, which contribute to endothelial dysfunction, are initiated during the early moments of reperfusion [21,22]. Hence, it can be hypothesized that the cardioprotective effects of postconditioning may involve the reduction of the peak generation of reactive oxygen species or the reduction in neutrophil-endothelial cell interactions occurring during the first minutes of reperfusion. The observations by Zhao et al. [9,10] suggest that the few minutes during which postconditioning is imposed are critical to its cardioprotection. However, it has not been shown previously that the cardioprotection by postconditioning is dependent on the early moments of reperfusion. Accordingly, the present study was designed to test the hypothesis that the brief period of postconditioning applied at the onset of reperfusion is critical to the cardioprotection of that intervention. In addition, the cardioprotection of postconditioning is compared to that of classical preconditioning in the rat model, which has been previously reported only for canine [10] and rabbit models [23].

2. Methods

2.1. Animal care

All animals received humane care in compliance with 'The Guide for the Care of Use of Laboratory Animals' published by the National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.2. Surgical preparation

Male Sprague–Dawley rats weighing 270–360 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg) followed by continuous inhalation of 0.5–1.5% isoflurane after endotracheal intubation with a 14 gauge tube. The animals were ventilated with oxygen-enriched room air using a rodent respirator (Harvard Rodent Ventilator Model 683, 45–60 breaths per minute, and tidal volume was set to 1.0 ml/100 mg body weight). Normal blood gas levels and acid–base status were maintained by adjusting the rate and tidal volume or by intravenous administration of sodium bicarbonate as necessary to correct for acidemia. The left carotid artery was cannulated with a 24-gauge angiocath connected to a fluid-filled pressure transducer to monitor mean arterial pressure and heart rate. The right external jugular vein was cannulated for fluid administration. The chest was opened via a left thoracotomy through the fourth or fifth intercostal space, and the ribs were gently retracted to expose the heart. After pericardiotomy, a 6–0 proline (Ethicon, NJ) ligature was placed under the left main coronary artery, and the ends of the tie were threaded through a small plastic (PE50) tube to form a snare for reversible left coronary artery (LAD) occlusion. Heparinization was maintained during the experimental period with a bolus injection of 100 U/kg sodium heparin. The body temperature was monitored by rectal thermometer and maintained constant between 37 and 38 °C by a heating pad. Cardiac function was analyzed using SPECTRUM cardiovascular acquisition and analysis software (Wake Forest University, Winston-Salem, NC, USA).

2.3. Experimental protocol

In all rats, the left coronary artery (LCA) was occluded for 30 min, and then reperfused by loosening the ligature for 3 h. The rats were assigned to one of six groups based upon the intervention ($n = 6–8$ in each group, Fig. 1): (1) Control: there was no intervention either before or after LCA occlusion; (2) Ischemic preconditioning (IPC): the LCA was occluded for 5 min followed by 10 min of reperfusion before the prolonged occlusion; (3) Post-con 1: immediately at the onset of
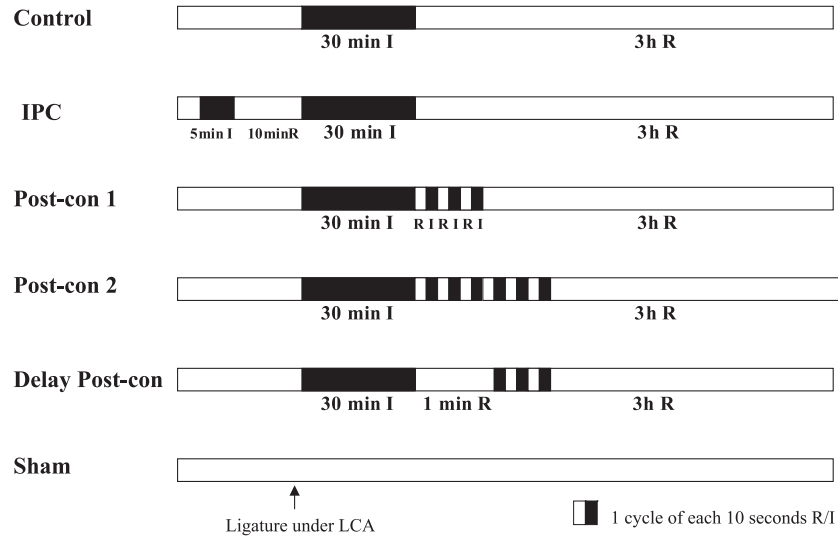
Fig. 1. Experimental protocol: Control ($n = 8$); occlusion of LCA for 30 min (dark bar), was followed by 3 h of reperfusion (open bar). Ischemic preconditioning (IPC, $n = 8$); the LCA was occluded (I, dark bar) for 5 min followed by 10 min of reperfusion (R, open bar) before the prolonged occlusion; Post-con 1 ($n = 8$); 10 s of full R and 10 s of re-occlusion I were repeated for three cycles; Post-con 2 ($n = 8$); 6 cycles 10 s R and 10 s (I) was applied; the remainder of the protocol was identical to Post-con 1; Delayed Post-con (delayed postconditioning, $n = 8$); the three-cycle algorithm was applied after 1 min of full unbridled reperfusion; Sham ($n = 6$); a ligature was placed under the LCA (arrow) but was not tightened; a time course similar to control (1.5 h) was followed. Post-con = postconditioning.

reperfusion, reflow was initiated with 10 s of full coronary flow, followed by 10 s of re-occlusion, repeated twice more for a total of three cycles (1 min total intervention); (4) Post-con 2; the reflow–occlusion algorithm described above was repeated for six cycles (2 min total intervention); (5) Delayed Post-con; the ligature was completely released for full reperfusion for 1 min (the duration of the Post-con 1 algorithm), after which three cycles of occlusion–reperfusion were applied as in Post-con 1; and (6) Sham operation; after placing the ligature under the LCA, the total experimental time course from the other groups was followed.

2.4. Area at risk and infarct size

At the end of experiment the area at risk was determined by injecting 1 ml of 20% unisperse blue dye via the external jugular vein after the LAD was ligated. Extra-left ventricular tissue was removed and the left ventricle was sliced transversely into five to six slices. The non-stained area at risk (AAR) was separated from the blue-stained non-ischemic zone myocardium, and the AAR was incubated in a 37 °C 1% solution of buffered (pH 7.4) triphenyltetrazolium chloride (TTC) for 15 min to identify the area of necrosis (AN) within the AAR. The AAR was expressed as a percentage of the left ventricular mass (AAR/LV), and the AN was expressed as a percentage of the AAR (AN/AR), the mass of each area being determined gravimetrically [24].

2.5. Plasma creatine kinase (CK) activity

Arterial blood samples (0.3 ml) were collected at baseline, end of ischemia, and after 180 min of reperfusion, and

centrifuged at $2500 \times g$ and 4 °C for 10 min. The plasma was analyzed spectrophotometrically for CK activity and protein concentration (CK-10 kit, Sigma, St. Louise, MO). Plasma CK activity was expressed as international units per gram of protein (IU/g).

2.6. Determination of tissue myeloperoxidase (MPO) activity

After determining infarct size, tissue samples were taken from non-ischemic and AAR zones for analysis of MPO activity, a marker of neutrophil accumulation in myocardium, by the method of Mullane et al. [25]. The samples were frozen and stored at -80 °C. The activity of MPO was measured spectrophotometrically at 460 nM (SPECTRA-max, Molecular Devices, Sunnyvale, CA) and expressed as units (U) per 100 g tissue as previous described [26].

2.7. Determination of plasma malondialdehyde (MDA) activity

Plasma malondialdehyde (MDA), a presumptive marker of oxidant-mediated lipid peroxidation, was quantified to estimate the extent of lipid peroxidation in the AAR myocardium [27]. Arterial blood samples (0.6 ml) were collected at baseline, the end of ischemia, and after 180 min of reperfusion. MDA product has a long half-life in plasma, and its levels are therefore cumulative. These samples were immediately centrifuged at $2500 \times g$ and 4 °C for 10 min, and the plasma stored at -80 °C until analyzed. The activity of MDA was measured using a commercial kit

(Lipid Peroxidation Assay Kit, Calbiochem, USA) and expressed as µM per ml plasma.

2.8. Post-experimental tissue superoxide anion by dihydroethidium fluorescence

Superoxide anion generation from ischemic-reperfused myocardium was determined using dihydroethidium (DHE) fluorescence [28]. The cell permeable DHE stain is rapidly oxidized to fluorescent ethidium by superoxide anions, which is then intercalated into DNA. Fluorescent ethidium is therefore a presumptive marker of intracellular superoxide anion generation at that point in time. Imme-

diately after the reperfusion, transmural tissue samples of the AAR were placed in cold saline, and embedded in OCT for cryosectioning. These samples were not subjected to TTC staining. About 20 µm tissue sections were cut using a Hacker-Bright cryostat, thaw-mounted on Fisher-Plus (Fisher Scientific) slides, and stained with 10 µM DHE at 37 °C for 30 min. The fluorescent image was obtained using a fluorescence microscope with a 585-nm long-pass filter attached to an image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). Superoxide generation in myocardium is expressed as red fluorescence. At least five determinations were performed in each group.
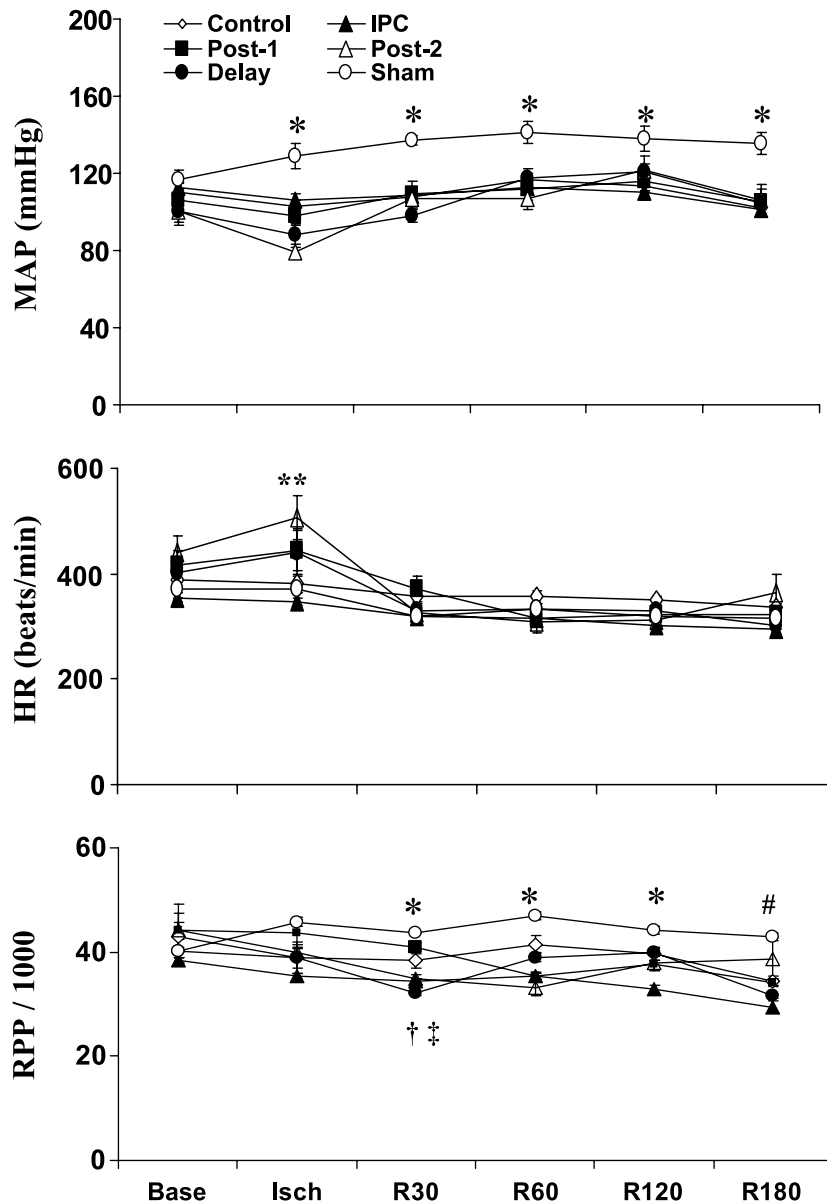

Fig. 2. Hemodynamic data during ischemia and reperfusion in the six groups. MAP=mean arterial pressure; HR=heart rate; RPP=rate-pressure product; base=baseline; isch=end ischemia; R30, 60, 120, 180=minutes of reperfusion. *$p<0.05$ Sham vs. other groups; **$p<0.05$ Post-2 vs. IPC; #$p<0.05$ Sham vs. control, Post-1 IPC and delay postconditioning groups; †$p<0.05$ Post-con 1 vs. Delayed postconditioning; ‡$p<0.05$ Control vs. Delayed Postconditioning; Group abbreviation as defined in Fig. 1. Values are means±SEM.
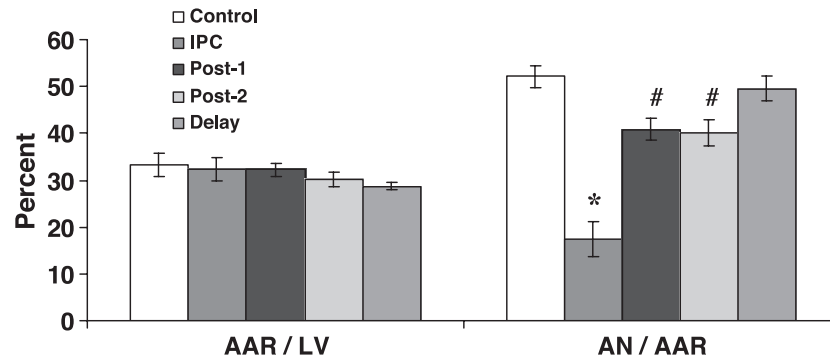
Fig. 3. Bar graph showing area at risk (AAR) expressed as a percentage of the left ventricle (LV) and area of necrosis expressed as a percentage of the AAR. IPC, Post-con 1 and 2 significantly reduced infarct size (AN/AAR). However, infarct size was not changed compared to Control in Delayed Post-con group. *$p < 0.001$ IPC vs. Control and Post-1 and Post-2 groups; #$p < 0.05$ Post-1 and Post-2 vs. Control and delayed postconditioning groups; Group abbreviations as defined in Fig. 1. Values are means ± SEM.
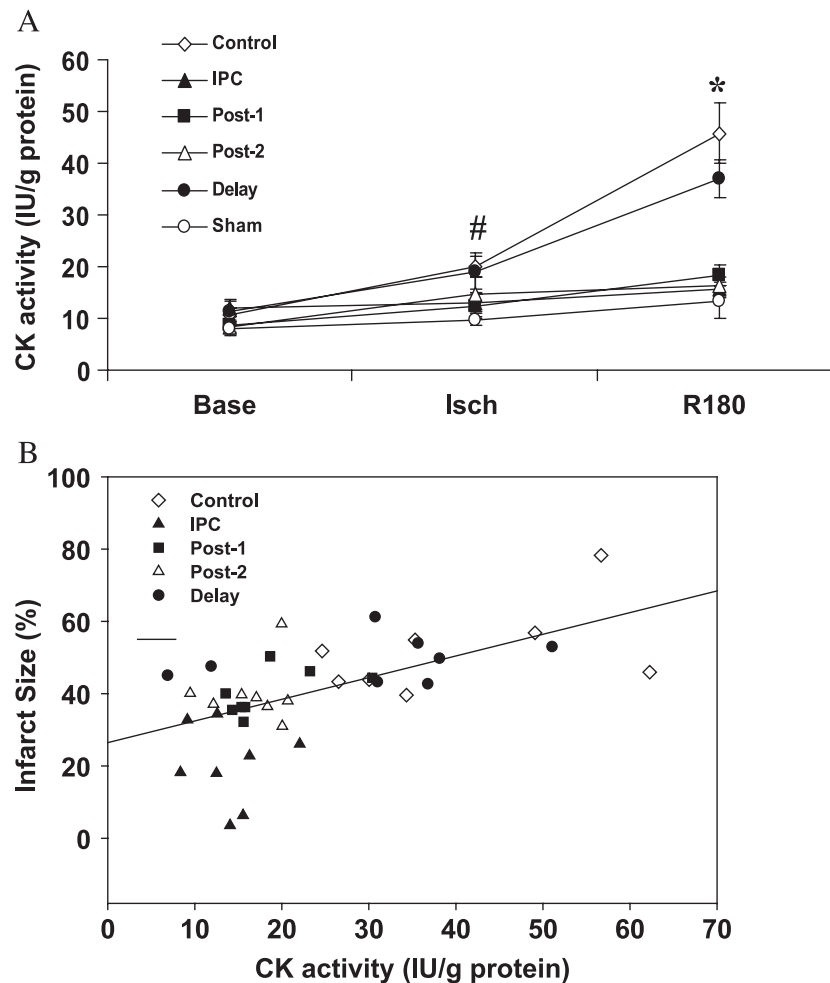

Fig. 4. Panel A: Plasma creatine kinase (CK) activity during the time course of the experiment. Base = baseline; isch = ischemia; and R180 = 180 min of reperfusion. CK activity at R180 was significantly lower in IPC, Post-con 1, 2 and Sham groups (without differences between these groups) compared to Control and Delayed postconditioning. CK activity in the Delayed Post-con group was comparable to that in the Control group at R180. *$p < 0.001$ Control vs. IPC, Post-con 1, 2 and Sham. #$p < 0.05$ Control vs. Sham; Group abbreviations as defined in Fig. 1. Values are means ± SEM. Panel B: Scatter plot showing the relation between plasma CK activity at 3 h of reperfusion and infarct size in each group. The relation was $y = 26.8 + 0.59_x$, $r = 0.57$.

2.9. Statistical analysis

All data are expressed as means ± standard error of the means. All data were analyzed using SigmaStat 2.0 for Windows statistical software package (SPSS, Chicago, IL). A one-way analysis of variance (ANOVA) and ANOVA for repeated measures were used as appropriate on data in all six groups, with post-hoc analysis between groups using the Student–Newman–Keuls test correcting for multiple comparisons. A *p*-value less than 0.05 was considered significant.

3. Results

3.1. Hemodynamic data

Hemodynamic data for mean arterial pressure (MAP), heart rate (HR), and rate pressure product (RPP) in the six groups are shown in Fig. 2. There were no significant differences among the six groups at baseline. However,

MAP and RPP of the Sham group were higher during the experiment than that of the other groups. During LCA occlusion, there were trends for a decrease in MAP and an increase in HR in all groups except for the Sham group. The RPP was significantly lower in the Delayed Post-con group than Control and Post-con 1 at 30 min of reperfusion.

3.2. Area at risk and infarct size

The area placed at risk by LCA occlusion was comparable among the six groups, averaging between 29% and 33% (Fig. 3). Infarct size, expressed as a percentage of the area at risk (AN/AAR), was significantly smaller in the IPC group, representing a 60% reduction from the control group. By ANOVA, infarct size was significantly smaller in the Post-con 1 ($p = 0.021$) and Post-con 2 ($p = 0.024$) groups compared to that in the Control group, but was significantly larger than that in the IPC group ($p < 0.001$). Delaying the postconditioning intervention reversed the infarct size reduction to values comparable to the Control group.
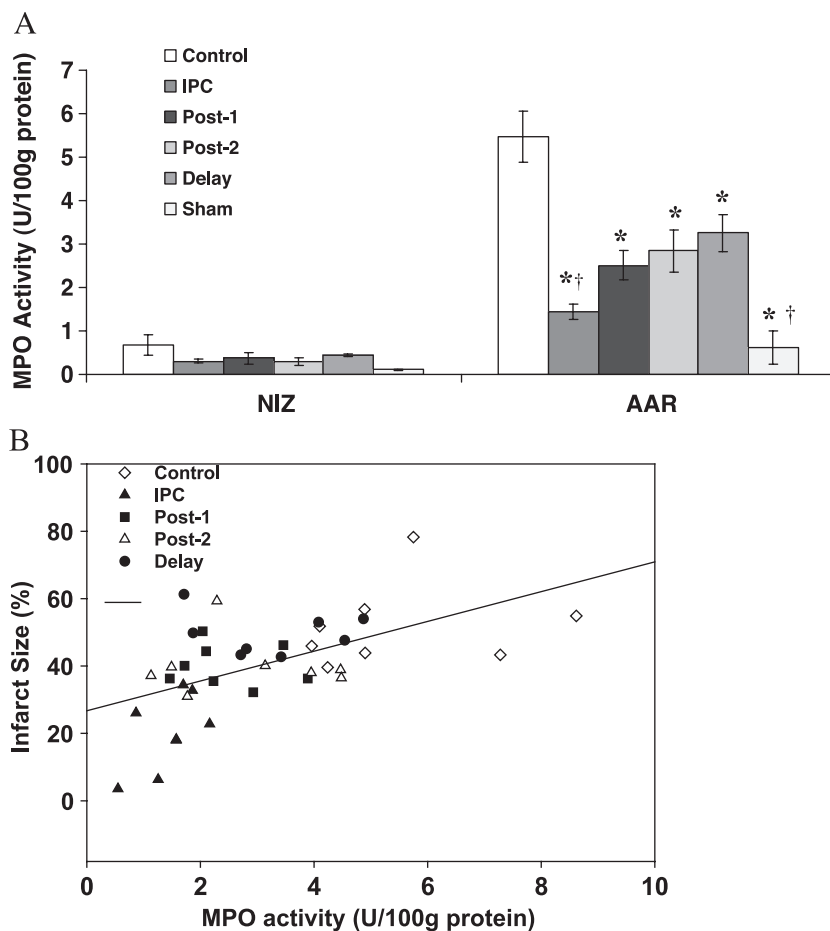

Fig. 5. Panel A: Myeloperoxidase (MPO) activity in the non-ischemic and area at risk (AAR) of left ventricular myocardium. MPO activity in the non-ischemic zone (NIZ) was low and relatively comparable among the six groups. MPO activity was significantly inhibited in IPC, Post-con 1, 2 and Delayed Post-con groups relative to that in the Control group. *$p<0.05$ vs. Control; †$p<0.05$ vs. Post-con groups. Group abbreviation as defined in Fig. 1. Values are mean±SEM. Panel B: Scatterplot showing the relation between MPO activity and infarct size at the end of the experiment in each group. The linear equation is $y=26.1+4.5\chi$, $r=0.55$.
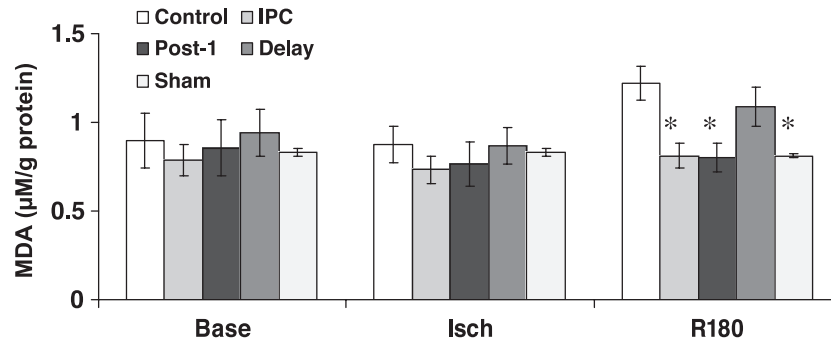
Fig. 6. Plasma malondialdehyde (MDA) activity during the course of the experiment in the five groups used for comparison ($n = 6$ for each group). Base = baseline; isch = ischemia; and R180 = 180 min of reperfusion. There were no statistical differences among the five groups at baseline or after ischemia. However, plasma MDA was significantly greater in both Control and Delayed Post-con at R180 compared to IPC, Post-con 1 and Sham, which remained significantly lower. *$p < 0.05$ vs. Control and Delayed Postcon. Values are means ± SEM.

3.3. Plasma creatine kinase (CK) activity

Plasma CK activity at baseline was comparable among the six groups (Fig. 4A). After 30 min of ischemia, plasma CK activity was significantly greater in Control compared to Sham. The plasma CK activity at 3 h of reperfusion was significantly less in the IPC, Post-con 1, Post-con 2 and Sham groups compared to Control. There was no significant difference between IPC, Post-1 and Post-2 groups. Plasma CK activity at 3 h of reperfusion in the Delayed Post-con was significantly greater than that in the other interventional groups, and was comparable to that in the Control group. The linear relationship between

CK activity at 3 h of reperfusion and infarct size was significant, with an $r = 0.57$, as shown in Fig. 4B.

3.4. Myocardial MPO activity

MPO activity in the non-ischemic zone was low and comparable among the six groups (Fig. 5A). MPO activity in the AAR after ischemia–reperfusion was significantly greater compared to the non-ischemic zone in all groups except for the Sham group in which there was no significant difference between AAR and non-ischemic zone. However, MPO activity in the AAR myocardium was significantly less in the IPC group, Post-con 1 group, the Post-con 2
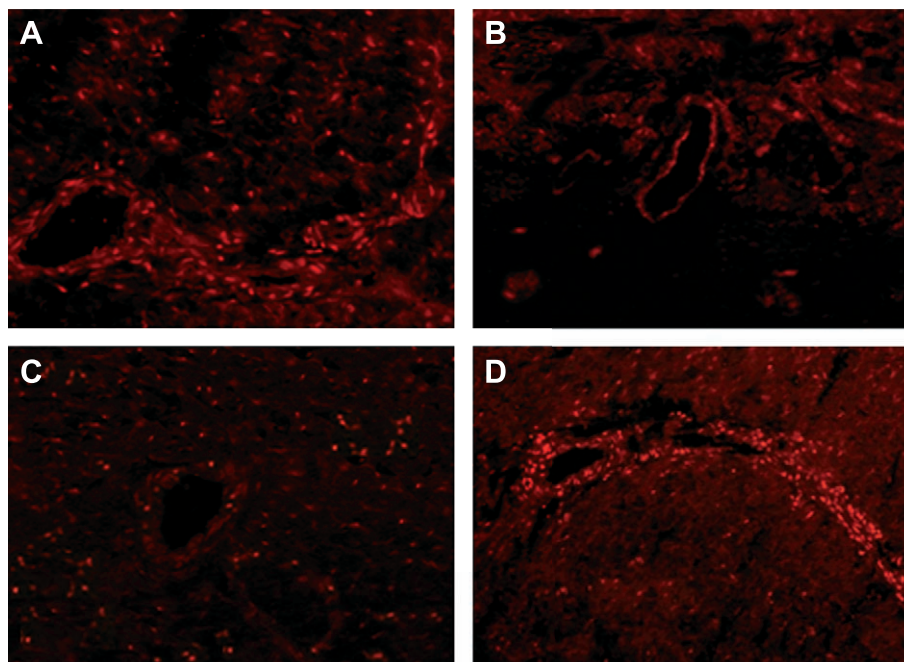

Fig. 7. Area at risk myocardium harvested after reperfusion and stained with DHE to visually assess generation of superoxide anions. Each panel shows one to two small blood vessels and adjacent myocardium. Panel A: Untreated ischemia–reperfusion (Control group); Panels B and C: area at risk myocardium from ischemic preconditioned and postconditioned hearts, respectively; Panel D: increased intensity of DHE staining in the area at risk from the Delayed Post-con group localized to perivascular and adjacent myocardium. Data consisted of at least five determinations in each group.

group and the Delayed Post-con group relative to that in the Control group ($p < 0.001$). There was no statistical difference in MPO activities between the two immediate postconditioning groups and the delayed postconditioning group. However, MPO activity in the AAR of the IPC group was significantly lower than that observed in the three postconditioning groups. The correlation between MPO activity and infarct size in each group at R180 is shown in Fig. 5B. The linear relationship was significant with an $r = 0.55$. The lowest values of both MPO and infarct size were in the IPC, Post-1 and Post-2 groups, while delaying postconditioning increased both CK activity and infarct size. The greatest values for infarct size and MPO activity were observed in the Control group.

3.5. Plasma MDA levels during ischemia and reperfusion

An additional 30 rats were used to obtain sufficient blood to measure plasma MDA in all groups; MDA was not measured in the Post-con 2 group because there was no difference in outcome variables compared to the Post-con 1 group. There were no statistical differences among these five groups at baseline and after 30 min of ischemia (Fig. 6), at which time the plasma MDA in IPC, Post-con 1 and Sham groups was significantly lower than that in the Control group; plasma MDA levels were comparable among these three groups. Plasma MDA at 180 min of reperfusion in the Delayed Post-con group was significantly higher than the other interventional groups, and was comparable to that in the Control group.

3.6. Post-experimental tissue superoxide anion generation

Dihydroethidium fluorescence, a marker of superoxide anion generation, in ischemic-reperfused myocardium is shown in Fig. 7. Robust fluorescence is observed in ischemic-reperfused myocardium from the Control group (Panel A), with a preponderance of fluorescence localized in the area surrounding and adjacent to an arteriole. In contrast, red fluorescence in myocardium from the IPC and Post-con groups showed less fluorescence in areas surrounding blood vessels, as well as in adjacent myocardium (Panels B and C). However, the red fluorescence signal was greater in myocardium in which postconditioning was delayed (Panel D), suggesting a robust generation of superoxide anions.

4. Discussion

4.1. General discussion

Previous studies by Zhao et al [9,10] reported that postconditioning in a canine model of coronary occlusion–reperfusion reduced multiple manifestations of ischemia–reperfusion injury, including infarct size and endothelial dysfunction. The results from that study suggested that the early moments of reperfusion were important in the pathogenesis of post-ischemic injury, and that manipulation of this early reperfusion phase could reduce these downstream physiological consequences of ischemia–reperfusion injury [10]. In the present study, we confirm in a rat myocardial ischemia–reperfusion model that three cycles of postconditioning applied over the first minute of reperfusion optimally reduced infarct size. Extending the postconditioning algorithm to six 10-second "on–off" cycles over the first two minutes of reperfusion did not further reduce infarct size. The reduction in infarct size with immediate postconditioning was corroborated by a decrease in CK activity. In addition, postconditioning attenuated neutrophil accumulation in the area at risk. Furthermore, postconditioning was associated with a reduction in plasma MDA activity indicative of oxidant-mediated injury to membrane phospholipids, and a decrease in fluorescence intensity of DHE staining from area at risk myocardium excised at the end of reperfusion. Importantly, the infarct sparing advantage of postconditioning and the reduction in MDA and DHE fluorescence intensity were lost when postconditioning was delayed for the one-minute period of time otherwise required to apply the three-cycle intervention, during which time reperfusion was unbridled and uncontrolled. These data support the general concept that postconditioning attenuates ischemia–reperfusion injury in agreement with our previous report in a canine model of acute ischemia–reperfusion [9]. In addition, these data suggest that the early moments of reperfusion during which immediate postconditioning is applied are critical to its protection. Whether deleterious mechanisms were attenuated, or whether beneficial mechanisms were triggered by postconditioning is not clear at this time. However, a preliminary report by Yang et al. [23] suggest that postconditioning triggers intracellular signaling mechanisms involving extracellular-signaling regulated kinases (ERK/Akt), mitochondrial K_{ATP} channels, and release of nitric oxide. In addition, this study lends further support to the concept that the early phase of reperfusion is critical in the pathogenesis of post-ischemic injury, as suggested previously by Tsao et al. [29] and others [12,30].

The reduction in infarct size observed in the rat model of ischemia–reperfusion was not as robust as that reported for the canine model [10]. The algorithm for postconditioning was more compressed in the rat model than in the canine model, in which an algorithm of three cycles of 30 s reperfusion and 30 s re-occlusion was applied during the first 3 min of reperfusion. The relatively modest reduction in infarct size in the rat compared to the canine contrasted to the large decrease in infarct size achieved with ischemic preconditioning, which showed an infarct size reduction of approximately 60%, similar in extent as observed in the canine model [10]. Rats are known to have greater xanthine oxidase activity

and greater production of oxygen radicals than other species [31,32]. The rat has 59 times the xanthine oxidase activity than rabbits, which are relatively deficient in xanthine oxidase activity [33]. In addition, the endogenous anti-oxidant defense system consisting of catalase, superoxide dismutase, and glutathione peroxidase is less robust in rats than in porcine and human myocardium [32]. The canine is reported to have significant xanthine oxidase in myocardium, but whether this contributes to ischemia–reperfusion injury and infarct size specifically is controversial [34–36]. The infarct sparing effect of postconditioning in the rabbit [23] was greater than that observed in the rat in the present study, and was consistent with that observed in the canine model by Zhao et al. [10]. However, whether the differences between species in infarct size reduction with postconditioning is related to species differences in xanthine oxidase activity or differences in the endogenous anti-oxidant systems remains to be determined.

Numerous studies support the concept that reactive oxygen species (ROS), such as superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicals, contribute to myocardial tissue injury secondary to ischemia and reperfusion [37–39]. There is a significant burst of oxygen-derived free radicals generated within the first minutes of reperfusion [18] peaking 4–7 min after the onset of reperfusion in some models, followed by a persistently elevated generation thereafter [19,39]. This oxidative burst may be derived in vivo to some extent from activated neutrophils [18]. Oxygen radicals trigger the release of pro-inflammatory mediators, transcription factors such as NF-κB, and stimulate the surface expression of adhesion molecules on coronary vascular endothelium [40]. The observations from the present study that DHE staining in the area at risk and plasma MDA after 3 h of reperfusion are reduced are in accord with the previous observations by Zhao et al. [9,10] in the canine model, and suggest that at least the later phase of ROS generation is attenuated by postconditioning. The larger infarct size observed when postconditioning was delayed was associated with more intense DHE fluorescence and elevated MDA levels. These observations would suggest that postconditioning attenuated ROS generation, and that this mechanism was an important component of the cardioprotection afforded by postconditioning. However, in the absence of direct measures of ROS, and the absence of data that ROS generators administered intracoronary during postconditioning prevents myocardial salvage with postconditioning, we cannot distinguish whether the reduction in ROS generation (DHE staining) or products (MDA) was a result of infarct limitation, or was a cause of infarct limitation. We speculate that postconditioning may reduce ROS generation by limiting the delivery of oxygen during the rapidly repetitive occlusions. However, the role of ROS in the pathogenesis of post-ischemic injury is still somewhat controversial since the use of

oxygen radical scavengers has yielded mixed results [41]. In addition, postconditioning may alter the washout or release patterns of autacoids such as adenosine and nitric oxide [23] that may affect ROS generation from neutrophils and other cell sources [42–44].

In the present study, postconditioning attenuated neutrophil accumulation in the area at risk myocardium as measured by MPO activity. Numerous studies have reported that neutrophils migrate into and accumulate in ischemic-reperfused myocardium [45–47]. The process of neutrophil accumulation within the area at risk begins immediately after the onset of reperfusion[29,46,48] and continues for 24 h in canine models [49]. Moreover, numerous studies have demonstrated that a reduction in neutrophil activity and accumulation is associated with a concomitant reduction in infarct size [50–52]. However, it is still controversial whether the presence of neutrophils in reperfused myocardium is causally related to subsequent injury during reperfusion, or if the accumulation of neutrophils is simply an inflammatory response to injury that results from other causes, such as oxygen radicals, cytokines, etc. [53]. In the present study, the reduction in neutrophil accumulation with postconditioning could be the result of either process. The attenuation of neutrophil accumulation by postconditioning may have directly reduced infarct size (cause and effect relationship), or have reduced other pathological processes such as chemotactic signals that attract neutrophils to sites of injury, but without having a direct effect on the pathogenesis of infarction. Alternatively, postconditioning could have attenuated neutrophil oxygen radical generation without altering adherence to coronary vascular endothelium and accumulation. Neutrophils may be activated to generate superoxide anions by soluble pro-inflammatory mediators such as platelet activating factor. This dissociation between neutrophil accumulation and infarct size reduction was also observed by Sato et al. [11] in which infarct size was reduced but neutrophil accumulation was significantly greater compared to untreated controls when reperfusion was gradually initiated over 30 min. In that study, it was suggested that low shear stress during the low-flow conditions of gradual reperfusion may have reduced the release of the anti-neutrophil autacoid nitric oxide [54,55] thereby permitting the adherence of neutrophils to the coronary vascular endothelium. This is clearly not the case with delayed postconditioning in which shear stress would be high during hyperemic blood flow during the first minute of reperfusion in the rat model. The reduction of neutrophil accumulation in the area at risk without a concomitant reduction in infarct size observed with delayed postconditioning would argue against inhibition of neutrophil adherence-dependent mechanisms, but would not rule out adherence-independent mechanisms. However, it is difficult to differentiate between adherence-dependent and -independent mechanisms in vivo, but such distinction could be made using

a hypoxia-reoxygenation model of cultured endothelium or cardiomyocytes co-incubated with neutrophils. Hence, further studies are warranted on the role of neutrophils in the cardioprotection exerted by postconditioning.

4.2. Limitations

The present study did not determine whether the early burst or the sustained elevated levels of oxidant generation during the first minutes of reperfusion were attenuated by postconditioning. However, the greater DHE fluorescence in the area at risk tissue implies that at least superoxide anion generation occurring at 3 h of reperfusion was reduced by postconditioning. In addition, the present study did not identify the species of oxidants, nor its sources, that were potentially attenuated by postconditioning. Furthermore, plasma MDA levels were measured in a separate group of rats in which infarct size was not concomitantly measured. Hence, potential variability between these two end points may preclude drawing direct conclusions on the relationship between plasma MDA and infarct size.

In summary, this study reports for the first time that ischemic postconditioning reduces infarct size in an in vivo rat model, in general agreement with the myocardial salvage shown in the in vivo canine model [9]. The less robust cardioprotection in the rat compared to the canine [10] or rabbit [23] model may be specific to the rodent species, a point which must be further pursued before implications regarding its clinical impact in humans should be raised. The reduction in plasma DHE and MDA fluorescent staining suggests that myocardial salvage may be mediated by a reduction in oxidant activity, potentially during the early minutes (the burst) and/or the prolonged phase of reperfusion, but a cause and effect relationship can not be definitively implied. In addition, the data suggest that direct attenuation of neutrophil accumulation within the area at risk myocardium may not be a primary mechanism of protection, but this does not rule out a role for other neutrophil-related functions such as generation of reactive oxygen species or protease enzymes, or generation of cytokines that may amplify or perpetuate the inflammatory-like response to ischemia–reperfusion. Finally, the advantage of postconditioning is lost when the maneuver is delayed for 1 min, suggesting that this early period of reperfusion is critical to the pathogenesis of infarction, and suggesting moreover that delaying the application of therapeutics beyond this window may not be optimally efficacious. It is not known whether postconditioning is less effective with longer ischemic times, as has been reported in the canine model [56].

Postconditioning is very simple to apply in the human catheterization laboratory, and can be initiated with intra-coronary balloon catheters during percutaneous transluminal coronary angioplasty (PTCA), and may be applied during coronary artery bypass grafting (CABG), especially during off pump CABG. The short duration of the intervention, as well as its timing at reperfusion when the patient presents to the catheterization laboratory for interventional procedures or for off-pump surgery, may make postconditioning a feasible clinical strategy to reduce ischemia–reperfusion injury. However, delaying the postconditioning intervention for even a few minutes while changing balloon catheters, or while allowing balloons to remain deflated beyond the period of time suggested by the algorithm, may abrogate the cardioprotective advantage of postconditioning.

Acknowledgements

References

[1] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–36.

[2] Cohen MV, Yang XM, Downey JM. Conscious rabbits become tolerant to multiple episodes of ischemic preconditioning. Circ Res 1994;74:998–1004.

[3] Hagar JM, Hale SL, Kloner RA. Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. Circ Res 1991;68:61–8.

[4] Kita H, Miura T, Tsuchida A, et al. Suppression of reperfusion arrhythmias by preconditioning is inhibited by an ATP-sensitive potassium channel blocker, 5-hydroxydecanoate, but not by protein kinase C blockers in the rat. J Cardiovasc Pharmacol 1998;32:791–7.

[5] Lu H, Remeysen P, De Clerck F. The protection by ischemic preconditioning against myocardial ischemia- and reperfusion-induced arrhythmias is not mediated by ATP-sensitive potassium channels in rats. Cor Art Dis 1993;4:649–57.

[6] Miura T, Ishimoto R, Sakamoto J, et al. Suppression of reperfusion arrhythmia by ischemic preconditioning in the rat: is it mediated by the adenosine receptor, prostaglandin or bradykinin receptor? Basic Res Cardiol 1995;90:240–6.

[7] Liu YG, Downey JM. Ischemic preconditioning protects against infarction in rat-heart. Am J Physiol 1992;263:H1107–11.

[8] Lott FD, Guo P, Toombs CF. Reduction in infarct size by ischemic preconditioning persists in a chronic rat model of myocardial ischemia–reperfusion injury. Pharmacology 1996;52:113–8.

[9] Zhao Z-Q, Corvera JS, Wang N-P, Guyton RA, Vinten-Johansen J. Reduction in infarct size and preservation of endothelial function by ischemic postconditioning: comparison with ischemic preconditioning. Circulation 2002;106:II314 [Suppl., Abstract].

[10] Zhao Z-Q, Corvera JS, Halkos ME, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol (Heart Circ Physiol) 2003;285:579–88.

[11] Sato H, Jordan JE, Zhao Z-Q, et al. Gradual reperfusion reduces infarct size and endothelial injury but augments neutrophil accumulation. Ann Thorac Surg 1997;64:1099–107.

[12] Vinten-Johansen J, Lefer DJ, Nakanishi K, et al. Controlled coronary hydrodynamics at the time of reperfusion reduces postischemic injury. Core Art Dis 1992;3:1081–93.

[13] Pisarenko OI, Shulzhenko VS, Studneva IM, et al. Effects of gradual reperfusion on postischemic metabolism and functional recovery of isolated guinea pig heart. Biochem Med Metabol Biol 1993;50: 127–34.

[14] Okamoto F, Allen BS, Buckberg GD, et al. Studies of controlled reperfusion after ischemia: XIV. Reperfusion conditons: importance of ensuring gentle versus sudden reperfusion during relief of coronary occlusion. J Thorac Cardiovasc Surg 1986;92:613–20.

[15] Hori M, Kitakaze M, Sato H, et al. Staged reperfusion attenuates myocardial stunning in dogs. Role of transient acidosis during early reperfusion. Circulation 1991;84:2135–45.

[16] Peng CF, Murphy ML, Colwell K, et al. Controlled versus hyperemic flow during reperfusion of jeopardized ischemic myocardium. Am Heart J 1989;117:515–22.

[17] Zweier JL, Flaherty JT, Weisfeldt ML. Direct measurement of free radicals generated following reperfusion of ischemic myocardium. Proc Natl Acad Sci 1987;84:1404–7.

[18] Duilio C, Ambrosio G, Kuppusamy P, et al. Neutrophils are primary source of O_2 radicals during reperfusion after prolonged myocardial ischemia. Am J Physiol (Heart Circ Physiol) 2001;280:H2649–57.

[19] Kevin LG, Camara AKS, Riess ML, et al. Ischemic preconditioning alters real-time measure of O_2 radicals in intact hearts with ischemia and reperfusion. Am J Physiol (Heart Circ Physiol) 2003;284: H566–74.

[20] Garlick PB, Davies MJ, Hearse DJ, et al. Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. Circ Res 1987;61:757–60.

[21] Lefer AM, Tsao PS, Lefer DJ, et al. Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. FASEB J 1991;5:2029–34.

[22] Tsao PS, Lefer AM. Time course and mechanism of endothelial dysfunction in isolated ischemic- and hypoxic-perfused rat hearts. Am J Physiol 1990;259:H1660–6.

[23] Yang X-M, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by activation of ERK and production of nitric oxide. Circulation (Suppl) 2003;108: IV745.

[24] Toombs CF, McGee DS, Johnston WE, et al. Myocardial protective effects of adenosine. Infarct size reduction with pretreatment and continued receptor stimulation during ischemia. Circulation 1992; 86:986–94.

[25] Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J Pharmacol Methods 1985;14:157–67.

[26] Zhao Z.-Q., Todd JC, Sato H, et al. Adenosine A_2-mediated cardioprotection during reperfusion does not involve K_{ATP} channel activation. Am J Physiol 1997;273:H1677–8.

[27] Di Pierro D, Tavazzi B, Lazzarino G, et al. Malondialdehyde is a biochemical marker of peroxidative damage in the isolated reperfused rat heart. Mol Cell Biochem 1992;116:193–6.

[28] Gu W, Weihrauch D, Tanaka K, et al. Reactive oxygen species are critical mediators of coronary collateral development in a canine model. Am J Physiol (Heart Circ Physiol) 2003;285:H1582–9.

[29] Tsao PS, Aoki N, Lefer DJ, et al. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation 1990;82:1402–12.

[30] Sato H, Zhao Z-Q, Jordan JE, Williams MW, Sarvotham SS, Vinten-Johansen J. Gradual restoration of reperfusion reduces coronary artery endothelial injury and myocardial infarction. FASEB J 1995;9:A421 [Abstract].

[31] Southard JH, Marsh DC, McAnulty JF, et al. Oxygen-derived free radical damage in organ preservation: activity of superoxide dismutase and xanthine oxidase. Surgery 1987;101:566–70.

[32] Janssen M, van der Meer P, de Jong JW. Antioxidant defences in rat, pig, guinea pig, and human hearts: comparison with xanthine oxidoreductase activity. Cardiovasc Res 1993;27:2052–7.

[33] Downey JM, Miura T, Eddy LJ, et al. Xanthine-oxidase is not a source of free-radicals in the ischemic rabbit heart. J Mol Cell Cardiol 1987;19:1053–60.

[34] Werns SW, Grum CM, Ventura A, et al. Xanthine oxidase inhibition does not limit canine infarct size. Circulation 1991;83:995–1005.

[35] Motoe M, Yoshida S. A useful canine model of ischemic myocardium with coronary retrograde flow diversion, and its application for the study of allopurinol on myocardial infarct size. Jpn Circ J 1991;55: 490–9.

[36] Reimer KA, Jennings RB. Failure of the xanthine oxidase inhibitor allopurinol to limit infarct size after ischemia and reperfusion in dogs. Circulation 1985;71(5):1069–75.

[37] Ambrosio G, Zweier JL, Flaherty JT. The relationship between oxygen radical generation and impairment of myocardial energy metabolism following post-ischemic reperfusion. J Mol Cell Cardiol 1991;23:1359–74.

[38] Bolli R. Oxygen-derived free radicals and myocardial reperfusion injury: an overview [Review]. Cardiovasc Drugs Ther 1991; 5(Suppl. 2):249–68.

[39] Jeroudi MO, Hartley CJ, Bolli R. Myocardial reperfusion injury: role of oxygen radicals and potential therapy with antioxidants [Review]. Am J Cardiol 1994;73:2B–7B.

[40] Fan H, Sun B, Gu Q, et al. Oxygen radicals trigger activation of NF-kB and AP-1 and upregulation of ICAM-1 in reperfused canine heart. Am J Physiol (Heart Circ Physiol) 2002;282:H1778–86.

[41] Miki T, Cohen MV, Downey JM. Failure of N-2-mercaptopropionyl glycine to reduce myocardial infraction after 3 days of reperfusion in rabbits. Basic Res Cardiol 1999;94:180–7.

[42] Lefer DJ, Nakanishi K, Johnston WE, et al. Anti-neutrophil and myocardial protecting action of SPM-5185, a novel nitric oxide (NO) donor, following acute myocardial ischemia and reperfusion in dogs. Circulation 1993;88:2337–50.

[43] Jordan JE, Thourani VH, Auchampach JA, et al. A_3 adenosine receptor activation attenuates neutrophil function and neutrophil-mediated reperfusion injury. Am J Physiol 1999;277:H1895–905.

[44] Zhao Z-Q, Sato H, Williams MW, et al. Adenosine A_2-receptor activation inhibits neutrophil-mediated injury to coronary endothelium. Am J Physiol 1996;271:H1456–64.

[45] Chatelain P, Latour J-G, Tran D, et al. Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. Circulation 1987;75:1083–90.

[46] Dreyer WJ, Michael LH, West MW, et al. Neutrophil accumulation in ischemic canine myocardium: insights into time course, distribution, and mechanism of localization during early reperfusion. Circulation 1991;84:400–11.

[47] Albertine KH, Weyrich AS, Ma X-L, et al. Quantification of neutrophil migration following myocardial ischemia and reperfusion in cats and dogs. J Leukoc Biol 1994;55:557–66.

[48] Mehta JL, Nichols WW, Mehta P. Neutrophils as potential participants in acute myocardial ischemia: relevance to reperfusion. J Am Coll Cardiol 1988;11:1309–16.

[49] Zhao Z.-Q., Nakamura M, Wang N-P, et al. Dynamic progression of contractile and endothelial dysfunction and infarct extension in the late phase of reperfusion. J Surg Res 2000;94:1–12.

[50] Curtis WE, Gillinov AM, Wilson IC, et al. Inhibition of neutrophil adhesion reduces myocardial infarct size. Ann Thorac Surg 1993;56: 1069–72 [discussion 1072-].

[51] Forman MB, Virmani R, Puett DW. Mechanisms and therapy of myocardial reperfusion injury. Circulation 1990;81(suppl. IV): IV-69–78.

[52] Hartman JC, Anderson DC, Wiltse AL, et al. Protection of ischemic/reperfused canine myocardium by CL18/6, a monoclonal an-

tibody to adhesion molecule ICAM-1. Cardiovasc Res 1995;30: 47–54.

[53] Baxter GF. The neutrophil as a mediator of myocardial ischemia–reperfusion injury: time to move on. Basic ResCardiol 2002;97: 268–75.

[54] Ma X-L, Tsao PS, Viehman GE, et al. Neutrophil-mediated vasoconstriction and endothelial dysfunction in low-flow perfusion-reperfused cat coronary artery. Circ Res 1991;69:95–106.

[55] Ma X-L, Weyrich AS, Lefer DJ, et al. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. Circ Res 1993;72: 403–12.

[56] Gumina RJ, Gross GJ. If ischemic preconditioning is the gold standard has a platinum standard of cardioprotection arrived? Comparison with NHE inhibition. J Thromb Thrombolysis 1999;8:39–44.