Molecular Medicine 7(6): 406–417, 2001

Myocardial Ischemic Preconditioning in Rodents Is Dependent on Poly (ADP-Ribose) Synthetase

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Accepted February 5, 2001

Abstract

Background: Activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS) in response to oxidant-mediated DNA injury has been shown to play an important role in the pathogenesis of reperfusion injury. Here we investigated the role of PARS in myocardial ischemic preconditioning (IPC).

Materials and Methods: Mice with or without genetic disruption of PARS and rats in the absence or presence of the PARS inhibitor 3-aminobenzamide underwent coronary occlusion and reperfusion with or without IPC.

Results: Both poly(ADP-ribose) synthetase (PARS) deficiency and ischemic preconditioning (IPC) induced protection from reperfusion injury, attenuated inflammatory mediator production, and reduced neutrophil infiltration when compared to the response in wild-type mice. Surprisingly, the protective effect of IPC not only disappeared in $PARS^{2\,y2}$ mice, but the degree of myocardial injury and inflammatory response was similar to the one seen in wild-type animals. Similarly, in the rat model of IPC, 3-aminobenzamide pretreatment blocked the beneficial effect of IPC. Myocardial NAD^{1} levels were maintained in the PARS-deficient mice during reperfusion, while depleted in the wild-type mice. The protection against reperfusion injury by IPC was also associated with partially preserved myocardial NAD^{1} levels, indicating that PARS activation is attenuated by IPC. This conclusion was further strengthened by poly(ADP-ribose) immunohistochemical measurements, demonstrating that IPC markedly inhibits PARS activation during reperfusion.

Conclusions: The mode of IPC's action is related, at least in part, to an inhibition of PARS. This process may occur either by self-auto-ribosylation of PARS during IPC, and/or via the release of endogenous purines during IPC that inhibit PARS activation during reperfusion.

Introduction

Poly (ADP ribose) synthetase (PARS, also known as poly [ADP ribose] polymerase or PARP) is an abundant nuclear enzyme of eukaryotic cells, which has been implicated in response to DNA injury (1). Free radical- and oxidant-induced cellular injury have been proposed to involve the activation of PARS. When activated by DNA single-strand breaks, PARS initiates an energy-consuming cycle by transferring ADP ribose units to nuclear proteins. The result of this process is a rapid depletion of the intracellular NAD^{1} and ATP pools which slows the rate of glycolysis and mitochondrial respiration, leading to cell necrosis (2,3).

Activation of PARS has recently been proposed to play an important role in the development of myocardial reoxygenation injury. We recently demonstrated that oxygen-derived free radicals and peroxynitrite (a reactive oxidant produced from nitric oxide and superoxide), which are known to be formed in myocardial reperfusion injury, activate PARS and induce resultant cytotoxicity in various cell types including cultured cardiac myoblasts (4–8). Furthermore, we demonstrated that absence of PARS prevents the functional alterations associated with hypoxia-reoxygenation injury in isolated work-performing mouse hearts (9). In addition, we demonstrated that the early phase of myocardial reperfusion injury is ameliorated by pharmacologic inhibition of PARS (10), or in genetically engineered mice, which lack functional PARS enzyme ($PARS^{2\,y2}$) (11,12). Similar findings have also been obtained in a number of other laboratories: genetic inactivation or pharmacologic inhibition of PARS was found to protect against myocyte oxidative injury in vitro, or myocardial ischemia and reperfusion in vivo (13–19).

Myocardial tolerance against infarction is substantially increased by exposing myocytes to short periods of transient ischemia. In this phenomenon, termed "ischemic preconditioning" (IPC), a number of pathways and mechanisms have been implicated, including the adenosine and its receptors; protein

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kinase C; opening of ATP-sensitive potassium (KATP) channels; and protein kinases, such as tyrosine kinase and protein kinase C (20–24). In the present study, by comparing the response to IPC followed by myocardial ischemia/reperfusion in wild-type and PARS-deficient animals and in animals with or without pharmacologic inhibition of PARS, we investigated the potential role of PARS in myocardial IPC. We hypothesized that inactivation of PARS due to endogenous processes during IPC contributes to the acute window of cardioprotection offered by IPC.

Materials and Methods

All parts of the current investigation conform to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and with the approval of the Institutional Animal Care and Use Committee.

Myocardial Ischemia and Reperfusion in Wild-Type and PARS-Deficient Mice

Breeding pairs for the PARS-deficient and wild-type colony of mice were kindly provided by Dr. Z.Q. Wang of the Institute of Molecular Pathology, Vienna, Austria. $PARS^{2\,y2}$ mice and littermate wild-type controls ($PARS^{1\,y1}$) (129/Sv 3 C57BL6, 20–30 g) were genotyped as previously described (25) and used in the myocardial ischemia-reperfusion experiments as described previously (12) and below. The isoform lacking in the PARS-deficient animals is the first discovered major PARS isoform currently known as PARS-1 (or PARP-1).

Animals were anesthetized with sodium pentobarbital (100 mg/kg, i.p.). The animals were placed in a supine position with their paws and tails taped to the operating table. Their heads were retracted by a thin rubber band fastened to the upper incisors. The upper portion of the trachea was exposed through a middle incision in the neck and the pretracheal muscles were bluntly dissected. A black-tipped endotracheal tube, made of PE-60 tubing, was inserted by way of the mouth into the trachea with the black tip placed 5–8 mm below the thyroid cartilage. Artificial respiration was maintained by a respirator with an Fio_2 of 0.80, a frequency of 100 strokes/min, and a tidal volume of 0.8–1.2 ml to maintain normal arterial Pao_2, $Paco_2$, and pH. The middle skin incision in the neck was extended down to the xiphoid. The left pectoris major and the muscle beneath it were dissected longitudinally, without cutting these muscles, to expose the left 3rd and 4th ribs. A parasternal incision was made to open the chest by cutting the left 3rd and 4th ribs and intercostal muscles with a cautery pen (General Medical Corporation, Richmond, VA, USA). The animal was slightly rotated to the right by releasing the left upper paw to expose the left ventricle. Coronary artery ligation was achieved by a balloon occluder fixed onto the left anterior descending artery (LAD) by a 7-0 silk suture passed with a tapered needle underneath the LAD and 1 mm inferior to the left auricle.

Coronary artery occlusion and reperfusion were achieved by inflating and deflating the balloon. Significant ECG changes, including widening of QRS complex (monitored by Maclab ETH-255 Bridge/Bio Amplifier, CB Science Inc., Dover, NH, USA) and elevation of ST segment, and color changes of the area at risk were considered indicative of successful coronary occlusion and reperfusion. Once the reperfusion started, the chest was closed in layers. The respirator was weaned and endotracheal tube was removed when the animal recovered spontaneous breathing and began to move. Dextrose (5%) at 0.5 ml/10 g body weight was injected i.p. after the animal was ventilated. Rectal temperature was monitored with a rectal probe (Homeothermic Blanket Control Unit, Harvard, MA, USA) throughout the operation and was maintained within 36.5–37.5°C.

Experimental Groups Animals were assigned to one of the four groups as follows. Group 1: Sham control ($PARS^{1\,y1}$ and $PARS^{2\,y2}$) groups, subjected to 45 min chest opening, 30 min LAD occlusion, and up to 24 hr reperfusion. Group 2: Ischemic preconditioning ($PARS^{1\,y1}$ IPC and $PARS^{2\,y2}$ IPC) groups, subjected to four cycles of 5 min occlusion of LAD and 5 min reperfusion prior to 30 min LAD occlusion, and up to 24 hr reperfusion. After the fourth occlusion of LAD, 10 min reperfusion was allowed before the 30 min occlusion. Before harvesting the hearts, animals were re-anesthetized and blood was drawn by puncturing the right ventricle. The hearts excised were either stained with TTC (2,3,5-triphenyltetrazolium chloride, Sigma, St. Louis, MO, USA) for measurements of myocardial infarct size or kept in 2 70°C for measurements of tissue myeloperoxidase (MPO) activity and myocardial NAD^1 content.

Altogether 85 mice ($PARS^{1\,y1}$ and $PARS^{2\,y2}$) were used in the present experiments. Six mice died perioperatively. There was no difference in mortality among the various groups. Two TTC staining to reveal the infarct size failed due to the unevenly distributed blue in nonischemic area or the blue entering the area at risk.

Analysis of Myocardial Infarction After excision, the hearts were cannulated through the ascending aorta by a 23-gauge needle and perfused with 2–3 ml of 37°C 0.9% sodium chloride solution, and then with 3–4 ml of 37°C 1.0% TTC in phosphate buffer (pH 7.4). After TTC staining, the LAD was re-occluded. Then the hearts were perfused with 2 ml of 2% Evens Blue to delineate the nonischemic tissue. The hearts were then frozen, and the right ventricle and atria were trimmed off. The left ven-

tricle was cut into 5–7 transverse slices, which were fixed in 10% neutral buffered formalin solution. Each slice was weighed and photographed under a dissective microscope. The pictures of both sides of each slice were traced by the borders of infarction area, noninfarction area, and nonischemic area. The corresponding areas were calculated with Adobe Photoshop software after scanning these tracing graphs. The sizes in weight of nonischemic area, area at risk, and infarction area of each slice were then calculated as a percentage of corresponding area multiplied by the weight of the slice. Infarct size is expressed by percentage of area at risk (26).

Determination of Myocardial Myeloperoxidase Activity Left ventricles, harvested 24 hr after reperfusion, were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mmol/L potassium phosphate buffer (pH 7.0) and centrifuged for 30 min at 20,000 g at 48C. An aliquot of the supernatant was allowed to react with a solution of tetramethylbenzidine (1.6 mmol/l) and 0.1 mmol/L H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 mmol/L of hydrogen peroxide per minute at 378C and expressed in units per 100 gram wet tissue (10).

Measurement of Serum Levels of Tumor Necrosis Factor Alpha, Interleukin-10, and Interlukin-12 Serum levels of immunoreactive murine IL-10, IL-12, and TNF-α were quantified using a commercially available ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA) as previously described (12,27).

Measurement of NAD^+ Levels in Heart Tissue Heart samples (left ventricles) were stored at 2 808C until analysis. The tissue was finely powdered in liquid nitrogen using a mortar and pestle, and the powder was transferred to a 50-ml tube. It was homogenized in 20 ml of chilled 1 N perchloric acid (PCA) using a polytron homogenizer at a speed of 4.5, twice for 30 sec each, and was precipitated at 48C for 1 hr. After centrifugation at 1000 g for 15 min (48C), 5 ml of the PCA supernatant is neutralized with 2.5 ml of 2 N KOH; 0.33 M KPO_4, pH 7.5. The $KClO_4$ precipitate was discarded after centrifugation at 2000 g for 15 min at 48C, and the supernatant was stored at 2 208C until NAD^+ analysis. Microassay for NAD^+ in heart tissue was carried out in a 96-microwell flat-bottom assay plate in the dark at 308C in a dry-air oven. In a total volume of 170 μl, 50 μl of sample (or 0–20 pmol standard NAD^+) was reacted with 100 μl of freshly prepared reagent mix (to obtain final concentrations of 0.1 M bicine, pH 7.8; 0.5 M ethanol; 4.17 mM EDTA-Na_4; 0.83 mg/ml BSA; 0.42 mM 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT); 1.66 mM phenazine ethosulfate). The reaction was started by the addition of 2 U of alcohol dehydrogenase (EC 1.1.1.1; Sigma Chemical Co., St. Louis, MO, USA; 20 μl of 0.335 mg/ml in 0.1 M bicine, pH 7.8). The color was developed in the dark at 308C for 30 min and the absorbance was monitored at 550 nm (A_{550}) in a microplate reader EAR 400AT Easy Reader (SLT Labinstruments, Vienna, Austria) (28).

Myocardial Ischemia and Reperfusion in Rats With and Without PARS Inhibition

Male Wistar rats (300–350 g) were used in this part of the study. The rats were anesthetized with pentobarbital (60 mg/kg i.p.), tracheostomized and mechanically ventilated with room air (75 strokes/min, Vt 5 8 ml/kg) using a Harvard model 683 rodent respirator (Harvard Apparatus, Holliston, MA, USA). The animals were placed on controlled heating pads, and core temperature measured via a rectal probe was maintained at 378C. Following a left thoracotomy at the fourth intercostal space, the pericardium was opened and the heart briefly exteriorized. Coronary artery ligation was achieved by a small piece of polyethylene tubing (PE 160) fixed onto the LAD by a 6-0 silk suture passed underneath the artery. Reperfusion was allowed by relieving the PE tubing applied against the artery.

Experimental Groups The rats were assigned to several treatment groups as follows. Group 1: MI group (n 5 15): rats were exposed to 1 hr LAD occlusion followed by 2 hr reperfusion. Group 2: 3-aminobenzamide (3-AB) MI group (n 5 15). Rats exposed to 1 hr LAD occlusion and 2 hr reperfusion were treated with 3-AB, 20 mg/kg i.p., 10 min before reperfusion. Group 3: IPC MI group (n 5 15). Rats were preconditioned by 4 cycles of 5 min of ischemia and 5 min of reperfusion (except from the first cycle where only 3 min of ischemia was induced). Group 4: 3-AB IPC MI (n 5 15). The rats were first treated with 3-AB (20 mg/kg i.p.), and 20 min later were subjected to preconditioning as above, followed by 1 hr LAD occlusion and 2 hr reperfusion. Group 5: IPC only (n 5 3). In this group, the animals were sacrificed immediately after preconditioning for histologic studies (see below). Group 6: 3-AB IPC only (n 5 3). Rats were first treated with 3-AB (20 mg/kg i.p.), followed by preconditioning 20 min later, and were sacrificed immediately after preconditioning. Group 7: IPC 3-AB MI (n 5 15). Rats were preconditioned as above and then subjected to 1 hr LAD occlusion followed by 2 hr reperfusion, with 3-AB given 10 min before reperfusion.

Measurement of Infarct Size and Area at Risk Except from groups 5 and 6 (IPC only and 3-AB IPC only), 12 rats from each group were used for the determi-

nation of the area at risk and infarct size. The procedure was the same as that applied in the mice (see above), except that instead of being processed for image analysis, the different portions of the left ventricle (area at risk [AAR], necrotic, and non-necrotic areas) were weighed after careful microsurgical dissection, as previously described (10).

Immunohistochemical Localization of PARS Activation and Nitrotyrosine Formation in the Myocardium Three to four rats from each experimental group were used for immunohistochemical studies. Hearts were harvested at the conclusion of the experiments and paraffin embedded for poly(ADP-ribose) and 3-nitrotyrosine (3-NT) immunohistochemistry, which were performed as previously described (29). Paraffin-embedded sections (3 μm) were deparaffinized in xylene and rehydrated in decreasing concentrations (100%, 95%, and 75%) of ethanol followed by a 10 min incubation in PBS (pH 7.4). Sections were treated with 0.3% hydrogen peroxide for 15 min to block endogenous peroxidase activity and then rinsed briefly in 10 mM PBS. Nonspecific binding was blocked by incubating the slides for 1 hr in PBS containing 2% horse (PAR) or goat (3-NT) serum. Mouse monoclonal anti-poly (ADP-ribose) antibody (Alexis, San Diego, CA, USA) or rabbit polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY, USA) was applied in a dilution of 1:100 (PAR) or 1:200 (3-NT) for 2 hr at room temperature. Following extensive washing (5 3 5 min) with PBS, immunoreactivity was detected with a biotinylated rabbit anti-mouse (PAR) or goat anti-rabbit (3-NT) secondary antibody and the avidin-biotinperoxidase complex (ABC) both supplied in the Vector Elite kit (Vector Laboratories, Burlingame, CA, USA). Color was developed using Ni-DAB substrate (95 mg diaminobenzidine, 1.6 g NaCl, 2 g nickel sulfate in 200 ml 0.1 M acetate buffer). Sections were then counterstained with nuclear fast red, dehydrated, and mounted in Permount. Photomicrographs were taken with a Zeiss Axiolab microscope equipped with a Fuji HC-300C digital camera.

Data Analysis

All values in the figures, tables, and text are expressed as mean 6 SEM of *n* observations, where *n* represents the number of mice. The results were analyzed by one-way ANOVA, followed by the Bonferroni correction post hoc test. A value of p , 0.05 was considered significant.

Results

Myocardial Postischemic Reperfusion Injury Is Reduced in PARS$^{2\,y2}$ Mice

In the PARS$^{1\,y1}$ mice, 30 min of occlusion and 24 hr of reperfusion induced massive myocardial injury, as indicated by an infarct size of 53.4% (percentage of area at risk). In PARS$^{2\,y2}$ mice, the infarct size was significantly reduced (36.7%, p , 0.01 when compared to wild-type; Figs. 1 and 2). There were no significant differences between the wild-type and PARS-deficient groups in the areas at risk (represented as percentage of the left ventricle).

Myeloperoxidase (MPO) content of the left ventricle, as an index of neutrophil infiltration into the reperfused myocardium, showed a massive increase in the wild-type mice. Neutrophil infiltration was significantly reduced in PARS$^{2\,y2}$ mice subjected to ischemia/reperfusion (9.7 6 1.4 versus 21.6 6 3.0 U/g, p , 0.05; Fig. 3). The inflammatory response, triggered by postischemic reperfusion, was already evident after 60 min reperfusion with a significantly increased production of the pro-inflammatory cytokine TNF-α (Fig. 4). The production of IL-10, an anti-inflammatory cytokine, was also enhanced in response to the inflammatory reaction in PARS$^{1\,y1}$ sham control mice (Fig. 5). Serum IL-12 levels significantly increased at 24 hr after reperfusion, in the PARS$^{1\,y1}$ mice (Fig. 6). The production of the above

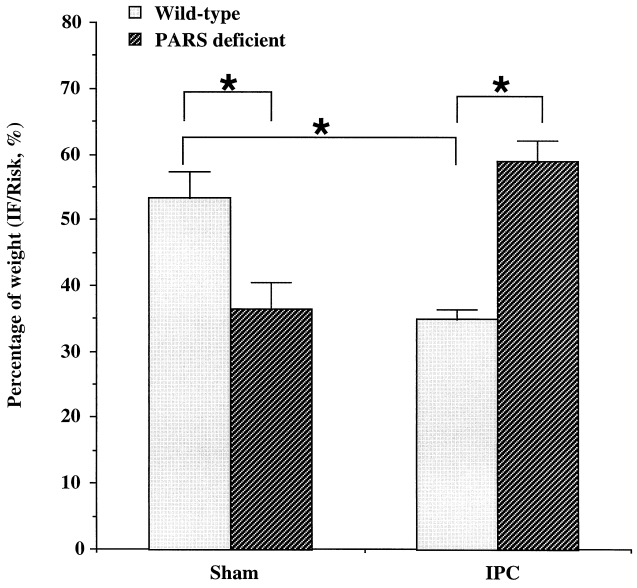

Fig. 1. Myocardial infarct size in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 24 hr reperfusion. Infarct size is represented as a ratio of weight of the infarct tissue and weight of the area at risk (IF/area at risk). No difference was found between the four groups in area at risk as characterized by a ratio of area at risk and left ventricle (p . 0.05). Ischemic preconditioning caused 35% reduction of infarction size in PARS$^{1/1}$ mice when compared with the response in nonpreconditioned animals (p , 0.05). Without preconditioning, PARS$^{2/2}$ mice developed a significant reduction in the infarct size after 30 min occlusion than the PARS$^{1/1}$ mice (p , 0.05). However, ischemic preconditioning markedly enhanced the infarct size in the PARS$^{2/2}$ mice (p . 0.05). Thus, there was no significant difference in infarct size between the preconditioned PARS$^{2/2}$ myocardial infarction group and the PARS$^{1/1}$ nonpreconditioned group. Data shown as mean 6 SEM; n 5 7–8 determinations per experimental group.

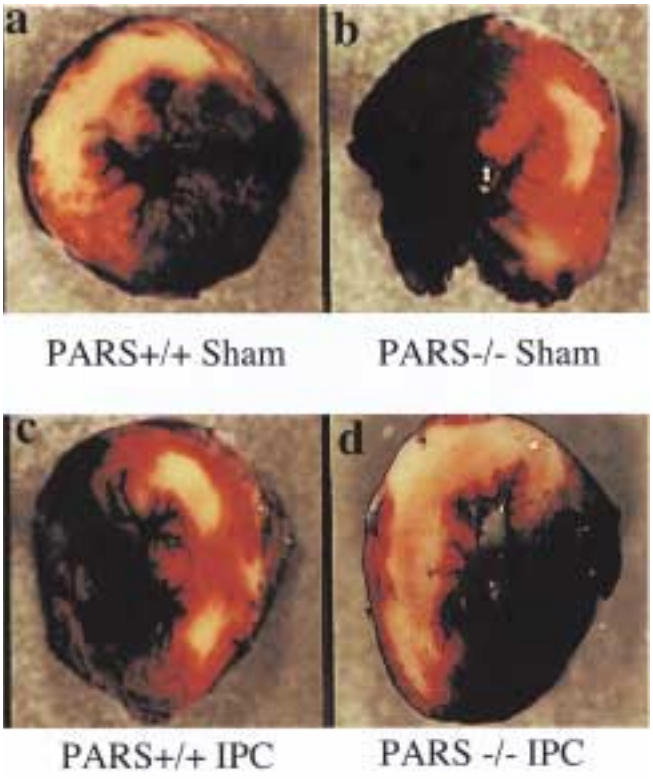

Fig. 2. Examples of TTC staining in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 24 hr reperfusion. The region stained with blue was the nonischemic portion of left ventricle. The rest portion was the area at risk, which was subjected to 30 min ischemia and 24 hr reperfusion. Within the area at risk, the viable myocytes was stained bright red and the infarcted area was light yellow.

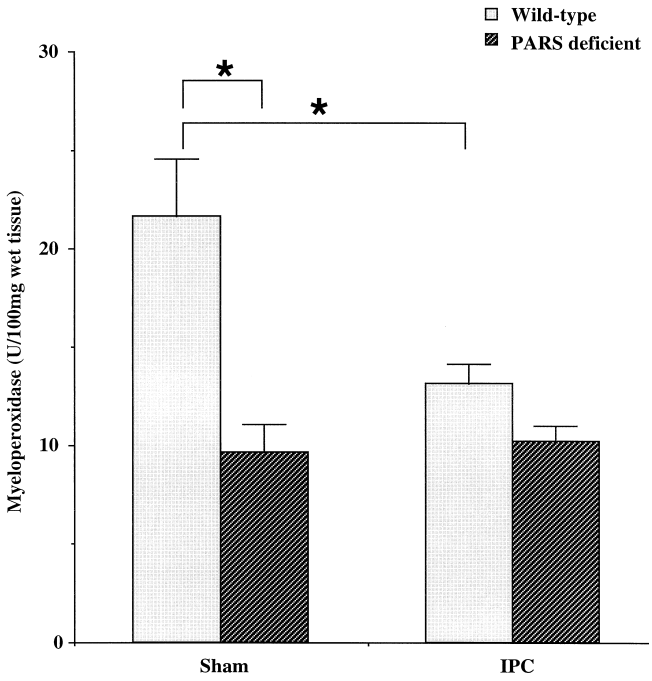

Fig. 3. Myeloperoxidase activity, as measured in homogenates from the left ventricle of the mouse heart in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 24 hr reperfusion. After 30 min occlusion of LAD and 24 hr reperfusion, myeloperoxidase activity was significantly enhanced in PARS$^{1\,y1}$ mice but reduced remarkably in PARS$^{2\,y2}$ mice. IPC significantly reduced MPO levels in the PARS$^{1\,y1}$ mice, but did not further suppress the (already reduced) levels in the PARS$^{2\,y2}$ mice. Basal MPO levels in the hearts of sham mice amounted to 4.2 6 0.8 U/100 mg wet tissue (n 5 8). Data are shown as mean 6 SEM; n 5 7–8 determinations per experimental group.

pro- and anti-inflammatory mediators was significantly reduced in PARS$^{2\,y2}$ mice (Figs. 4, 5, and 6).

Ischemic Preconditioning Protects the Hearts of PARS$^{1\,y1}$ Mice Against Reperfusion Injury

Four cycles of 5 min of occlusion, interrupted by 5-min periods of reperfusion, prior to the 30 min occlusion of LAD, significantly reduced the infarct size in PARS$^{1\,y1}$ mice. These data are consistent with the phenomenon of the early window of ischemic preconditioning. The degree of the protection provided by IPC was similar to the degree of protection seen in the absence of PARS (Figs. 1, 2, and 3). The production of inflammatory mediators was also significantly inhibited by IPC (Figs. 4, 5, and 6). However, the inhibitory effect of IPC on inflammatory mediator production was less pronounced, when compared with the degree of suppression seen in the PARS$^{2\,y2}$ mice (Figs. 4, 5, and 6).

Myocardial Ischemia/Reperfusion Activates PARS, Which Induces a Rapid Depletion of Intracellular NAD^1

The myocardial content of NAD^1 (presented as pmoles per mg protein) was not significantly different between PARS$^{1\,y1}$ and PARS$^{2\,y2}$ mice in normal conditions (without ischemia or reperfusion). After 15 min of reperfusion, the myocardial NAD^1 content was significantly reduced in PARS$^{1\,y1}$ mice (p , 0.05, compared with the content at the end of 30 min occlusion), whereas in the PARS$^{2\,y2}$ mice, no myocardial NAD^1 depletion was observed during reperfusion (Fig. 7).

Ischemic Preconditioning Ameliorates the Depletion of Myocardial NAD^1 Pools

Ischemic preconditioning, applied to the PARS$^{1\,y1}$ mice before the 30 min occlusion, mimicked the effects of PARS knockouts with respect to preserving the myocardial NAD^1 pools: IPC significantly, although only partially, attenuated (p , 0.05) the ischemia/reperfusion–induced decreases in the myocardial NAD^1 levels in the PARS$^{1\,y1}$ mice (Fig. 8).

In PARS Knockout Mice, The Protective Effect of IPC Disappears

The salutary effect of IPC on myocardial ischemia/reperfusion completely disappeared in the PARS$^{2\,y2}$ mice. In fact, the myocardial infarct size was similar, in extent, to the degree of infarction seen in the

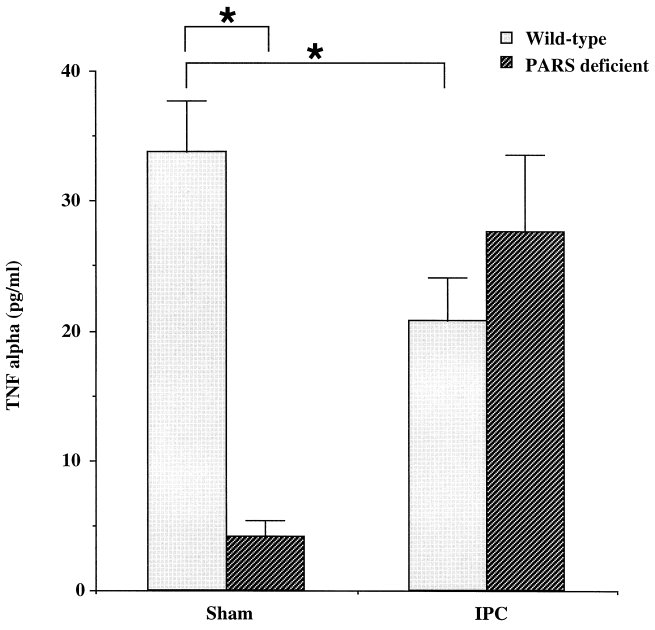

Fig. 4. Serum levels of TNF-α, in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 60 min reperfusion. The production of TNF-α was significantly reduced in PARS$^{2\,y2}$ animals after myocardial infarction (p , 0.05). Ischemic preconditioning applied to PARS$^{1\,y1}$ mice before the 30 min occlusion significantly reduced the production of TNF-α when compared to the response in nonpreconditioned animals (p , 0.05). No detectable levels of the cytokine were measured in wild-type or PARS-deficient mice without myocardial occlusion and reperfusion (data not shown). Data are shown as mean 6 SEM; n 5 7–8 determination per experimental group.

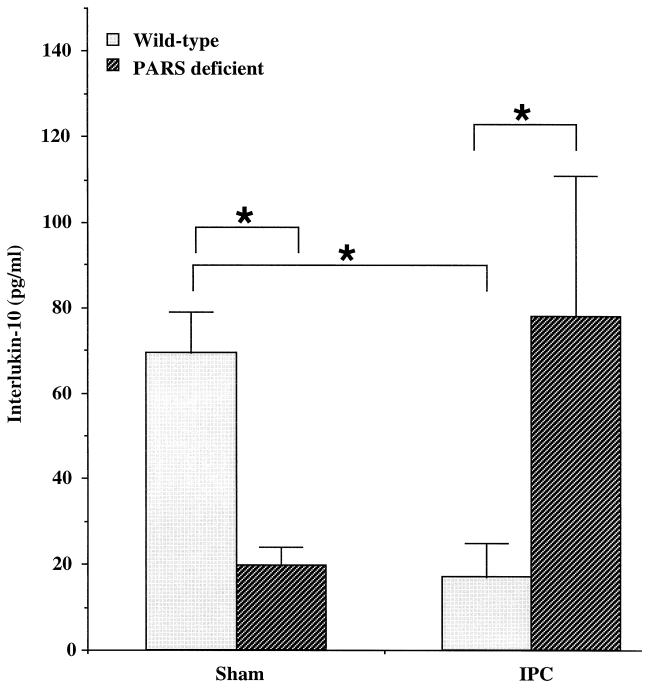

Fig. 5. Serum levels of IL-10, in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 24 hr reperfusion. The production of IL-10 was significantly reduced in PARS$^{2\,y2}$ sham control mice when compared with the response in wild-type animals (p , 0.05). Ischemic preconditioning applied to PARS$^{1\,y1}$ mice before the 30 min occlusion significantly reduced the production of IL-10, when compared to the response in the sham control (p , 0.05). However, ischemic preconditioning markedly enhanced the production of IL-10 in the PARS$^{2\,y2}$ mice (p , 0.05). Thus, there was no significant difference in the plasma IL-10 levels between the preconditioned PARS$^{2\,y2}$ myocardial infarction group and the PARS$^{1\,y1}$ nonpreconditioned group. No detectable levels of the cytokine were measured in wild-type or PARS-deficient mice without myocardial occlusion and reperfusion (data not shown). Data are shown as mean 6 SEM; n 5 7–8 determinations per experimental group.

wild-type mice subjected to myocardial ischemia/reperfusion without IPC. In other words, although IPC induced a 35% reduction of myocardial infarction in PARS$^{1\,y1}$ mice, the same procedure caused a 60% increase of myocardial infarction in PARS$^{2\,y2}$ mice (Figs. 1 and 2). There was no significant increase in myocardial MPO activity in the PARS-deficient mice subjected to IPC, indicating that the massive enhancement of the infarction occurred via a mechanism independent of neutrophil infiltration (Fig. 3). However, an enhanced inflammatory response did ensue after applying the IPC procedures to PARS$^{2\,y2}$ mice, as characterized by increased plasma levels of various inflammatory mediators (Figs. 4, 5, and 6).

Pretreatment With the PARS Inhibitor 3-AB Mimics the Effect of PARS Deficiency in Eliminating the Protective Effect of IPC

Figure 9 illustrates the area at risk (A), and infarct size expressed either as a percentage of the AAR (B), or of the left ventricle (C) in rats treated with the PARS inhibitor 3-AB, or subjected to IPC, or its combination. No significant differences were noted between the AAR of the different groups. As expected, preconditioning (IPC MI group) and also treatment with 3-AB before reperfusion (3-AB MI group) reduced the infarct size when compared to the vehicle treated control MI group (Fig. 9). The benefit of preconditioning was cancelled when animals were pretreated with 3-AB prior to preconditioning (3-AB IPC MI group). On the other hand, the effect of IPC was not affected when the PARS inhibitor was given after preconditioning (IPC 3-AB-MI group).

Immunohistochemical Evidence That IPC Inhibits PARS Activation During Myocardial Reperfusion Injury

As shown in Figure 10, no activation of PARS was detected in the nonischemic myocardium in all treatment groups (left column). In contrast, positive PAR immunostaining, indicative of PARS activa-

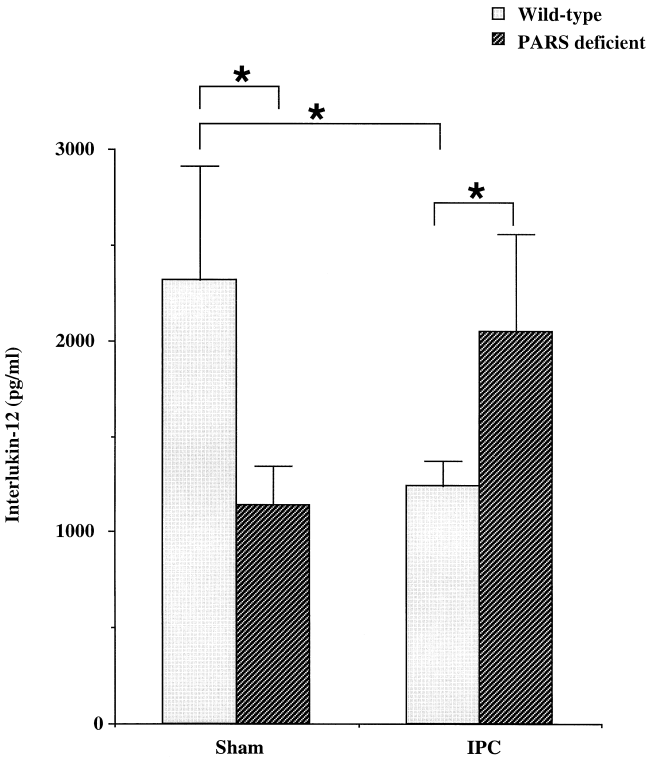

Fig. 6. Serum levels of IL-12, in PARS$^{+/+}$ and PARS$^{-/-}$ sham control animals and PARS$^{+/+}$ and PARS$^{-/-}$ animals subjected to IPC after 30 min occlusion of LAD and 24 hr reperfusion. The production of IL-12 was significantly reduced in PARS$^{2\,y2}$ sham control mice when compared with the response in wild-type animals (p , 0.05). Ischemic preconditioning applied to PARS$^{1\,y1}$ mice before the 30 min occlusion significantly reduced the production of IL-12, when compared to the response in the sham control (p , 0.05). However, ischemic preconditioning markedly enhanced the production of IL-12 in the PARS$^{2\,y2}$ mice (p , 0.05). Thus, there was no significant difference in the plasma IL-12 levels between the preconditioned PARS$^{2\,y2}$ myocardial infarction group and the PARS$^{1\,y1}$ nonpreconditioned group. No detectable levels of the cytokine were measured in wild-type or PARS-deficient mice without myocardial occlusion and reperfusion (data not shown). Data are shown as mean 6 SEM; n 5 7–8 determinations per experimental group.

tion, was noted in the ischemic/reperfused myocardium (right column), with noticeable differences between the various experimental groups. Numerous positively stained nuclei were detected in the myocytes of rats from the MI group. The degree of PARS staining was clearly reduced in nonpreconditioned animals treated with 3-AB (3-AB MI) and in rats exposed to preconditioning (IPC MI). In contrast, PAR staining was enhanced in preconditioned rats treated with 3-AB, administered either before (3-AB IPC MI) or after preconditioning (IPC 3-AB MI). In animals exposed to preconditioning only (IPC only), several scattered positive cells were detected, a phenomenon that was inhibitable with 3-AB (3-AB IPC only) (Fig. 10).

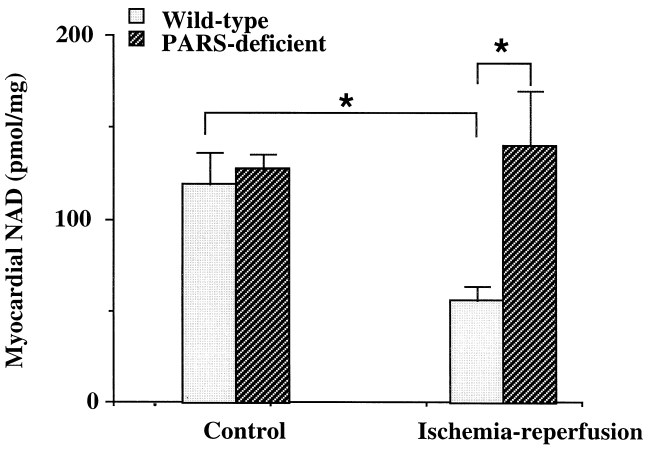

Fig. 7. Effect of myocardial infarction on the myocardial tissue content of NAD^{+}. Myocardial NAD^{1} content decreased in the PARS$^{1\,y1}$ mice at 15 min of reperfusion (p , 0.05), while myocardial NAD^{1} levels remained unchanged in the PARS^{-y-} mice. Data are shown as mean 6 SEM; n 5 4–5 determinations per experimental group.

IPC and PARS Inhibition Affects Tyrosine Nitration Patterns (Evidence of Reactive Nitrogen Species Generation) in the Reperfused Myocardium

Figure 11 illustrates the results of nitrotyrosine immunostaining, and depicts three distinct patterns of positive staining in the ischemic/reperfused myocardium: in cardiomyocytes, blood vessel walls, and infiltrating inflammatory cells. In rats from the MI group, a positive staining was noted in numerous myocytes, both in the cytoplasm and (predominantly) in the sarcolemma. An intense nitrotyrosine staining was also detected in the wall of blood vessels, as well as in mono- and polynuclear cells infiltrating the ischemic myocardium. As observed

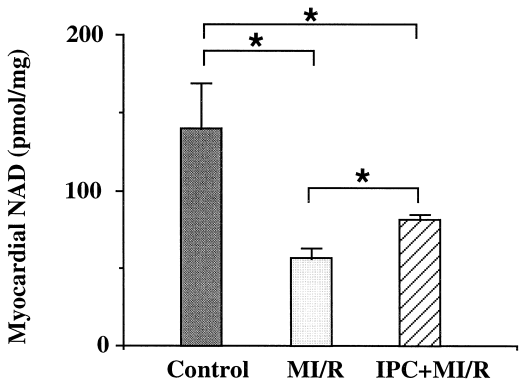

Fig. 8. Effect of IPC on the myocardial tissue content of NAD^{+} in PARS$^{+/+}$ mice during myocardial ischemia/reperfusion (MI/R). Ischemic preconditioning significantly attenuated the depletion of the myocardial NAD^{1} pool, as measured at 15 min after reperfusion (p , 0.05). Myocardial NAD levels, nevertheless, still showed a significant decrease when compared to baseline values in the IPC-subjected mice subjected to coronary ischemia and reperfusion (p , 0.05). Data are shown as mean 6 SEM; n 5 42 5 determinations per experimental group.

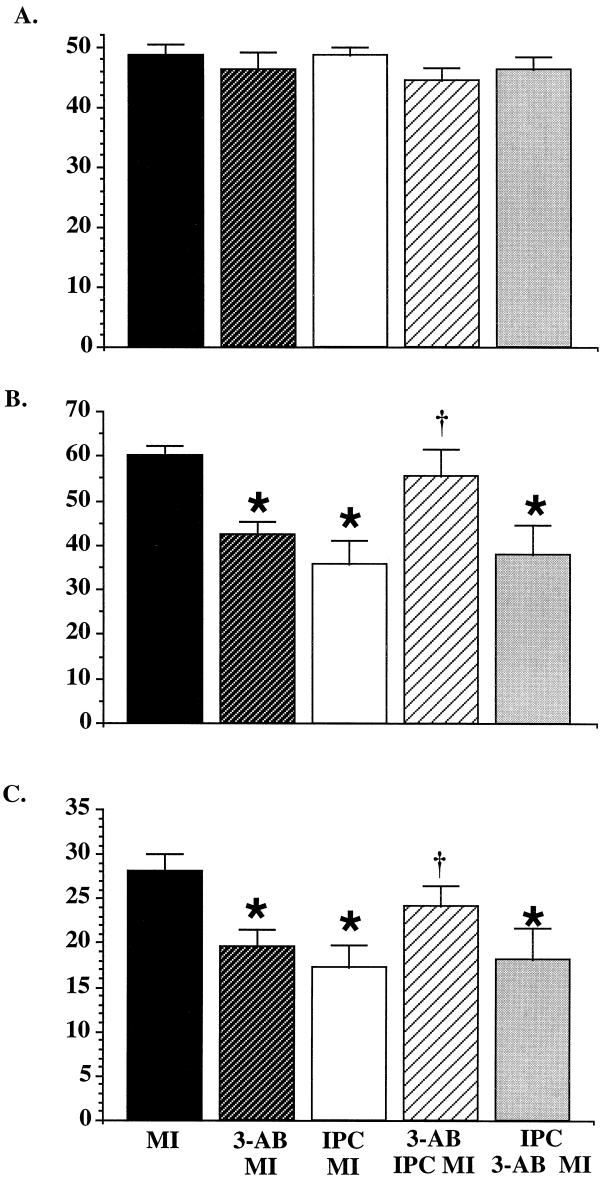

Fig. 9. AAR (A) and infarct size in percent of the AAR (B) or of the left ventricle (C) in preconditioned and nonpreconditioned rats treated with vehicle or the PARS inhibitor 3-AB before or after IPC. Both 3-AB treatment (3-AB MI) and preconditioning (IPC MI) reduced infarct size in comparison to untreated rats (MI group). The benefit of preconditioning was lost when rats were treated with 3-AB before preconditioning (3-AB IPC MI), and it was not influenced when 3-AB was administered after preconditioning (IPC 3-AB MI). Data are shown as mean 6 SEM. *p , 0.05 versus MI group. †p , 0.05 versus IPC group.

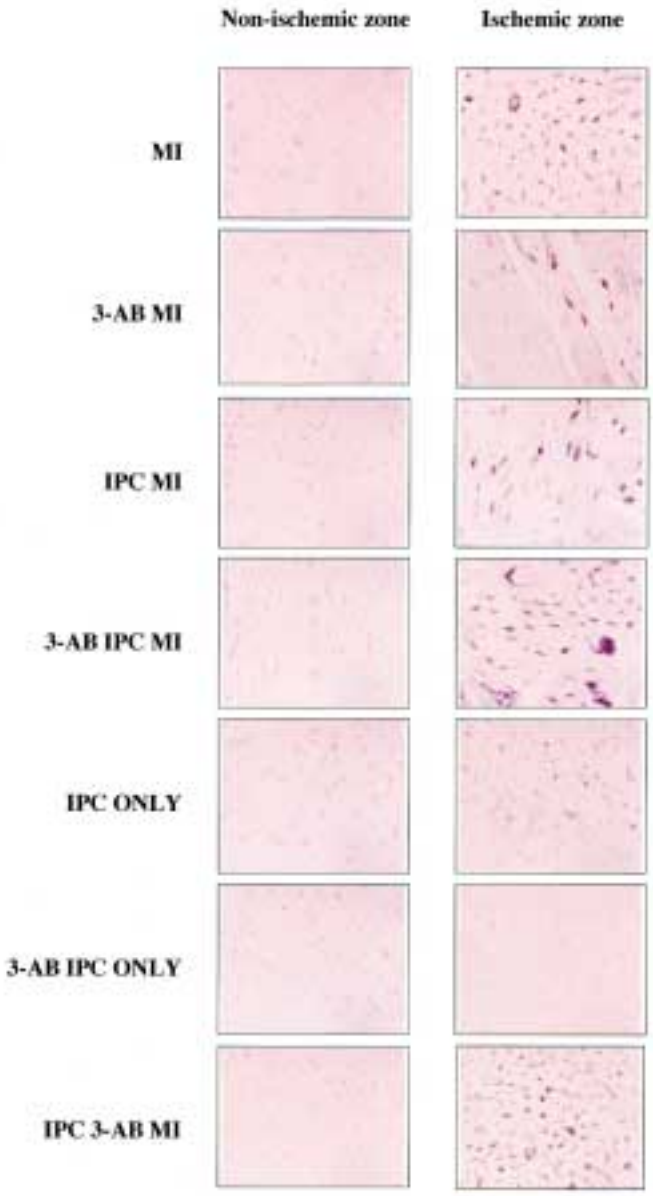

Fig. 10. Immunohistochemical localization of PARS activation in hearts subjected to coronary ischemia/reperfusion with or without preconditioning or PARS inhibition, or preconditioning alone. Representative staining patterns from the following respective experimental groups are shown (from top to bottom): MI, myocardial ischemia/reperfusion alone; 3-AB MI, 3-AB pretreatment in hearts subjected to myocardial ischemia and reperfusion; IPC MI, ischemic-preconditioned hearts subjected to myocardial ischemia and reperfusion; 3-AB IPC MI, 3-AB treatment, followed by ischemic preconditioning and subsequent to myocardial ischemia and reperfusion; IPC only, ischemic preconditioning without subsequent ischemia or reperfusion; 3-AB IPC only, 3-AB pretreatment followed by ischemic preconditioning without subsequent ischemia or reperfusion; and IPC 3-AB MI, ischemic-preconditioned hearts, treated with 3-AB subsequent to IPC, and then subjected to myocardial ischemia and reperfusion. Representative sections from the different groups of rats show areas from the nonischemic myocardium (left column) and ischemic myocardium (right column). Positive staining is indicated by the black stained nuclei of cardiomyocytes in the ischemic myocardium. Magnification 3 400.

before (10), in rats treated with 3-AB, cardiomyocyte staining was reduced and only detected in discrete areas surrounding blood vessels. A significant degree of staining still persisted, however, in blood vessels and inflammatory cells. In preconditioned rats, there was also a clear reduction in the nitrotyrosine staining of cardiomyocytes, while blood vessels and scattered inflammatory cells were positively stained. In animals exposed to preconditioning only (IPC only),

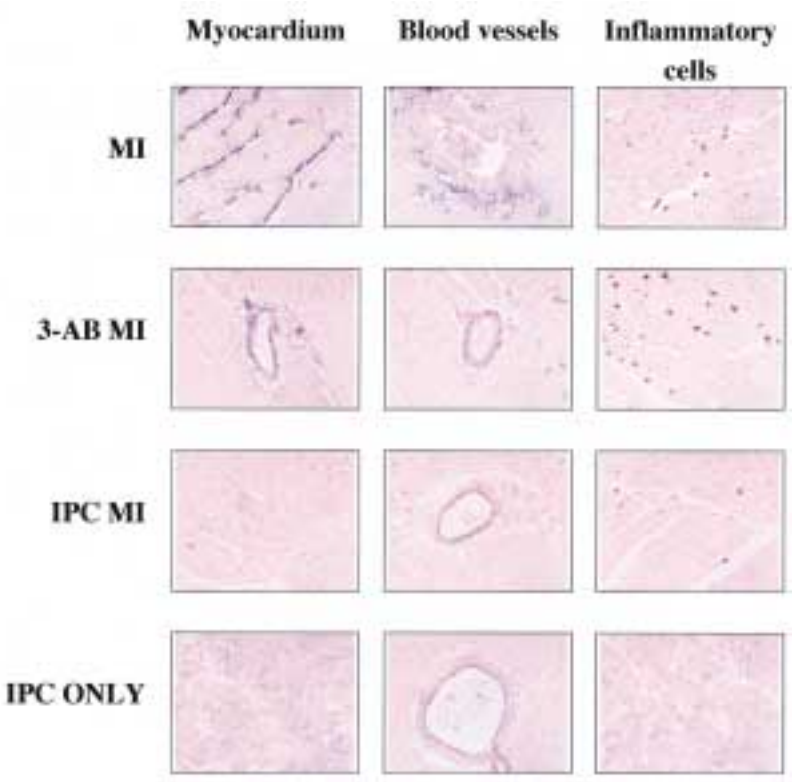

Fig. 11. Immunohistochemical localization of nitrotyrosine formation in ischemic and reperfused myocardial sections. The various groups or rats are identified to a scheme identical to the one described in the legend to Figure 10. Representative sections from different groups of rats showing several patterns of nitrotyrosine immunostaining in the ischemic left ventricle (myocytes, left; blood vessel walls, middle; and inflammatory cells, right). See text ("Results") for details. Magnification 3 400.

the majority of cardiomyocytes from the left ventricle showed an intense cytoplasmic formation of nitrotyrosine. Positive blood vessels were also detected, and there was no evidence of infiltrating cells.

Discussion

PARS Deficiency Inhibits Myocardial Reperfusion Injury, Down-regulates the Inflammatory Response, and Maintains Myocardial NAD^1 Levels in Reperfusion Injury

Comparison of the responses to myocardial ischemia and reperfusion in wild-type and PARS-deficient mice confirm previous findings demonstrating that pharmacologic PARS inhibition or PARS deficiency have myocardial protective effects in hypoxia-reoxygenation and ischemia/reperfusion injury (9,11–15,19). Furthermore, the results demonstrate an attenuated inflammatory mediator response in the absence of PARS. This latter phenomenon may either be a consequence of the reduced myocardial injury and neutrophil infiltration/activation, and/or a direct consequence of a suppression, in the absence of PARS, of pro-inflammatory signal transduction processes governed by the transcription factor nuclear factor kappa B (30).

There was a significant suppression of myocardial NAD^1 levels during reperfusion, and there was a complete maintenance of NAD^1 levels in the hearts of the PARS-deficient mice. Based on previous work by our group and others (4,18), these changes in the NAD^1 levels are consistent with (A) the activation of PARS at the early stage of postischemic reperfusion, followed by cellular NAD^1 depletion during the reperfusion stage and (B) with a complete maintenance of the myocardial NAD^1 pools in hearts lacking functional PARS. These data also underline the central role of PARS in regulating myocardial NAD^1 levels. Although in the current study we have not measured myocardial ATP levels, previous studies have presented a good correlation between PARS-regulating changes in cellular NAD and ATP in myocardial tissues, as well as in other cell types (4,9,31).

IPC Protects Against Myocardial Reperfusion Injury by Inhibiting PARS Activation

The similar degree of protection by IPC and PARS deficiency (or pharmacologic PARS inhibition), the similar effects on inflammatory mediator production, the similar improvements in myocardial NAD^1 content, and, finally, the lack of IPC-induced protection against reperfusion injury in the absence of functional PARS are all consistent with the proposal that IPC leads to an inhibition of PARS activation, which mediates, at least in part, its cardioprotective effects. This conclusion is further strengthened by our direct measurement of PAR formation in the reperfused myocardium, which was markedly attenuated in the animals subjected to IPC prior to myocardial ischemia and reperfusion (Fig. 10).

One conceivable mechanism that may provide explanation for these observations may be that the short periods of ischemia and reperfusion (during IPC) induce a small degree of oxidant generation, and subsequent PARS activation. Because PARS activation leads to auto-ribosylation (and subsequent

auto-inhibition) of PARS (32,33), such a process could, in turn, protect against the deleterious effects of the ischemia and reperfusion, via inhibition of the subsequent, massive activation of PARS, which occurs in naïve (nonpreconditioned wild-type) animals during the phase of reperfusion. This hypothesis is supported by our observation demonstrating significant degree of reactive nitrogen species generation (Fig. 11) and PARS activation (Fig. 10) in response to IPC alone. The finding that treatment with the short-acting PARS inhibitor 3-AB before IPC (but not after IPC) cancels the cardioprotective effect of IPC is consistent with this hypothesis as well: one can hypothesize that there is an inactivation of PARS during IPC due to a transient low-level activation during IPC, which is inhibited by 3-AB.

An additional possibility is that the inhibition of PARS activation during IPC is related to the release of ischemic mediators released during IPC. There is overwhelming evidence for increased myocardial levels of these purines in myocardial ischemia and reperfusion (34–36). Certain purines, released during IPC, appeared as conceivable candidates for such an effect. We have recently demonstrated that some of these compounds (e.g., inosine and hypoxanthine)—in the concentration range that occurs in vivo during ischemia and early reperfusion—dose dependently inhibit PARS activation (37,38). The fact that the protection by IPC against the reperfusion-induced depletion of myocardial NAD^+ levels was only partial, can be explained by (A) a partial myocardial NAD^+ depletion during the PARS activation during IPC, and/or (b) a partial nature of the inhibition of PARS during ischemia/reperfusion in the animals previously subjected to IPC. The latter possibility is supported by direct immunohistochemical measurements demonstrating a residual degree of PARS activation in the reperfused myocardium of animals subjected to IPC (Fig. 10). Further work is required to investigate the potential relationship between PARS, and the various previously proposed mechanisms of IPC-induced cardioprotection (such as calcium, zinc, nitric oxide, and potassium channels). It is noteworthy that PARS activation can be linked to many of these mechanisms (3).

It is also noteworthy that two major pathways have been proposed to explain the cytoprotective effects seen in the absence or functional PARS: (1) the energetic hypothesis, which attributes the effects to preservation in cellular NAD and ATP levels, and subsequent alterations in cell viability, and (2) the pro-inflammatory hypothesis, which attributes some of the protection due to down-regulation of pro-inflammatory pathways in the absence of functional PARS (1–19,30). It is conceivable that, in order to explain the findings presented in the current work, a combination of both of these modes of action plays a significant role.

It is important to note that recent work demonstrated that the mode of PARS inhibition's cytoprotection against oxidant-induced cell death is related to inhibition of cell necrosis (which is related to cellular energetic depletion and mitochondrial injury) (39,40), which can occur at the expense of accelerated apoptosis. Further work should address whether accelerated apoptosis occurs in the reperfused PARS-deficient hearts after IPC, and whether the combination of PARS and caspase inhibition provides a more effective cardioprotection than either of the two approaches alone.

It is interesting to note that in animals exposed to preconditioning only, we observed a significant cytoplasmic formation of nitrotyrosine (Fig. 11). Positive blood vessels were also detected, although there was no evidence of infiltrating cells. It is conceivable that this early oxidative stress during IPC triggers the activation or expression of defensive antioxidant systems during the phase of the longer ischemia and reperfusion, which may contribute to the cardioprotective effects of IPC. In various experimental systems, there are a number of reports demonstrating that low-level oxidative stress induces various protective mechanisms, which protects against a subsequent, more severe oxidant injury (41–44). It was somewhat unexpected for us to find that there is a marked tyrosine nitration in response to IPC alone, which is then decreased by the end of the myocardial reperfusion (Fig. 11). However, one must keep in mind that tyrosine nitration and denitration is an active process, and in fact tyrosine denitrase is an inducible enzyme (45). Therefore, it is conceivable that one of the antioxidant enzymes induced during IPC is tyrosine denitrase. Further studies are required to address this possibility.

Although the mechanism of the enhanced infarction after IPC in the PARS-deficient hearts has not been clarified by the current study, two statements can be made with respect to its mechanisms: Obviously, the mechanism of this enhancement is independent of PARS activation; and the enhancement is not directly related to neutrophil-mediated myocardial injury, because it occurred despite the reduced number of neutrophils that infiltrated the hearts of the PARS-deficient animals subjected to IPC and myocardial infarction. One potential hypothesis to explain the enhancement of myocardial injury in PARS-deficient mice subjected to IPC may be related to endogenously released purines. These compounds (e.g., inosine, hypoxanthine, or adenosine) accumulate during ischemia, and are released in high concentrations into the previously hypoxic tissue during the early phase of reperfusion. Some of the purines' actions are cardioprotective (e.g., via activation of specific adenosine receptor [46,47], and via inhibition of PARS [37,38], while others are deleterious (e.g., hypoxanthine feeding the xanthine oxidase pathway with substrate, leading to enhanced oxidant generation [48]). It is possible that in the absence of PARS, the balance of these actions

tilts toward the detrimental side. Further experiments are required to address this hypothesis.

Taken together, the results of the current study allow the following conclusions and suggestions. (1) IPC and PARS deficiency are both cardioprotective. (2) The mechanism of cardioprotection is associated with similar alterations in neutrophil infiltration, cytokine production, and changes in myocardial NAD levels. Based on these similarities, coupled with direct demonstration that IPC inhibits the degree of PAR immunostaining in the reperfused myocardium, one can conclude that the mechanism of IPC-induced cardioprotection involves inhibition of PARS activation. (3) There is an unexpected enhancement of myocardial injury in the PARS deficient animals subjected to IPC and myocardial ischemia/reperfusion injury. This phenomenon of enhancement of myocardial infarction, which can be brought about by a relatively simple process such as IPC (but only in the absence of functional PARS), point to the necessity of identification of these pathways of myocardial injury.

Acknowledgment

This work was supported by grants from the National Institutes of Health (R01HL59266 and R01GM60915) to C.S. Lucas Liaudet is on leave from the Critical Care Division, Department of Internal Medicine, University Hospital, Lausanne, Switzerland, and is supported by a grant from the ADUMED Foundation (Switzerland).

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