Free Rad. Res., Vol. 29, pp. 235–245
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Preconditioning with Hydrogen Peroxide (H_2O_2) or Ischemia in H_2O_2-Induced Cardiac Dysfunction

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Accepted by Prof. B. Halliwell

(Received 23 March 1998; In revised form 11 May 1998)

The possible cardioprotective effects of preconditioning by ischaemia (IPC) or a low dose of H_2O_2 (HPC) prior to a high dose of H_2O_2 was investigated. Langendorff-perfused rat hearts ($n = 10$ in each group) were subjected to 10 min of 140 μmol/L H_2O_2 and 30 min recovery after either (1) control perfusion, (2) 20 μmol/L H_2O_2 for 10 min, recovery 10 min, or (3) 2 × 2 min global ischaemia and 5 min reperfusion. 140 μmol/L H_2O_2 increased left ventricular end-diastolic pressure from 0 to 68 ± 8 mmHg in controls (mean ± SEM), which was attenuated by IPC (46 ± 9 mmHg, $p < 0.001$) and HPC (18 ± 4 mmHg, $p < 0.001$ compared to controls, $p < 0.01$ compared to IPC). HPC, but not IPC, improved coronary flow ($p < 0.02$) and left ventricular developed pressure ($p < 0.001$) during recovery. Troponin T release was similar in all groups. Tissue thiobarbituric acid reactive substances, antioxidant capacity, catalase, and glutathione peroxidase were not influenced by 140 μmol/L H_2O_2. H_2O_2 decreased the level of tissue glutathione. This reduction was augmented by HPC ($p < 0.02$) and attenuated by IPC ($p < 0.02$). H_2O_2 increased superoxide dismutase ($p < 0.04$). The increase was attenuated by IPC ($p < 0.05$), but not influenced by HPC. HPC efficiently protected cardiac function in H_2O_2-induced cardiac injury, while IPC had only a small protective effect. The functional protection cannot be explained by reduction of irreversible injury, attenuation of lipid peroxidation, or modification of tissue antioxidant parameters.

Keywords: Catalase, glutathione, glutathione peroxidase, ischaemic preconditioning, superoxide dismutase, rat, thiobarbituric acid reactive substances

Abbreviations: CAT, catalase; CF, coronary flow; GSH, glutathione; GSH-Px, glutathione peroxidase; H_2O_2, hydrogen peroxide; HPC, preconditioning with 20 μmol/L H_2O_2; HR, heart rate; IPC, ischaemic preconditioning; LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; ROI, reactive oxygen intermediates; SOD, superoxide dismustase; TAS, total antioxidant status; TBARS, thiobarbituric acid reactive substances; TnT, troponin T

INTRODUCTION

Ischaemic preconditioning reduces infarct size and improves cardiac function in experimental models of global and regional ischemia.[1,2] Despite intensive investigations into the underlying

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mechanisms of the preconditioning response, this is far from clarified and appears to differ between species. Reactive oxygen intermediates (ROI), including hydrogen peroxide, have been suggested as possible mediators.[3] ROI are produced during short episodes of ischaemia and reperfusion.[4] Some investigators have found antioxidants to reduce the acute, beneficial effects of preconditioning,[5–8] while others did not.[9–11] Ischaemic preconditioning is reported to reduce the lipid peroxidation product malone dialdehyde[12] and influence myocyte antioxidant status,[5,13,14] but these findings were not confirmed in other models.[15,16] The effect of preischaemic exposure to low doses of exogenous ROI on the preconditioning response is controversial.[17–19]

Preconditioning has been suggested to be an universal mode of myocardial protection.[20] If so is the case, ischaemic preconditioning should protect against cardiac injury induced by other agents than ischaemia. Ischaemic preconditioning has been shown to protect the heart against cardiac dysfunction induced by anthracyclin toxicity,[21] but not against dysfunction induced by hypoxia[22] or exogenous ROI.[23]

The present study investigates whether a low concentration of ROI prior to a higher concentration triggers the preconditioning response. Cardiac function and release of troponin T were investigated in Langendorff-perfused rat hearts subjected to either a low dose of hydrogen peroxide or ischaemic preconditioning prior to exposure to a higher dose of hydrogen peroxide. Furthermore the possibility that reduction of lipid peroxidation or influence on tissue levels of endogenous antioxidants mediated the protection provided by hydrogen peroxide in this model was investigated.

MATERIALS AND METHODS

Heart Perfusion

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. Male Sprague Dawley rats (200–300 g) were anaesthetized with diethyl ether, and 200 IU heparin was injected into the femoral vein. The hearts were then rapidly excised through a median sternotomy and placed in ice-cold buffer during preparation for aortic cannulation. The hearts were retrogradely perfused with gassed (5% CO_2, 95% O_2) Krebs Henseleit buffer (NaCl 118.5 mmol/L, $NaHCO_3$ 25.0 mmol/L, KCl 4.7 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4 \cdot 7H_2O$ 1.2 mmol/L, Glucose·H_2O 11.1 mmol/L, $CaCl_2 \cdot 2H_2O$ 1.8 mmol/L) as a modified Langendorff-preparation. The perfusion pressure (100 cm H_2O) was kept constant. Water jackets round the perfusate reservoirs and heart chamber kept the temperature at 37°C throughout the experiments. A balloon was inserted into the left ventricle via the left atrium for isovolumetric recordings of left ventricular systolic (LVSP) and end-diastolic (LVEDP) pressures. Coronary flow (CF) was measured by timed collections of the coronary effluent. Heart rate (HR) was counted from the pressure curves. Left ventricular developed pressure (LVDP) was calculated (LVDP = LVSP – LVEDP). Two perfusate reservoirs were employed in order to rapidly change between buffer and H_2O_2-perfusion. H_2O_2 (3% solution) was purchased at the Karolinska Hospital's Pharmacy, and mixed directly into the buffer to achieve concentrations of either 20 or 140 μmol/L. Global ischaemia was induced by clamping the inflow tubing.

Experimental Protocol

The hearts were stabilized for 20 min before start of the experiments (defined as time 0). Only hearts with LVSP between 60–160 mmHg, LVEDP 0 mmHg, CF 8–16 ml/min, and HR 240–360 beats/min at the end of stabilization were included in one of the experimental groups below:

Group 1: ($n = 10$) Control perfusion for 20 min before 10 min perfusion with 140 μmol/L H_2O_2 and recovery with buffer only for 30 min.

Group 2: ($n = 10$) Ischaemic preconditioning with 2 episodes of 2 min global ischaemia and 5 min reperfusion before H_2O_2 and recovery as group 1.
Group 3: ($n = 10$) Pretreatment with 20 μmol/L H_2O_2 for 10 min, followed by 10 min buffer perfusion only before H_2O_2 and recovery as in groups 1 and 2.

LVSP, LVEDP, LVDP, HR, and CF were measured after 15 min stabilization (S), at time 0 immediately before infusion of 140 μmol/L H_2O_2, after 5 and 10 min of H_2O_2-infusion, and after 5, 10, 20, and 30 min recovery. Samples of the coronary effluent were collected for measurement of troponin T (TnT) at time 0, at the end of H_2O_2-infusion, and at the end of recovery. Additional hearts were perfused and freeze-clamped in liquid nitrogen cooled thongs at time 0 ($n = 10$) and at the end of infusion of 140 μmol/L H_2O_2 ($n = 10$ in all 3 groups) for analysis of cardiac antioxidative parameters and lipid peroxidation products. The freeze-clamped hearts were not included in the haemodynamic comparisons, or in measurements of TnT. Tissue reduced glutathione content (GSH), glutathione peroxidase (GSH-Px), total antioxidant status (TAS), catalase (CAT), superoxide dismutase (SOD), and antioxidant capacity were measured. Lipid peroxidation was evaluated by tissue contents of thiobarbituric acid reactive substances (TBARS).

Laboratory Methods

Samples (1 mL) of the coronary effluent were collected in precooled tubes, stored on ice, and rapidly frozen at −70°C. The freeze-clamped hearts were immediately stored at −70°C, transported on dry ice to Estonia, and kept frozen (−70°C) until analysis. Heart tissue was homogenized in 10 volumes of ice-cold 1.15% KCl, and filtered to get a homogenous mixture. Butylated hydroxytoluene (1.1 μmol/L) was added in 1:1000 v/v ratio to one aliquot of homogenate to suppress artefacts during handling of the samples in the TBARS measurements. Protein was measured according to Lowry *et al.*[24]

TBARS were evaluated according to Okhawa *et al.*,[25] with modifications previously described by us.[26] Assays were performed in triplicates with mean values used for comparisons. Tetraethoxypropane was used as internal standard for malone dialdehyde. TBARS are expressed in nmol/g wet weight.

Antioxidant capacity was determined by the ability of the samples to inhibit linolenic acid peroxidation *in vitro*.[26] The reaction was initiated by 100 μmol/L $FeSO_4$, and the products of peroxidation was assessed by contents of TBARS. The results are expressed as percent of sample induced inhibition of linolenic acid peroxidation. For the control value linolenic acid peroxidation was measured at the presence of 0.9% NaCl.

Total antioxidant status was measured by the commercially available kit (Randox Laboratories Ltd, Ardmore, United Kingdom) for assay of total antioxidant status. The values are expressed in mmol/L.

Tissue content of reduced glutathione was measured with Ellman reagent as described by Beutler *et al.*[27] Briefly, tridistilled water (1800 μL) and 100 μL of 500 mmol/L perchloric acid were added to 500 μL sample to precipitate proteins before the assessment. After 5 min incubation the samples were centrifuged (3000 rpm, 10 min), and 300 μL of protein-free supernatant removed. Subsequently it was mixed with 600 μL of 300 m Na_2HPO_4 and 150 μL Ellman reagent, and incubated for 10 min at 37°C. The absorbance of yellow dye was measured at 412 nm. GSH content was calculated by means of a standard plot (1–20 mg GSH/dL).

Superoxide dismutase activity of the sample was measured by a commercial kit (RANSOD, Randox Laboratories Ltd, Ardmore, United Kingdom). The method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red

formazan dye (at pH 7.0). The SOD activity is measured as the degree of inhibition of this reaction. The results are reflecting the sum activity of different SOD types, and are expressed as SOD units/mg protein.

Glutathione peroxidase activity was measured by a commercial kit (RANSEL, Randox Laboratories Ltd, Ardmore, United Kingdom). The method is based on reaction of GSH with cumene hydroperoxide catalysed by GSH-Px and yielding GSSG. The latter is converted by glutathione reductase and NADPH to GSH and NADP. The concentration of GSH-Px is assessed from the decrease in absorption at 340 nm due to oxidation of NADPH to NADP. Results are expressed as GSH-Px units/mg protein.

Catalase activity was measured with the method described by Goth,[28] and expressed as CAT units/mg protein.

Troponin T was measured with the second generation of the cardiac troponin T ELISA.[29] The assays were performed using the Enzymum-Test Troponin-T (Boehringer Mannheim GmbH, Mannheim, Germany) on ES300 Enzymum Immunoassay system (Boehringer Mannheim). TnT in the coronary effluent was calculated as amount released per minute [TnT (μg/L) × CF (mL/min) × 10^{-3} = ng/min].

Statistics

A Mann Whitney U-test was employed to compare differences between groups, and a Wilcoxon Signed rank sum test for differences within groups. $p < 0.05$ was considered significant. Data are presented as mean ± SEM.

RESULTS

Left Ventricular Developed Pressure

LVDP was reduced by preconditioning with both 20 μmol/L H_2O_2 and ischaemia compared to controls before perfusion with 140 μmol/L H_2O_2 ($p < 0.02$ and $p < 0.03$, respectively) (Figure 1). LVDP decreased during H_2O_2-perfusion in controls. Hearts preconditioned with H_2O_2 had higher LVDP than controls during perfusion with 140 μmol/L H_2O_2 and the subsequent recovery ($p < 0.01$ at all time points), and higher than ischaemic preconditioned hearts after 5 min of H_2O_2-perfusion ($p < 0.005$) and 30 min recovery ($p < 0.03$). Ischaemic preconditioned hearts were not significantly different from controls after start of intervention (Figure 1).

Left Ventricular End-Diastolic Pressure

Neither preconditioning with ischaemia nor H_2O_2 influenced LVEDP before perfusion with 140 μmol/L H_2O_2 (Figure 1). 140 μmol/L H_2O_2 increased LVEDP in controls, with a maximal level after 5 min recovery (15 min observation). Ischaemic preconditioning attenuated this increase at the end of H_2O_2-perfusion ($p < 0.0004$), and after 5 min recovery ($p < 0.03$). Preconditioning with 20 μmol/L H_2O_2 attenuated the H_2O_2-induced increase of LVEDP at all time points compared to H_2O_2 controls ($p < 0.0006$ at all time points) and compared to ischaemic preconditioning ($p < 0.01$ at all time points) (Figure 1).

Coronary Flow

Preconditioning with 20 μmol/L H_2O_2 reduced CF before intervention compared to controls ($p < 0.02$), and compared to ischaemic preconditioning ($p < 0.002$) (Figure 1). 140 μmol/L H_2O_2 increased CF both during infusion and recovery. Hearts pretreated with H_2O_2 had higher CF than controls after 10 min infusion of 140 μmol/L H_2O_2, and after 5 and 10 min recovery ($p < 0.02$ at all times). Ischaemic preconditioning did not significantly influence CF at any time point (Figure 1).

Heart Rate

HR was 290 ± 7, 296 ± 11, and 288 ± 9 beats/min in controls, IPC and HPC at time 0, respectively.

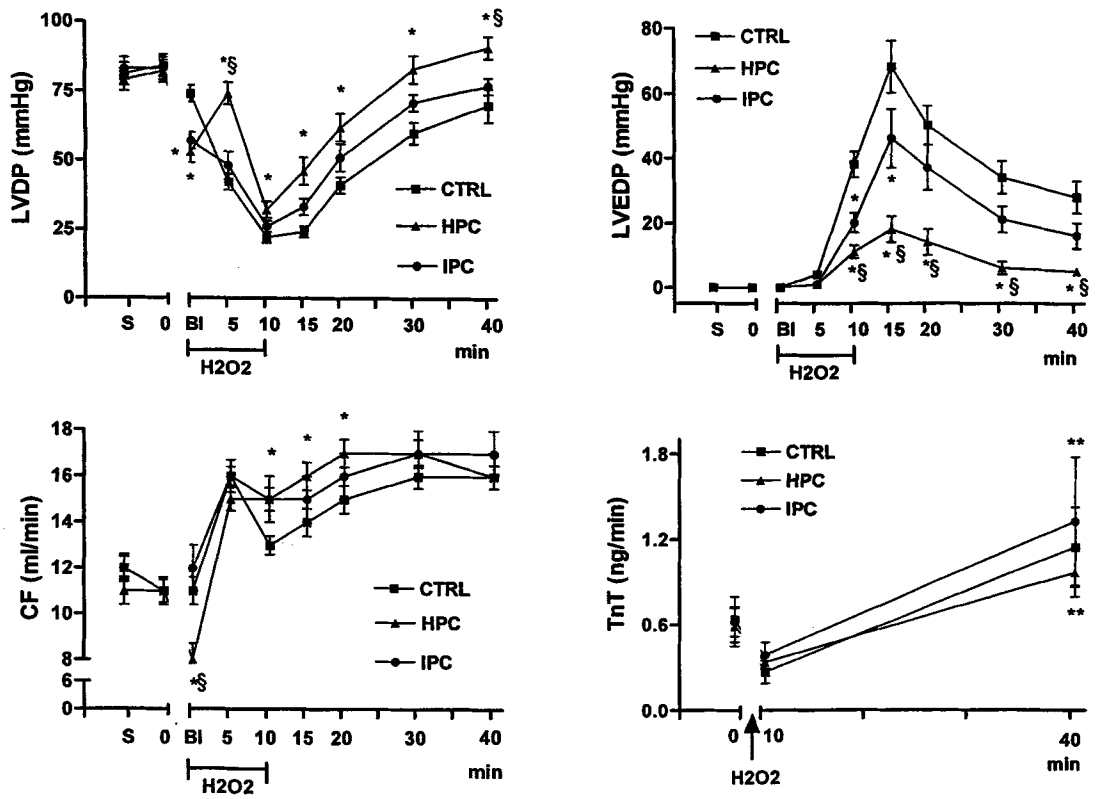

FIGURE 1 Left ventricular developed (LVDP) and end-diastolic (LVEDP) pressures, coronary flow (CF), and release of troponin T (TnT) into the coronary effluent of Langendorff-perfused rat hearts subjected to control perfusion (CTRL, $n = 10$), pretreated with 20 μmol/L H_2O_2 for 10 min followed by 10 min recovery (HPC, $n = 10$), or ischaemic preconditioning with 2 episodes of 2 min global ischaemia and 5 min reperfusion (IPC, $n = 10$) before 10 min perfusion with 140 μmol/L H_2O_2 and 30 min recovery (10–40 min). Values are mean ± SEM. * denotes $p < 0.05$ compared to CTRL, ** denotes $p < 0.05$ compared to value at end of H_2O_2-perfusion (TnT), § denotes $p < 0.05$ between HPC and IPC. S = stabilization (see Methods), 0 = start of the experiments, BI = immediately before intervention with 140 μmol/L H_2O_2.

HR was 321 ± 12, 313 ± 19, and 303 ± 9 beats/min at the end of recovery, with no significant difference within or between groups during preconditioning or perfusion with 140 μmol/L H_2O_2.

Troponin T

Release of TnT tended to decrease at the end of H_2O_2-perfusion, and to increase during recovery (Figure 1). In controls the increase was not significant compared to either time 0 or to the end of H_2O_2-perfusion. In ischaemic preconditioned hearts TnT at the end of recovery was not different from time 0, but increased compared to release at the end of H_2O_2-perfusion ($p < 0.03$). Likewise, TnT release after pretreatment with H_2O_2 increased compared to the end of H_2O_2-perfusion ($p < 0.004$), but not compared to time 0 (Figure 1). There was no difference between groups at any time point.

Tissue Contents of Antioxidants

Reduced glutathione (GSH) The baseline contents of GSH in cardiac tissue before preconditioning was 6.5 ± 0.3 mg/g wet weight, and decreased to 4.0 ± 0.3 mg/g wet weight in

controls at the end of H_2O_2-perfusion ($p < 0.004$) (Figure 2). Preconditioning with H_2O_2 decreased GSH compared to baseline value at the end of H_2O_2-infusion (3.4 ± 0.5 mg/g wet weight, $p < 0.002$), and compared to H_2O_2 controls ($p < 0.02$). In hearts preconditioned with ischaemia GSH decreased compared to baseline value (5.1 ± 0.2 mg/g wet weight, $p < 0.008$), but GSH was increased at the end of H_2O_2-perfusion compared to H_2O_2 controls ($p < 0.02$) (Figure 2).

Glutathione peroxidase (*GSH-Px*) Baseline GSH-Px was 112 ± 8 U/g protein, and did not change significantly in any group (Figure 2).

Total antioxidant status (*TAS*) Baseline TAS in cardiac tissue was 3.5 ± 0.3 µmol/g wet weight. TAS was not significantly reduced by H_2O_2-perfusion, and was not influenced by preconditioning with H_2O_2 or ischaemia (Figure 2).

Catalase (*CAT*) Baseline CAT was 55.9 ± 6.3 U/g protein, and did not change significantly within or between groups (Figure 2).

Superoxide dismutase (*SOD*) Baseline SOD was 2.5 ± 0.2 U/mg protein. 140 µmol/L H_2O_2 increased SOD to 3.1 ± 0.2 U/mg protein ($p < 0.04$). The increase was not modified by H_2O_2-preconditioning, but ischaemic preconditioning abolished the H_2O_2-induced increase of SOD ($p < 0.04$) (Figure 3).

Antioxidant capacity Antioxidant capacity was not influenced by H_2O_2-perfusion or preconditioning (Figure 3).

Thiobarbituric Acid Reactive Substances (*TBARS*) Baseline TBARS were 34 ± 2 nmol/g

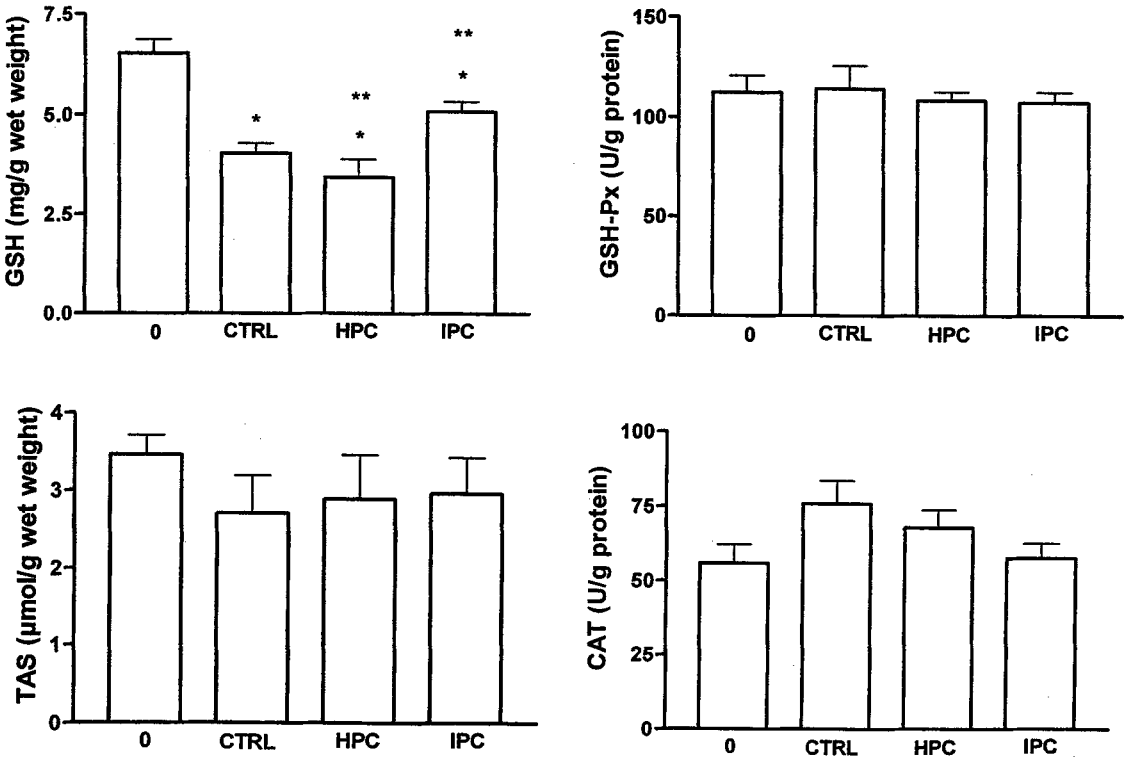

FIGURE 2 Tissue contents of glutathione (GSH), glutathione peroxidase (GSH-Px), total antioxidant status (TAS), and catalase (CAT) in Langendorff-perfused rat hearts before intervention (0, $n = 10$), after 20 min control perfusion and 10 min perfusion with 140 µmol/L H_2O_2 (CTRL, $n = 10$), after pretreatment with 20 µmol/L H_2O_2 for 10 min followed by 10 min recovery and 10 min perfusion with 140 µmol/L H_2O_2 (HP C, $n = 10$), or ischaemic preconditioning with 2 episodes of 2 min global ischaemia and 5 min reperfusion and 10 min perfusion with 140 µmol/L H_2O_2 (IPC, $n = 10$). Values are mean ± SEM. * denotes $p < 0.05$ compared to 0, ** denotes $p < 0.05$ compared to CTRL.

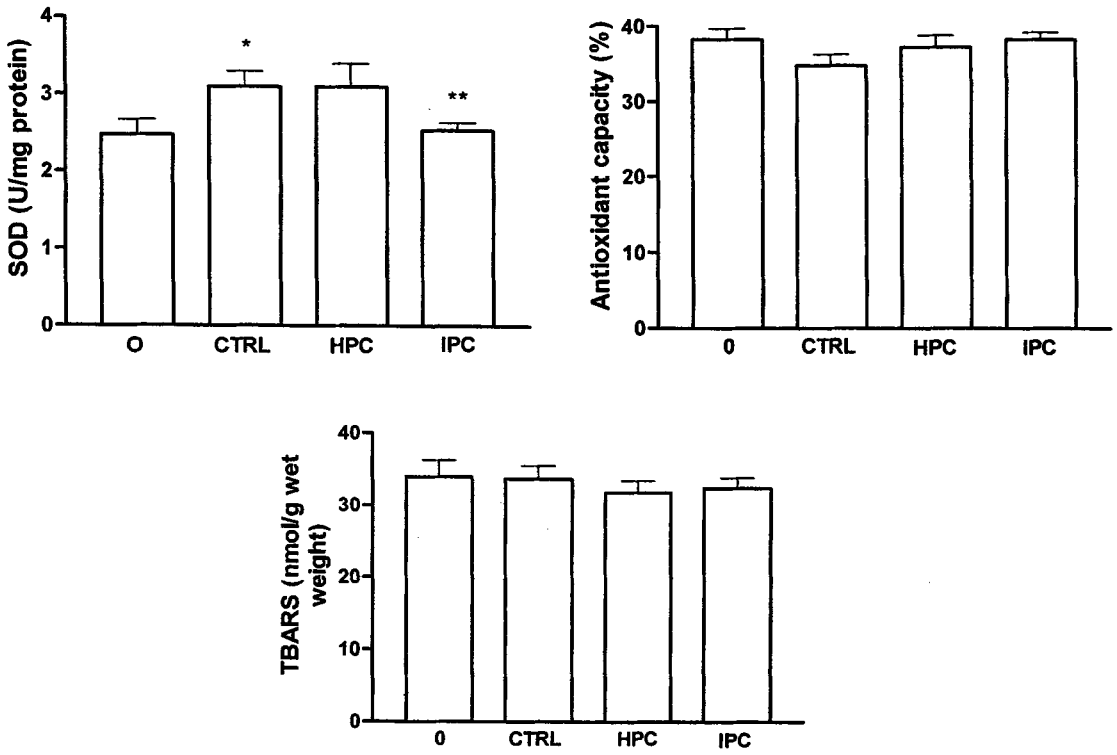

FIGURE 3 Tissue contents of superoxide dismutase (SOD), antioxidant capacity as measured by *in vitro* inhibition of linoleic acid peroxidation, and thiobarbituric acid reactive substances (TBARS) in Langendorff-perfused rat hearts before intervention (0, $n = 10$), after 20 min control perfusion and 10 min perfusion with 140 μmol/L H_2O_2 (CTRL, $n = 10$), after pretreatment with 20 μmol/L H_2O_2 for 10 min followed by 10 min recovery and 10 min perfusion with 140 μmol/L H_2O_2 (HPC, $n = 10$), or ischaemic preconditioning with 2 episodes of 2 min global ischaemia and 5 min reperfusion and 10 min perfusion with 140 μmol/L H_2O_2 (IPC, $n = 10$). Values are mean ± SEM. * denotes $p < 0.05$ compared to 0, ** denotes $p < 0.05$ compared to CTRL.

wet weight, and did not change after H_2O_2-perfusion or preconditioning (Figure 3).

DISCUSSION

The main findings of the present study were that cardiac dysfunction induced by H_2O_2 was only minimally influenced by ischaemic preconditioning, while preconditioning with a low dose of H_2O_2 prior to the higher dose of H_2O_2 attenuated the increase of LVEDP, the decrease of LVDP, and increased CF during recovery. There was no difference between groups in the cardiac release of TnT during recovery, indicating that reduction of myocyte injury was not an important mechanism for the functional protection afforded. One previous study has investigated the effect of ischaemic preconditioning prior to hydrogen peroxide. In accordance with the present findings, Gan *et al.* found that ischaemic preconditioning prior to 30 min 200 μmol/L H_2O_2 had only marginally beneficial effects on the function of isolated rat hearts.[23]

We have previously shown that the ischaemic preconditioning model employed in the present study protects isolated rat hearts against global ischaemia, and that preconditioning episodes of 2 min duration optimally preserved cardiac function during reperfusion.[19,30] Moderate oxidative stress as a possible cardioprotective intervention is not well studied. 20 μmol/L H_2O_2 was selected

for preconditioning, as this concentration was the only one of several tested which improved post-ischaemic performance in globally ischaemic rat hearts.[19] However, this concentration only protected against severe reperfusion arrhythmias, but not against left ventricular dysfunction.[18,19] Ischaemic preconditioning provided far better protection than hydrogen peroxide in this model of ischaemia-reperfusion injury.[19] Consequently when ischaemia-reperfusion was the cause of injury the preconditioning response was stronger by ischaemia than by hydrogen peroxide.[19] When hydrogen peroxide caused the main injury as in the present study, the protection induced by hydrogen peroxide was more evident than protection provided by ischaemia.

The exact mechanisms of cardiac dysfunction induced by high doses of H_2O_2 are not fully clarified. H_2O_2 itself may be the main injurious agent, since catalase inhibits its functional as well as biochemical effects.[31–33] The hydroxyl radical may have been generated, as hydroxyl radical scavengers partially attenuate the cardiac dysfunction induced by H_2O_2.[31,32] In the present study 140 μmol/L H_2O_2 did not induce lipid peroxidation. We have previously found that perfusing rat hearts with 180 μmol/L H_2O_2 for 10 min increased TBARS at the end of intervention.[34] The lipid peroxidation product malone dialdehyde increased in isolated rat hearts perfused with 600 μmol/L H_2O_2,[35] and was released into the coronary effluent of hearts perfused with either 200 or 300 μmol/L, but not 100 μmol/L H_2O_2.[36] In rat hearts perfused with xanthine oxidase/hypoxanthine, tissue contents of malone dialdehyde was increased at the end of a 10 min intervention, as were contents of H_2O_2.[37] The concentration of H_2O_2 employed in the present study was evidently not high enough to induce a significant lipid peroxidation as evaluated by TBARS and indirectly by antioxidative markers. Consequently, lipid peroxidation may not explain the functional impairment after ROI-induced injury in the present model.

Total antioxidant status and capacity were unchanged, as were myocardial contents of CAT and GSH-px. H_2O_2 increased tissue SOD, and this increase was abolished by ischaemic preconditioning. In a previous study, perfusing rat hearts with hypoxanthine/xanthine oxidase reduced tissue contents of SOD at the end of intervention.[37] This finding may be due to augmented production of the superoxide anion by hypoxanthine/xanthine oxidase, and subsequent depletion of cardiac SOD to protect against superoxide-induced injury. A possible depletion of SOD by superoxide anion production could have taken place in hearts subjected to ischaemic preconditioning in the present study, explaining why these hearts had lower SOD than hearts exposed to H_2O_2 only at the end of intervention.

Tissue glutathione was decreased by H_2O_2, suggesting that in the present model, thiol-groups were more easily exposed to H_2O_2-induced injury than myocardial lipids. The reduction was augmented by pretreatment with H_2O_2, and was attenuated by ischaemic preconditioning. Perfusing rat hearts with H_2O_2 has previously been found to increase release of glutathione into the coronary effluent,[38] which may explain the reduced tissue contents found in the present study. Depletion of endogenous glutathione seemed not to be critical for cardiac function in our study, as the most depleted hearts had the best function. Accordingly, Steare and Yellon found that increased endogenous glutathione levels prior to perfusion with H_2O_2 in isolated rat hearts did not protect against H_2O_2-induced functional injury or release of lactate dehydrogenase.[39] However, exogenous glutathione attenuated hypoxia-induced stunning in another isolated rat heart model.[40] Loss of glutathione has been found in conjunction with ischaemic preconditioning.[8] N-acetylcysteine blocked both the loss of glutathione and the beneficial effects of preconditioning on cardiac function.[8] It may be speculated that changes in glutathione levels are adaptive in a certain range, but impaired myocardial function may be

evident when glutathione decreases below a critical concentration. On the other hand, the availability of glutathione in cellular compartments might be of importance. The depletion of glutathione could reflect improved protection of protein SH-groups at the expense of glutathione oxidation, and, consequently, could explain the improved contractile function in the HPC group.

We can only speculate on the possible mechanisms of attenuating H_2O_2-induced injury by preconditioning, as the beneficial functional effects were not due to reduction of irreversible cell injury evident as release of TnT, inhibition of lipid peroxidation, or increase of antioxidant levels. We have previously found that perfusing isolated rat hearts with low concentrations of H_2O_2 induces a vasodilation which is partly dependent on nitric oxide, and inhibition of nitric oxide aggravated functional effects of H_2O_2.[41] Thus, release of nitric oxide in hearts pretreated with H_2O_2 may have contributed to the protective effects in the present study. However, before inducing injury with 140 µmol/L H_2O_2, CF was lowest in hearts preconditioned with H_2O_2. A possible role of endogenous adenosine in ischaemic preconditioning prior to injury induced by exogenous H_2O_2 has been suggested by Gan *et al.*,[23] but exogenous adenosine did not significantly reduce dysfunction caused by H_2O_2.[42] We have previously found that vasoactive eicosanoids play a role in H_2O_2-induced cardiac injury.[43] As prostacyclin has been suggested as a mediator of ischaemic preconditioning,[20] it may possibly have contributed to protection against H_2O_2-induced dysfunction. H_2O_2 may activate phospholipases,[44] which may activate protein kinase C, which is proposed as a major mechanism of myocardial protection afforded by ischaemic preconditioning.[20] Indeed, Miyawaki *et al.*[45] pretreated isolated rat hearts with 20 µmol/L H_2O_2, and attenuated the calcium paradox through protein kinase C activation. However, if this were a mechanism in the present study, ischaemic preconditioning should be expected to improve cardiac function as well.

In summary, ischaemic preconditioning offered only marginal protection against dysfunction induced by H_2O_2 in isolated rat hearts, while pretreatment with a low dose of H_2O_2 attenuated cardiac dysfunction. The mechanism of protection was not through reduction of myocyte injury, limitation of lipid peroxidation, or influence on cardiac antioxidant activity.

Acknowledgements

This work was supported by grants from The Swedish Society of Medicine, The Family Blix' Foundation (Norway), the Swedish Medical Research Council (11235), the Estonian Scientific Foundation (1371) and the Karolinska Institute and Hospital. The excellent technical assistance of Kersti Zilmer, Ceslava Kairane, and Aune Rehemaa, Institute of Biochemistry, University of Tartu, is gratefully acknowledged.

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