Ischemic preconditioning enhances scavenging activity of reactive oxygen species and diminishes transmural difference of infarct size

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Ischemic preconditioning enhances scavenging activity of reactive oxygen species and diminishes transmural difference of infarct size. Am J Physiol Heart Circ Physiol 290: H577–H583, 2006. First published July 22, 2005; doi:10.1152/ajpheart.00817.2004.—Reactive oxygen species (ROS) enhance myocardial ischemia-reperfusion (I/R) injury. Ischemic preconditioning (PC) provides potent cardioprotective effects in I/R. However, it has not been elucidated whether PC diminishes ROS stress in I/R and whether PC protects the myocardium from ROS stress transmurally and homogeneously. Ischemic PC diminishes ROS stress in I/R and whether PC protects the myocardium from ROS stress transmurally and homogeneously. More specifically, it is not known whether ischemic PC cancels the potentially harmful effects of ROS transmurally.

Accordingly, the primary goal of the current investigation was to determine whether the effects of ischemic PC were transmurally homogeneous. A second goal was to determine whether ischemic PC modified oxidative stress, in particular, whether ischemic PC salvaged ROS-mediated myocardial damage and enhanced the myocardial scavenging action of ROS transmurally. To accomplish these goals, we investigated the effects of ischemic PC in isolated perfused rabbit hearts. The effects of ischemic PC on myocardial oxidative stress were compared with and without the enhanced ROS stress of H$_2$O$_2$ infusion in the first minute of early reperfusion. We studied ROS-mediated myocardial injury using hydroxylated deoxyguanosine residues of 8-hydroxydeoxyguanosine (8-OHdG) (19), an indicator of DNA damage, which has been reported to initiate and mediate ischemic PC and its cardioprotective mechanism (13, 14, 22, 23, 24). Recently, Vanden Hoek et al. (37) showed that ischemic PC was associated with the attenuation of an oxidant burst at reperfusion in cultured cardiac cells. It remains unclear whether ischemic PC attenuates ROS-mediated myocardial injury in vivo.

Ischemic myocardial damage is not transmurally homogeneous. The subendocardium is more vulnerable to ischemia than is the subepicardium. Myocardial damage of infarction and stunning is more prominent in the subendocardium than in the subepicardium (16, 17, 28). However, it is not known whether the cardioprotective effect of ischemic PC is exerted transmurally and homogeneously. More specifically, it is not known whether ischemic PC cancels the potentially harmful effects of ROS transmurally.

METHODS

Isolated perfused heart preparation. Japanese White male rabbits weighing 3.0–3.8 kg were anesthetized with intravenous pentobarbital sodium (40 mg/kg). The rabbit was mechanically ventilated with 100% oxygen via a positive-pressure respirator (model SN-460-6, Shinano). The chest was opened and the heart was quickly removed. Retrograde perfusion was started on a Langendorff apparatus with a Krebs-Henseleit solution, gassed with 95% O$_2$–5% CO$_2$. The initial perfusion pressure was set at 100 cmH$_2$O. Hearts were paced at 200 beats/min, and the left ventricular (LV) pressure was continuously monitored with an intraventricular latex balloon connected to a transducer (AP-601G, Nihon-Kohden). The initial end-diastolic pressure of the left ventricle was adjusted to 5 mmHg. The coronary blood flow was monitored by an electromagnetic flow probe (model FF-060T, Nihon-Kohden).
The animals used in the present study were treated in accordance with the guidelines of the Committee on Laboratory Animals, and the study protocol was approved by the Ethical Committee of Asahikawa Medical College.

Experimental protocol. The experimental protocol is summarized in Fig. 1. Rabbids were assigned to four groups. The control group (n = 18 rabbits) underwent 30 min of global ischemia and 60 min of reperfusion. The PC group (n = 20) underwent a single cycle of ischemic PC, 5 min of ischemia, and 10 min of reperfusion before ischemia-reperfusion. The H2O2 group (n = 19) underwent ischemia-reperfusion with H2O2 infusion (50 μM, 15 ml/min) started in the first minute of reperfusion with a syringe pump (syringe infusion pump 22, Harvard Apparatus, Holliston, MA) to enhance oxidative stress during the early reperfusion period. We determined this dose of H2O2 infusion by preliminary experiments testing 10 to 100 μM H2O2 infusion. We found that 1 min of infusion of H2O2 in these doses did not cause significant myocardial damage 60 min after its application by using the triphenyltetrazolium chloride (TTC) method, but two of three hearts with 100 μM H2O2 infusion could not complete the protocol of 60-min reperfusion. Thus we elected to use 50 μM H2O2 infusion, which could cause a modifiable enhancement of LV dysfunction, i.e., ~20% reduction in the developed pressure (DP) after 60-min reperfusion. The H2O2+PC group (n = 20) underwent a PC with H2O2 protocol. The detailed hemodynamic data were analyzed in 12 of 18 rabbits in the control group, 13 of 20 rabbits in the PC group, 14 of 19 rabbits in the H2O2 group, and 13 of 20 rabbits in the H2O2+PC group. In the rest of the experiments, we monitored the hemodynamic variables at selected time points to confirm the stability of the hemodynamic conditions. In each group, eight hearts were used for the myocardial infarct size measurement, and the rest were used for the following analysis.

Measurement of myocardial infarct size. After 60 min of reperfusion, five hearts from each group were sliced into 2-mm-thick transverse sections across the long axis. The slices were incubated in 1% TTC in phosphate buffer (pH 7.4) at 37°C for 15 min. Viable tissue stains brick red and infarct tissue is left unstained, allowing the differentiation of damaged tissue (11). The volume of the left ventricle and the area of infarct tissue were measured blindly by an independent observer using computerized planimetry (NIH Image, Research Service Branch, National Institutes of Health). Each slice was mechanically divided into the subendocardium and subepicardium parts by tracing the middle points of radius. The infarct and noninfarct areas in each part were separately measured, and the volumes were calculated by multiplying by the slice thickness. Myocardial infarct size was expressed as a percentage of the LV volume of the subendocardium and subepicardium.

Reduced GSH and GSH peroxidase activity assay. Myocardial samples from the subendocardium and subepicardium were separately stored at −80°C before assay and acidified in an ice-cold 5% metaphosphoric acid working solution to reduce oxidation of GSH to GSSG. Reduced GSH was measured by using nonenzymatic two-step colorimetric assay (Bioxytech GSH-400, Oxis International, Portland, OR). The derived thioether obtained from GSH mixed with a reagent (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulfate) was then transformed into a chromophoric thione by the addition of 30% NaOH. Its absorbance at 400 nm was measured, and the concentration of GSH in samples was expressed as nanomoles per gram of wet weight.

The tissue GSH peroxidase (GPX) activity was measured spectrophotometrically in absorbance at 340 nm with the oxidation of NADPH to NADP by tert-butyl hydroperoxide. GPX activity was expressed in millimoles of NADPH oxidized per milliliter (mU/mg protein).

Isolation and hydrolysis of myocardial DNA and quantification of 8-OHdG. Myocardial samples were separated into the subendocardium and subepicardium, rapidly frozen in liquid N2, and later homogenized and digested by protease K under an argon atmosphere. The resulting solutions were mixed with NaI and isopropyl alcohol according to the method of Kaneko and Tahara (18). The pelleted DNA was treated by ribonuclease T1, ribonuclease A, and Sevag. The aqueous phase of centrifugation was mixed with polyethylene glycol overnight at 4°C. The concentration and purity of DNA were determined by ultraviolet (UV) absorption.

Acetate buffer (pH 4.8) containing 20 μg DNA and nuclease P1 was incubated, and 1 M Tris•HCl (pH 7.4) and 0.3 units of alkaline phosphatase were then added under an argon atmosphere. The mixture was filtered and applied to HPLC equipped with a Pegasil 0DS column (4.6 × 150 mm, Senshu Scientific). Detection of 8-OHdG was performed by electrochemical detection (ECD) with the use of ECD-100 (Eicom). Oxidative damage of DNA is expressed as the molar ratio of 8-OHdG to 106 deoxyguanosine. The amount of deoxyguanosine in each sample was measured with a UV detector at 260 nm (UV-484MS; Waters, Milford, MA).

Immunohistochemistry. The cardiac sections were blocked in 10% normal pig serum (Cosmo Bio) and incubated with mouse monoclonal anti-8-OHdG antibodies (Nikkken Foods) overnight at 4°C. Subsequently, sections were incubated with peroxidase conjugate rabbit anti-mouse antibodies (Dako) for 30 min and the chromogen substrate dianimobenzene for color development. Nuclei were counterstained with hematoxylin. Slices were observed with the use of the BX-FLA-1 system (Olympus).

Statistical analysis. All data are expressed as means ± SE. Statistical analysis was performed by two-way repeated-measures ANOVA followed by Bonferroni’s post hoc test or Scheffé’s post hoc test when appropriate. Differences with P < 0.05 were considered significant.

### RESULTS

#### Hemodynamic parameters

LV systolic pressure (LVP), LV end-diastolic pressure (LVEDP), DP, and coronary flow in the four groups are summarized in Table 1 and Fig. 2. There were no significant differences in the baseline hemodynamic parameters among the four groups. After 60 min of reperfusion, the LVEDP in the PC group was slightly greater than 86 ± 5 mmHg compared with 78 ± 7 mmHg of the control group. H2O2 decreased LVEDP, markedly increased LVEDP, and consequently diminished a transient hyperdynamic response of DP just after reperfusion (Fig. 2). DP after 60 min of reperfusion was significantly lower than 50 ± 5 mmHg in the H2O2 group.
Myocardial infarct size. Figure 3A shows the individual data of myocardial infarct size, and Fig. 3B summarizes the transmural differences of infarct size. The myocardial infarct size of the control group was 37 ± 4% and was significantly reduced by 19 ± 2% in the PC group (P < 0.05; Fig. 3A). The myocardial infarct size was significantly increased by 59 ± 4% in the H2O2 group (P < 0.05) and was significantly reduced by 31 ± 4% in the H2O2 + PC group (P < 0.01). There was no significant difference between the PC and H2O2 + PC groups.

As shown in Fig. 3B, the myocardial infarct size was significantly reduced in the subendocardium as well as the subepicardium in the PC group compared with the control group (P < 0.05), particularly in the subendocardium. Consequently, the transmural difference in infarct size was diminished by PC. H2O2 significantly increased the infarct size transmurally (P < 0.05), and this enhancement was totally abolished in the H2O2 + PC group (P < 0.01).

GSH content and GPX activity. GSH content was significantly preserved in the PC group compared with the control group in the subendocardium (130.2 ± 10.0 vs. 98.8 ± 6.1 nmol/g wet wt; P < 0.01) and subepicardium (149.3 ± 5.7 vs. 118.6 ± 12.0 nmol/g wet wt; P < 0.05) (Fig. 4). GSH content was significantly reduced in H2O2 in the subendocardium (30.2 ± 3.0 nmol/g wet wt; P < 0.01) and subepicardium (42.3 ± 6.9 nmol/g wet wt; P < 0.01) and increased in the

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Values are means ± SE. LVSP, left ventricular (LV) systolic pressure; LVEDP, LV end-diastolic pressure; DP, developed pressure; CF, coronary flow; PC, preconditioning; n, no. of rabbits. *P < 0.05, †P < 0.01 vs. baseline; ††P < 0.01 vs. control; †††P < 0.01 vs. PC; and ††††P < 0.01 vs. reactive oxygen species.

(P < 0.05) and was significantly preserved in the H2O2 + PC group (80 ± 5 mmHg; P < 0.05).

Fig. 2: Time course of changes in left ventricular developed pressure (%DP) in control (○; n = 12) and PC (●; n = 13) (top) and H2O2 (□; n = 14) and H2O2 + PC (■; n = 13) (bottom). Note that the transient hyperdynamic response of %DP just after reperfusion in control and PC groups was totally abolished in H2O2 and H2O2 + PC groups. After 60 min of reperfusion, %DP was significantly suppressed in H2O2 compared with H2O2 + PC. Values are means ± SE. *P < 0.05 vs. control or H2O2; **P < 0.01 vs. H2O2. Shaded area indicates H2O2 50 μM infusion.

Fig. 3: A: individual (○) and average (●) data of myocardial infarct size as a percentage of left ventricular volume. Infarct size was significantly reduced in PC (n = 8) compared with control (n = 8). Infarct size of H2O2 (n = 8) was significantly increased compared with control and was significantly reduced in H2O2 + PC (n = 8). B: transmural difference of infarct size in subendocardium (solid bars) and subepicardium (open bars). Myocardial infarct size was significantly smaller in subendocardium in control (n = 8). PC (n = 8) reduced infarct size, particularly in subendocardium, and diminished transmural difference in infarct size. H2O2 (n = 8) significantly increased infarct size transmurally, and it was totally abolished in H2O2 + PC (n = 8). Values are means ± SE. *P < 0.05 vs. control; †P < 0.01 vs. PC.
protocol were potentially modest compared with those of multiple cycles of PC protocol (29), the scope of the present study was to evaluate the effects of ischemic PC on the modifiable myocardial damage with a focus on oxidative stress. The subendocardium is more vulnerable to ischemia (16, 17, 28) because of greater oxygen demand due to greater wall motion (39), greater extravascular compression (31), and greater mechanical stress compared with the subepicardium (39). As we expected, the myocardial infarct size was significantly greater in the subendocardium. Importantly, ischemic PC abolished the transmural difference of infarct size. We investigated the impact of oxidative stress on the transmural differences of infarct size and effects of ischemic PC.

We used 8-OHdG as a fingerprint of myocardial oxidative DNA damage in ischemia-reperfusion hearts. There are a few reports on myocardial oxidative DNA damage. Cordis et al. (6) demonstrated that 8-OHdG content was increased after ischemia-reperfusion in rat hearts. Recently, Tsutsui et al. (35) reported that the increased generation of ROS was associated with mitochondrial DNA damage and cardiac dysfunction in failing hearts. However, myocardial DNA damage has never been investigated in ischemic PC. We first demonstrated the effects of ischemic PC and enhanced oxidative stress on myocardial oxidative DNA damage using 8-OHdG levels in vivo.

The changes in 8-OHdG levels were similar to those in the infarct size but not identical. The myocardial 8-OHdG levels were relatively greater in the subendocardium but were not statistically significant being different from the infarct size. The major reason for this discrepancy may be because oxidative DNA damage does not directly indicate necrosis of tissue (6, 18, 40). We used 1 min of infusion of H2O2, started just after reperfusion. This short duration of exogenous H2O2 application was sufficiently powerful to abolish the transient hyperdynamic response of DP just after reperfusion, shown in Fig. 2. Although we did not have direct evidence of transmural equal distribution of H2O2, we confirmed that the infused H2O2 enhanced the infarct size with maintaining the transmural difference of infarct size as in the controls, suggesting that the effects of infused H2O2 were expressed transmurally. In the present study, the myocardial protective effects of ischemic PC were observed transmurally, but they were more prominent in
the subendocardium. Consequently, the transmural difference in the myocardial infarct size was diminished by PC. To the best of our knowledge, this is the first report demonstrating that ischemic PC cancels not only endogenous but also exogenous oxidative stress with enhancement of ROS scavenging activity, facilitates the functional recovery of DP, and reduces the myocardial infarct size. It was accompanied by the preservation of GSH levels.

Intracellular GSH scavenges -OH, HOCl, peroxynitrate and O2• radicals (4), and preserves myocardial lipophilic antioxidant under oxidative stress conditions (21). Thus preservation of myocardial GSH content in the present study may indicate enhancement of scavenging ability in hearts. Alterations in myocardial antioxidants after ischemia-reperfusion remain controversial (1, 7, 8, 10, 15, 27). Only a study by Turrens et al. (36) reported the effects of ischemic PC on myocardial antioxidants of GSH levels. Turrens et al. (36) found better preservation of GSH levels in preconditioned hearts, which was consistent with our results. They speculated that it was because of less injury in preconditioned hearts. In contrast, Vanden Hoek et al. (37) demonstrated that attenuation of oxidative stress is a crucial mechanism of ischemic PC. In the present study, two findings suggested that greater oxidative stress leads to a greater consumption of GSH in myocardium. The first was the fact that greater myocardial infarct size accompanied the lower GSH levels, and the second was the significant reduction of GSH levels in H2O2 group. Interestingly, we found a significantly greater increase in GSH levels in the H2O2+PC group compared with the H2O2 group. This discrepancy suggested another possibility, which is that ischemic PC not only reduced consumption of GSH but also actively preserved GSH levels during ischemia-reperfusion in the presence of enhanced oxidative stress.

GSH is an important cofactor for the GPX family. GPX removes H2O2 by coupling its reduction to H2O with oxidation of GSH (20) and plays a crucial role in LV remodeling and failure after myocardial infarction (30). Some studies reported increased activity of myocardial enzymatic antioxidants in ischemia-reperfusion (1), whereas other studies reported decreased (10, 27) or unchanged (7, 37) activity. In the present study, GPX activity was significantly suppressed by H2O2 infusion but was not affected by ischemic PC per se. Thus the changes in GPX activity were not likely the major determinant of preserved GSH levels in the present study. GSH is enzymatically synthesized and degraded (20). The changes in metabolic pathways of GSH may facilitate preservation of GSH levels during ischemia-reperfusion in the presence of enhanced oxidative stress. Further studies are required to clarify these enzymatic changes of GSH metabolism in ischemia-reperfusion.

**Study limitation.** Although the normal range of 8-OHdG level has never been determined in myocardium, the 8-OHdG levels measured in the present study were relatively lower...
comparing with the data of Cordis et al. (6) and You et al. (40) for rat hearts, Cordis et al. (6) applied a UV detection method instead of the ECD-UV detection method, which we used in the present study. The major difference was the method for DNA extraction with the use of phenol, which potentially increases artificial DNA damage and shows 20-fold overestimation of 8-OHdG compared with that of the phenol-free method (5). We used NaI instead of phenol for extraction of DNA to lessen artificial oxidative damage. This can be the major cause of relatively lower 8-OHdG levels in the present study.

The effect of myocardial ischemia, which was inevitably required for setting up the isolated perfused heart, was not completely negligible for the assessment of myocardial damage induced by ischemia-reperfusion. Although hearts were readily removed and set on the Langendorff apparatus as quickly as possible, it required ~15 to 20 s of cessation of perfusion. However, it was equally applied to all experimental conditions in the present study. The correlation of the measurements of the TTC method after 60 min of reperfusion and the histological findings has not been confirmed in the present study. Although a relatively short duration of reperfusion potentially underestimates the histological infarct size, 60 min of reperfusion have been used in previous studies (33, 34) to evaluate the myocardial infarct size by TTC. We evaluated the effects of ischemic PC on the modifiable myocardial damage with a focus on oxidative stress by applying the same protocol of 60 min of reperfusion on four different experimental ischemic conditions.

In conclusion, ischemic PC reduced myocardial infarct size, particularly in the subendocardium, and consequently diminished the transmural difference in myocardial infarction. Exogenous ROS of H2O2 infusion transmurally aggravated oxidative myocardial damage. Preserved GSH content leading to preserved scavenging activity against ROS contributed, at least in part, to the mechanism of myocardial protection of ischemic PC.

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GRANTS

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REFERENCES


