Ischemic Preconditioning Protects Against Coronary Endothelial Dysfunction Induced by Ischemia and Reperfusion

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Background. Repetitive, brief periods of ischemia and reperfusion ("preconditioning") increase the resistance of myocardial tissue to subsequent prolonged ischemic episodes and limit infarct size. We investigated whether preconditioning also protects against coronary endothelial dysfunction induced by ischemia and reperfusion.

Methods and Results. Experiments were performed in four groups of rats (n=8 in each group): group 1 rats underwent sham surgery, group 2 rats were subjected to 20 minutes of left coronary artery occlusion without reperfusion, group 3 rats underwent 20 minutes of occlusion followed by 1 hour of reperfusion, and group 4 rats (preconditioning group) underwent the same protocol as group 3 rats, preceded by three cycles of 5 minutes of ischemia and 5 minutes of reperfusion. At the end of the experiments, coronary segments (internal diameter, 250 to 300 μm) were removed distal to the occlusion site and mounted in wire myographs for isometric tension recording. Relaxations induced by increasing concentrations of acetylcholine, the calcium ionophore A23187, or the nitric oxide (NO) donor SIN-1 were determined in arteries precontracted by serotonin. Basal NO release was estimated by measuring contractions to Nω-nitro-L-arginine methyl ester (L-NAME). In addition, we determined the effect of preconditioning on infarct size in two additional groups that were subjected to the same protocols as those of groups 3 and 4. In those animals, area at risk (India ink injection) and infarct size (triphényltetrazolium stain) were determined by computerized analysis of enlarged sections after video acquisition. Preconditioning markedly limited infarct size (percent of area at risk: controls, 57±2; preconditioning, 2.2±0.6; P<.01). Ischemia (without or with reperfusion) or preconditioning did not affect the coronary responses to L-NAME, serotonin, A23187, or SIN-1. Ischemia without reperfusion did not modify the relaxations to acetylcholine (maximal relaxation: sham, 58±4%; ischemia, 56±7%; P=NS). In contrast, ischemia followed by reperfusion markedly impaired the response to acetylcholine (26±6%; P<.01 versus sham). This impaired response was restored by preconditioning (maximal relaxation: 59±9%; P=NS versus sham; P<.01 versus ischemia/reperfusion).

Conclusions. In addition to protecting myocardial cells, preconditioning also protects coronary endothelial cells against ischemia/reperfusion injury. (Circulation. 1994;89:1254-1261.)

Key Words: • endothelium • circulation • nitric oxide • ischemia • reperfusion

Repetitive, short periods of ischemia, separated by intermittent reperfusion, render the heart more tolerant to subsequent ischemic episodes and induce a marked limitation of infarct size.1 Such a cardioprotective effect, which has been called "ischemic preconditioning," has been shown to occur in various species, including dogs,1-4 pigs,3 rabbits,5,7 and rats.8-12 A similar protection recently has been suggested to also occur in humans during angioplasty13,14 or coronary bypass surgery.15

Initially, much attention has been paid to the infarct size–limiting properties of preconditioning. More recently, the concept of preconditioning has been expanded to include protection against other consequences of ischemia, such as reperfusion-induced arrhythmias.8,16 Moreover, it is well known that ischemic injury of the heart is not limited to myocardial cells but extends to coronary vascular cells, including endothelial cells. However, although the beneficial effects of preconditioning have been well characterized at the level of the myocytes, whether a similar effect extends to coronary vascular cells, and especially endothelial cells, is largely unknown.

The endothelium regulates coronary vascular tone through the production of various vasodilating and vasoconstricting substances that can profoundly affect cardiovascular function.17,18 One of the most important of these substances is the endothelium–derived relaxing factor (EDRF),19 which has been identified as nitric oxide (NO)20 or a NO-containing molecule.21 NO is continuously produced from endothelial cells both in vitro and in vivo, and this permanent production contributes to maintain a permanent dilator tone in the circulation.22 In addition to being a vasodilator, NO inhibits platelet aggregation and adhesion and possesses antiaggregatory properties on neutrophils.22 Various experiments suggested that reperfusion after prolonged episodes of ischemia is associated with an impaired endothelium-dependent vasodilation that can be observed at the level of the coronary microcirculation23-26 and at the level of large conduit coronary arteries,27-32 although other studies failed to detect an impairment at
this level.\textsuperscript{25,33} However, despite this accumulating evidence, it is not known whether such an impaired endothelium-dependent vasodilation of large coronary arteries can be prevented by preconditioning.

Therefore, the present study was designed to assess whether ischemia followed by reperfusion induces coronary endothelial dysfunction in rats and whether this endothelial dysfunction could be prevented by preconditioning.

**Methods**

**Experimental Preparation**

The study was performed with male Wistar rats (Charles River) weighing between 300 and 400 g that were anesthetized with 40 mg \( \cdot \) kg\(^{-1} \) sodium pentobarbital IP. A midline incision was made in the neck, and a tracheotomy was performed. The rats were mechanically ventilated with room air supplemented with low-flow oxygen using a small-rodent ventilator (Apleex) at a rate of 60 cycles per minute and a tidal volume of 1 mL/100 g body wt. The respiratory rate and tidal volume were adjusted to maintain arterial blood gases within a normal range. Body temperature was maintained at 37°C using a thermostated heating blanket connected to a rectal thermometer. The left carotid artery was cannulated, and a small Millar Mikrotip catheter (model SPR407, Millar Instruments) was inserted in the artery to measure arterial blood pressure. An ECG was also obtained using standard limb electrodes. Heart rate and arterial pressure were monitored continuously on a Gould ES2000 recorder. In the experiments on infarct size, the right jugular vein was cannulated for injection of India ink to delineate the area at risk (see below).

A left thoracotomy was performed, and the heart was exposed. A 7-0 polypropylene suture was passed around the proximal left coronary artery, and the ends were passed through a small plastic tube to form a snare. The artery was occluded by pulling the snare, which was kept in place with a hemostatic clamp. Myocardial ischemia was confirmed by visual cyanosis. Reperfusion was induced by releasing the snare. Sham-operated rats were subjected to the same protocol, except the snare was not tied.

**Experimental Protocol**

Rats were assigned to one of six groups. Two groups (one control and one preconditioned) were used for determination of the effect of preconditioning on infarct size, and four groups were used in the in vitro vascular experiments. In all experiments, preconditioning was induced by three cycles of 5 minutes of coronary occlusion, each followed by 5 minutes of reperfusion, whereas prolonged ischemia consisted of a 20-minute period of coronary occlusion, followed by 60 minutes of reperfusion.

The following groups of rats were used for the vascular studies (Fig 1). Group 1 rats (sham) were killed after a 110-minute open chest period without occlusion of the artery. Group 2 rats (ischemia only) were subjected to 20 minutes of ischemia without reperfusion. Group 3 rats (ischemia/reperfusion) were subjected to 20 minutes of coronary occlusion followed by 60 minutes of reperfusion. Group 4 rats (preconditioning) were preconditioned with three cycles of 5 minutes of coronary occlusion, each followed by 5 minutes of reperfusion. At the end of the last 5-minute period of reperfusion, hearts were subjected to the same 20-minute coronary occlusion–60-minute reperfusion cycle as in group 3.

The two groups used for the studies on the effect of preconditioning on infarct size were subjected to the same protocols as in groups 3 (controls) and 4 (preconditioning).

**Measurements of Area at Risk and Infarct Size**

In the two groups from the infarct size study, the artery was briefly reocluded at the end of the 60-minute reperfusion, and 0.7 mL of India ink was injected slowly into the jugular catheter to delineate the area at risk of infarction. The heart was excised, the right ventricle and the atria were dissected away with small surgical scissors, and the remaining left ventricle was frozen in cold isopentane and kept in isopentane at \(-10^\circ\text{C}\) for 1 hour. We have previously verified that this freezing procedure does not affect histochemical determination of necrosis compared with fresh tissue.\textsuperscript{12} The frozen ventricle was then sliced from apex to base into seven or eight sections. The slices were immersed in 1\% triphenyltetrazolium chloride (TTC, Sigma Chimie) in phosphate buffer (pH 7.4) for 20 minutes at 37°C to delineate the infarcted tissue. The sections were then fixed in phosphate-buffered Formalin at room temperature for a minimum of 4 days. After fixation, each section was weighed and placed under a microscopic video camera (Microwatcher VS-30H, Mitsubishi Kasei Corp) with a 20-fold enlargement lens. The camera was connected to an electronic color digitalization card (Matrox Illuminator 16) coupled to an IBM-compatible computer. The digitized color images were enlarged fivefold (final enlargement, 100-fold), and the resulting images were stored as bitmap files for later analysis. These stored images were later displayed on a 1024\(\times\)768-pixel color screen using a Windows-based image analysis software (Cybervision, Cervus Int.), and the edges of the nonischemic (India ink stained), viable (triphenyltetrazolium chloride positive), and infarcted (triphenyltetrazolium chloride negative) tissue were traced on the computer screen using a mouse. The areas (mm\(^2\)) of nonischemic, ischemic, and infarcted tissue were then calculated using the same image analysis software, and infarct and area at risk weights were calculated with knowledge of the individual weight of each section. Area at risk size was then expressed as a percentage of the left ventricle, and the infarct size was expressed as a percentage of the left ventricle and as a percentage of the area at risk.

**In Vitro Vascular Studies**

At the end of the experiment, the heart was removed and immediately placed into cold, oxygenated physiological saline (control solution) or the following composition (mmol/L): NaCl 118.3, KCl 4.7, CaCl\(_2\) 2.5, NaHCO\(_3\) 25, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, EDTA 0.02, and glucose 11.1. The left coronary artery was carefully dissected free with a dissecting microscope. Artery segments 1.5 to 2 mm long (internal diameter, 250 to 300 \(\mu\)m) were taken distal to the occlusion site and mounted in a small-vessel myograph for isometric tension recording (JP Trading). For this purpose, the segments were threaded onto two 40-\(\mu\)m stainless-steel wires; the ends of the wires were then fastened to two stainless-steel support blocks.\textsuperscript{38} One block was mounted on a tension transducer, and...
the other was mounted on a displacement device operated with a micromanometer. Care was taken during the dissection procedure to avoid damage to the endothelium. During the mounting process, the myograph chamber was filled with cold, oxygenated (95% O2, 5% CO2, pH 7.4) solution. Vessel length was measured using a calibrated lens placed in the dissection microscope. After equilibration, the vessels were progressively stretched by 1-mN increments, using the micromanometer. The corresponding measured force was read on the recorder, and wall tension was calculated by dividing this measured force by vessel length. Internal circumference (IC) of the arterial segment was also calculated for each level of stretch using the micromanometer reading. From these measurements, the Laplace law was used to calculate the effective pressure (ie, the pressure that would be necessary to extend the vessel to the measured IC)\textsuperscript{35}:

\[
\text{Effective Pressure} = \frac{\text{Wall Tension} \times 2\pi}{\text{IC}}
\]

The stepwise distension was stopped when the effective pressure was more than 100 mm Hg. The relation between effective pressure and IC was then fitted to an exponential curve using a computer program, and the circumference of the vessels corresponding to a transmural pressure of 100 mm Hg (IC\textsubscript{100}) was calculated from the fitted curve.\textsuperscript{35} The vessels were then set to a normalized IC equal to 0.9 \times IC\textsubscript{100}, which corresponds to the IC for which the active contraction is maximal.\textsuperscript{34} Internal diameters of the arteries were then calculated as IC\textsubscript{100}/\pi. After normalization, the vessels were allowed to equilibrate for 30 minutes, during which chamber temperature was progressively increased to 37°C.

After another 60-minute equilibration period during which the vessels were washed, segments were exposed to increasing concentrations of serotonin (10\textsuperscript{-10} to 10\textsuperscript{-2} mol/L). The following concentration-response curves were then studied in each ring after preconstriction by serotonin: the NO donor SIN-1 (10\textsuperscript{-4} to 10\textsuperscript{-2} mol/L; endothelium-independent relaxation), the calcium ionophore A23187 (10\textsuperscript{-4} to 10\textsuperscript{-2} mol/L; receptor-independent, endothelium-dependent relaxation), and acetylcholine (10\textsuperscript{-4} to 10\textsuperscript{-2} mol/L; receptor-mediated, endothelium-dependent relaxation). Rings were washed twice and allowed to equilibrate for 30 minutes between each concentration-response curve, after which serotonin was again added to the bath. At the end of these experiments, the L-arginine analogue N\textsuperscript{G}-nitro L-arginine methyl ester (L-NAME, 10\textsuperscript{-3} mol/L) was added to the chamber to inhibit NO synthesis. Finally, the role of NO in the relaxing response to acetylcholine was also studied in six additional sham-operated rats, in which the response to increasing concentrations of acetylcholine was tested before and after addition of the NO synthase inhibitors L-NAME or N\textsuperscript{H}-nitro L-arginine (L-NA). In those experiments, the reversal of the inhibitory effect of L-NA was also tested by addition of excess concentrations of L-arginine or D-arginine (both 10\textsuperscript{-2} mol/L) in the presence of L-NAME (10\textsuperscript{-3} mol/L) and acetylcholine (10\textsuperscript{-2} mol/L).

**Drugs**

The following drugs were used: acetylcholine chloride, L-NAME, L-NA, 5-hydroxytryptamine (serotonin; as creatine sulfate complex), A23187, triphenyltetrazolium chloride (all from Sigma) and SIN-1 (a gift from Laboratoires Hoechst). All drugs were dissolved in distilled water, except A23187, which was prepared as a stock solution in DMSO. Further dilutions were made in distilled water. The concentrations are expressed as final molar concentration in the chamber.

**Data Analysis**

All results are expressed as mean±SEM. Area at risk and infarct size were compared using an unpaired Student’s t test.

In all in vitro experiments, n refers to the number of animals from which the arteries were taken. Contractions to serotonin and to L-NAME were normalized to vessel length and expressed in millinewtons (mN) per millimeter\textsuperscript{35}; contractions were also expressed as percent maximal contraction to serotonin. For relaxations, the negative logarithm of the effective molar concentration of agonist causing 50% inhibition (IC\textsubscript{50}) of the contraction to serotonin was calculated from concentration-response curves after adjusting to a sigmoidal curve using a curve-fitting software (ORIGIN, MicroCal Software, Inc.), and the mean±SEM of these values are presented. Results were then compared using a one-way ANOVA followed when ANOVA was significant by a Tukey’s test for multiple comparisons. A value of P\textless .05 was considered statistically significant.

**Results**

**Area at Risk and Infarct Size**

The effect of preconditioning on infarct size is shown in Fig 2. The size of the area at risk, an important baseline determinant of infarct size, did not differ between the two groups (control, 46.9±2.7; preconditioning, 44.6±2.1; n=8 in each group; P=NS).

In control animals, coronary occlusion and reperfusion were associated with the development of an infarct that involved 57±2% of the area at risk (n=8). Compared with controls, preconditioning induced a marked limitation of infarct size (2.2±0.6% of the area at risk; n=8; P<.01 versus controls).

![Fig 3](http://circ.ahajournals.org/) Bar graph of contractile responses of rat coronary arteries to the nitric oxide synthase inhibitor L-NAME, expressed in millinewtons (mN) per mm of artery. Values are mean±SEM of six or seven animals in each group. No significant differences were observed between the four experimental groups. I indicates ischemia; I/R, ischemia/reperfusion; and PC, preconditioning.
In Vitro Vascular Studies

**Contraction to L-NAME**

Responses of rat coronary arteries to the NO synthase inhibitor L-NAME (10^{-5} mol/L) are shown in Fig 3. No significant differences in the response to this inhibitor were seen among the four groups (sham, 0.78±0.19; ischemia, 0.70±0.21; ischemia/reperfusion, 0.73±0.19; preconditioning, 0.64±0.29 mN/mm, n=7).

**Contraction to Serotonin**

Responses of rat coronary arteries to increasing concentrations of serotonin (10^{-9} to 10^{-3} mol/L), expressed in absolute values (mN/mm) or as a percentage of maximal contraction, are shown in Fig 4 (n=8 in each group). There were no significant differences in the contractile responses to serotonin in the four groups at any concentration. In addition, there were no significant differences in the EC_{50} values for serotonin in the four groups (sham, 0.67±0.19; ischemia, 0.64±0.18; ischemia/reperfusion, 0.65±0.12; preconditioning, 0.54±0.11 μmol/L; n=8 in each group; ANOVA: F=.14, P=NS).

**Relaxation to SIN-1**

Responses of rat coronary arteries to increasing concentrations of the NO donor SIN-1 (10^{-4} to 10^{-6} mol/L) are shown in Fig 5 (n=8 in each group). In all groups, SIN-1 induced concentration-dependent relaxations that reached 100% at higher concentrations (10^{-5} to 10^{-4} mol/L). No statistically significant differences were observed among the four groups. In addition, there were no significant differences in the EC_{50} values for SIN-1 in the four groups (sham, 3.1±1.8; ischemia, 2.6±0.8; ischemia/reperfusion, 2.8±0.5; preconditioning, 2.2±0.4 μmol/L; n=8 in each group; ANOVA: F=.09, P=NS).

**Relaxation to A23187**

Arterial responses to the calcium ionophore A23187 are shown in Fig 5 (n=8 in each group). In this model, significant relaxations were observed only at high concentrations (3×10^{-6} to 10^{-5} mol/L) of A23187. L-NAME inhibited the maximal responses to the ionophore by about 60% in all groups (data not shown). In addition, no significant differences were observed in the response to A23187 in the four groups.

**Relaxation to Acetylcholine**

In sham-operated animals, acetylcholine induced concentration-dependent relaxations that reached 60% at the highest dose (10^{-3} mol/L; Figs 6 and 7). This response to acetylcholine was lower than that obtained in arteries taken from normal, nonoperated rats (maximal response, 81±9%; n=4; data not shown). The relaxations induced by acetylcholine were virtually abolished by L-NA and L-NAME (both 10^{-5} mol/L; Fig 6). Furthermore, the inhibitory effects of L-NAME could be reversed by addition of excess concentrations of L-arginine but not D-arginine (both 10^{-3} mol/L; Fig 6).

Ischemia without reperfusion did not affect the coronary response to acetylcholine (Fig 7, left, maximal response, 56±7%; P=NS versus sham). In contrast, the relaxation to acetylcholine was markedly reduced after
ischemia and reperfusion (Fig 7, right, maximal response, 26±6%; P<.01 versus sham and versus ischemia without reperfusion). Compared with sham-operated rats, the response to acetylcholine was significantly reduced by ischemia/reperfusion at all concentrations from 3×10⁻⁷ to 10⁻⁵ mol/L. However, coronary arteries isolated from preconditioned hearts exhibited normal responses to acetylcholine (Fig 7, right, maximal relaxation, 59±9%; P<.01 versus ischemia/reperfusion; P=NS versus sham or versus ischemia without reperfusion). Compared with ischemia/reperfusion, the response to acetylcholine was significantly improved by preconditioning at all concentrations starting at 10⁻⁶ mol/L. Thus, preconditioning prevented the ischemia/reperfusion-induced impairment of endothelium-dependent response to acetylcholine.

Discussion

The present study was performed to examine whether preconditioning, in addition to protecting myocardial cells, also prevents endothelial cells from the dysfunction induced by ischemia/reperfusion. The major findings of the study were (1) that coronary occlusion followed by reperfusion induced a significant decrease in the coronary artery responses to acetylcholine; (2) that a similar impairment was not found on coronary arteries obtained from hearts subjected to ischemia without reperfusion, suggesting that this endothelial dysfunction was a manifestation of reperfusion injury; and (3) that preconditioning with short periods of ischemia separated by intermittent reperfusion induced a marked limitation of infarct size and completely prevented the reperfusion-induced endothelial dysfunction. Thus, our experiments suggest that in addition to protecting myocardial cells, preconditioning protects coronary endothelial cells against ischemia/reperfusion injury.

Effect of Preconditioning on Infarct Size

In the present study, preconditioning induced a marked limitation of infarct size, in agreement with previous studies in rats performed by our group and others. However, although it is clear from those experiments that rat hearts can be preconditioned similarly to dogs, pigs, or rabbits, the mechanism involved in rats appears to differ from that of other species. Indeed, although adenosine appears to be a major mediator of preconditioning in dogs, pigs, and rabbits, experiments from different groups have suggested that it was probably not the case in rats. In addition, other potential mechanisms of preconditioning, such as opening of ATP-sensitive potassium channels, inhibition of mitochondrial ATPase, and production of oxygen-derived free radicals, also appear not to be operative in rats. Thus, the mechanism by which preconditioning limits infarct size in this species remains unknown.

One potential limitation of our infarct-size study is that determination of infarct size was performed after a short (1 hour) duration of reperfusion. We limited the duration of reperfusion to 1 hour to perform the infarct size and vascular studies under identical conditions. However, it is unlikely that limiting reperfusion to 1 hour significantly affected the delineation of infarcts based on triphenyltetrazolium chloride staining. Indeed, in some of our previous experiments in which we used similar experimental conditions (ie, 20 minutes of ischemia followed by 1 hour of reperfusion), we showed that extending the duration of reperfusion to 6 hours did not modify the infarct size measured in controls and did not affect the infarct size-limiting effect of preconditioning. Thus, this suggests that despite the short duration of reperfusion used in the present experiments, our measurements of infarct size are valid.

In the present study, we used a short (20 minutes) duration of ischemia. Nevertheless, such ischemia still resulted in the development of large myocardial infarcts, averaging 60% of the area at risk. In the present study, myocardial salvage was mainly detectable in the subepicardium, with small bands of salvaged tissue along the borders of the area at risk as well as in the subendocardium. We used such a short duration of ischemia because pilot experiments with longer (30 to 45 minutes) episodes always resulted in the development of transmural infarcts. It must be noted that this duration of ischemia is shorter than that used in previous studies of the effect of preconditioning on infarct size in rats. However, despite this shorter duration of ischemia, infarcts in the present study are larger than those obtained by Li and Kloner, who used a 90-minute period of occlusion, and of similar size as those of Liu and Downey and Yellow et al, who used 30- and 45-minute periods of occlusion, respectively. The reasons for these differences in the rate of development of necrosis could be in part the result of differences in the anesthetics used (eg, ketamine/xylazine in the study of Li and Kloner versus pentobarbital in other studies, including ours) or of differences in the strains of rats used (Wistar in the present study versus Sprague-Dawley in other studies).

Effect of Ischemia/Reperfusion on In Vitro Coronary Vascular Responses

In the present study, we showed that ischemia with or without reperfusion did not affect the contractile response to L-NAME. L-NAME is a potent inhibitor of endothelial NO synthase. In vitro, it inhibits endothelium-dependent relaxations induced by acetylcholine and induces endothelium-dependent contractions that can be reversed by addition of L-arginine. In vivo,
L-NAME induces an increase in mean arterial pressure and an endothelium-dependent constriction of large epicardial coronary arteries. Thus, contractions to L-NAME are usually accepted as an estimate of basal release of NO from coronary endothelial cells. Our data agree with those of Pearson et al., who showed that the contractile response of dog coronary arteries to hemoglobin (which inactivates NO) was unaffected by ischemia/reperfusion. However, Ma et al. showed that reperfusion markedly reduced the contractile response of isolated cat coronary arteries to L-NAME. These differences cannot be explained by differences in the durations of reperfusion; in the study of Ma et al., contractile response to L-NAME was already significantly depressed after 10 to 20 minutes of reperfusion. The reasons for these differences are not known but could be because of differences in the species used.

Our experiments also show that ischemia with or without reperfusion did not affect the contractile response to serotonin. Although serotonin has been shown to release NO in porcine and canine coronary arteries, previous experiments have shown that rat coronary arteries do not express endothelial serotonin receptors coupled to the release of EDRF. Thus, serotonin can be considered as a pure vasoconstrictor in our preparation. In this context, such an unaffected contractile response is in agreement with most studies, which show that ischemia and reperfusion do not affect contractions of coronary arteries to prostaglandin F2α, the thromboxane analogue U46619, and potassium chloride. Given the well-known role of basal release of NO as a modulator of vascular contraction (which can be evidenced by the observation that inhibitors of NO synthase potentiate the response to various contracting agents, eg, phenylephrine), the fact that ischemia/reperfusion does not affect contractile responses of the coronary arteries in various experimental models is another indirect indicator that basal release of NO is not affected in this pathological situation.

One limitation of the present study, as well as of all studies in which the effects of ischemia on basal NO release were assessed in vitro, is that these conditions probably do not reflect the situation present in vivo, in which case “basal” release of NO is the combination of true permanent activity of NO synthase (similar to that found in vitro), flow (or shear stress)-dependent release of NO from endothelial cells, and NO release form “nitroxidergic” nerve terminals. Whether ischemia affects these other aspects of NO release is not known.

Response to the calcium ionophore A23187 is usually considered a receptor-independent, NO-dependent relaxation. In the present study, this response was not affected by ischemia with or without reperfusion, suggesting that the capacity of endothelial cells to release NO was conserved in these conditions. This is in contrast with previous studies in other species, in which the response to A23187 was significantly impaired after reperfusion. However, it must be noted that in our experiments, rat coronary arteries were relatively insensitive to A23187. Significant relaxations were obtained only at very high concentrations ($3 \times 10^{-6}$ to $10^{-5}$ mol/L), the maximal relaxations induced being only 76%–9% in arteries taken from sham-operated rats. In dog experiments, ischemia and reperfusion do not affect the maximal response to A23187 (although they induce a significant shift of the concentration-response curve).

Thus, because of this insensitivity of rat coronary arteries to A23187, results obtained in the present study with this compound must be interpreted cautiously.

In sham-operated animals, the response to acetylcholine was virtually abolished by both L-NA and L-NAME. Furthermore, the inhibitory effect of L-NA could be reversed by addition of an excess concentration of $\text{L}$-arginine, whereas $\text{D}$-arginine was inactive. This suggests that in our experimental preparation, the relaxing response to acetylcholine is entirely mediated through the release of NO from $\text{L}$-arginine.

Our experiments clearly show that ischemia/reperfusion induced a significant decrease in the coronary response to acetylcholine. Such an endothelial dysfunction confirms previous results obtained in other species. Moreover, because a similar alteration was not observed in arteries obtained from hearts subjected to ischemia without reperfusion, this suggests that this coronary endothelial dysfunction is a manifestation of reperfusion injury. Although we have not tested this hypothesis in rats, there is evidence in other species that this reperfusion-induced endothelial injury is the consequence of the production of oxygen-derived free radicals, because impaired endothelium-dependent relaxation can be restored by treatment with superoxide dismutase and catalase in both large coronary arteries and the microcirculation.

In the present study, only one receptor-dependent, endothelium-dependent vasodilator was used (acetylcholine). We attempted to study other potential endothelium-dependent vasodilators, such as adenosine diphosphate, $\alpha_2$-adrenergic agonists, and bradykinin, and found in pilot studies that none of them induced significant endothelium-dependent relaxations. In addition, substance P and serotonin, which induce endothelium-dependent relaxations in other species, also do not relax rat coronary arteries. Thus, to our knowledge, acetylcholine is the only endothelium-dependent relaxing agent active on isolated rat coronary arteries. Because of this limitation, we could not determine whether the impairment observed in the present study reflects a true defect in NO synthase activity or a specific impairment of the transduction pathway linking muscarinic receptors to NO synthase. Such a selective impairment has already been shown to occur in other pathological situations such as hypercholesterolemia or atherosclerosis, which selectively affect $\text{G}_\text{i}$ protein-mediated transduction pathways and in canine experiments involving chronic reperfusion.

The main result of our study is that preconditioning was able to completely prevent the endothelial dysfunction induced by ischemia/reperfusion in the present model, as evidenced by a restored relaxation to acetylcholine. To our knowledge, this is the first report of such an effect of preconditioning at the level of epicardial coronary arteries. In a recent report, DeFily and Chilian showed in dogs that preconditioning could also protect coronary arteriolar endothelium against ischemia/reperfusion injury, assessed in vivo as a maintained dilation of $<100\mu$m arteries to acetylcholine and serotonin. In contrast, Bauer et al. failed to detect any protective effect of preconditioning against the
progressive loss of vasodilatory response to acetylcholine during reperfusion in dogs. However, in this last study, the depressed response to acetylcholine could not be considered an index of endothelial dysfunction because under these conditions, a similar depressed response was seen with the endothelium-independent vasodilator nitroglycerin. Such an unspecific loss of coronary vasodilatory reserve is probably the consequence of reperfusion-induced microvascular injury, causing progressive deterioration of tissue perfusion and extension of the areas of "no-reflow." Because such coronary microvascular injury appears to be at least in part the consequence of myocyte injury (eg, through capillary compression resulting from myocyte edema or influx of neutrophils), it is likely that this microvascular injury and its consequences, such as loss of coronary vasodilator reserve, are highly dependent on the extent of myocardial necrosis. Because of this, the effects of treatments such as preconditioning on coronary microvascular injury and vasodilator reserve assessed in vivo are at least in part indirectly dependent on their infarct size–limiting properties.

In the present study, the effect of preconditioning on endothelial dysfunction was studied at only one time point (1 hour of reperfusion). Thus, based on these experiments, we cannot rule out the hypothesis that preconditioning actually accelerates recovery of endothelial function after reperfusion compared with control animals. However, there is evidence that posts ischemic endothelial injury is not a transient mechanism, because an impairment of endothelium-dependent relaxation can be observed even after 12 weeks of reperfusion. Thus, it is likely that preconditioning is actually protecting the endothelium against reperfusion injury rather than accelerating its recovery from transient dysfunction.

The mechanism by which preconditioning prevents posts ischemic endothelial dysfunction in this model is not clear. As mentioned, there is evidence that this dysfunction is mediated through the production of oxygen-derived free radicals. Two of the major sources of free radicals—xanthine oxidase and activated neutrophils—are present in reperfused coronary arteries; xanthine oxidase is mainly located in endothelial cells, whereas there is evidence in dogs that neutrophils accumulate in the walls of large epicardial coronary arteries during reperfusion. Superoxide anions are potent inactivators of EDRF and may alter endothelial binding sites for endothelium-dependent vasodilators such as acetylcholine, thus reducing the capacity of the endothelium to release EDRF in response to these agonists. Thus, one likely explanation is that preconditioning somehow limits the vascular production of oxygen-derived free radicals during reperfusion. In theory, preconditioning could limit the production of free radicals through limitation of ischemia-induced, calcium-dependent conversion of xanthine dehydrogenase to xanthine oxidase (eg, through prevention of ischemia-induced increase in intracellular calcium or prevention of neutrophil accumulation during reperfusion. Unfortunately, to our knowledge, neither of these hypotheses has been tested experimentally. Finally, preconditioning could also reduce free radicals through an increase in myocardial antioxidant activity. However, the effect of preconditioning on this activity has been controversial; although preconditioning increased mitochondrial manganese–superoxide dismutase activity in dog hearts, similar changes could not be detected in rabbit hearts. Whether antioxidant activity is affected by preconditioning in rats is not known.

In our experiments, preconditioning induced a marked protection at both the endothelium and at myocytes. Thus, one could hypothesize that endothelial protection might somehow contribute to the beneficial effect of preconditioning on myocytes and thus to its anti-infarct properties (eg, if prevention of endothelial function could somehow improve tissue perfusion or if endogenous NO production was protective during ischemia/reperfusion). However, this is probably not the case in the present experiments. Endothelial dysfunction was clearly a manifestation of reperfusion injury, because no alteration was observed in arteries taken from nonreperfused hearts. In contrast, previous studies in dogs have shown that the beneficial effect of preconditioning on myocytes was already present within the first minutes of ischemia (as evidenced by electron microscopy as delayed ultrastructural damage of the myocytes). Thus, because a beneficial effect of preconditioning on myocytes could be observed before endothelial dysfunction had occurred, the endothelial protective effect is quite unlikely to be the only explanation for the anti-infarct properties of preconditioning.

Our results could have important clinical implications. In view of the known major role of endothelium-derived NO in the regulation of coronary tone, as well as on platelet and neutrophil function, it is likely that long-term impairment of endothelial function after reperfusion could favor smooth muscle contraction as well as platelet and neutrophil adhesion, thus increasing the risk of subsequent vasospasm and thrombosis. Thus, the endothelial protection afforded by preconditioning could be associated with long-term protection of the coronary vascular wall against these adverse consequences of reperfusion, which would add to the now well-known beneficial effect of preconditioning on ischemic myocytes.

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