Classic Preconditioning Decreases the Harmful Accumulation of Nitric Oxide During Ischemia and Reperfusion in Rat Hearts

Csaba Csonka, MD; Zoltán Szilvássy, MD, PhD; Ferenc Fülöp, PhD, DSc; Tibor Páli, PhD; Ingolf E. Blasig, PhD; Arpad Tosaki, PhD; Richard Schulz, PhD; Péter Ferdinandy, MD, PhD

Background—The role of NO in the mechanism of preconditioning is not understood. Therefore, we studied the effect of preconditioning and subsequent ischemia/reperfusion on myocardial NO content in the presence of an NO synthase (NOS) inhibitor.

Methods and Results—Isolated working rat hearts were subjected to preconditioning protocols of 3 intermittent periods of rapid pacing or no-flow ischemia of 5 minutes’ duration each followed by a test 30 minutes of global no-flow ischemia and 15 minutes of reperfusion. Test ischemia/reperfusion resulted in a deterioration of myocardial function and a considerable increase in cardiac NO content as assessed by electron spin resonance. Preconditioning improved postischemic myocardial function and markedly decreased test ischemia/reperfusion-induced NO accumulation. In the presence of 4.6 μmol/L N G-nitro-L-arginine (LNA), basal cardiac NO content decreased significantly, although test ischemia/reperfusion-induced functional deterioration and NO accumulation were not affected in nonpreconditioned hearts. However, the protective effects of preconditioning on both test ischemia/reperfusion-induced functional depression and NO accumulation were abolished. When 4.6 μmol/L LNA was administered after preconditioning, it failed to block the effect of preconditioning. In the presence of 46 μmol/L LNA, ischemia/reperfusion-induced NO accumulation was significantly decreased and postischemic myocardial function was improved in nonpreconditioned hearts.

Conclusions—Our results show that (1) although NO synthesis by the heart is necessary to trigger classic preconditioning, preconditioning in turn attenuates the accumulation of NO during ischemia/reperfusion, and (2) blockade of ischemia/reperfusion-induced accumulation of cardiac NO by preconditioning or by an appropriate concentration of NOS inhibitor alleviates ischemia/reperfusion injury as demonstrated by enhanced postischemic function. (Circulation. 1999;100:2260-2266.)

Key Words: nitric oxide ■ preconditioning ■ ischemia ■ reperfusion ■ electron spin resonance

Although the effectiveness of ischemic preconditioning might be attenuated in the heart during some disease states, such as hyperlipidemia and diabetes (see Reference 1 for review), preconditioning confers a remarkable cardioprotection in a variety of species as well as in humans (see References 2 and 3 for reviews). The early phase of preconditioning (“classic preconditioning”) is manifested within minutes after the ischemic stress and has a duration of <3 hours. The late phase is characterized by a slower onset (≥20 hours) and a duration of up to 72 hours. Both phases of preconditioning involve reduction of necrotic tissue mass, improvement of cardiac performance after ischemia and reperfusion, and reduction of arrhythmias (see References 2 and 3 for reviews). There is considerable debate regarding the biochemical mechanism of ischemic preconditioning. The discrepancies are generally attributed to species differences, different preconditioning stimuli such as no-flow ischemia or rapid pacing models,6 and different study end points, ie, myocardial function, arrhythmias, or infarct size (see References 3 and 5 for reviews).

In the normal heart, nitric oxide (NO) is synthesized by Ca 2+ -dependent NO synthases (NOS) in cardiac myocytes, vascular and endocardial endothelium (NOS III), and specific cardiac neurons (NOS I) and plays an important role in the regulation of coronary circulation and cardiac contractile function.6 Myocardial function leads to increased activity of Ca 2+ -dependent NOS7 and accumulation of NO, which might contribute to ischemia/reperfusion injury.8–10
Use of inhibitors of NOS has indicated that NO is involved in both the early11–13 and the late phases of preconditioning.14,15 However, no studies have followed changes in myocardial NO content during preconditioning and subsequent ischemia/reperfusion, although this could possibly be key missing information necessary to elucidate the role of NO in the biochemical mechanism of preconditioning.

Therefore, the aim of the present study was to determine the effects of classic preconditioning in the presence and absence of the NOS inhibitor N\textsuperscript{G}-nitro-L-arginine (LNA) on changes in cardiac NO content both during preconditioning and after subsequent test ischemia and reperfusion.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Isolated Heart Preparation

Hearts of male Wistar rats (300 to 360 g, anesthetized with diethyl ether and given 500 U/kg heparin) were isolated and perfused at constant pressure (9.8 kPa) in Langendorff mode or in working mode at 37°C with Krebs-Henseleit bicarbonate buffer at a constant preload (1.7 kPa) and afterload (9.8 kPa) as described.12,16 Heart rate, left ventricular developed pressure (LVDP), +dP/dt\textsubscript{max}, −dP/dt\textsubscript{max}, and left ventricular end-diastolic pressure (LVEDP) were derived from the online-digitized left intraventricular pressure recording as described.12 Coronary flow was measured by collection of effluent from the right atrium for a timed period, and aortic flow was measured by rotameter (KDG Flowmeters).4,12

Experimental Groups

A time-matched nonpreconditioning protocol (C, control), pacing-induced preconditioning protocol (C-VOP, control–ventricular overdrive pacing), and no-flow ischemia–induced preconditioning protocol (C-NFlow, control–no-flow ischemia) were applied before induction of test ischemia/reperfusion (Figure 1). The same protocols were applied in the presence of 4.6 μmol/L LNA (Sigma) to test whether NO is necessary to trigger preconditioning (LNA-C, LNA-VOP, and LNA-NFlow groups). This concentration of LNA was selected for these groups because according to our previous results,12,15 it did not significantly affect coronary flow or any of the myocardial functional parameters during preconditioning protocols and test ischemia/reperfusion, except for an 80% increase in preischemic LVEDP. However, it decreased basal cardiac NO content near the detection limit (0.05 nmol NO/g wet tissue weight)19 as assessed by electron spin resonance (ESR) spectroscopy.12,13 To test whether NO is a mediator of preconditioning, in 2 separate groups, LNA was added to the perfusion medium immediately after the preconditioning protocols, ie, late treatment (LNA-Late-VOP, LNA-Late-NFlow). In these groups, test ischemia was applied 20 minutes after termination of the pacing/no-flow period to allow for LNA to exert its inhibitory effect on NO synthesis in the heart.12,17 According to our preliminary experiments (data not shown) and our previous studies,12 this 15-minute extension of the perfusion protocol did not affect protection by preconditioning. A higher concentration of LNA (46 μmol/L) that was able to decrease NO accumulation during ischemia and reperfusion (see Results) was also tested in nonpreconditioning (LNA46-C), pacing-induced preconditioning (LNA46-VOP), and no-flow ischemia-induced preconditioning (LNA46-NFlow) protocols (n = 7 in each group).

Preconditioning Protocols

After 10 minutes of aerobic perfusion as working hearts, the hearts were subjected to either 3 intermittent periods (4.75 minutes each) of Langendorff-mode perfusion without pacing (nonpreconditioning protocol) or with rapid pacing at 10 Hz, or to no-flow ischemia, each separated by 0.5 minute of Langendorff perfusion followed by 4.75 minutes of aerobic working perfusion (Figure 1). The 0.5 minute of Langendorff perfusion allowed for the spontaneous restoration of sinus rhythm before switching to working-mode perfusion between pacing or no-flow periods. Pacing and no-flow were performed as described.4,12 According to our previous studies12,13 as well as this study, all cardiac functional parameters recovered within 3 minutes after the termination of pacing or no-flow ischemia, indicating that they induced a completely reversible ischemia (data not shown).

Test Ischemia/Reperfusion

After nonpreconditioning and preconditioning protocols, test ischemia/reperfusion was induced by 30 minutes of global no-flow ischemia followed by 15 minutes of reperfusion. In the first 5 minutes of reperfusion, Langendorff perfusion was applied to allow spontaneous recovery of sinus rhythm before switching to the working perfusion for another 10 minutes. In the first minute of reperfusion, the incidence of ventricular fibrillation (VF) was determined by ECG,7 and the hearts were mechanically defibrillated when necessary to avoid VF-induced free radical release, which might interfere with the ischemia/reperfusion-induced depression of myocardial function.19 Hearts with unsucceful defibrillation were excluded from the measurement of cardiac function and LDH.
release. Functional parameters were recorded before and after preconditioning and after 15 minutes of reperfusion. LDH release was assayed from coronary effluents collected in the first 3 minutes of reperfusion as described.16

Design of ESR Studies

The spin-trap for NO, the complex of N-methylglucamine dithiocarbamate (MGD, synthesized as described)20 with ferrous ion ([Fe²⁺(MGD)]), was prepared fresh before each experiment. MGD 175 mg and FeSO₄ 50 mg dissolved in distilled water (pH 7.4, volume 6 mL) was infused into the aortic cannula under Langendorff perfusion for 5 minutes at a rate of 1 mL/min before the preconditioning protocol was begun to measure basal myocardial NO content. In separate experiments, the infusion of the spin trap commenced immediately after the preconditioning protocol and was maintained for 5 minutes to assess the effect of preconditioning on cardiac NO content. A tissue sample from the apex of the heart (∼150 mg) was collected at the end of the infusion of Fe²⁺(MGD), placed into a quartz ESR tube, and frozen in liquid nitrogen. To measure the accumulation of NO during the test ischemia, in separate studies, Fe²⁺(MGD), infusion was started 5 minutes before the induction of test ischemia to load the heart with the spin-trap before the perfusion line was clamped, and tissue samples were collected at the end of ischemia. To assess the generation of NO during early reperfusion, the spin trap was infused for 5 minutes starting at the onset of reperfusion, and tissue samples were collected at the end of the infusion. ESR spectra of the NO-Fe²⁺(MGD) adduct were recorded with a Bruker ECS106 spectrometer (parameters: X band; modulation frequency, 100 kHz; temperature, 160 K; microwave power, 10 mW; modulation amplitude, 2.85 G; central field, 3356 G) and analyzed for NO signal intensity as described.13,18,21

Statistics

Data were expressed as mean±SEM. All groups were analyzed with Fischer’s exact test or 1-way ANOVA followed by Bonferroni test. Significant difference was established when P<0.05.

Results

Preconditioning in the Absence of LNA

In control (C) hearts, test ischemia/reperfusion resulted in a marked decrease in coronary flow (Figure 2A), aortic flow (Figure 2B), LVDP (Figure 3A), and both +dP/dt max and −dP/dt max (Table), a considerable increase in LVEDP (Figure 3B) and LDH release (Figure 4A), and 100% incidence of VF (Figure 4B). When test ischemia was preceded by preconditioning elicited by ventricular overdrive pacing (C-VOP) or no-flow ischemia (C-NFlow), coronary flow, aortic flow, LVDP, +dP/dt max and −dP/dt max improved and LVEDP, LDH release, and the incidence of VF decreased, showing the protective effect of preconditioning against acute ischemia/reperfusion injury (Table; Figures 2 to 4).

In the control (C) hearts, NO content was not changed when measured before or after preconditioning (Figure 5A). However, test ischemia induced a marked increase in cardiac NO signal intensity (Figure 5B). On reperfusion, NO content was significantly lower than that measured at the end of ischemia, but it was still significantly higher than the basal cardiac NO content (Figure 5B). Both preconditioning protocols significantly attenuated the ischemia/reperfusion-induced increase in cardiac NO signal (Figure 5B).

Preconditioning in the Presence of 4.6 μmol/L LNA

When LNA treatment (4.6 μmol/L) was commenced before preconditioning, cardiac functional parameters (Figures 2 and
reduced the incidence of VF (Figure 4B) after test ischemia/reperfusion (LNA46-C group). In the presence of 46 μmol/L LNA, preconditioning protocols did not significantly affect the protective effect of 46 μmol/L LNA itself on test ischemia/reperfusion.

LNA 46 μmol/L significantly decreased basal NO content as well as the NO signal during test ischemia and reperfusion (LNA46-C group) compared with the control group (C). Similar changes of NO content were seen in the LNA46-VOP and LNA46-NFlow groups (Figure 5).

Discussion

This is the first demonstration that classic preconditioning inhibits ischemia- and reperfusion-induced accumulation of cardiac NO, resulting in an improvement of postischemic myocardial function and reduction of arrhythmias. Furthermore, our results strongly suggest that NO biosynthesis during preconditioning is necessary to trigger preconditioning but that NO itself does not mediate the protective effect of classic preconditioning.

**Figure 3.** Effects of preconditioning on LVDP (A) and LVEDP (B) before and after test ischemia/reperfusion. Values are mean±SEM (n=7 in each group). *P<0.05 vs C group.

**Figure 4.** Effects of preconditioning on LDH release (A, n=7 in each group) and incidence of VF (B, n=9 to 13) on reperfusion after test ischemia. Values are mean±SEM. *P<0.05 vs C group.

### Effects of Preconditioning on Heart Rate and ±dP/dt\(_{max}\) or -dP/dt\(_{max}\) Before and After 30 Minutes of Ischemia Followed by 15 Minutes of Reperfusion

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<th>-dP/dt(_{max}), kPa</th>
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Values are mean±SEM (n=7 in each group).

*P<0.05 vs C group.
Numerous earlier studies that used different concentrations of NOS inhibitors or NO donors, without attempting to measure actual changes in myocardial NO content, led to contradictory conclusions about endogenous NO being either protective, neutral, or a destructive player in ischemia/reperfusion.6 –30 Recent studies suggest that the harmful effects of NO in the heart and in the vasculature are not due to NO itself but rather to peroxynitrite, a reaction product of NO and superoxide.10,31 (see Reference 32 for review). A seminal study addressing this controversy by Yasmin et al10 demonstrated that the NO-dependent ischemia/reperfusion injury is mediated by peroxynitrite in the heart. Nω-monomethyl-L-arginine 10 μmol/L abolished peroxynitrite generation and was cardioprotective at this concentration, whereas this beneficial effect was lost at 100 μmol/L as a result of a marked decrease in coronary flow.10 The NO donor S-nitroso-N-acetyl-penicillamine (0.2 μmol/L) was also cardioprotective in their model,10 because NO in itself is an antioxidant that interferes with peroxynitrite-mediated radical chain propagation reactions.33 Wang and Zweier31 reported that NOS inhibitors at concentrations that decreased cardiac NO during ischemia/reperfusion alleviate peroxynitrite generation and ischemia/reperfusion injury. These results, including our present data, suggest that to draw valid conclusions from studies using NOS inhibitors during ischemia/reperfusion, the selection of an appropriate concentration of an NOS inhibitor to reduce the excessive generation of NO during ischemia/reperfusion and protect the heart from subsequent injury requires the measurement of myocardial NO content.

**NO and Preconditioning**

Our results show that although preconditioning with rapid pacing or no-flow ischemia did not significantly change basal cardiac NO content, it markedly decreased NO accumulation during test ischemia and reperfusion, improved postischemic myocardial function, and decreased the release of LDH and the incidence of VF. In the presence of 4.6 μmol/L LNA, which reduced basal NO synthesis, preconditioning failed to protect against ischemia/reperfusion and failed to attenuate test ischemia/reperfusion-induced NO accumulation. When LNA was applied after the preconditioning protocol, the effect of preconditioning on myocardial function and NO content was not affected. When preconditioning was applied in the presence of the higher concentration of LNA (46 μmol/L), which protected nonpreconditioned hearts, no further improvement of postischemic myocardial function and LDH release was seen, nor did it further decrease the NO signal during ischemia and reperfusion. These results show that intact NO synthesis is required to elicit preconditioning but that NO is not a mediator of the cardioprotective effect of classic preconditioning. On the contrary, it suggests that the cardioprotection provided by preconditioning induced by either pacing or no-flow ischemia involves a mechanism that decreases the accumulation of NO in the myocardium during ischemia and reperfusion. The nature of this mechanism is not known. Preconditioning may decrease the rate of enzymatic6 or nonenzymatic22 NO production during ischemia/reperfusion by altering pH and the availability of cofactors and/or substrate for NO synthesis, or it may possibly stimulate the formation of endogenous NOS inhibitors.34

Our present results support those of Vegh et al,11 who demonstrated that 10 mg/kg LNA methyl ester abolished the antiarrhythmic effect of preconditioning in a coronary occlusion model in anesthetized dogs. However, studies by Lu et al15 using 10 mg/kg Nω-monomethyl-L-arginine or LNA methyl ester in anesthetized rats with coronary occlusion/
reperfusion and by Weselcouch et al\textsuperscript{30} using 30 \(\mu\text{mol/L}\) LNA methyl ester in isolated rat hearts showed, surprisingly, that the different NOS inhibitors did not interfere with the outcome of ischemia/reperfusion with or without preceding preconditioning as assessed by postischemic myocardial function, LDH release,\textsuperscript{30} and arrhythmias.\textsuperscript{35} Because NO generation was not determined and only a single dose of NOS inhibitor was used in these studies, it is difficult to interpret these negative results.

**Limitations of the Study**

Although the present results clearly demonstrate marked changes in the ESR signal of NO during ischemia/reperfusion with or without a previous period of preconditioning, the cellular sources of cardiac NO are not demonstrated, because ESR analysis of total cardiac tissue was performed. Absolute quantification of NO concentration by ESR detection in tissue samples is not possible\textsuperscript{12,18,21}; therefore, we expressed NO signal intensity in arbitrary units, which allowed us to demonstrate relative changes in cardiac NO.\textsuperscript{12,13,21} The isolated rat heart with test global ischemia and reperfusion used in this study is a convenient model to follow changes in cardiac NO content by ESR; however, this model does not allow direct measurement of necrotic tissue mass, the traditional marker of preconditioning. Therefore, we assessed the cardioprotective effect of preconditioning by measuring post-ischemic myocardial function, arrhythmias, and LDH release during preconditioning in rat hearts: role of nitric oxide and cholesterol-enriched diet. J Mol Cell Cardiol. 1997;29:3321–3333. This study is the first attempt to follow changes in cardiac NO content during preconditioning and subsequent test ischemia/reperfusion.

**Conclusions**

This is the first demonstration that preconditioning protocols do not change basal cardiac NO content but effectively reduce the accumulation of NO during subsequent ischemia and reperfusion, resulting in an improvement of postischemic myocardial function. Furthermore, our results suggest that cardiac NO biosynthesis is necessary to trigger preconditioning but that NO itself does not mediate the protective effect of classic preconditioning. In terms of the role of NO, it appears that there is no difference between the mechanism of pacing-induced or no-flow ischemia--induced classic preconditioning. Finally, we suggest that pharmacological inhibition of cardiac NO biosynthesis, although it may confer protection to the ischemic heart, may also abolish the development of endogenous cardioprotective mechanisms.

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**References**


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