

# Nitric Oxide Synthase Is the Mediator of Late Preconditioning Against Myocardial Infarction in Conscious Rabbits

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**Background**—Despite intense investigation, the effector of the infarct-limiting protection observed during the late phase of ischemic preconditioning (PC) remains unknown. The goal of this study was to test the hypothesis that late PC against myocardial infarction is mediated by the activity of nitric oxide synthase (NOS).

**Methods and Results**—Conscious rabbits underwent a 30-minute coronary occlusion followed by 3 days of reperfusion. In group I (control group,  $n=10$ ), infarct size (tetrazolium staining) averaged  $56.8\pm 5.3\%$  of the risk region, which was decreased to  $27.6\pm 2.5\%$  ( $P<0.05$ ) in rabbits preconditioned 24 hours earlier with a sequence of six 4-minute occlusion/4-minute reperfusion cycles (group II,  $n=10$ ). When preconditioned rabbits were given the nonselective NOS inhibitor *N*<sup>ω</sup>-nitro-L-arginine (L-NA, 13 mg/kg IV [group III,  $n=8$ ]) or the selective iNOS inhibitor aminoguanidine (AG, 150 mg/kg SC [group V,  $n=7$ ]) before the 30-minute occlusion, the protective effect of late PC was completely abrogated; that is, infarct size ( $59.9\pm 4.5\%$  and  $65.8\pm 3.3\%$ , respectively) was similar to that measured in the control group. Measurements of systolic wall thickening (sonomicrometry) demonstrated that L-NA and AG also abolished the improved recovery of myocardial function effected by late PC in group II. When rabbits were given L-NA or AG without prior PC (group IV [ $n=8$ ] and group VI [ $n=6$ ], respectively), infarct size did not differ from that observed in controls ( $53.8\pm 4.3\%$  and  $59.8\pm 4.3\%$ , respectively), demonstrating that L-NA and AG do not increase the extent of cell death in nonpreconditioned myocardium.

**Conclusions**—Taken together, these results indicate that in the conscious rabbit, the infarct-sparing effect of the late phase of ischemic PC is mediated by the activity of NOS and suggest that the specific isoform primarily responsible for this cardioprotective phenomenon is iNOS. Thus, NO appears to be a pivotal component of the pathophysiological cascade of late PC. (*Circulation*. 1998;98:441-449.)

**Key Words:** nitric oxide ■ ischemia ■ reperfusion ■ myocardial infarction

In addition to an immediate infarct-sparing effect,<sup>1-5</sup> ischemic PC elicits a late phase of protection against myocardial infarction, which becomes apparent 24 hours after the initial ischemic stress and persists up to 72 hours.<sup>5-17</sup> Therapeutic exploitation of this sustained cardioprotection would be conceptually appealing but is hindered by the paucity of current knowledge regarding its mechanism.<sup>18,19</sup> In this regard, it is important to distinguish the molecular species that initiates the development of late PC (trigger or initiator of late PC) from that which confers cardioprotection 24 to 72 hours later (mediator or effector of late PC). The pathophysiological roles of these 2 species are completely different. Recent studies<sup>17</sup> indicate that NO is the trigger of late PC against infarction. However, despite vigorous investigative endeavors, the species that accounts for the delayed infarct-limiting effects of ischemic PC (ie, the mediator of late PC) remains unknown.

In conscious rabbits, the late phase of ischemic PC protects both against a mild, reversible ischemic insult (myocardial

stunning)<sup>8,16</sup> and against a severe, irreversible ischemic insult (myocardial infarction).<sup>15,17</sup> Recent studies<sup>16</sup> have suggested that the protective effects of late PC against stunning are mediated by augmented formation of NO, apparently via the inducible isoform of nitric oxide synthase (iNOS). It is unknown, however, whether NOS also mediates late PC against infarction. Data obtained in the setting of late PC against stunning<sup>16</sup> cannot be extrapolated to late PC against infarction for 2 main reasons. First, myocardial stunning and infarction are 2 very different types of injury, so that the effect of a putative cardioprotective agent on one may not be applicable to the other. For example, in several experimental models, the beneficial effects of ischemic PC are apparently restricted to either reversible or irreversible ischemic injury but do not seem to apply to both (eg, in dogs, the early phase of PC does not protect against the stunning induced by a 10- or 15-minute coronary occlusion,<sup>18,20</sup> although it is highly effective in protecting against infarction,<sup>4</sup> whereas in pigs, the late phase of PC fails to protect against the infarction induced

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**Selected Abbreviations and Acronyms**

AG	= aminoguanidine
eNOS	= endothelial nitric oxide synthase
iNOS	= inducible nitric oxide synthase
L-NA	= <i>N</i> <sup>o</sup> -nitro-L-arginine
LV	= left ventricle
NOS	= nitric oxide synthase
PC	= preconditioning
WTh	= wall thickening

by a 40-minute occlusion,<sup>5</sup> although it is highly effective in protecting against stunning<sup>12-14</sup>). The second reason is that studies of the role of NO in *in vivo* models of myocardial infarction have yielded conflicting results, concluding that this radical has either a salutary<sup>21,22</sup> or a detrimental<sup>23,24</sup> effect on cell death. *In vitro* studies have suggested that the effects of NO on ischemia/reperfusion injury may depend on the timing of its formation (ie, NO was suggested to be beneficial before and during ischemia but deleterious after reperfusion<sup>25</sup>). In the present investigation, we found no effect of NOS inhibition on infarct size in nonpreconditioned myocardium. Therefore, it remains controversial whether augmented activity of NOS (such as that which occurs during late PC) represents a protective phenomenon in the setting of acute myocardial infarction.

In principle, there are several plausible NOS-independent pathways whereby late PC may limit infarct size, because myocardial ischemia triggers a complex array of cellular adaptations, including activation of various receptors, kinases, and transcription factors and upregulation of numerous genes.<sup>2,26</sup> Indeed, a number of mechanisms other than NO biosynthesis (eg, upregulation of heat stress proteins and antioxidant enzymes) have been proposed to account for the late phase of ischemic PC.<sup>10,11,19</sup> In view of all of the above considerations, one cannot assume that the mechanism uncovered for late PC against reversible injury (stunning)<sup>16</sup> will necessarily apply to late PC against cell death.

Accordingly, the present study was undertaken to explore the role of NOS as a mediator of the late phase of PC against myocardial infarction in conscious rabbits. To investigate whether NO (irrespective of its source) contributes to the protective effects of late PC, we tested the effects of the nonselective NOS inhibitor L-NA, given before sustained ischemia, on myocardial infarct size. Having found that L-NA abrogated late PC, we then tested the effects of the selective iNOS inhibitor AG. In addition to measuring infarct size, we also evaluated the recovery of myocardial function (assessed as systolic WTh) after the lethal ischemic insult, because this is an additional index of myocardial protection that is independent of histochemical measurements of cell death. The study was conducted in conscious animals to eliminate the potentially confounding influence of factors associated with open-chest preparations, such as anesthesia, surgical trauma, abnormal hemodynamics, elevated catecholamine levels, fluctuations in body temperature, exaggerated formation of reactive oxygen species, and release of cytokines, which could, in themselves, induce iNOS<sup>27</sup> and could also interfere with myocardial infarction or ischemic

PC.<sup>1,3,28,29</sup> The results provide evidence for the first time that late PC against infarction is mediated by the activity of NOS.

**Methods**

The conscious rabbit model of myocardial ischemia has been described in detail previously<sup>8,16,17</sup> and will be briefly summarized here.

**Experimental Preparation**

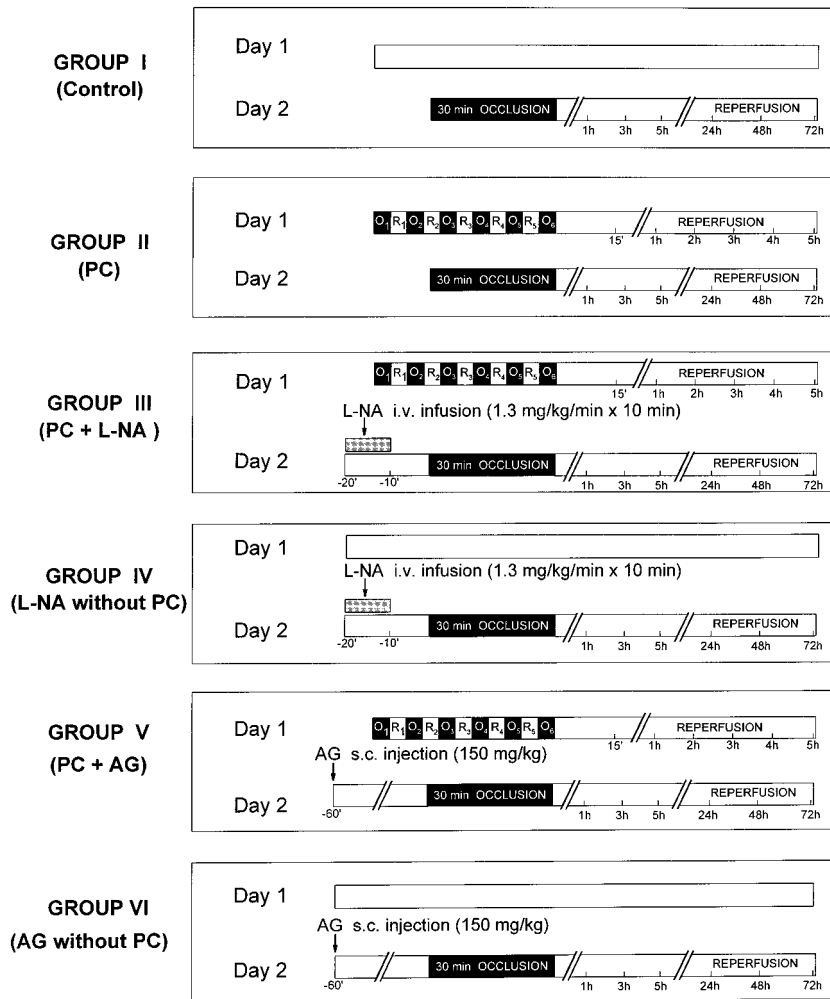
New Zealand White male rabbits (weight, 2.4±0.1 kg) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed Doppler ultrasonic crystal in the center of the region to be rendered ischemic, bipolar pacing leads in the left atrial appendage, and bipolar ECG leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for 3 days to evacuate air and fluids postoperatively. Gentamicin was administered before surgery and on the first and second postoperative days (0.7 mg/kg IM each day). Rabbits were allowed to recover for a minimum of 10 days after surgery.

**Experimental Protocol**

Throughout the experiments, rabbits were kept in a cage in a quiet, dimly lit room. LV systolic WTh, range gate depth, and the ECG were continuously recorded on a thermal array chart recorder. Regional myocardial function was assessed as systolic thickening fraction by use of the pulsed Doppler probe, as previously described.<sup>8</sup> All rabbits were subjected to a 30-minute coronary artery occlusion followed by 3 days of reperfusion. We verified the performance of successful coronary occlusion by observing the development of ST-segment elevation and changes in the QRS complex on the ECG and the appearance of paradoxical wall thinning on the ultrasonic crystal recordings. Diazepam was administered 20 minutes before the onset of ischemia (4 mg/kg IV) to relieve the stress caused by the coronary occlusion. No antiarrhythmic agents were given at any time.

Rabbits were assigned to 6 groups (Figure 1). Group I (control group) underwent the 30-minute occlusion with no PC protocol and no drug treatment. Group II (PC group) underwent a sequence of six 4-minute coronary occlusions interspersed with 4-minute intervals of reperfusion 24 hours before the 30-minute coronary occlusion. Group III (PC+L-NA group) underwent the same protocol as group II except that the rabbits received an intravenous infusion of L-NA at a rate of 1.3 mg·kg<sup>-1</sup>·min<sup>-1</sup> for 10 minutes starting 20 minutes before and ending 10 minutes before the 30-minute coronary occlusion (total dose, 13 mg/kg). L-NA (Sigma Chemical Co) was dissolved in normal saline (total volume infused, 20 mL). Group IV (L-NA group) underwent the same protocol as group III except that the rabbits were not preconditioned. Group V (PC+AG group) underwent the same protocol as group II except that the rabbits received a subcutaneous injection of AG (150 mg/kg) 1 hour before the 30-minute coronary occlusion. AG hydrochloride (Aldrich Chemical Co) was dissolved in 2 mL of water, and the pH of the solution was adjusted to 7.4 with 0.1 NaOH. In group VI (AG group), rabbits underwent the same protocol as in group V except that they were not preconditioned. The solutions of L-NA and AG were filtered through a 0.2-μm filter to ensure sterility.

AG was given 1 hour before ischemia because, on the basis of the slow onset of its vascular actions<sup>30,31</sup> and the increase in its potency with time of preincubation,<sup>31-33</sup> this agent is thought to enter the intracellular space relatively slowly.<sup>34</sup> The reason for administering AG by subcutaneous injection was that in our previous studies,<sup>16</sup> we found that a dose of 100 mg/kg of this drug given intravenously caused a sustained (>2 hours) decrease in both heart rate and arterial pressure, possibly due to inhibition of NOS in the central nervous system<sup>35,36</sup> by high initial circulating levels of AG resulting from intravenous administration. Because several investigations have successfully used the subcutaneous route to block iNOS activity with AG,<sup>37-40</sup> we selected this route in an effort to achieve lower and more sustained circulating levels of the drug. Previous hemodynamic



**Figure 1.** Experimental protocol. Six groups of rabbits were studied. On day 2, all groups underwent a 30-minute coronary occlusion followed by 3 days of reperfusion. On day 2, rabbits in groups I ( $n=10$ , control group) and II ( $n=10$ , PC group) received no treatment. On day 1, rabbits in group II underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles. Rabbits in group III ( $n=8$ , PC+L-NA group) underwent the same protocol as group II on day 1; on day 2, they received an intravenous infusion of L-NA at a rate of  $1.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  starting 20 minutes before and ending 10 minutes before the 30-minute coronary occlusion (total dose,  $13 \text{ mg/kg}$ ). Rabbits in group IV ( $n=8$ , L-NA group) underwent the same protocol as group III except that they were not preconditioned on day 1. Rabbits in group V ( $n=7$ , PC+AG group) underwent the same protocol as group II on day 1; on day 2, they received a subcutaneous injection of AG ( $150 \text{ mg/kg}$ ) 1 hour before the 30-minute coronary occlusion. Rabbits in group VI ( $n=6$ , AG group) underwent the same protocol as group V except that they were not preconditioned on day 1.

measurements<sup>16</sup> have demonstrated that subcutaneous injection of  $150 \text{ mg/kg}$  AG has no effect on heart rate or arterial pressure in conscious rabbits.

### Postmortem Tissue Analysis

At the conclusion of the study, the size of the occluded-reperfused coronary vascular bed was determined by a previously described postmortem perfusion technique.<sup>17</sup> Briefly, the coronary artery was tied at the site of the previous occlusion, and the aortic root was perfused for 2 minutes with a 5% solution of Phthalo blue dye (Heucotech LTD) in normal saline at a pressure of  $70 \text{ mm Hg}$  by use of a Langendorff apparatus. The heart was then cut into 6 to 7 transverse slices, which were incubated for 10 minutes at  $37^\circ\text{C}$  in 1% triphenyl tetrazolium chloride (pH 7.4). The slices were weighed, fixed in a 10% formaldehyde solution, and photographed (Nikon AF N6006). Transparencies were projected onto a paper screen at a 10-fold magnification, and the borders of the infarcted, ischemic-reperfused, and nonischemic regions were traced. The corresponding areas were measured by computerized planimetry (Adobe Photoshop, version 4.0), and from these measurements, infarct size was calculated as a percentage of the region at risk.<sup>17</sup>

### Measurement of Regional Myocardial Function

Regional myocardial function was assessed as systolic thickening fraction by use of the pulsed Doppler probe, as previously described.<sup>41</sup> Percent systolic thickening fraction was calculated as the ratio of net systolic WTh to end-diastolic wall thickness, multiplied by 100.<sup>41</sup> We calculated the total deficit of systolic WTh over the 3-day reperfusion period (an integrative assessment of the overall

severity of contractile dysfunction during this time interval) by measuring the area between the systolic WTh-versus-time line and the baseline (100% line) during the 3-day recovery phase after the 30-minute coronary occlusion.<sup>17</sup> In all animals, measurements were averaged from  $\geq 10$  beats at baseline and from  $\geq 5$  beats at all subsequent time points.

### Statistical Analysis

Data are reported as mean  $\pm$  SEM. Heart rate and thickening fraction were analyzed by a 2-way repeated-measures ANOVA (time and group). Infarct sizes and risk region sizes were analyzed with a 1-way ANOVA followed by Student's *t* tests for unpaired data with the Bonferroni correction. The relationship between infarct size and risk region size was compared among groups with an ANCOVA, with size of the risk region used as the covariant. The correlation between infarct size and risk region size was assessed by linear regression analysis using the least squares method.

## Results

### Exclusions

Of the 57 rabbits instrumented for this study, 13 were assigned to the control group (group I), 12 to the PC group (group II), 8 to the PC+L-NA group (group III), 8 to the L-NA group (group IV), 10 to the PC+AG group (group V), and 6 to the AG group (group VI). Of the 13 rabbits assigned to the control group, 2 died of ventricular fibrillation during coronary occlusion and 1 was excluded because of failure of

## Heart Rate (bpm) During Coronary Occlusion and Reperfusion

	Baseline	Preocclusion	Occlusion		Reperfusion					
			6 th	15 min	1 h	3 h	5 h	24 h	48 h	72 h
Group I (control)										
Day 2	...	245±10	...	256±6	257±9	245±8	249±8	252±6	248±6	251±6
Group II (PC)										
Day 1	...	251±9	253±12	...	255±10	245±10	246±7	...	...	...
Day 2	...	250±4	...	264±6	265±10	262±9	271±10	263±8	262±4	259±6
Group III (PC+L-NA)										
Day 1	...	235±11	239±15	...	222±14	228±11	231±12	...	...	...
Day 2	245±11	185±7*#	...	182±7*#	182±4*#	183±4*#	188±3*#	231±9	243±9	244±10
Group IV (L-NA)										
Day 2	240±5	199±5*#	...	175±3*#	192±7*#	192±9*#	195±6*#	237±8	257±6	258±8
Group V (PC+AG)										
Day 1	...	250±9	250±9	...	236±5	238±2	239±7	...	...	...
Day 2	266±6	257±11	...	273±8	278±9	273±12	257±7	260±6	259±3	261±7
Group VI (AG)										
Day 2	249±9	256±9	...	265±10	270±13	267±10	261±6	259±2	254±4	254±4

In groups I, IV, and VI, rabbits underwent a 30-minute coronary occlusion on day 2 followed by a 72-hour observation period. Heart rate was measured at baseline (before treatment), 1 minute before occlusion (preocclusion), at 25 minutes into the 30-minute coronary occlusion, and at selected times after reperfusion. In groups II, III, and V, rabbits underwent a sequence of six cycles of 4-minute coronary occlusion/4-minute reperfusion followed by a 5-hour observation period on day 1. Heart rate was measured 1 minute before occlusion (preocclusion), at 3 minutes into the 6th occlusion, and at selected times after the 6th reperfusion. On day 2, the rabbits underwent a 30-minute coronary occlusion followed by a 72-hour observation period; heart rate was measured at baseline (before treatment), 1 minute before occlusion (preocclusion), at 15 minutes into the 30-minute coronary occlusion, and at selected times after reperfusion.

Data are mean±SEM. \* $P<0.05$  vs baseline within the same group; # $P<0.05$  vs corresponding value in group I.

the balloon occluder. Of the 12 rabbits assigned to the PC group, 2 died of ventricular fibrillation during the 30-minute coronary occlusion. Of the 10 rabbits assigned to the PC+AG group, 2 were excluded because of ventricular fibrillation during the 30-minute occlusion and 1 because of failure of the balloon occluder. None of the rabbits assigned to the PC+L-NA group, the L-NA group, or the AG group were excluded. Therefore, a total of 10 rabbits completed the protocol in the control group, 10 in the PC group, 8 in the PC+L-NA group, 8 in the L-NA group, 7 in the PC+AG group, and 6 in the AG group. No rabbit included in the final analysis was subjected to defibrillation.

### Hemodynamic Variables

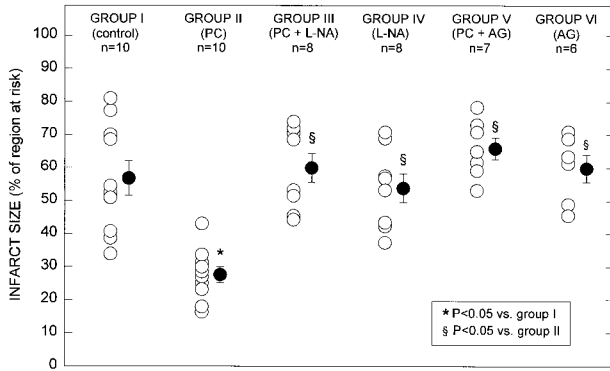
Previous studies in conscious rabbits have shown that the dose of L-NA used in the present study does not alter systemic arterial pressure or systolic thickening fraction<sup>8,16,17</sup> and that the dose of AG used does not affect heart rate, arterial pressure, or systolic thickening fraction.<sup>16</sup> On the day of the 30-minute coronary occlusion, baseline (pretreatment) heart rate did not differ among the 6 groups (Table). Consistent with our previous studies,<sup>8,16,17</sup> administration of L-NA produced a sustained decrease in heart rate that persisted up to 5 hours after the 30-minute occlusion. As a result, heart rate was significantly ( $P<0.05$ ) lower in groups III and IV than in group I (control group) after treatment (preocclusion) (185±7 and 199±5 versus 245±10 bpm, respectively), at 15 minutes of occlusion (182±7 and 175±3 versus 256±6 bpm), at 1 hour of reperfusion (182±4 and 192±7 versus 257±9 bpm), at 3 hours of reperfusion (183±4 and 192±9

versus 245±8 bpm), and at 5 hours of reperfusion (188±3 and 195±6 versus 249±8 bpm) (Table). The decreases in heart rate elicited by L-NA in groups III and IV were similar (Table). Heart rate did not differ significantly in groups II, V, and VI versus group I during the 30-minute occlusion or the ensuing 5 hours of reperfusion (Table).

As expected,<sup>8,16,17</sup> WTh before ischemia was not altered by either L-NA (groups III and IV) or AG (groups V and VI): systolic thickening fraction averaged 35.0±4.1% and 34.3±3.0% before L-NA versus 35.8±4.0% and 34.0±2.8% after L-NA in groups III and IV, respectively, and 31.1±2.1% and 33.8±2.8% before AG versus 31.6±1.6% and 33.2±3.2% after AG in groups V and VI, respectively.

### Region at Risk and Infarct Size

There were no significant differences among groups I, II, III, IV, V, and VI with respect to LV weight (4.4±0.1, 4.3±0.2, 4.5±0.2, 4.8±0.2, 4.6±0.6, and 5.4±0.5 g, respectively) or weight of the region at risk (0.8±0.1 g [18.5±2.3% of LV weight], 0.7±0.1 g [15.6±1.3% of LV weight], 0.8±0.1 g [18.8±1.7% of LV weight], 1.0±0.1 g [20.1±1.9% of LV weight], 0.8±0.1 g [16.8±1.9% of LV weight], and 1.1±0.1 g [21.8±3.1% of LV weight], respectively). Average infarct size was 51% smaller in group II than in control animals (group I) (27.6±2.5% versus 56.8±5.3% of the region at risk, respectively;  $P<0.05$  [Figure 2]), indicating a late PC effect against myocardial infarction. In group III, however, infarct size (59.9±4.5% of the region at risk) was significantly greater than in group II ( $P<0.05$ ) and essentially indistinguishable from controls (Figure 2), indicating that L-NA

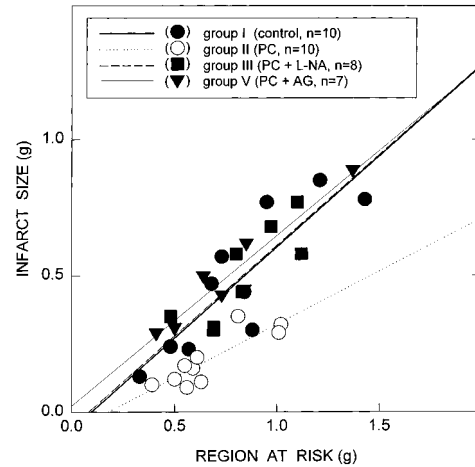


**Figure 2.** Myocardial infarct size in groups I (control group), II (PC group), III (PC+L-NA group), IV (L-NA group), V (PC+AG group), and VI (AG group). Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent mean  $\pm$  SEM. \* $P < 0.05$  versus group I (controls); § $P < 0.05$  versus group II (PC group).

abrogated the late PC effect against infarction. In group IV, infarct size ( $53.8 \pm 4.3\%$  of the region at risk) did not differ significantly from that in controls (Figure 2), indicating that administration of L-NA did not affect the extent of cell death in nonpreconditioned myocardium. Similar to the results obtained in group III, the infarct size in group V ( $65.8 \pm 3.3\%$  of the region at risk) was significantly greater than in group II ( $P < 0.05$ ) and comparable to that measured in controls (Figure 2), indicating that AG abrogated the late PC effect against infarction. In group VI, infarct size ( $59.8 \pm 4.3\%$  of the region at risk) did not differ significantly from that in controls (Figure 2), indicating that administration of AG in and of itself had no effect on the magnitude of lethal injury in the absence of ischemic PC. In all 6 groups, the size of the infarction was positively and linearly related to the size of the region at risk ( $r = 0.86, 0.86, 0.85, 0.86, 0.95,$  and  $0.75$ , respectively). As expected, the regression line was significantly shifted to the right in group II compared with group I ( $P < 0.05$  by ANCOVA) (Figure 3). In contrast, in groups III and V, regression lines were indistinguishable from that in group I and significantly ( $P < 0.05$  by ANCOVA) different from that in group II, indicating that for any given size of the region at risk, the resulting infarction was greater in preconditioned rabbits treated with L-NA or AG than in untreated preconditioned rabbits (Figure 3). (Regression equations are given in the legend to Figure 3).

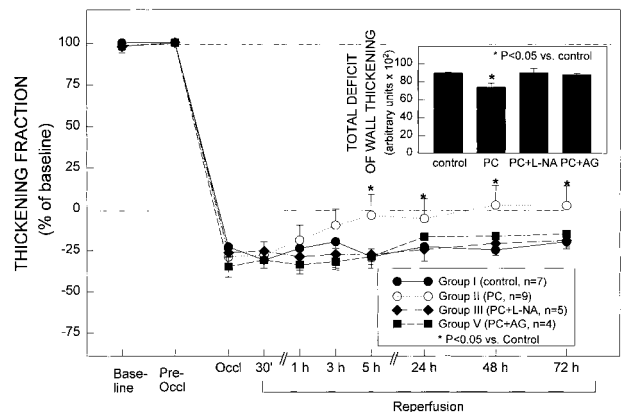
**Regional Myocardial Function**

Because of Doppler probe malfunction, complete measurements of WTh for 3 days after reperfusion could be obtained in only 7 of 10 rabbits in group I, 9 of 10 rabbits in group II, 6 of 8 rabbits in group III, and 4 of 7 rabbits in group V. On the second day of the protocol, baseline systolic fraction averaged  $38.7 \pm 4.5\%$  in group I,  $37.7 \pm 2.5\%$  in group II,  $35.0 \pm 4.1\%$  in group III, and  $31.1 \pm 2.0\%$  in group V ( $P = NS$ ). After release of the 30-minute occlusion, control rabbits (group I) exhibited essentially no recovery of WTh even at 3 days (Figure 4). In preconditioned rabbits (group II), recovery of WTh was significantly ( $P < 0.05$ ) improved compared with controls at 5 hours, 1 day, 2 days, and 3 days after reperfusion



**Figure 3.** Relationship between size of the region at risk and size of myocardial infarction. Illustrated are both individual values and regression lines obtained by linear regression analysis for group I (control group), group II (PC group), group III (PC+L-NA group), and group V (PC+AG group). In all groups, infarct size was positively and linearly related to risk region size. Linear regression equations were as follows: group I,  $y = 0.67x - 0.06$  ( $r = 0.86$ ); group II,  $y = 0.39x - 0.07$  ( $r = 0.86$ ); group III,  $y = 0.67x + 0.06$  ( $r = 0.85$ ); group V,  $y = 0.62x + 0.02$  ( $r = 0.95$ ). ANCOVA demonstrated that the slope of the regression line for group II was significantly different from that for groups I, III, or V ( $P < 0.05$  for each comparison), indicating that for any given risk region size, infarct size was smaller in group II than in other groups. These data demonstrate that late PC reduced infarct size independently of risk region size and that this effect was abrogated by both L-NA and AG.

(Figure 4). The total deficit of WTh over the 3-day reperfusion period (an integrative assessment of the overall severity of contractile dysfunction during this time interval<sup>17</sup>) was decreased by 17% in group II versus group I ( $P < 0.05$ ) (Figure 4). In groups III (L-NA treated) and V (AG treated), recovery of WTh was indistinguishable from that in the control group (Figure 4), indicating that both L-NA and AG



**Figure 4.** Systolic thickening fraction in the ischemic-reperused region in groups I, II, III, and V. Measurements were obtained at baseline, at 15 minutes into the 30-minute occlusion (Occl), and at 30 minutes and 1, 3, 5, 24, 48, and 72 hours after reperfusion. Thickening fraction is expressed as a percentage of baseline values. Total deficit of WTh was calculated by measuring the area between the systolic WTh-versus-time line and the baseline (100% line) during the 3-day reperfusion period after the 30-minute occlusion.<sup>17</sup> Data are mean  $\pm$  SEM. \* $P < 0.05$  versus group I (controls).

abrogated the salutary actions of late PC on recovery of myocardial function.

### Discussion

One of the most critical, if not the most critical, unresolved issues pertaining to the mechanism of late PC is the nature of the cellular mediator that is responsible for conferring increased tolerance to lethal ischemic injury 24 to 72 hours after a brief ischemic challenge. The search for this mediator has been intense, and many hypotheses have been formulated (reviewed in Reference 19). The implications of this issue are potentially vast, because identification of the key cytoprotective protein(s) is critical not only for understanding the pathophysiology of the delayed myocardial adaptations to stress, but also for formulating therapeutic strategies aimed at mimicking these adaptations with pharmacological agents capable of inducing a sustained cardioprotective effect similar to that afforded by the late phase of ischemic PC.

The present study provides new insights into this issue. The results reported herein demonstrate that the nonselective NOS inhibitor L-NA completely abrogates late PC against myocardial infarction in conscious rabbits, indicating that formation of NO is essential for this cardioprotective phenomenon to become manifest. The present study also demonstrates that the selective iNOS inhibitor AG is as effective as L-NA in abrogating the late PC effect, suggesting that iNOS is the major source of the NO that protects against infarction. The inhibition of the infarct-sparing action of late PC by L-NA and AG is further corroborated by our measurements of WTh in the ischemic-reperfused region (a tetrazolium-independent index of myocardial protection). These measurements demonstrated that the enhanced recovery of WTh after the 30-minute coronary occlusion brought about by late PC in group II was abolished by both of the NOS inhibitors. The effects of L-NA and AG cannot be ascribed to an inherent detrimental action of these agents on myocardial infarction because neither of them had any discernible influence on infarct size in nonpreconditioned myocardium (ie, in groups IV and VI, respectively). Taken together, these results demonstrate that the infarct-sparing effects of late PC are mediated by the activity of NOS and specifically implicate iNOS as the primary mediator. To the best of our knowledge, this is the first evidence that NO generation is responsible for the protection against lethal injury observed during the late phase of ischemic PC.

The mechanism of the late phase of ischemic PC involves 2 key components: the molecular species that initiates this slow adaptive response during the first ischemic challenge (trigger of late PC) and the species that increases the resistance to infarction during the second ischemic challenge (mediator of late PC). In principle, these 2 species could be different. Previous observations have shown that NO generated during the first ischemic insult serves as a trigger for the development of late PC against myocardial infarction.<sup>17</sup> The present data expand our understanding of the late phase of ischemic PC by demonstrating that NO also serves as a mediator of the increased resistance to infarction during the second ischemic insult. Therefore, the present study reveals a much more complex, multifaceted role of NO as a pivotal

component of the entire pathophysiological process underlying late PC.

The dose of L-NA used in the present study has been shown to inhibit NOS activity by >70%,<sup>42</sup> to markedly decrease exhaled NO (measured by chemiluminescence),<sup>35</sup> and to blunt acetylcholine-induced vasodilation in rabbits.<sup>8</sup> The decrease in heart rate observed after L-NA in the present study is consistent with previous reports<sup>8,16,17,35,36</sup> and is thought to reflect the central regulatory function of NO on the sympathetic and parasympathetic tone.<sup>35</sup> The decrease in heart rate elicited by L-NA, if anything, would be expected to decrease infarct size, an effect opposite to that observed in group III. The conclusion that the action of L-NA on heart rate did not contribute to the abrogation of late PC in group III is further corroborated by the results obtained in group IV, in which the same decrease in heart rate after L-NA administration produced no detectable change in infarct size.

Having found that L-NA abolished late PC against infarction, we then tested the effect of AG to specifically interrogate the role of iNOS. AG was selected among the iNOS inhibitors currently available because it has the highest selectivity for this isoform,<sup>34,43,44</sup> with an IC<sub>50</sub> of 160.0 μmol/L for constitutive NOS versus 5.4 μmol/L for iNOS.<sup>33,43</sup> In accordance with these pharmacological data *in vitro*, AG is ≈40 times less effective than N<sup>G</sup>-monomethyl-L-arginine in raising arterial blood pressure *in vivo*.<sup>45</sup> The fact that our dose of AG has no effect on arterial blood pressure in conscious rabbits<sup>16</sup> further supports the notion that it does not inhibit NO production by vascular eNOS. Our results demonstrate that AG was as effective as L-NA in abrogating late PC against infarction, suggesting that the primary isoform involved in this cardioprotective phenomenon is iNOS. To the best of our knowledge, these are the first data to implicate iNOS as a mediator of the late phase of ischemic PC against infarction. However, because the iNOS-versus-eNOS selectivity of any currently available NOS inhibitor is only relative,<sup>34</sup> a role of eNOS as a possible mediator cannot be ruled out. In this regard, the recent finding that brief ischemia induces a delayed upregulation of coronary eNOS in conscious dogs<sup>46</sup> suggests that this enzyme may also contribute to the protective effects of late PC. Definitive identification of the specific isoforms of NOS responsible for late PC will necessitate a molecular approach, such as the use of transgenic and gene-targeted murine models of ischemic PC. The primary objective of this investigation was to explore the role of NOS in general, not to determine the specific NOS isoforms involved. The concordant results obtained with 2 unrelated NOS inhibitors provide cogent evidence to support the conclusion that NOS activity plays a pivotal role in late PC against infarction.

The enhancement in recovery of WTh after the 30-minute occlusion effected by late PC (Figure 4) was relatively modest compared with the reduction in infarct size (Figure 2). However, we assessed WTh only for the first 3 days of reperfusion. It is probable that the reduction in the deficit of WTh would have been greater if we had monitored the rabbits for a longer interval, sufficient for myocardial stunning in the surviving tissue to resolve. The WTh data provide an inde-

pendent confirmation of the results obtained with tetrazolium staining, because the effects of L-NA and AG on WTH paralleled those on infarct size.

Although the influence of NO on lethal ischemic injury has been addressed in several investigations, with conflicting results,<sup>21-25</sup> no previous study has examined the role of NO as the possible mediator of the protective effects of the late phase of ischemic PC against infarction. Zhao et al<sup>40</sup> have recently shown in open-chest rabbits that the administration of monophosphoryl lipid A (MLA) elicits a delayed infarct-sparing effect that becomes apparent 24 hours later; this protection was associated with an increase in iNOS activity and was inhibited by AG, suggesting that it is mediated by iNOS. It appears, therefore, that the mechanism of pharmacological PC with MLA resembles that of the late phase of ischemic PC. Ischemic PC might induce expression of iNOS via activation of protein kinase C,<sup>2,47</sup> mitogen-activated protein kinases,<sup>48</sup> and/or nuclear factor  $\kappa$ B,<sup>49</sup> ie, through signaling pathways analogous to those involved in the induction of iNOS by cytokines.<sup>50</sup> Because these pathways can be stimulated by reactive oxygen species,<sup>51-59</sup> the proposal that iNOS mediates the late phase of ischemic PC is compatible with evidence supporting an important role of reactive oxygen metabolites in the development of this phenomenon.<sup>13,60</sup> The concept that iNOS protects against infarction may seem paradoxical, or even counterintuitive, in view of the well-known detrimental role of this enzyme in various pathological conditions.<sup>50,61</sup> However, because of the differences in species, tissues, and perhaps most importantly, type and severity of injury, it is difficult to compare previous studies of iNOS in other systems<sup>50,61</sup> with our present findings. We suggest that the effects of NO are likely to be dose dependent, so that although massive NO formation is toxic, less-robust generation of NO may be protective to the ischemic myocardium. Many actions of NO have been identified that would be expected to be beneficial during acute myocardial ischemia. For example, NO (or its second messenger, cGMP) has been shown to inhibit the influx of calcium into myocytes,<sup>62,63</sup> to antagonize the effects of  $\beta$ -adrenergic stimulation,<sup>64,65</sup> to decrease myocardial contractility,<sup>50,65-67</sup> to reduce myocardial oxygen consumption,<sup>68-71</sup> and to open  $K_{ATP}$  channels.<sup>72-74</sup> These actions may alleviate the calcium overload and depletion of high-energy phosphates associated with acute myocardial ischemia, which are 2 of the major mechanisms of tissue injury in this setting.

In conclusion, the present study supports the novel concept that the infarct-sparing effect of the late phase of ischemic PC in conscious rabbits is due to the activity of NOS and specifically the iNOS isoform. Induction of iNOS after cellular stress generally has been viewed as a deleterious process.<sup>50,61</sup> The apparent contradiction between this concept and the present observations should stimulate a critical reassessment of current views regarding the functional significance of iNOS induction in disease states. We propose a more complex paradigm in which iNOS activity can play both a beneficial and a detrimental role depending on the type of injury and, perhaps, the intensity of iNOS induction.

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