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Biphasic response of cardiac NO synthase isoforms to ischemic preconditioning in conscious rabbits

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Xuan, Yu-Ting, Xian-Liang Tang, Yumin Qiu, Supratim Banerjee, Hitoshi Takano, Hui Han, and Roberto Bolli. Biphasic response of cardiac NO synthase isoforms to ischemic preconditioning in conscious rabbits. *Am J Physiol Heart Circ Physiol* 279: H2360–H2371, 2000.—In conscious rabbits, a sequence of six 4-min coronary occlusion/4-min reperfusion cycles, which elicits late preconditioning (PC), caused rapid activation of calcium-dependent nitric oxide (NO) synthase (NOS) [cNOS; endothelial NOS (eNOS) and/or neuronal NOS (nNOS)], whereas calcium-independent NOS [inducible NOS (iNOS)] activity remained unchanged. The enhanced cNOS activity was associated with increased myocardial levels of NO_2 and/or NO_3 (NO_x). Twenty-four hours after ischemic PC was induced, the opposite pattern was observed, i.e., there was a pronounced increase in cytosolic iNOS activity but no change in cNOS activity. The initial burst of ischemia-induced cNOS activity was not affected by pretreatment with the antioxidant *N*-2-mercaptopropionyl glycine (MPG), the protein kinase C (PKC) inhibitor chelerythrine, or the tyrosine kinase inhibitor lavendustin A, indicating that it is independent of the generation of oxidant species and the activation of PKC and tyrosine kinases. In contrast, the delayed upregulation of iNOS 24 h after PC was prevented by pretreatment with N^{ω}-nitro-L-arginine, MPG, or chelerythrine before the PC ischemia, indicating that it is triggered by a signaling mechanism that involves the generation of NO, the formation of oxidant species, and the activation of PKC. Taken together, these results demonstrate that, in conscious animals, ischemic PC elicits a biphasic response in cardiac NOS activity, i.e., an immediate activation of cNOS (most likely eNOS) followed 24 h later by a delayed upregulation of iNOS. To our knowledge, this is the first study to directly measure NOS activity after brief myocardial ischemia in vivo. In conjunction with previous functional studies, the data support a distinctive role of NOS isoforms in late PC, with eNOS serving as the trigger on *day 1* and iNOS as the mediator on *day 2*.

nitric oxide synthase; chelerythrine; N^{ω}-nitro-L-arginine; reactive oxygen species; protein tyrosine kinases

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MUCH ATTENTION HAS RECENTLY FOCUSED on the cellular mechanisms that underlie the development of delayed cardioprotection after a sublethal ischemic stress [late phase of ischemic preconditioning (PC)] (5, 23). Pharmacological studies support the concept that this adaptive mechanism is triggered by enhanced biosynthesis of nitric oxide (NO) during the initial ischemic stress. Specifically, it has been demonstrated that the administration of the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NNA) before the initial ischemic stimulus prevents the development of late PC against both myocardial stunning (4) and myocardial infarction (30) and, conversely, that administration of NO donors in lieu of ischemia mimics the cardioprotective effects of the late phase of ischemic PC (40). However, a number of important issues remain to be addressed regarding the role of NO as a trigger of late PC. First, to date, there is no direct evidence that ischemic PC activates NOS in the heart. Second, no information is available regarding which NOS isoform(s) is(are) involved in triggering the late phase of ischemic PC, because the studies that implicated NO as a trigger used L-NNA, which is a nonselective inhibitor of all three NOS isoforms (37). Endothelial NOS (eNOS) is constitutively expressed in cardiomyocytes and endothelial cells (8, 20, 24). Neuronal NOS (nNOS) has been described in cardiac neurons (20, 35, 44) and in cardiomyocytes (8, 36, 48). In addition, a number of studies (24, 46) support the presence of inducible NOS (iNOS) in normal myocardium. In keeping with these studies, we have recently observed the presence of iNOS protein and calcium-independent NOS (iNOS) activity in normal murine hearts (17), indicating that iNOS is constitutively expressed in this species. Similar findings were obtained in control rabbits in the present study (vide infra), indicating that iNOS is normally present in the rabbit heart as well. In view of this constitutive expression of iNOS in cardiac tissue, it is theoretically possible that iNOS could serve as a trigger of late PC. Third, the role of various signaling elements known to be recruited by ischemic PC [oxidant species, protein kinase C (PKC), and protein tyrosine kinases (PTKs)] in the activation of NOS during the initial ischemic stimulus remains to be determined. This could be elucidated by measuring NOS activity in the presence and absence of inhibitors of PKC and

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PTKs and scavengers of oxidant species. Finally, the notion that NO triggers late PC is predicated upon the assumption that L-NNA is indeed effective in blocking the activation of NOS by ischemic PC in vivo, but no direct evidence for this is available. Therefore, it remains unknown whether the dose of L-NNA that blocks the development of late PC (13 mg/kg iv; see Refs. 4 and 30) also blocks the activation of NOS associated with the initial ischemic stimulus.

There are also important unresolved issues regarding the role of NO in mediating late PC 24 h after the PC stimulus. First, although iNOS appears to be the mediator of this phenomenon in rabbits (6, 39), the cellular signaling mechanisms that underlie the upregulation of this enzyme after a sublethal ischemic challenge in the heart remain unclear. A previous study (49) has shown that ischemic PC activates nuclear factor-κB (NF-κB) (a major modulator of iNOS expression; see Refs. 1 and 47) and that this activation is dependent upon a signaling pathway that includes NO, oxidant species, and PKC. Accordingly, it seems plausible to postulate that the upregulation of iNOS activity on *day 2* may also be orchestrated by a signaling mechanism that includes NO, oxidant species, and PKC. Second, the role of iNOS as the mediator of late PC in rabbits has been inferred exclusively from the outcome of administering the iNOS inhibitors aminoguanidine (AG) and *S*-methylisothiourea (SMT) (6, 39). It was assumed in those studies (6, 39) that the doses of AG and SMT given to rabbits selectively inhibited iNOS without affecting eNOS activity. However, the selectivity of AG and SMT for iNOS versus eNOS is modest (IC_{50} ratio of ~ 30:1; see Ref. 37), and the tissue concentrations of these drugs achieved in vivo are unknown, raising the possibility that they may also have affected eNOS. In order for the abrogation of late PC by AG and SMT (6, 39) in rabbits to be construed as an indication that iNOS mediates late PC in this species, it is necessary to show that the doses of AG and SMT utilized in those previous studies do indeed inhibit iNOS but not eNOS.

The overall goal of the present study was to address these unresolved issues. We sought to provide direct evidence for the involvement of NOS in both the initiation (*day 1*) and mediation (*day 2*) of the late phase of ischemic PC and to elucidate the mechanisms that control the upregulation of iNOS during this process. The following specific questions were addressed. *1*) Does an ischemic PC stimulus cause immediate activation of NOS in vivo? *2*) If so, which isoform (iNOS or cNOS) is activated? *3*) What is the time-course of NOS activation after ischemic PC? *4*) Is NOS upstream of PKC and PTKs in the genesis of late PC? *5*) Is the upregulation of iNOS activity 24 h after sublethal ischemia modulated by the formation of NO and oxidants and by the activation of PKC during the ischemic stimulus? *6*) Do the doses of AG and SMT previously found to abrogate late PC on *day 2* (6, 39) actually inhibit iNOS without interfering with eNOS in vivo? To address these issues, the activity of iNOS and cNOS as well as the myocardial levels of NO_2 and/or NO_3 (NO_x; the stable byproducts of NO) were measured in the presence or absence of a number of interventions (NOS inhibitors, oxidant scavengers, PKC and PTK inhibitors) that are targeted at the main elements of the known signaling cascade of late PC (5). These experiments were conducted in the same rabbit model of late PC in which previous investigations have demonstrated an essential role of NO (4, 6, 30, 39), oxidant species (5, 42), PKC (26, 28, 29), and PTKs (12, 27), so that the changes in NOS activity could be correlated with the activation of these signaling elements and with the presence or absence of cardioprotection. All studies were performed in conscious animals to obviate the confounding effects of factors associated with open-chest preparations, such as anesthesia, surgical trauma, fluctuations in temperature, elevated catecholamine levels, excessive oxidant formation, release of cytokines, etc. (3, 10, 14, 18, 22, 34, 43). The results demonstrate, for the first time, that two different NOS isoforms are sequentially recruited during the development of the late phase of ischemic PC, with activation of cNOS occurring immediately after the ischemic stimulus (*day 1*) and activation of iNOS occurring 24 h later (*day 2*), and that the upregulation of iNOS on *day 2* is dependent upon a signaling mechanism that involves formation of NO and reactive oxygen species (ROS) and activation of PKC and PTKs on *day 1*.

METHODS

Experimental Preparation

The experimental preparation has been described in detail previously (4, 6, 12, 29, 30, 39, 40, 49). Briefly, New Zealand White male rabbits (weight 2.0 to 2.5 kg, age 3–4 mo) 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 (7) in the center of the region to be rendered ischemic, and bipolar electrocardiogram leads on the chest wall. All rabbits were allowed to recover for a minimum of 10 days after surgery. Throughout the experiments, rabbits were kept in a cage in a quiet, dimly lit room. Left ventricular (LV) systolic wall thickening (WTh), the range gate depth, and the electrocardiogram were continuously recorded on a thermal array chart recorder (Gould TA6000, Valley View, OH). No antiarrhythmic agents were given at any time.

Experimental Protocol

The study consisted of two successive *phases* (*A* and *B*) (Figs. 1 and 2). The objective of *phase A* was to investigate whether ischemic PC induces activation of cNOS on *day 1* and if so, to elucidate the signaling factors responsible for such activation. The objective of *phase B* was to investigate the mechanisms whereby brief ischemia on *day 1* upregulates NOS on *day 2*.

Phase A: effect of PC on NOS activity on day 1. Rabbits were assigned to eleven groups (Fig. 1). *Group I* (nonischemic control) did not receive any treatment and did not undergo coronary artery occlusion. To investigate the time-dependent effect of PC on NOS activity, rabbits in *groups II–VI* underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles [a protocol that induces late PC against both myocardial stunning (4, 6, 29, 40, 49) and infarction (30, 39,

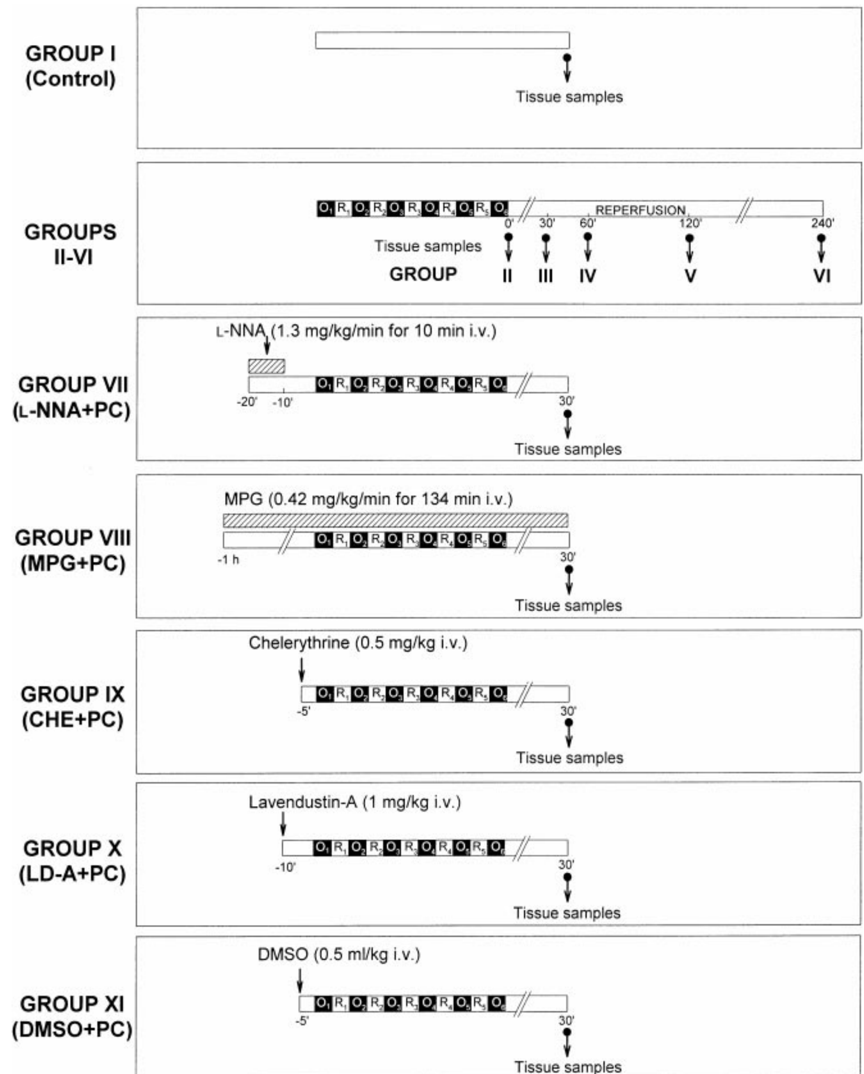

Fig. 1. Diagram of the experimental protocol for *phase A*. O, 4-min coronary occlusion; R, 4-min reperfusion; PC, preconditioning. L-NNA, N^{ω}-nitro-L-arginine; MPG, *N*-2-mercaptopropionyl glycine; CHE, chelerythrine; LD-A, lavendustin A.

40)] and were euthanized 0, 30, 60, 120, or 240 min after the last occlusion, respectively. The rabbits were given heparin (1,000 U iv), after which they were anesthetized with pentobarbital sodium (50 mg/kg iv) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (~0.5 g) were rapidly removed from the ischemic-reperfused region (whose boundaries had been marked with sutures at the time of instrumentation) and from the nonischemic region (posterior LV wall), put in liquid nitrogen, and stored at −70°C until used. To verify whether the dose of L-NNA that blocks late PC (4, 30) inhibits the activation of NOS associated with PC, rabbits in *group VII* (L-NNA + PC) received an intravenous infusion of L-NNA at a rate of 1.3 $mg \cdot kg^{-1} \cdot min^{-1}$ for 10 min, starting 20 min before and ending 10 min before the first occlusion (total dose 13 mg/kg, total volume infused 20 ml). To investigate the role of NO-derived reactive species in ischemia-induced NOS activation, *group VIII* (MPG + PC) received an intravenous infusion of MPG at 0.42 $mg \cdot kg^{-1} \cdot min^{-1}$ starting 60 min before and ending 30 min after the sequence of six 4-min coronary occlusion/4-min reperfusion cycles. To interrogate the role of PKC in ischemia-induced NOS activation, *group IX* (chelerythrine + PC) received a bolus iv of chelerythrine (5 mg/kg) 5 min before the first occlusion. To examine the role of PTKs in ischemia-induced NOS activation, *group X* [lavendustin A (LD-A) + PC] received a bolus iv of LD-A (1 mg/kg iv) 10 min before the first occlusion. L-NNA (Sigma Chemical, St. Louis, MO) was dissolved in normal saline. MPG (Sigma) was dissolved in sterile water, and the pH was adjusted to 7.5 using 0.1 mmol/l NaOH. Chelerythrine chloride (Research Biomedicals International, Natick, MA) was dissolved in 0.5 ml/kg of DMSO plus 0.5 ml/kg of normal saline. LD-A (CalBiochem, San Diego, CA) was dissolved under sterile conditions in 10% (vol/vol) DMSO in normal saline (total volume infused 1 ml/kg). These doses of L-NNA, MPG, chelerythrine, and LD-A have previously been shown to block late PC in conscious rabbits (4, 12, 29, 30, 40, 42). Because both chelerythrine and LD-A were dissolved in DMSO, an additional group of rabbits, *group XI* (DMSO + PC), was studied to examine any possible influence of DMSO itself on NOS activity. Five minutes before they were first occluded, these rabbits received a bolus iv of the vehicle used in *group IX* for chelerythrine (0.5 ml/kg of DMSO + 0.5 ml/kg of saline). The dose of DMSO used in *group IX* to dissolve chelerythrine (0.5 ml/kg) was higher than that used in *group X* to dissolve LD-A (0.1 ml/kg); consequently, *group XI* received the dose used in *group IX*. All rabbits in *groups VII–XI* were euthanized 30 min after the last occlusion. Myocardial samples were re-

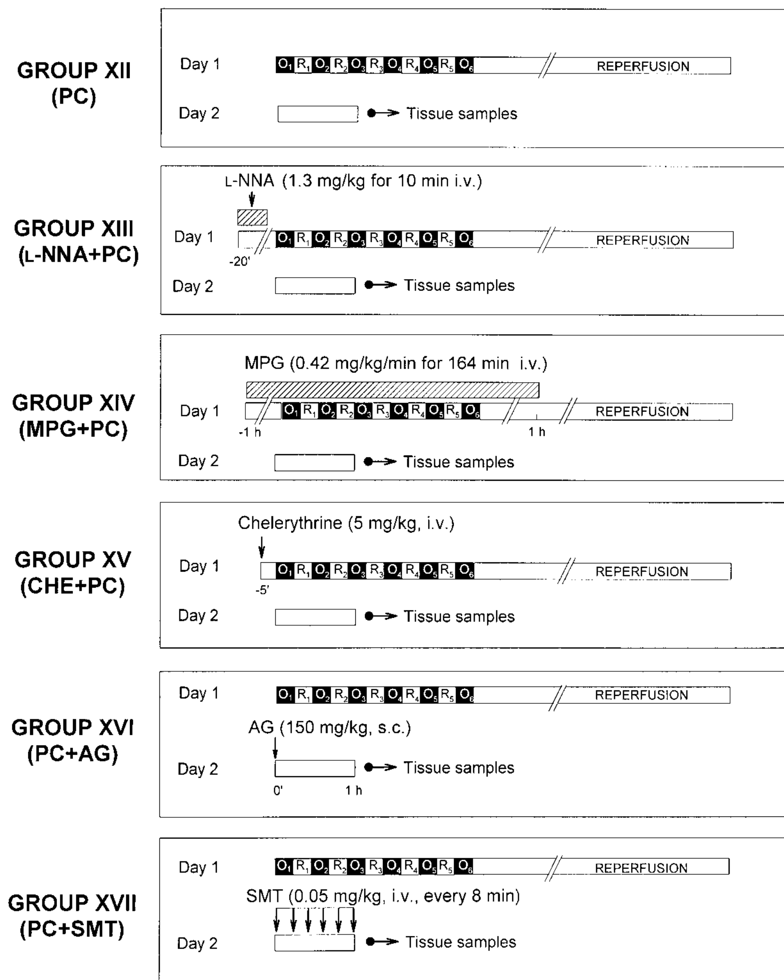

Fig. 2. Diagram of the experimental protocol for *phase B*. AG, aminoguanidine; SMT, *S*-methylisothiourea.

moved from the ischemic-reperfused region and the nonischemic region as described above and stored at −70° C until used.

Phase B: effect of PC on NOS activity on day 2. Rabbits were assigned to five groups (Fig. 2). All groups underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles and were euthanized 24 h later. *Group XII* (PC) did not receive any treatment. *Groups XIII–XV* were studied to investigate the signaling mechanisms on *day 1* that modulate the increase in iNOS activity on *day 2*. These rabbits received L-NNA (*group XIII*), MPG (*group XIV*), or chelerythrine (*group XV*) at the same doses used in *phase A* in *groups VII, VIII*, and *IX*, respectively. *Groups XVI* and *XVII* were studied to investigate the ability of putative iNOS inhibitors to block iNOS activity in this model. *Group XVI* was given AG 1 h before euthanasia (150 mg/kg sc), whereas *group XVII* received SMT (6 consecutive boluses iv of 0.05 mg/kg every 8 min ending 4 min before euthanasia, corresponding to a total dose of 0.3 mg/kg). These doses of AG and SMT have previously been shown to abrogate late PC against both myocardial stunning and myocardial infarction when given on *day 2* (6, 39). [The dose of SMT shown to abrogate late PC against stunning after 6 4-min occlusion/4-min reperfusion cycles on *day 2* was 10 0.05 mg/kg boluses (see Ref. 6); the rationale for the SMT protocol in *group XVII* was to assess the effect of SMT on NOS activity at a time corresponding to approximately the midpoint during the 6 occlusion/reperfusion cycles so as to obtain an estimate of the effect of the drug on NOS over the 44 min encompassed by the 6 cycles. Because in our previous study (see Ref. 6) only 6 boluses of SMT were given by the 3rd reperfusion (halfway through the sequence of 6 cycles), in the present study we examined the effect of 6 boluses of SMT on NOS activity.] Twenty-four hours after the six 4-min occlusion/4-min reperfusion cycles, the rabbits were given heparin (1,000 U iv), after which they were anesthetized with pentobarbital sodium (50 mg/kg iv) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples (~0.5 g) were rapidly removed from the ischemic-reperfused region (whose boundaries had been marked with sutures at the time of instrumentation) and from the nonischemic region (posterior LV wall), put in liquid nitrogen, and stored at −70°C until used.

Preparation of Cytosolic and Membranous Fractions

Cytosolic and membranous proteins were prepared as previously described (12, 17). Briefly, myocardial samples were homogenized in *buffer A* [25 mmol/l Tris·HCl (pH 7.5), 0.5 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 2 μmol/l leupeptin, 1 μmol/l pepstatin, 1 μmol/l aprotinin, 10 mmol/l NaF, and 100 μmol/l dephostatin]. The homogenates were centrifuged at 14,000 *g* for 15 min, and the resulting supernatants were collected as cytosolic fractions. The pellets were incubated in a lysis buffer (*buffer A* + 1% Nonidet P-40) for 4 h, centrifuged, and the resulting supernatants used as membranous fractions. The protein content

in the cytosolic and membranous fractions was determined using the Bradford technique (Bio-Rad).

Measurement of NOS Activity

NOS activity was determined by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline as previously described (12, 17). Briefly, isolated cytosolic or membranous proteins (80–120 μg) were incubated in assay buffer (total volume 80 μl) containing 50 mmol/l Tris·HCl (pH 7.4), 1 mmol/l NADPH, 5 μmol/l FAD, 5 μmol/l flavin mononucleotide (FMN), 10 μmol/l tetrahydrobiopterin, 10 μmol/l L-arginine, and purified L-[^{14}C]arginine [~220,000 counts/min (~0.1 μCi) per tube; New England Nuclear-DuPont] at 30°C for 60 min. To determine Ca^{2+}-dependent NOS (cNOS) activity, 2 mmol/l $CaCl_2$, and 100 nmol/l calmodulin were included in the assay. To determine Ca^{2+}-independent NOS (iNOS) activity, the assay was conducted in the presence of 1 mmol/l EGTA without Ca^{2+} and calmodulin. In both cases, duplicate assays were performed in the presence or absence of 1 mmol/l N^{ω}-nitro-L-arginine methyl ester, and the differences in counts per minute were used to calculate NOS activity. The reaction was quenched by adding 1 ml of ice-cold stop buffer [20 mmol/l HEPES (pH 5.5), 2 mmol/l EDTA, and 2 mmol/l EGTA], and the reaction mixture passed over a 1-ml column containing Dowex AG 50WX-8 (Na^+ form) resin (preequilibrated in stop buffer), washed with 1 ml of water, and collected into a 20-ml liquid scintillation vial. NOS activity was expressed as picomoles of citrulline per minute per milligram protein.

Measurement of iNOS-Dependent Activation of Cytosolic Guanylate Cyclase

iNOS-dependent formation of cGMP through the activation of cytosolic guanylate cyclase was determined as an additional indicator of iNOS activation using previously described methods (21). To rule out the involvement of particulate guanylate cyclase, only the cytosolic fractions were used. The cytosolic fractions (~200 μg of protein) were incubated with 50 mmol/l Tris·HCl (pH 7.6), 1 mmol/l NADPH, 5 μmol/l FAD, 5 μmol/l FMN, 10 μmol/l tetrahydrobiopterin, 10 μmol/l L-arginine, 1 mmol/l EGTA without Ca^{2+} and calmodulin (to prevent cNOS activation), 4 mmol/l $MgCl_2$, 1 mmol/l GTP, 15 mmol/l creatine phosphate, and 5 U creatine kinase at 30°C for 30 min. The reaction was terminated by adding trichloroacetic acid at a final concentration of 6%. The samples were centrifuged, and the supernatants were extracted three times with water-saturated ether. The extracted samples were lyophilized and subjected to radioimmunoassay for cGMP content. Because, under these assay conditions, only iNOS can be activated to generate NO, which in turn activates cytosolic guanylate cyclase, the formation of cGMP observed in this assay reflects the activity of iNOS.

Measurement of NO_x

Tissue samples were homogenized in a buffer containing 25 mmol/l Tris·HCl (pH 7.5), 0.5 mmol/l EDTA, and 0.5 mmol/l EGTA and centrifuged at 14,000 *g* for 15 min, and the resulting supernatants were collected as cytosolic fractions. The supernatants were loaded to a Centricon-30 filtrator and centrifuged to remove substances larger than 30 kDa. Nitrite was assayed by using the Griess reaction as modified by Gilliam et al. (15). Nitrate content was determined after conversion of nitrate to nitrite with *Aspergillus* nitrate reductase (EC1.6.6.2) (15). All assays were performed in duplicate. Tissue NO_x levels were expressed as nanomoles per milligram of protein.

Statistical Analysis

Data are reported as means ± SE. Differences among groups with respect to iNOS and cNOS activity, cGMP content, and NO_x levels were analyzed using a one-way or two-way ANOVA, as appropriate. If the ANOVA showed an overall difference, post hoc contrasts were performed with Student's *t*-tests for either paired or unpaired data, and the resulting *P* values were adjusted with the Bonferroni correction.

RESULTS

A total of 87 conscious rabbits were used in this study. All animals completed the experimental protocol and were included in the final analysis.

Phase A: Effect of PC on NOS Activity on Day 1

The purpose of these studies was to determine whether the sequence of six 4-min coronary occlusion/4-min reperfusion cycles activates NOS and, if so, to elucidate the

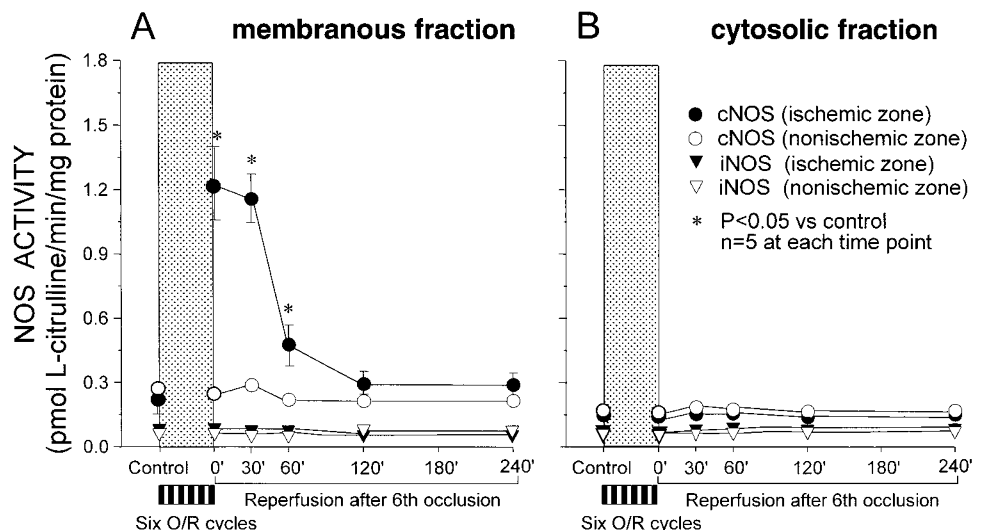

Fig. 3. Time course of calcium-dependent nitric oxide synthase (NOS) [cNOS; endothelial NOS (eNOS) and/or neuronal NOS (nNOS)] and calcium-independent NOS [inducible NOS (iNOS)] activity in myocardial samples obtained from the ischemic-reperfused or nonischemic regions of conscious rabbits that were preconditioned with a sequence of six 4-min coronary occlusion (O)/4-min reperfusion (R) cycles and were euthanized at serial time points thereafter [0, 30, 60, 120, and 240 min (*groups II*, *III*, *IV*, *V*, and *VI*, respectively)]. Control rabbits did not undergo coronary occlusion/reperfusion (*group I*). *A*: cNOS and iNOS activity in the membranous fraction of the homogenate; *B*: cNOS and iNOS activity in the cytosolic fraction. Data are means ± SE.

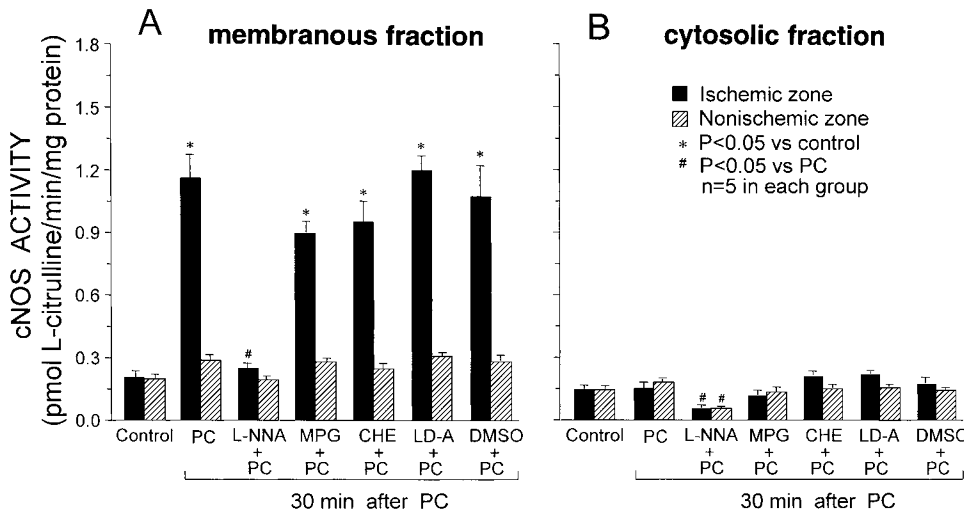

Fig. 4. cNOS (eNOS and/or nNOS) activity in myocardial samples obtained from the ischemic-reperfused or nonischemic regions of the heart of rabbits that were preconditioned with a sequence of six 4-min coronary occlusion/4-min reperfusion cycles without any treatment [PC (*group II*)], after pretreatment with L-NNA [L-NNA + PC (*group VII*)], MPG [MPG + PC (*group VIII*)], CHE [CHE + PC (*group IX*)], LD-A [LD-A + PC (*group X*)], or vehicle [DMSO + PC (*group XI*)]. Control rabbits (*group I*) did not receive any treatment, nor did they undergo occlusion/reperfusion cycles. *A*: cNOS activity in the membranous fraction; *B*: cNOS activity in the cytosolic fraction. Data are means ± SE.

mechanisms that regulate this activation. As illustrated in Fig. 3*A*, the sequence of six 4-min coronary occlusion/4-min reperfusion cycles resulted in a time-dependent increase in cNOS activity in the membranous fraction of the ischemic-reperfused region. cNOS activity increased 4.8-fold at the end of the sixth occlusion (time = 0 min, *group II*; $P < 0.05$ vs. control), remained elevated (4.4-fold increase) at 30 min (*group III*; $P < 0.05$), declined but was still significantly ($P < 0.05$) elevated (1.2-fold increase) at 60 min (*group IV*), and returned to values not significantly different from control values by 120 min (*group V*). As expected, no increase in cNOS activity was observed in the cytosolic fraction (Fig. 3*B*). cNOS activity in the nonischemic region (posterior LV wall) did not change appreciably in either the membranous (Fig. 3*A*) or the cytosolic (Fig. 3*B*) fraction. In contrast to cNOS, iNOS activity was unaffected by the ischemic PC protocol (Fig. 3).

On the basis of these results, we selected the 30-min time point to assess the effects of pharmacologic manipulations on NOS. As illustrated in Fig. 4*A*, in rabbits treated with L-NNA before the first coronary occlusion (L-NNA + PC, *group VII*), cNOS activity in the membranous fraction was similar to that measured in control rabbits (*group I*), confirming the effectiveness of this dose of L-NNA (which in previous studies has been shown to inhibit the development of late PC; see Refs. 4 and 30) in blocking the activation of cNOS elicited by ischemic PC. In contrast to L-NNA, infusion of MPG (MPG + PC, *group VIII*) or administration of chelerythrine (chelerythrine + PC, *group IX*) before the first coronary occlusion had no significant effect on cNOS activity (Fig. 4*A*), indicating that the activation of cNOS induced by ischemia is not dependent on MPG-sensitive oxidant species (such as $ONOO^-$ and/or ·OH) or on PKC. Similarly, administration of LD-A before the first coronary occlusion (LD-A + PC, *group X*) did not affect ischemia-induced cNOS activation (Fig. 4*A*), suggesting that this phenomenon is independent of PTKs. Administration of the vehicle used for chelerythrine and LD-A, DMSO (DMSO + PC, *group XI*), was without effect (Fig. 4*A*). Consistent with the results obtained in untreated rabbits (*groups II–VI*; Fig. 3), in the treated groups (*groups VII–XI*) there was no discernible increase in cNOS activity in the cytosolic fraction (Fig. 4*B*). None of the interventions tested in *phase A* affected iNOS activity 30 min after the ischemic PC protocol (Table 1).

Table 1. *iNOS activity 30 min after the six 4-min coronary occlusion/4-min reperfusion cycles on day 1*

Group	iNOS Activity, pmol L-citrulline · min^{-1} · mg $protein^{-1}$	
	Membranous fraction	Cytosolic fraction
Control		
IZ	0.063 ± 0.008	0.065 ± 0.003
NIZ	0.062 ± 0.006	0.065 ± 0.006
PC		
IZ	0.068 ± 0.010	0.084 ± 0.007
NIZ	0.073 ± 0.006	0.064 ± 0.003
L-NNA + PC		
IZ	0.059 ± 0.007	0.091 ± 0.007
NIZ	0.050 ± 0.002	0.077 ± 0.007
MPG + PC		
IZ	0.045 ± 0.004	0.089 ± 0.015
NIZ	0.041 ± 0.004	0.102 ± 0.009
CHE + PC		
IZ	0.046 ± 0.003	0.085 ± 0.012
NIZ	0.045 ± 0.010	0.059 ± 0.006
LDA + PC		
IZ	0.044 ± 0.002	0.082 ± 0.011
NIZ	0.044 ± 0.004	0.064 ± 0.005
DMSO + PC		
IZ	0.054 ± 0.010	0.072 ± 0.007
NIZ	0.044 ± 0.003	0.063 ± 0.006

Data are means ± SE; $n = 5$ rabbits in each group. Calcium-independent nitric oxide synthase (NOS) [inducible NOS (iNOS)] activity in the membranous and cytosolic fractions of myocardial samples obtained from the ischemic-reperfused (IZ) or nonischemic (NIZ) regions of the heart of rabbits preconditioned (PC) with a sequence of six 4-min coronary occlusion/4-min reperfusion cycles without any treatment [PC (*group II*)], or after pretreatment with N^{ω}-nitro-L-arginine (L-NNA) [L-NNA + PC (*group VII*)], *N*-2-mercaptopropionyl (MPG) [MPG + PC (*group VIII*)], chelerythrine (CHE) [CHE + PC (*group IX*)], lavendustin A (LD-A) [LD-A + PC (*group X*)], or vehicle [DMSO + PC (*group XI*)]. Control rabbits (*group I*) did not receive any treatment, nor did they undergo occlusion/reperfusion cycles.

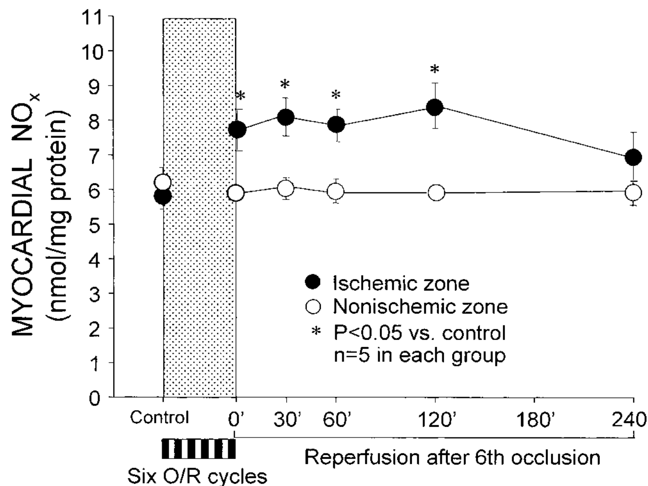

Fig. 5. Time course of changes in total myocardial content of NO_2 and/or NO_3 (NO_x) in samples obtained from the ischemic-reperfused or nonischemic regions of conscious rabbits that were preconditioned with a sequence of six 4-min coronary occlusion/4-min reperfusion cycles and were euthanized at serial time-points thereafter [0, 30, 60, 120, and 240 min (*groups II*, *III*, *IV*, *V*, and *VI*, respectively)]. Control rabbits did not undergo coronary occlusion/reperfusion cycles (*group I*). Nitrite was assayed by using the Griess reaction and nitrate was determined after conversion of nitrate to nitrite with *Aspergillus* nitrate reductase, as described in METHODS. Data are means ± SE.

Similar to the results obtained with measurements of cNOS activity, the sequence of six 4-min occlusion/4-min reperfusion cycles induced a significant ($P < 0.05$) increase in the myocardial levels of NO_x (the stable oxidation products of NO) at the end of the sixth occlusion (time = 0 min) (Fig. 5). Myocardial NO_x levels remained elevated until 120 min after the sixth occlusion and returned to baseline values by 240 min (Fig. 5). There was no change in NO_x levels in the nonischemic region at any time point (Fig. 5).

Phase B: Effect of PC on NOS Activity on Day 2

As shown in Fig. 6, the sequence of six 4-min coronary occlusion/4-min reperfusion cycles resulted in augmented iNOS activity 24 h later (*group XII*). Both membranous (Fig. 6*A*) and cytosolic (Fig. 6*B*) iNOS activity in the ischemic-reperfused region increased significantly ($P < 0.05$) above the levels measured in the anterior LV wall of control rabbits (*group I*). The increase in iNOS activity was more pronounced in the cytosolic fraction (+194%) than in the membranous fraction (+62%), consistent with the notion that iNOS is expressed predominantly in the cytosol (1, 16, 24). No change in iNOS activity was detected in the nonischemic region (Fig. 6). In contrast to iNOS activity, cNOS (eNOS and/or nNOS) activity in the ischemic-reperfused region did not change appreciably 24 h after ischemic PC, in either the membranous (Fig. 7*A*) or cytosolic (Fig. 7*B*) fraction.

When rabbits were given L-NNA before the ischemic PC protocol on *day 1* (L-NNA + PC, *group XIII*), the increase in iNOS activity on *day 2* was inhibited (Fig. 6), indicating that increased NO generation on *day 1* is necessary for the upregulation of iNOS on *day 2*. Similarly, when rabbits were given MPG during the six 4-min occlusion/4-min reperfusion cycles on *day 1* (MPG + PC, *group XIV*), the rise in iNOS activity on *day 2* was markedly blunted (Fig. 6), implicating reactive species as triggers of the upregulation of iNOS. The increase in iNOS activity on *day 2* was also suppressed by pretreatment of rabbits with chelerythrine (chelerythrine + PC, *group XV*) before the ischemic protocol on *day 1* (Fig. 6). None of these pharmacological manipulations had any appreciable effect on cNOS activity on *day 2* (Fig. 7).

In rabbits given the iNOS inhibitors AG (PC + AG, *group XVI*) or SMT (PC + SMT, *group XVII*) 24 h after the ischemic PC protocol, the activity of iNOS was markedly reduced compared with preconditioned untreated rabbits (*group XII*; Fig. 6), confirming the effectiveness of these doses of AG and SMT (which have been previously shown to abrogate late PC; see Refs. 6 and 39) in inhibiting iNOS activation during the late phase of PC. In all groups studied in *phase B* (*groups XII–XVII*), the changes in iNOS

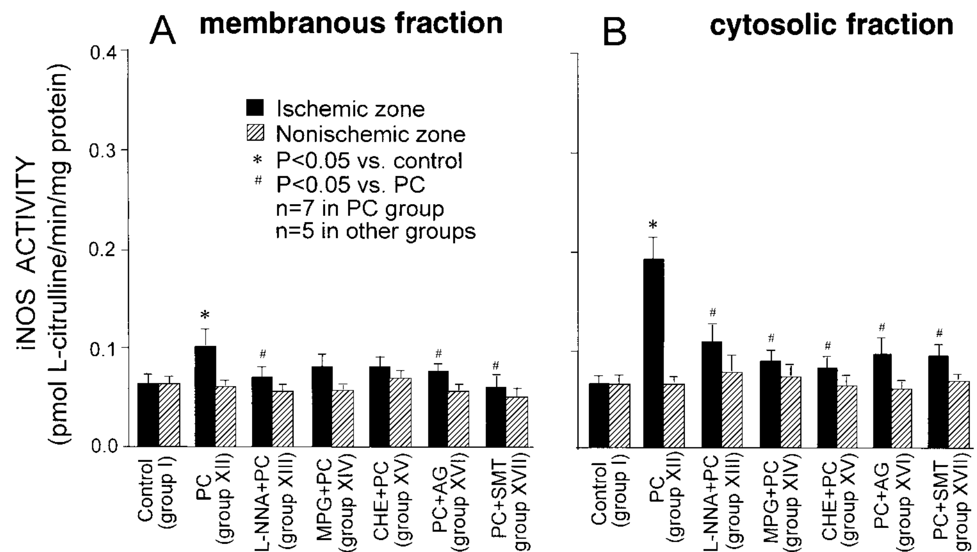

Fig. 6. Calcium-independent NOS (iNOS) activity in myocardial samples obtained from the ischemic-reperfused or nonischemic regions of the heart of rabbits preconditioned 24 h earlier (*day 1*) with six 4-min coronary occlusion/4-min reperfusion cycles (*groups XII–XVII*). Before the PC ischemia (*day 1*), rabbits received no treatment [PC (*group XII*)], L-NNA [L-NNA + PC (*group XIII*)], MPG [MPG + PC (*group XIV*)], or CHE [CHE + PC (*group XV*)]. In *groups XVI* and *XVII*, no treatment was given on *day 1*. On *day 2* (24 h after the PC ischemia), rabbits received either AG [PC + AG (*group XVI*)] or SMT [PC + SMT (*group XVII*)]. Control rabbits did not receive any treatment and did not undergo coronary occlusion/reperfusion cycles. *A*: iNOS activity in the membranous fraction; *B*: iNOS activity in the cytosolic fraction. Data are means ± SE.

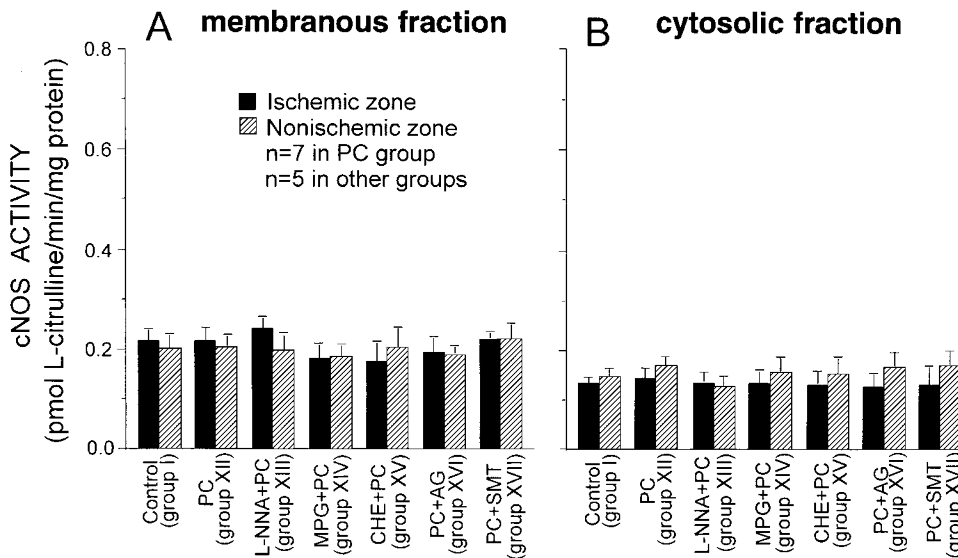

Fig. 7. cNOS (eNOS and/or nNOS) activity in myocardial samples obtained from the ischemic-reperfused or nonischemic regions of the heart of rabbits preconditioned 24 h earlier (*day 1*) with six 4-min coronary occlusion/4-min reperfusion cycles (*groups XII–XVII*). Before the PC ischemia (*day 1*), rabbits received no treatment [PC (*group XII*)], L-NNA [L-NNA + PC (*group XIII*)], MPG [MPG + PC (*group XIV*)], or CHE [CHE + PC (*group XV*)]. In *groups XVI* and *XVII*, no treatment was given on *day 1*. On *day 2* (24 h after the PC ischemia), rabbits received either AG [PC + AG (*group XVI*)] or SMT [PC + SMT (*group XVII*)]. Control rabbits did not receive any treatment and did not undergo coronary occlusion/reperfusion cycles. *A*: cNOS activity in the membranous fraction; *B*: cNOS activity in the cytosolic fraction. Data are means ± SE.

activity were paralleled by directionally similar changes in myocardial NO_x levels (Fig. 8) and in cGMP formation (Fig. 9).

DISCUSSION

The salient findings of this study can be summarized as follows: *1*) in conscious rabbits, brief, reversible episodes of myocardial ischemia induce rapid activation of cNOS, which lasts for 30–60 min and then abates, whereas iNOS activity is unaffected; *2*) this initial burst of ischemia-induced cNOS activity is not affected by pretreatment with MPG, chelerythrine, or LD-A, indicating that it is independent of the genera-

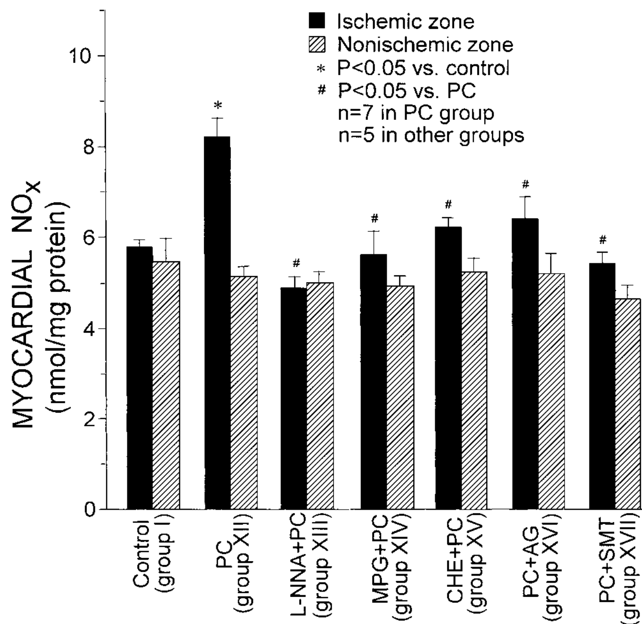

Fig. 8. Total myocardial content of NO_x in samples obtained from the ischemic-reperfused or nonischemic regions of the heart of rabbits preconditioned 24 h earlier (*day 1*) with six 4-min coronary occlusion/4-min reperfusion cycles (*groups XII–XVII*). Before the PC ischemia (*day 1*), rabbits received no treatment [PC (*group XII*)], L-NNA [L-NNA + PC (*group XIII*)], MPG [MPG + PC (*group XIV*)], or CHE [CHE + PC (*group XV*)]. In *groups XVI* and *XVII*, no treatment was given on *day 1*. On *day 2* (24 h after the PC ischemia), rabbits received either AG [PC + AG (*group XVI*)] or SMT [PC + SMT (*group XVII*)]. Control rabbits did not receive any treatment and did not undergo coronary occlusion/reperfusion cycles. Data are means ± SE.

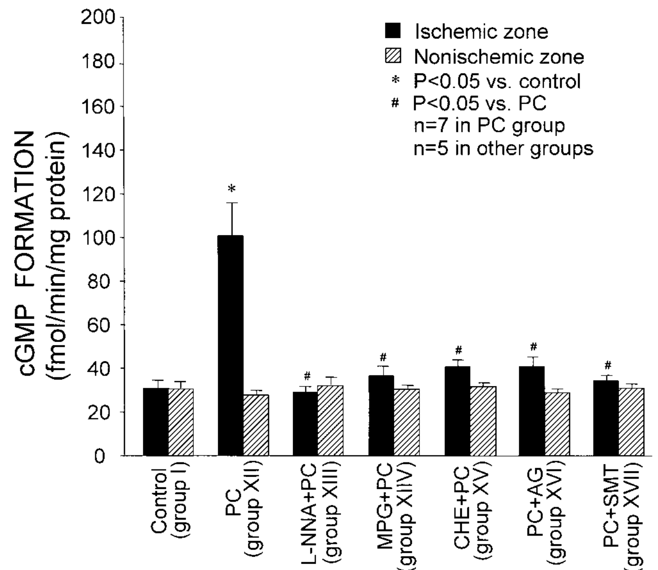

Fig. 9. iNOS-dependent formation of cGMP in vitro in cytosolic fractions isolated from myocardial samples obtained from the ischemic-reperfused or nonischemic regions of the heart of conscious rabbits preconditioned 24 h earlier (*day 1*) with six 4-min coronary occlusion/4-min reperfusion cycles (*groups XII–XVII*). Before the PC ischemia (*day 1*), rabbits received no treatment [PC (*group XII*)], L-NNA [L-NNA + PC (*group XIII*)], MPG [MPG + PC (*group XIV*)], or CHE [CHE + PC (*group XV*)]. In *groups XVI* and *XVII*, no treatment was given on *day 1*. On *day 2* (24 h after the PC ischemia), rabbits received either AG [PC + AG (*group XVI*)] or SMT [PC + SMT (*group XVII*)]. Control rabbits did not receive any treatment and did not undergo coronary occlusion/reperfusion cycles. iNOS-dependent formation of cGMP was assessed by activating cytosolic guanylate cyclase through generation of NO in the absence of calcium and calmodulin, as described in METHODS. Data are means ± SE.

tion of oxidant species and of the activation of PKC and PTKs; *3*) 24 h after the ischemic stress, the opposite pattern is observed, i.e., the heart exhibits a pronounced increase in iNOS activity with no change in cNOS activity; *4*) this delayed upregulation of iNOS is prevented by pretreatment 24 h earlier with L-NNA, MPG, or chelerythrine, indicating that it is triggered by a mechanism that involves generation of NO, oxidant species, and activation of PKC; and *5*) administration of doses of AG and SMT previously shown to abrogate late PC (6, 39) suppresses the delayed increases in iNOS activity 24 h after ischemic PC but does not affect cNOS activity, demonstrating the effectiveness and selectivity of these doses in targeting iNOS in vivo in rabbits. Taken together, the results of this investigation demonstrate that, in conscious animals, ischemic PC elicits a biphasic response in cardiac NOS activity, with an immediate activation of cNOS (eNOS and/or nNOS) followed by a delayed upregulation of iNOS. This differential regulation of cNOS versus iNOS provides a framework for identifying the specific function of individual NOS isoforms in the pathogenesis of the late phase of ischemic PC. In conjunction with the previous finding that late PC is abrogated by NOS inhibitors given on either *day 1* (4, 30) or *day 2* (6, 39), the present results indicate that cNOS (most likely the eNOS isoform) serves as the trigger, whereas iNOS serves as the mediator, of this cardioprotective phenomenon.

Prior studies (13, 25, 45, 50) have reported activation of NOS during myocardial ischemia in isolated, buffer-perfused hearts in vitro. To our knowledge, this is the first study to directly measure the effect of brief myocardial ischemia on NOS activity in vivo, to define the time-course of cNOS activation immediately after the ischemic PC challenge, and to examine the role of ROS, PKC, and PTKs in modulating this process. This is also the first indication that NO, ROS, and PKC are involved in the delayed activation of iNOS elicited by a sublethal ischemic stress. Our findings support the novel idea that the recruitment of one NOS isoform (eNOS) results in delayed activation of another isoform (iNOS) (eNOS-induced upregulation of iNOS).

Effect of Ischemic PC on NOS Activity on Day 1

We have previously shown that administration of the nonselective NOS inhibitor L-NNA on *day 1* before the PC ischemia abrogates the development of late PC against both myocardial stunning (4) and infarction (30), suggesting that generation of NO plays a critical role in triggering the development of this delayed cardioprotective phenomenon. However, our understanding of the role of NOS in triggering late PC is limited by a number of issues. First, there is no direct evidence that cardiac NOS is activated by sublethal ischemia in vivo. Such activation has been inferred from the functional consequences of infusing L-NNA but has never been actually demonstrated. Similarly, the ability of L-NNA to prevent ischemia-induced activation of NOS in intact animals has never been shown. Second, it is unknown whether the activation of NOS during the ischemic PC stimulus involves cNOS or iNOS, because L-NNA blocks both with similar potency (37). The need for examining the potential role of iNOS on *day 1* is underscored by the finding that control rabbits exhibited consistent, albeit modest, iNOS activity in the absence of ischemic PC or any other stress (Fig. 3), which is congruent with our previous finding (17) that both iNOS protein expression and iNOS activity are detectable in apparently unstressed mouse hearts. The presence of this "constitutive" iNOS activity in the rabbit heart raises the possibility that iNOS may also contribute to triggering late PC.

The present results address these issues by providing direct evidence that a brief ischemic stress causes rapid activation of cNOS in the heart (Fig. 3) and that the activation of cNOS is completely blocked (Fig. 4) by the same dose of L-NNA (13 mg/kg) previously shown to block the development of late PC (4, 30). These results strongly corroborate the hypothesis that cNOS acts to trigger the development of late PC after an ischemic stress. Our results also argue against a role of iNOS in triggering late PC, because iNOS activity did not exhibit any detectable change during ischemic PC (Fig. 3) and L-NNA (which blocks late PC; see Refs. 4 and 30) had no effect on iNOS activity (Table 1). The finding in *group VII* that L-NNA completely blocked the activation of cNOS (Fig. 4) but had no effect on iNOS (Table 1) is important, because it verifies that the dose of L-NNA (13 mg/kg), which has previously been found to prevent late PC in this model (4, 30), is indeed effective in blocking cNOS without interfering with iNOS activity. Although our measurements of cNOS activity do not distinguish nNOS from eNOS, it is likely that the observed changes are caused primarily by eNOS. nNOS is expressed at very low levels in the heart (8). Accordingly, the activity of this isozyme would have to increase several orders of magnitude to account for the fourfold increase in cNOS activity induced by ischemic PC (Fig. 3).

L-NNA completely blocked the increase in cNOS activity associated with ischemic PC but had little effect on cNOS activity in the nonischemic zone (Fig. 4). This biochemical result is congruent with a previous functional study (4) in rabbits documenting that this same dose of L-NNA (13 mg/kg) blunts acetylcholine-induced hypotension without raising arterial blood pressure. In conjunction with these previous data, the present results suggest that the dose of L-NNA we selected is sufficient to prevent increased cNOS activity during ischemia-reperfusion without decreasing endothelial NO release.

The mechanism responsible for the rapid activation of cNOS in response to brief myocardial ischemia is unknown. Increased synthesis of NO by this enzyme could be caused by the rise in cytosolic calcium levels and augmented availability of NADPH associated with myocardial ischemia. The increased shear stress during reactive hyperemia and/or the release of bradykinin with activation of endothelial B_2 receptors could also contribute to cNOS activation (5). Because previ-

ous studies have implicated PKC (26, 28, 29) and PTKs (12, 27) in the development of late PC, in the present investigation we sought to elucidate the role of these kinases in the activation of cNOS. We found that neither chelerythrine nor LD-A, given at doses that block both the development of late PC (12, 26, 29) and the activation of PKC (26, 29) and PTKs (27) in this conscious rabbit model, had any significant effect on cNOS activity (Fig. 4). Thus cNOS must be upstream of both PKC and PTKs in the signaling cascade that underlies late PC. Because ROS are known to trigger late PC (38, 41, 42), we examined whether they contribute to the increased cNOS activity. Our finding that the antioxidant MPG had no significant effect on cNOS activation (Fig. 4) implies that this event is not caused by oxidant stress. On the basis of these data, we propose that brief ischemia generates both NO (via cNOS) and ROS, which then interact to form $ONOO^-$ and/or other reactive species that engage the signaling cascade of late PC, including PKC and PTKs (5).

The present results significantly expand our understanding of ischemia-induced modulation of NOS in the heart. No previous study has measured NOS activity during myocardial ischemia-reperfusion in vivo. Depre et al. (13) reported in isolated, buffer-perfused rabbit hearts that NOS activity increased after 5 min of ischemia and remained elevated throughout a 60-min low-flow ischemic period. Surprisingly, in that study, the increase in NOS activity was found only in the cytosolic fraction with no change in the membranous fraction. Although the authors claimed that the increased NOS activity was accounted for by activation of eNOS, this conclusion is problematic, because most eNOS activity is membrane-bound (9, 33), as confirmed in the present study (Fig. 3). It is therefore difficult to conceptualize how activation of eNOS would not increase membranous NOS activity. Furthermore, in that study (13), Ca^{2+}-independent NOS activity was not measured, and the protocol used (60-min ischemia) was not a protocol of ischemic PC, so that it is arduous to compare those data with our present results. Node et al. (25) reported increased levels of NO_x in coronary venous blood during 10–90 min of coronary hypoperfusion in dogs, and studies by Zweier and colleagues (45, 50) and others (11) have shown increased formation of NO during myocardial ischemia and/or reperfusion in vitro. However, these studies (11, 25, 45, 50) did not examine which NOS isoform was involved and did not use a PC protocol. Because of the numerous fundamental differences between the buffer-perfused working isolated heart subjected to global ischemia in vitro and the blood-perfused working heart subjected to regional ischemia in vivo, a direct comparison of our data with these previous findings is not possible. In this regard, it is important to emphasize that most in vitro studies have concluded that NO is detrimental during myocardial ischemia, whereas the opposite has been found in vivo, underscoring the importance of measuring NOS activity in vivo.

Effect of Ischemic PC on NOS on Day 2

The results in *group XII* provide direct evidence that iNOS activity is upregulated 24 h after ischemic PC, whereas cNOS activity is unchanged (Figs. 6 and 7), consistent with previous reports in rabbits (12) and mice (17). The signaling mechanisms responsible for the upregulation of iNOS activity during late PC remain incompletely understood. In the present study, we examined the effect of pharmacological manipulations targeted at NOS, oxidant species, and PKC. To test the hypothesis that formation of NO- and O_2^-·-derived oxidants (e.g., $ONOO^-$ and/or ·OH) after ischemic PC is an important signaling mechanism leading to the induction of iNOS, we examined the effects of the cell-permeant antioxidant MPG. Because MPG reacts avidly with both $ONOO^-$ and ·OH by virtue of its thiol group (31), and because it abrogates the cardioprotective effects of late PC (38, 40, 42), we reasoned that this antioxidant would be useful to interrogate the role of $ONOO^-$ and ·OH in the upregulation of iNOS during late PC. We found that pretreatment with MPG on *day 1* effectively prevented the delayed increase in iNOS activity (Figs. 6 and 9) and NO_x levels (Fig. 8) elicited by ischemic PC on *day 2*, indicating that MPG-sensitive oxidants (such as $ONOO^-$ and/or ·OH) play an important role in ischemic PC-induced upregulation of iNOS.

Compelling evidence has implicated activation of PKC as an early signaling event leading to late PC (26, 28, 29). To investigate the role of PKC in iNOS upregulation after ischemic PC, we examined the effects of chelerythrine, given at a dose that has previously been shown to block both the activation of PKC (28, 29) and the cardioprotective effects of late PC (26, 29) in this model. The findings that pretreatment with chelerythrine before the sequence of six 4-min coronary occlusion/4-min reperfusion cycles on *day 1* prevented the increase in iNOS activity (Figs. 6 and 9) and NO_x levels (Fig. 8) 24 h later indicates that PKC participates in the development of late PC by modulating iNOS upregulation. Taken together, the results obtained with MPG and chelerythrine provide a mechanism (upregulation of iNOS) by which oxidative stress and PKC play a critical role in the late phase of ischemic PC.

Previous studies in conscious rabbits have shown that the iNOS inhibitors AG and SMT abrogate late PC against myocardial stunning (6) and myocardial infarction (39), suggesting that iNOS is the mediator of this protection on *day 2*. However, the selectivity of both AG and SMT for iNOS versus cNOS is only relative, with a ratio of IC_{50} of ~30:1 (37). Because the concentrations of AG and SMT attained in vivo are not known, the possibility that administration of these inhibitors to intact rabbits may result in inhibition of cNOS as well cannot be completely ruled out. Thus a role of iNOS (as opposed to eNOS) as a mediator of late PC in rabbits cannot be conclusively established without verifying the selectivity of the doses of AG and SMT used in prior investigations (6, 39). The present study demonstrates, for the first time, that the same doses of

AG and SMT that block late PC (6, 39) also block the delayed increase in iNOS activity and NO_x levels (Figs. 6 and 8) but have no effect on cNOS activity (Fig. 7), indicating that the functional consequences of AG and SMT administration (abrogation of cardioprotection) can indeed be ascribed to iNOS rather than eNOS activity. These results are important to interpret previous studies of pharmacological inhibition of NOS during late PC (6, 39). The selectivity of AG and SMT demonstrated herein is congruous with the finding that these agents fail to block the development of late PC when given on *day 1* (6) (at a time when cNOS but not iNOS is activated; Fig. 3).

Methodological Considerations

Besides measuring L-arginine to L-citrulline conversion, we sought to examine two additional parameters of NOS activity. First, we measured iNOS-dependent formation of cGMP (via activation of cytosolic guanylate cyclase). This assay was done in the absence of Ca^{2+} and calmodulin through the use of isolated cytosolic fractions to rule out the involvement of eNOS activity and of particulate guanylate cyclase. As described in METHODS, under the assay conditions employed, only Ca^{2+}-independent NOS (iNOS) can be activated to generate NO, which in turn activates cytosolic guanylate cyclase. The data obtained with this assay provide another indication of iNOS activity besides formation of L-citrulline. We also measured myocardial levels of NO_2 and/or NO_3 (NO_x), the stable end products of NO metabolism. The measurements of NO_x are conceptually different from those of L-citrulline or cGMP formation, because they reflect NO generation occurring in vivo rather in vitro. Accordingly, the finding that NO_x levels increased after ischemic PC (Figs. 5 and 8) confirms that increased NOS activity did occur in myocardial tissue in the intact animal before the harvesting of tissue samples. These data provide an important in vivo correlate of the in vitro analyses of NOS activity.

Conclusions

In summary, the present study provides significant new information regarding the cellular mechanisms responsible for the development of the late phase of ischemic PC in conscious animals. The results demonstrate, for the first time, that two different NOS isoforms are sequentially involved in the genesis of late PC: cNOS is recruited immediately after the initial ischemic stress, when the late PC response develops, whereas iNOS is mobilized 24 h later, when the protection becomes manifest. In conjunction with previous functional studies (4, 6, 12, 30, 39, 40), these data support a distinctive role of NOS isoforms in late PC, with eNOS serving as the trigger on *day 1* and iNOS as the mediator on *day 2*. The present data also imply that the activation of cNOS on *day 1* is in parallel to or upstream of ROS, PKC, and PTKs. Finally, the observations reported herein indicate that the delayed upregulation of iNOS on *day 2* is dependent upon the activation of cNOS on *day 1* and the subsequent formation of ROS and activation of PKC. On the basis of these results and of previous studies (2, 4, 12, 17, 19, 26–30, 32, 40, 42, 49), we propose a pathophysiological paradigm in which brief myocardial ischemia rapidly activates eNOS, leading to increased biosynthesis of NO that reacts with $O_2^{-}\cdot$ to generate oxidant species, which activate downstream signaling pathways including PKC and PTKs, culminating in increased activity of iNOS and development of cardioprotection 24 h later. The concept that brief activation of eNOS leads to delayed upregulation of iNOS in the heart (eNOS-triggered iNOS induction) is novel and has potentially wide implications for many other pathophysiological situations in which eNOS is mobilized.

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