Cardioprotection During the Final Stage of the Late Phase of Ischemic Preconditioning Is Mediated by Neuronal NO Synthase in Concert With Cyclooxygenase-2

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Abstract—The infarct-sparing effect of the late phase of ischemic preconditioning (late PC) lasts for 72 hours. Upregulation of both cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) has been shown to be essential to the protection in the initial stage of late PC (24 hours after PC); however, the mechanisms underlying the protection in the final stage of late PC (48 to 72 hours after PC) are unknown. Conscious rabbits were preconditioned with six cycles of 4-minute coronary occlusion/4-minute reperfusion. At 72 hours after PC, powerful protection against infarction was associated with increased myocardial levels of COX-2 mRNA, protein, and cardioprotective prostaglandins (PGI₂ and PGE₂). The COX-2–selective inhibitor NS-398 completely blocked the protection. Surprisingly, iNOS expression was not increased at 72 hours; instead, upregulation of neuronal NO synthase (nNOS) was evident at both the mRNA (+266±20%, P<0.005) and the protein levels (+195±66%, P<0.005), which was accompanied by an increase in myocardial nitrite/nitrate (+20±4%, P<0.05). The nNOS-selective inhibitors N-propyl-L-arginine or S-ethyl N-[4-(trifluoromethyl)phenyl]isothiourea completely blocked the protection of late PC at 72 hours, whereas the iNOS-selective inhibitor S-methylisothiourea had no effect. In line with these findings, the disappearance of protection at 120 hours after PC was associated with the return of nNOS mRNA, protein, and activity to control levels. Although expression of COX-2 protein was still elevated at 120 hours, only a marginal increase in PGI₂ and PGE₂ levels was detected. In contrast, 72 hours after PC, nNOS was not upregulated at 24 hour after PC. We conclude that (1) the cardioprotection observed in the final stage of late PC (72 hour) is mediated by nNOS, not by iNOS, in contrast with COX-2, and (2) nNOS-derived NO is required to drive COX-2 activity. These data identify, for the first time, a cardioprotective role of nNOS and demonstrate, surprisingly, that the mechanism of late PC differs at 72 hours (nNOS) versus 24 hours (iNOS). (Circ Res. 2004;95:000-000.)

Key Words: heart • gene expression • nitric oxide • prostaglandins • pharmacology

Ischemic preconditioning (PC) is an endogenous protective mechanism whereby episodes of sublethal ischemia render the myocardium more resistant to subsequent ischemic stress.1 The late phase of ischemic PC (late PC) is a cardioprotective state that becomes manifest at 12 to 24 hours after the PC stimulus and lasts until 72 hours.2,3 Because late PC persists for a substantial period of time and protects the heart against both myocardial infarction and myocardial stunning, it has potential clinical relevance.4,5 In the past decade, considerable research has focused on understanding the cellular and molecular mechanisms underlying late PC in the hope that this powerful cardioprotective phenomenon can be replicated for therapeutic purposes.4,5 From these studies, it is now appreciated that late PC is a complex process that involves a network of intricate regulatory mechanisms at the levels of cell signaling and gene expression. In particular, a central role has emerged for NO and the NO synthase (NOS) gene family in the development of late PC. For instance, the inducible NOS (iNOS) has been identified as an obligatory mediator of the cardioprotection afforded by late PC.5 This is based on the fact that iNOS is upregulated in cardiac myocytes after ischemic PC,4,5 and that pharmacological inhibition of iNOS activity or genetic inactivation of the iNOS gene completely abrogates the protective effects of late PC at 24 hours after the PC stimulus.9,10 NO derived from the endothelial NOS (eNOS), on the other hand, appears to be required during the initial ischemic challenge11,12 to activate the cellular signaling cascade that leads to late PC.6 Curiously, the potential involvement in late PC of the neuronal NOS (nNOS), the third member of the three-member gene family, has so far not been investigated, although this enzyme has emerged as a key regulator of critical cardiac functions such as heart rate,13,14 calcium cycling,15-17 sodium transport,16 and energy metabolism.19,20

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In addition to NOS, convincing evidence has shown that the cyclooxygenase-2 (COX-2) pathway is also essential in conferring the cardioprotection afforded by late PC. COX-2 is co-upregulated with iNOS after ischemic PC, resulting in increased synthesis of cardioprotective prostaglandins (PGs) such as PGI₂ and PGE₂. At 24 hours after ischemic PC, administration of COX-2–selective inhibitors abolishes the increase in PGI₂ and PGE₂ as well as the cardioprotection.21,22 More recently, it has become apparent that, at 24 hours after PC, iNOS and COX-2 do not act independently of one another but, rather, in concert. In particular, although the upregulation of COX-2 parallels that of iNOS, the enzymatic activity of newly expressed COX-2 protein requires the presence of iNOS-derived NO.23 Thus, COX-2 is located downstream of iNOS in the cascade of cardioprotective proteins induced by ischemic PC.

In spite of these remarkable advances in the field, a conspicuous gap of knowledge remains, in that so far virtually all mechanistic studies have focused on the cellular and molecular events occurring in the initial stage of late PC (24 hours after the stimulus), whereas the cardioprotective effects of late PC persist for 72 hours.2,3 To date, the mechanisms underlying the final stage of late PC (48 to 72 hours after the stimulus) remain completely unknown. The present investigation was designed to systematically address this issue by (1) measuring the expression of all three NOS isoforms (iNOS, eNOS, and nNOS), NOS activity, COX-2 expression, and COX-2–derived prostanoids at serial times after ischemic PC, (2) determining the effect of iNOS, nNOS, and COX-2 inhibition on the cardioprotective properties of late PC at 72 hours, and (3) elucidating the hierarchical relationship between nNOS and COX-2 at 72 hours. All studies were performed in a well-established conscious rabbit model of late PC so as to avoid the potentially confounding influences of anesthesia, trauma, and associated abnormal conditions.9,11,12,21,23 Surprisingly, we found that at 72 hours after PC, the cardioprotection is mediated by nNOS, not iNOS. These results reveal a heretofore unrecognized difference in the mechanism of late PC at 72 hours versus 24 hours and identify, for the first time, a cardioprotective role of nNOS.

Materials and Methods

The conscious rabbit model of late PC9,11,12,21,23 and expanded Materials and Methods are described in the online data supplement available at http://circres.ahajournals.org.

RNase Protection Assay

RNase protection assay (RPA) was used to assess the steady-state level of COX-2 mRNA in rabbit hearts. A 294-bp HincII/ScaI fragment of the rabbit COX-2 cDNA was used as template for an antisense riboprobe.24

RT-PCR/Southern Blot Analysis

Semi-quantitative RT-PCR was used to assess the steady-state level of nNOS mRNA in rabbit hearts. The following primers (derived from GenBank Accession No. U91584) were used in PCR amplifications of the nNOS common region (25 cycles): sense 5'-CTTGCTCAACCGAATACCT-3', antisense 5'-GTCGCCCGCAACTTTCGAG-3'. An internal primer (5'-AACAGCGGCAGTTTGACAT-3') was used as a probe for subsequent Southern blot analysis. Amplification of a rabbit GAPDH fragment was used as a quantitative control. In addition, the following primers were used to amplify the nNOS region harboring the exon-μ insertion: sense 5'-TGACACCTGGAACAGAACC-3', antisense 5'-GAACAGCGGAACCTCCTACAT-3'. Internal primers 5'-CACAGACCTTTGGCAATGGAG-3' and 5'-GCAAGGCTTCGCGGAGTCAG-3' were used as probes for common and nNOSμ-specific sequences, respectively.

Real-Time RT-PCR

nNOS mRNA levels were further assessed with real-time RT-PCR using the following primers and probe: sense 5'-TTAGCCCTC-AAAACCATCAGAG-3', antisense 5'-CTCTGACTCCCCTGGTGGTGT-3', probe 5'-CCACGGCTTCACATTTCTGCAG-3'. PCR products for nNOS were continuously measured by the sequence detector and normalized by the endogenous reference (GAPDH). Three replicates for each RNA sample were performed.

Cloning of the Rabbit nNOSμ

The RT-PCR fragment containing exon μ as described was gel-purified, subcloned into the pCRII vector (Invitrogen), and subjected to DNA sequencing analysis on both strands.

Western Blot Analysis

Total cellular protein samples were separated on polyacrylamide/SDS gels and transferred onto nitrocellulose membranes. The following primary antibodies were used for Western blot analyses: an anti–COX-2 monoclonal antibody (BD Biosciences), an anti–COX-1 polyclonal antibody (Santa Cruz Biotechnology), an anti-iNOS polyclonal antibody (Upstate Biotechnology), an anti–μ-NOS polyclonal antibody (Affinity Bioreagents), and an anti-nNOS monoclonal antibody (BD Biosciences).7,8

Measurement of Prostaglandins (PGs)

Myocardial 6-keto-PGF₁α and PGE₂ were measured using enzyme immunoassays as described previously.21

Measurement of Nitrite and Nitrate

Myocardial nitrite and nitrate were measured using a nitrate/nitrite colorimetric assay kit (Cayman Chemical) according to the manufacturer’s protocol.

Infarct Size

Twelve groups of rabbits were studied (online Figure 1, available in the online data supplement). Group I (control) underwent a 30-minute coronary occlusion without any pretreatment. Groups II (PC 24 hour) and III (PC 72 hour) were preconditioned 24 hours and 72 hours before the 30-minute occlusion, respectively, with six 4-minute occlusion/reperfusion cycles.9,11,12,21,23 Group IV (PC 72 hour + NS-398) was preconditioned 72 hours earlier and received the COX-2–specific inhibitor NS-398.21 Group V (PC 72 hour + L-NA) was preconditioned 72 hours earlier and received the nonselective NOS inhibitor N’-nitro-l-arginine (L-NA).9 Group VI (PC 24 hour + NPA) and VII (PC 72 hour + NPA) were preconditioned 24 and 72 hours earlier, respectively, and received the nNOS-selective inhibitor N-propyl-l-arginine (NPA). Group VIII (NPA) was given NPA before the 30-minute occlusion without PC to exclude the possible effect of NPA itself on infarct size. Group IX (PC 72 hour + SEPT) was preconditioned 72 hours earlier and received the nNOS-selective inhibitor S-ethyl N-[4-(trifluoromethyl)phenyl]isothiourea (SEPT). Group X (SEPT) was given SEPT without PC to exclude the possible effect of SEPT itself on infarct size. Groups XI (PC 24 hour + SMT) and XII (PC 72 hour + SMT) were preconditioned 24 and 72 hour earlier, respectively, and received the iNOS-selective inhibitor S-methylisothiourea sulfate (SMT).12

Statistical Analysis

Data are reported as mean±SEM. Results were analyzed by either a one-way or a two-way repeated-measures ANOVA, as appropriate, followed by unpaired Student’s t tests with the Bonferroni correction.
Results
A total of 131 rabbits were instrumented for this study. The reasons for exclusion are detailed in online Table 1 (in the online data supplement).

Upregulation of COX-2 Expression and Activity
COX-2 mRNA was expressed at low levels in the heart of control rabbits. After ischemic PC, the steady-state levels of COX-2 mRNA in the ischemic/reperfused region increased markedly at 1 hour (352 ± 85% of control), remained elevated at 3 hours, and then decreased but were still elevated at 24 hours (212 ± 25% of control) (Figure 1A and 1B). The COX-2 mRNA levels appeared to reach a second peak at 72 hours (317 ± 81% of control), which eventually returned to control levels at 120 hours after ischemic PC (Figure 1A and 1B). In accordance with these data, an increase in COX-2 protein was observed in the ischemic/reperfused region at 24 and 72 hours after PC (Figure 1C and 1D). Surprisingly, COX-2 protein levels remained elevated at 120 hours after PC, a time when the cardioprotective effects of ischemic PC are known to have disappeared.2,3 COX-1 protein was detected at low levels in control hearts and did not change in preconditioned hearts at any time points (data not shown).

The myocardial content of cardioprotective prostanooids (PGI2 and PGE2) was measured to assess COX-2 enzymatic activity. We found that both 6-keto-PGF1α (the stable metabolite of PGI2) and PGE2 levels were increased in the ischemic/reperfused region at 24 and 72 hours after PC (Figure 2). These changes are consistent with the upregulation of COX-2 protein and the time-course of cardioprotection afforded by ischemic PC. At 120 hours after PC, 6-keto-PGF1α and PGE2 levels declined and were only marginally higher than in control rabbits (295 ± 66% of control) but not at 120 hours after PC (Figure 4C and 4D). A trend toward upregulation in nNOS protein could already be observed at 24 hours, but it did not reach statistical significance (Figure 4D). Interestingly, the nNOS protein found in the heart was slightly larger than that in the brain (Figure 4C), in line with the size of the nNOSα variant.

Upregulation of nNOS Expression and Activity
As shown in our previous studies in mice,7,8 iNOS protein was present in control rabbit hearts at low but detectable levels and increased in the ischemic/reperfused region 24 hours after ischemic PC (195 ± 29% of control, Figure 3A and 3B). Surprisingly, however, iNOS was not upregulated at 72 hours, when the protection of late PC against infarction was still evident. In contrast, a significant upregulation of nNOS in the ischemic/reperfused region was evident at 72 hours after ischemic PC. The steady-state levels of nNOS mRNA did not change at 24 hours but increased significantly at 72 hours (366 ± 20% of control), as assessed by both RT-PCR/Southern blotting and real-time RT-PCR, and returned to control levels at 120 hours (Figure 4A and 4B). In accordance with these data, an increase in nNOS protein levels in the ischemic/reperfused region was observed at 72 hours (295 ± 66% of control) but not at 120 hours after PC (Figure 4C and 4D). A trend toward upregulation in nNOS protein could already be observed at 24 hours, but it did not reach statistical significance (Figure 4D). Interestingly, the nNOS protein found in the heart was slightly larger than that in the brain (Figure 4C), in line with the size of the nNOSα variant.
that was previously reported in rat hearts.\textsuperscript{24} As previously reported,\textsuperscript{8} eNOS protein was not significantly upregulated at any time-point after ischemic PC (Figure 3C and 3D).

The myocardial content of the stable NO metabolites nitrite and nitrate (NO\textsubscript{x}) was measured to determine NOS activity. At 24 hours after PC, NO\textsubscript{x} increased significantly in the ischemic/reperfused region (127\textpm{}6\% of control), but not in the nonischemic region (Figure 4E). This increase in NO\textsubscript{x} coincided with the increase in iNOS protein, and was most likely a reflection of iNOS enzymatic activity. NO\textsubscript{x} in the ischemic/reperfused region remained elevated at 72 hours after ischemic PC (120\textpm{}4\% of control) (Figure 4E). Based on the expression profile of NOS isoforms at this time-point, this increase in NO\textsubscript{x} was likely a consequence of nNOS enzymatic activity. At 120 hours after PC, NO\textsubscript{x} returned to control levels.

An unexpected finding was that unlike COX-2 and iNOS, whose upregulation was limited to the ischemic/reperfused region (Figures 1C, 1D, 3A, and 3B), nNOS protein levels increased also in the nonischemic region (Figure 4C and 4D). This increase, however, was not accompanied by an increase in NO\textsubscript{x} (Figure 4E).

**Preferential Induction of nNOS\textsubscript{\mu}**

Exon-specific Southern blotting was used to determine the nNOS transcript(s) that accounted for ischemic PC-induced upregulation of the nNOS gene. We found that both the nNOS\textsubscript{\alpha} and the nNOS\textsubscript{\mu} variants were expressed in the heart of control rabbits but at levels much lower than those in the brain and skeletal muscle (Figure 5A and 5B). At 72 hours after ischemic PC, both variants were upregulated; however, the increase in nNOS\textsubscript{\mu} levels was more prominent (7.0-fold versus 2.9-fold for nNOS\textsubscript{\alpha}; Figure 5C and 5D). Sequence analysis after cDNA cloning demonstrated that the rabbit nNOS\textsubscript{\mu} was highly homologous to the human nNOS\textsubscript{\mu},\textsuperscript{25} sharing 33/34 similarity at the amino acid level (Figure 5E). In comparison, the rat (identical to the mouse) nNOS\textsubscript{\mu}\textsuperscript{24} shares only 28/34 similarity with the human nNOS\textsubscript{\mu}. Noticeably, only the carboxy-end serine residue (Ser31), among the five that were originally identified in the rat/mouse nNOS\textsubscript{\mu} and thought to be potential phosphorylation sites, was conserved in all four species; in contrast, Ser20 in rat/mouse was replaced by a threonine in human/rabbit, implying potentially differential regulation in nNOS\textsubscript{\mu} phosphorylation among these species. In addition, the potential N-myristoylation site GLAAAR, which could affect subcellular localization of this protein, was conserved only between humans and rabbits. Based on the deduced amino acid sequences, the nNOS\textsubscript{\mu} protein is 3.8 kDa larger than nNOS\textsubscript{\alpha} in rabbits (164.5 versus 160.7 kDa), consistent with the Western blot results (Figure 4C).
Infarct Size

As expected, infarct size was significantly smaller in rabbits preconditioned 24 hours earlier than in control rabbits (33.5±3.0% versus 56.6±4.6% of the risk region; *P*<0.05) (Figure 6). A similar infarct-sparing effect was observed in rabbits preconditioned 72 hours earlier (35.4±2.1% of the risk region). Administration of the COX-2–selective inhibitor NS-398 blocked the infarct-sparing effect of late PC at 72 hours (52.8±3.3% of the risk region). This finding is consistent with our previous observation at 24 hours after PC and suggests that COX-2 is an essential factor in mediating the cardioprotective effect of late PC at both 24 and 72 hours. Administration of the nonselective NOS inhibitor L-NA completely blocked the infarct-sparing effect of late PC at 72 hours (55.5±3.6% of the risk region), indicating that the protection at this time-point was NOS-dependent. Isoform-selective inhibitors were then used to discern the role of NOS from that of iNOS. Administration of the nNOS-selective inhibitor NPA failed to block cardioprotection at 24 hours after ischemic PC (infarct size, 30.5±4.6% of the risk region). However, the same dose of NPA completely blocked the infarct-sparing effect of late PC at 72 hours (57.5±3.7% of the risk region) (Figure 6). Similar results were obtained with SEPT, a structurally different nNOS-selective inhibitor at both 1.0 and 0.5 mg/kg (Figure 6) (because the results obtained with the two doses were similar, the data are combined in Figure 6). The iNOS-selective inhibitor SMT had effects diametrically opposite to those of NPA. SMT completely blocked cardioprotection at 24 hours after ischemic PC (58.3±3.7% of the risk region), an effect similar to that of another iNOS inhibitor, aminoguanidine, but failed to block the infarct-sparing effect of late PC at 72 hours (37.4±2.7% of the risk region) (Figure 6).

**Discussion**

Although the cardioprotective effects of late PC last for 72 hours, almost all investigations have focused on the first 24 hours after the PC stimulus. At present, essentially nothing is known regarding the mechanism of the final stage of late PC (48 to 72 hours after PC). In most investigators’ minds, a tacit assumption has been made that late PC is a monolytic phenomenon, underlain by the same mechanism throughout its duration. This assumption, however, has never been tested and is shown to be incorrect by our present results.

The salient findings of this study can be summarized as follows: (1) at 72 hours after the PC stimulus, a powerful protection against myocardial infarction is still present, equivalent to that observed at 24 hours; (2) this state of cardioprotection is associated with upregulation of COX-2 and is abrogated by COX-2 inhibitors, indicating that COX-2 activity plays a necessary role; (3) the infarct-sparing effect observed at 72 hours is associated with increased NO bio-synthesis and is abolished by L-NA, indicating that it is dependent on a NOS isoform; (4) neither iNOS nor eNOS protein expression is upregulated at 72 hours, and pharmacological treatment with various pharmacological agents. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent mean±SEM.
logical inhibition of iNOS fails to block the infarct-sparing effect at this time-point (although it effectively does so at 24 hours), suggesting that the NOS isoform involved at 72 hours is neither eNOS nor iNOS; (5) in contrast, nNOS is upregulated at 72 hours, both at the mRNA and at the protein levels, and inhibition of nNOS completely abrogates the infarct-sparing effects of late PC, indicating an important role of this isoform; (6) in line with this concept, the disappearance of protection at 120 hours after PC is associated with a return of nNOS mRNA, protein, and activity to control levels; and (7) although the expression of COX-2 protein at 120 hours is similar to that seen at 72 hours, COX-2–derived prostanooid levels are much lower, indicating that the enzyme is only marginally active at this time-point, when NOS is no longer upregulated. Taken together, these findings indicate that upregulation of both COX-2 and nNOS is required for the protection against myocardial infarction to become manifest during the final stage of late PC (72 hours after the stimulus) and that, at this stage, the activity of newly-synthesized COX-2 protein is driven by NO derived from nNOS.

Previous studies have demonstrated an obligatory role of iNOS7,8 and COX-221 at 24 hours after PC. However, to our knowledge, this is the first evidence that COX-2 is required for the final stage of late PC, whereas iNOS is not. Furthermore, this is the first report that nNOS is involved in the phenomenon of late PC, and that the mechanism responsible for this adaptation differs at 72 versus 24 hours (ie, nNOS is the mediator at 72 hours, whereas iNOS is the mediator at 24 hours). The recognition that nNOS serves as an obligatory mediator of late PC at 72 hours reveals a new facet of the function of this poorly understood enzyme in the cardiovascular system.

**Role of COX-2 in Late PC**

It has been known for more than two decades that prostanooids such as PGE2 and PGI2, or their analogues, protect the myocardium against ischemia/reperfusion injury26–28 (see review8). Recent studies have provided cogent evidence that COX-2–derived prostanooids play an essential role in conferring cardioprotection in the initial stage of late PC (24 hours after ischemic PC) in both rabbits31 and mice.22 The present results corroborate these previous findings and expand them by demonstrating that COX-2 activity is also essential for the protection observed during the final stage of late PC (72 hours after the PC stimulus). Thus, COX-2 serves as an obligatory mediator of protection throughout the entire duration of late PC.

Besides being activated at the gene level, the COX-2 pathway is also regulated at the level of enzymatic activity. Previous studies have shown that COX-2 is activated by NO,29,30 although it is still debatable whether the effector is NO itself or an NO-derived species, such as peroxynitrite.30 We have recently shown that NO derived from iNOS drives prostanooid synthesis by COX-2 in a cGMP-independent manner in rabbit hearts 24 hours after ischemic PC.23 Our present findings show that at 72 hours, when myocardial levels of NOx were increased, PGI2 and PGE2 levels were elevated and cardioprotection was present. In contrast, at 120 hours, when myocardial levels of NOx were no longer increased, PGI2 and PGE2 levels were only marginally elevated (much less than at 72 hours) despite the fact that the upregulation of COX-2 protein expression was almost the same, and cardioprotection was absent. These observations support the concept that enhanced biosynthesis of NO is required to drive production of cytoprotective prostanooids by COX-2 and to effect protection at 72 hours, in analogy with our previous data obtained at 24 hours.21,23 The major difference appears to be the source of the NO that stimulates COX-2 activity, ie, nNOS at 72 hours versus iNOS at 24 hours.

**Role of nNOS in Late PC**

The nNOS inhibitor used in this study, NPA, is reported to be 3158- and 149-times more selective for nNOS than for iNOS and eNOS, respectively.31 We further confirmed the specificity of this inhibitor at the dose used herein by showing that NPA had no effect on iNOS, because it did not block iNOS-mediated cardioprotection at 24 hours after ischemic PC, and had no effect on eNOS, because it did not affect eNOS-mediated vasodilation in response to acetylcholine (online Figure 2). Administration of a second nNOS inhibitor, SEPIIT, which is structurally different from NPA and is reported to be 115- and 29-times more selective for nNOS than for iNOS and eNOS, respectively,32 produced similar results. In contrast, in this rabbit model, iNOS inhibitors effectively block cardioprotection at 24 hours9 and L-NA (a nonselective NOS inhibitor) blunts acetylcholine-dependent vasodilation.31 Thus, the abrogation of protection after administration of NPA or SEPIT at 72 hours indicates a critical role of nNOS in the infarct-sparing effects observed during the final stage of late PC. Involvement of iNOS or eNOS in the final stage of late PC seems unlikely because neither gene was upregulated at this stage and the iNOS-selective inhibitor SMT (used at doses that block protection at 24 hours after ischemic PC25) failed to block protection at 72 hours. eNOS-selective inhibitors are not available at present.

The findings that the protection at 72 hours was blocked by NS-398 and that both prostanooid synthesis and protection had largely dissipated at 120 hours, when NOS was no longer upregulated but COX-2 protein expression was still increased, strongly suggest that nNOS confers infarct-sparing effects by enhancing the synthesis of COX-2–dependent cardioprotective prostanooids. It remains possible that nNOS may also mediate the cardioprotection afforded by late PC through other mechanisms. For example, the mitochondrial NOS (mtNOS) has been molecularly cloned and confirmed to be nNOS (with possible alternative splicing).20 Activation of mitochondrially localized nNOS may lead to transient inhibition of mitochondrial ATP production,19,20 which may, in turn, modulate myocardial contractility.19 Moreover, recent studies have localized nNOS to the cardiac sarcoplasmic reticulum.15 Compartmentalized release of NO in this important subcellular organelle is believed to play a key role in regulating calcium kinetics in cardiac myocytes and, in turn, myocardial contractility; however, it remains controversial whether nNOS-derived NO facilitates or reduces contractility.16,17 Lastly, nNOS in cardiac nerves may act to reduce heart rate and, in turn, oxygen consumption. Interestingly, hearts
subjected to regional cardiac denervation developed greater myocardial stunning than controls in a swine model of coronary artery stenosis, which appeared to involve increased oxidative stress secondary to activation of iNOS in the myocardium.

An interesting finding in the present study was that although only one predominant protein resembling nNOS was detected in the heart, consistent with what was reported previously in rabbits and rats, the mRNA transcripts coding for the nNOSα and nNOSμ variants were expressed at comparable levels under control conditions (Figure 5A and 5C). This phenomenon may reflect the fact that the half-life of nNOSα is only 24% of that of nNOSμ. Alternatively, it may result from a regulatory mechanism at the translational level that favors the expression of the nNOSμ protein. In this regard, it is noteworthy that nNOS is one of those genes whose tissue- and developmental stage–specific expression are extensively regulated by translational mechanisms. Regardless of the underlying mechanisms, it appears conceivable based on the current and previous studies that nNOSμ confers nNOS-mediated functions in the heart. The preferential induction of mRNA transcripts coding for nNOSμ after ischemic PC (Figure 5) further supports this notion. Although nNOSμ and nNOSα have been reported to possess similar enzymatic activities in vitro, their behavior in vivo under various conditions is completely unknown and warrants future investigation.

Conclusions

The present observations expand our understanding of both the mechanism of late PC and the function of nNOS in the heart. Our findings demonstrate that the antioxidant effects observed at 72 hours after ischemic PC are associated with, and depend on, upregulation of COX-2 and nNOS, whereas iNOS is not required at this stage. Thus, these results identify, for the first time, a cardioprotective role of nNOS and demonstrate that the mechanism for the late phase of ischemic PC differs at 72 hours (nNOS) versus 24 hours (iNOS).

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DETAILED METHODS

Animal preparation

The conscious rabbit model of late PC has been described in detail previously.1-5 Briefly, male New Zealand White rabbits (body 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. Rabbits were allowed to recover for a minimum of 21 days after surgery. Ischemic PC was produced with a sequence of six cycles of 4-min coronary occlusion/4-min reperfusion. Instrumented rabbits that did not undergo ischemic PC were used as controls.

Phase I. Studies of NOS and COX expression and activity

Rabbits were euthanized at 1, 3, 24, 72, or 120 h after ischemic PC and their hearts were removed. Myocardial tissue samples were rapidly excised from the ischemic-reperfused region (anterior wall of the left ventricle) and the nonischemic region (posterior wall of the left ventricle) and snap-frozen in liquid nitrogen for the molecular and biochemical analyses detailed below.

RNase protection assay

Total cellular RNA was extracted from frozen tissue samples using the TRI REAGENT (Molecular Research Center Inc.) according to the manufacturer’s protocol. A 294-bp HincII/ScaI fragment of the rabbit COX-2 cDNA (kindly supplied by Dr. M.D. Breyer, Vanderbilt University, Nashville, TN) was subcloned into the pCRII vector (Invitrogen) at the EcoRV site and linearized with EcoRI for use as a template in the RPA. A 359-nt antisense riboprobe was
transcribed with T7 RNA polymerase (MAXIscript™ kit, Ambion) using [α-32P]UTP (DuPont-NEN, specific activity, 800 Ci/mmol). RPAs were carried out using the RPAIII™ kit (Ambion).

Briefly, gel-purified riboprobes (10^5 cpm for COX-2 and 3x10^4 cpm for GAPDH) were hybridized with 30 μg total cellular RNA extracted from rabbit hearts or tRNA at 45°C for 18 h followed by RNase A/T1 digestion at 37°C for 30 min. Protected fragments were heat-denatured and separated on 5% denaturing polyacrylamide gels. A rabbit GAPDH antisense probe was used as a control. Radioactive signals were recorded and quantitated using a PhosphorImager (STORM 840 and ImageQuant 1.2 software, Molecular Dynamics). Each COX-2 signal was normalized to the GAPDH signal from the same sample and the normalized values were expressed as a percentage of the average control value.

**RT-PCR/Southern blot analysis**

Semi-quantitative RT-PCR was utilized to assess the steady-state level of nNOS mRNA in rabbit hearts. First strand cDNA was synthesized with total cellular RNA (5 μg) using random primers and superscript II reverse transcriptase (Invitrogen). The following primers were used in PCR amplification of the nNOS common region based on published rabbit nNOS cDNA sequence (GenBank Accession No. U91584): sense 5'-CTT GGC TCA ACC GAA TAC CT-3; antisense 5'-GTC CCC GCA CAC GTA GAT G-3. PCR amplifications were performed in a total volume of 50 μl for 25 cycles. A 764-bp fragment of rabbit GAPDH cDNA was amplified as a quantitative control (sense primer: 5'-CGG AGC CAA AAG GGT CAT CA-3'; antisense primer: 5'-CAG CGT GGT GGG ACT GAG TG-3'). PCR products were size-fractionated by agarose gel electrophoresis and transferred to GeneScreen Plus membranes (DuPont). Southern blots were hybridized with a [γ-32P]ATP-labeled oligonucleotide positioned internally to flanking PCR primers (5'-AAC AGC GGC AGT TTG ACA T-3'). In addition, the following primers were used to amplify the nNOS region harboring the exon- insertion: sense 5'-TGC ACC TGG AAC ACG AAA CT-3; antisense 5'-GAA CAC GGA GAA CCT CAC AT-3'. Internal primers 5'-CAG CAC...
CTT TGG CAA TGG AG-3 and 5'-GCA AGG GTT CCG GGT ACT-3 were used as probes for common and nNOS-specific sequences, respectively.

**Real-Time RT-PCR**

nNOS mRNA levels were further assessed with real-time RT-PCR using the Mx4000 Multiple Quantitative PCR System (Stratagene) and the Brilliant™ quantitative PCR core reagent (Stratagene) containing SureStart™ Taq DNA polymerase 1.25 U, MgCl2 5 mM, dNTP 200 M, TaqMan probe 200 M, and primers 400 M in 1xPCR buffer. cDNA derived from 100 ng total RNA was used as template. The following primers and TaqMan probe were used: sense 5'-TTA GCC GTC AAA ACC TCC AGA G-3' antisense 5'-CTC CTG ACT CCC GTT GGT GT-3' probe 5'-CCA GCC GCT CCA CCA TCT TCG TGC-3'. The TaqMan probe (Biosearch Technologies, Inc.) was labeled at the 5' end with a fluorescent reporter (6-carboxy-fluorescin, 6-FAM) and at the 3' end with a quencher dye (Black Hole Quencher™, BHQ-1). The PCR was performed for 40 cycles (30 s at 95°C and 30 s at 60°C) following denaturing for 10 min at 95°C. PCR products for the target gene were continuously measured by the sequence detector and normalized by the endogenous reference (GAPDH). Three replicates for each RNA sample were performed. Results are expressed as percentages of the average control value.

**Cloning of the rabbit nNOS**

A cDNA fragment containing exon was PCR-amplified from both the heart and the skeletal muscle using the primers described above. The 374-bp PCR product was gel-purified, subcloned into the pCRII vector (TA cloning, Invitrogen), and subjected to DNA sequencing analysis on both strands.

**Western blot analysis**
Frozen tissue samples were powdered in a prechilled pulverizer and homogenized on ice with a T25 Basic homogenizer (IKA Works) in sample buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 g/ml phenylmethylsulfonyl fluoride, 10 g/ml aprotinin, 10 g/ml leupeptin, 10 g/ml pepstatin A, and 10% glycerol (vol/vol). The homogenate was then incubated on a rocking platform in the presence of 20 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) at 4°C for 4 h. Following centrifugation at 14,000 g for 30 min, the supernatant was collected as total cellular protein. Protein concentration was determined using the modified Bradford method (Bio-Rad, Protein Assay Kit II). Protein samples were electrophoresed under reducing, denaturing conditions in 7% polyacrylamide/SDS gels and transferred by electroblotting onto nitrocellulose membrane. Equal loading and transfer efficiency were carefully recorded by making photocopies of membranes dyed with reversible Ponceau staining. After being blocked in 5% nonfat milk (Bio-Rad) overnight, membranes were incubated with either an anti-COX-2 monoclonal antibody (BD Biosciences), an anti-COX-1 polyclonal antibody (Santa Cruz Biotechnology), an anti-iNOS polyclonal antibody (Upstate Biotechnology), an anti-uNOS polyclonal antibody (Affinity Bioreagents), or an anti-nNOS monoclonal antibody (BD Biosciences), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (BD Biosciences). Signal detection was facilitated with enhanced chemiluminescence (ECL kit, Amersham).

Immunosignals and corresponding records of Ponceau stains of membranes were quantitated using an image scanning densitometer (Personal PI, Molecular Dynamics). Immunosignals were normalized to the Ponceau stain from the same lane and the normalized values were expressed as percentages of the average control value.

**Enzyme immunoassay (EIA) for prostaglandins (PGs)**

PGs were extracted from myocardial tissue samples by using ODS-silica reverse-phase columns (Sep-Pak C18, Waters) as described previously. Using [3H]PGE2 as an internal
standard, percent recovery was estimated to be >85%. The myocardial content of 6-keto-PGF$_{1\alpha}$ (stable metabolite of PGI$_{2}$) and PGE$_{2}$ was determined by using EIA kits (6-keto-PGF$_{1\alpha}$ kit from Amersham Life Science; PGE$_{2}$ kit from Cayman Chemical) according to the manufacturers protocols and expressed as picogram per milligram of protein.

**Measurement of nitrite and nitrate**

Myocardial tissue samples were homogenized in phosphate buffer (pH 7.4) and centrifuged at 14,000 g for 20 min. The resulting supernatants were then loaded in a Centricon-30 filtrator and centrifuged at 100,000 g for 20 min to remove substances larger than 30 kDa. Nitrite and nitrate were measured using a commercial kit (Nitrate/Nitrite Colorimetric Assay Kit, Cayman Chemical) according to the manufacturer s protocol. Briefly, nitrate content in the sample was first converted to nitrite using nitrate reductase. The total nitrite in the sample was then measured using the Griess reagents. All assays were performed in duplicate. Tissue nitrite/nitrate levels were expressed as nanomoles per milligram of protein.

**Phase II. Studies of infarct size**

To produce infarction, rabbits were subjected to a 30-min coronary artery occlusion followed by 3 days of reperfusion. Diazepam was administered 20 min before the occlusion (4 mg/kg i.p.) to relieve the stress caused by the procedure. Ischemic PC was induced by subjecting rabbits to a sequence of six cycles of 4-min coronary occlusion/4-min reperfusion at 24 or 72 h prior to the 30-min coronary occlusion. Rabbits were assigned to twelve groups.

Group I (control) underwent the 30-min coronary occlusion without any pretreatment. Groups II (PC 24 h) and III (PC 72 h) were preconditioned 24 h and 72 h prior to the 30-min occlusion, respectively. Group IV (PC 72 h+NS-398) was preconditioned 72 h earlier and received NS-398
(5 mg/kg i.p.) 30 min prior to the 30-min occlusion. This dose of NS-398 has been shown to abolish the increase in COX-2 activity and the concomitant protection against myocardial stunning and infarction 24 h after ischemia PC without causing any hemodynamic changes in this rabbit model. Previous studies in this rabbit model have also shown that NS-398 itself does not exert any effect on infarct size. Group V (PC 72 h+L-NA) was preconditioned 72 h earlier and received an i.v. infusion of \( N^\omega \)-nitro-L-arginine (L-NA; a nonselective inhibitor of all three NOS isoforms) at a rate of 1.3 mg/kg/min for 10 min, starting 20 min before and ending 10 min before the 30-min coronary occlusion (total dose, 13 mg/kg). This dose of L-NA has been shown to have no effect on infarct size by itself in this rabbit model. Groups VI (PC 24 h+NPA) and VII (PC 72 h+NPA) were preconditioned 24 and 72 h earlier, respectively, and received the selective nNOS inhibitor \( N \)-propyl-L-arginine (NPA, 0.1 mg/kg i.v.) 10 min prior to the 30-min occlusion. This dose of NPA was chosen based on pilot studies (see online supplement Fig. 1). Group VIII (NPA) was given NPA (0.1 mg/kg i.v.) 10 min before the 30-min occlusion without PC to exclude the possibility that NPA itself might affect infarct size. Group IX (PC 72 h+SEPIT) was preconditioned 72 h earlier and received the selective nNOS inhibitor \( S \)-ethyl \( N \)-[4-(trifluoromethyl)phenyl]isothiourea (SEPIT) at 2 doses (1.0 mg/kg i.v., n=7; 0.5 mg/kg i.v., n=4) 10 min prior to the 30-min occlusion. These doses of SEPIT were chosen based on pilot studies (see online supplement, Fig. 1). Group X (SEPIT) was given the same doses of SEPIT (1.0 mg/kg i.v., n=6; 0.5 mg/kg i.v., n=2) 10 min before the 30-min occlusion without PC to exclude the possibility that SEPIT itself might affect infarct size. Groups XI (PC 24 h+SMT) and XII (PC 72 h+SMT) were preconditioned 24 and 72 h earlier, respectively, and received the selective iNOS inhibitor \( S \)-methylisothiourea sulfate (SMT, 0.5 mg/kg i.v.) 10 min prior to the 30-min occlusion. This dose of SMT has previously been shown to block the activity of iNOS (without affecting eNOS and nNOS) 24 h after ischemic PC and to have no appreciable hemodynamic effects by itself in this rabbit model. NPA (Cayman Chemicals), SEPIT (Cayman
Chemicals), SMT (Aldrich Chemical Co.), and L-NA (Sigma Chemical Co.) were dissolved in normal saline. NS-398 (Cayman Chemicals) was dissolved in DMSO (20 mg/ml) and diluted in normal saline (final concentration, 20% DMSO in saline). All solutions were filtered through a 0.2 μm Millipore filter to ensure sterility.

At the conclusion of the study, the rabbits were euthanized and the hearts excised. The size of the ischemic-reperfused region (region at risk) was determined by perfusing the aortic root for 2 min with a 5% solution of Phthalo blue dye in normal saline at a pressure of 70 mmHg using a Langendorff apparatus after tying the coronary artery at the site of the previous occlusion.\(^{2,3}\) The heart was then cut into 6-7 transverse slices, which were incubated for 10 min at 37°C in a 1% solution of triphenyltetrazolium chloride in phosphate buffer (pH 7.4). All atrial and right ventricular tissues were excised. The slices were weighed, fixed in a 10% neutral buffered formaldehyde solution, and photographed (Nikon AF N6006). Transparencies were projected onto a paper screen at a ten-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 5.0) and infarct size was calculated from these measurements as a percentage of the region at risk.\(^{2,3}\)

**Statistical analysis**

Data are reported as mean–SEM. Results were analyzed by either a one-way or a two-way repeated-measures ANOVA, as appropriate, followed by unpaired Student's t-tests with the Bonferroni correction. The relationship between infarct size and risk region size was compared among groups with an ANCOVA using the size of the risk region as the covariate. The correlation between infarct size and risk region size was assessed by linear regression analysis using the least-squares method.
References


## Supplemental Table 1. Reasons for exclusion

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Supplemental Table 2. LV weight, risk region, and infarct.

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Data are mean – SEM.
IF: infarct; A@R: area at risk; LV: left ventricle.
### Supplemental Table 3. Heart rate at baseline, during occlusion, and during the 72 hour reperfusion period.

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In studies of infarct size, all groups underwent a 30-min coronary occlusion followed by 72-h of reperfusion. Heart rate was measured 5 min before any treatment (baseline), just before occlusion (preocclusion), at 15 min into the 30-min coronary occlusion, and at selected times after reperfusion. *P<0.05 vs. Baseline; ⁄P<0.05 vs. group I (control); P<0.05 vs. groups III and I. Data are means – SEM.
Supplemental Fig. 1. Experimental groups for the infarct size studies.
Supplemental Fig. 2. Specificity of nNOS inhibitors. Pilot studies were conducted to ensure that the doses of NPA and SEPIT to be used would have no inhibitory effect on endothelial NOS (eNOS). We determined the effect of NPA and SEPIT on the hypotensive response to acetylcholine (Ach). Arterial pressure was measured by cannulating the ear dorsal artery with a 22-gauge angiocatheter under local anesthesia (benzocaine). a. NPA had no effect on eNOS-mediated vasorelaxation at 0.1 mg/kg, although it had a minor inhibitory effect at 1.0 mg/kg. The former dose was thus selected for the main study. b. SEPIT had no effect on eNOS-mediated vasorelaxation at either 1.0 mg/kg or 0.5 mg/kg. Both doses were used in the main study.