

R. Bolli
B. Dawn
X.-L. Tang
Y. Qiu
P. Ping
Y.-T. Xuan
W. K. Jones
H. Takano
Y. Guo
J. Zhang

The nitric oxide hypothesis of late preconditioning

Received: 2 July 1998
Returned for 1. revision: 2 July 1998
1. Revision received: 10 July 1998
Accepted: 14 July 1998

Abstract Ischemic preconditioning (PC) occurs in two phases: an early phase, which lasts 2–3 h, and a late phase, which begins 12–24 h later and lasts 3–4 days. The mechanism for the late phase of PC has been the focus of intense investigation. We have recently proposed the “NO hypothesis of late PC”, which postulates that NO plays a prominent role both in initiating and in mediating this cardioprotective response. The purpose of this essay is to review the evidence supporting the NO hypothesis of late PC and to discuss its implications. We propose that, on *day 1*, a brief ischemic stress causes increased production of NO (probably via eNOS) and $\cdot\text{O}_2^-$, which then react to form ONOO $^-$. ONOO $^-$, in turn, activates the ϵ isoform of protein kinase C (PKC), either directly or via its reactive byproducts such as $\cdot\text{OH}$. Both NO and secondary species derived from $\cdot\text{O}_2^-$ could also stimulate PKC ϵ independently. PKC ϵ activation triggers a complex signaling cascade that involves tyrosine kinases (among which Src and Lck appear to be involved) and probably other kinases, the transcription factor NF- κ B, and most likely other as yet unknown components, resulting in increased transcription of the iNOS gene and

increased iNOS activity on *day 2*, which is responsible for the protection during the second ischemic challenge. Tyrosine kinases also appear to be involved on *day 2*, possibly by modulating iNOS activity. According to this paradigm, NO plays two completely different roles in late PC: on *day 1*, it initiates the development of this response, whereas on *day 2*, it protects against myocardial ischemia. We propose that two different NOS isoforms are sequentially involved in late PC, with eNOS generating the NO that initiates the development of the PC response on *day 1* and iNOS then generating the NO that protects against recurrent ischemia on *day 2*. The NO hypothesis of late PC puts forth a comprehensive paradigm that can explain both the initiation and the mediation of this complex phenomenon. Besides its pathophysiological implications, this hypothesis has potential clinical reverberations, since NO donors (i.e., nitrates) are widely used clinically and could be used to protect the ischemic myocardium in patients.

Key words Ischemic preconditioning – nitric oxide – reactive oxygen species – protein kinase C – nuclear factor kappa B – tyrosine kinase

R. Bolli (✉) · B. Dawn · X.-L. Tang · Y. Qiu
P. Ping · Y.-T. Xuan · W. K. Jones · H. Takano
Y. Guo · J. Zhang
Division of Cardiology
University of Louisville
Louisville, Kentucky 40292
USA
e-mail: rbolli@louisville.edu

The heart reacts to a mild ischemic stress by becoming more resistant to a subsequent ischemic stress. This phenomenon, termed ischemic preconditioning (PC), occurs in two phases: an early phase, which develops within minutes from the initial ischemic insult and lasts 2–3 h, and a late (or delayed) phase, which becomes apparent 12–24 h later and lasts for 3–4 days (reviewed in refs. 6, 9, 13, 22). Unlike the early phase, the late phase of ischemic PC protects not only against myocardial infarction, but also against myocardial stunning (34). Because of this, and because of its sustained duration, the late phase may have considerable clinical relevance (6, 9, 22). In the past two years, a series of studies have provided cogent evidence that nitric oxide (NO) serves both as the trigger and as the mediator of the late phase of ischemic PC – a new paradigm that we refer to as the “NO hypothesis of late PC”. The purpose of this review is to succinctly summarize the evidence supporting the NO hypothesis of late PC and to discuss its implications.

The components of the mechanism of late preconditioning

Despite intense research, the mechanism of the late phase of ischemic PC remains elusive (reviewed in refs. 6, 9, 22). It is now apparent that this phenomenon is the result of a complex cascade of cellular events that probably represents an archeotypical response of the heart to different stressful stimuli (Fig. 1). For didactic purposes, it is useful to subdivide this cascade into three major components: (i) the molecular species that is generated during the first ischemic challenge and is respon-

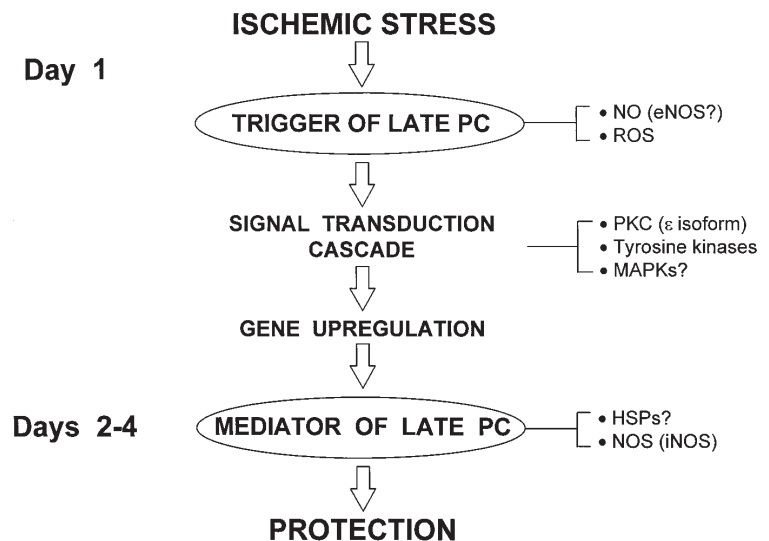
sible for initiating the response (“trigger” of late PC); (ii) the molecular species that is expressed in the heart 24–72 h later and is responsible for conferring protection during the second ischemic challenge (“mediator” of late PC); and (iii) the signaling pathway that is activated by the trigger and culminates in the expression of the mediator (Fig. 1). The NO hypothesis encompasses each of these three main components of late PC.

Role of NO as the trigger of late PC

A number of chemical signals have been proposed to trigger the development of the late phase of ischemic PC. Although activation of adenosine A₁ receptors can initiate late PC against infarction in rabbits (3), we have found that the development of late PC against myocardial stunning in conscious rabbits cannot be prevented with two different nonselective adenosine receptor antagonists [8-(p-sulfophenyl) theophylline (SPT) and PD 115,199 (both of which block A₁, A₂, and A₃ receptors)], and conversely, cannot be induced with large doses of the adenosine A₁ receptor agonist 2-chloro-*N*⁶-cyclopentyl-adenosine (CCPA) (21). Analogous results have been obtained in a conscious pig model of late PC against stunning (34). Thus, adenosine A₁ receptors do not trigger late PC against stunning.

Another potential trigger of late PC is the generation of reactive oxygen species (ROS). In conscious pigs, administration of antioxidant therapy (*viz.*, superoxide dismutase [SOD] plus catalase plus mercaptopropionyl glycine [MPG]) during the initial ischemic challenge on day 1 completely

Fig. 1 Schematic representation of the pathways involved in the genesis of late preconditioning against myocardial stunning. A brief episode of myocardial ischemia/reperfusion causes increased production of NO and reactive oxygen species (ROS), which serve as triggers for the development of late PC. NO and ROS activate a complex signal transduction cascade (which involves PKC (most likely PKC ϵ), tyrosine kinases, and possibly MAPKs), which leads to activation of transcription factors, upregulation of cardioprotective genes, and increased activity of NOS (specifically, iNOS) 24–72 h later. This increased NOS activity confers protection during the second ischemic stress.



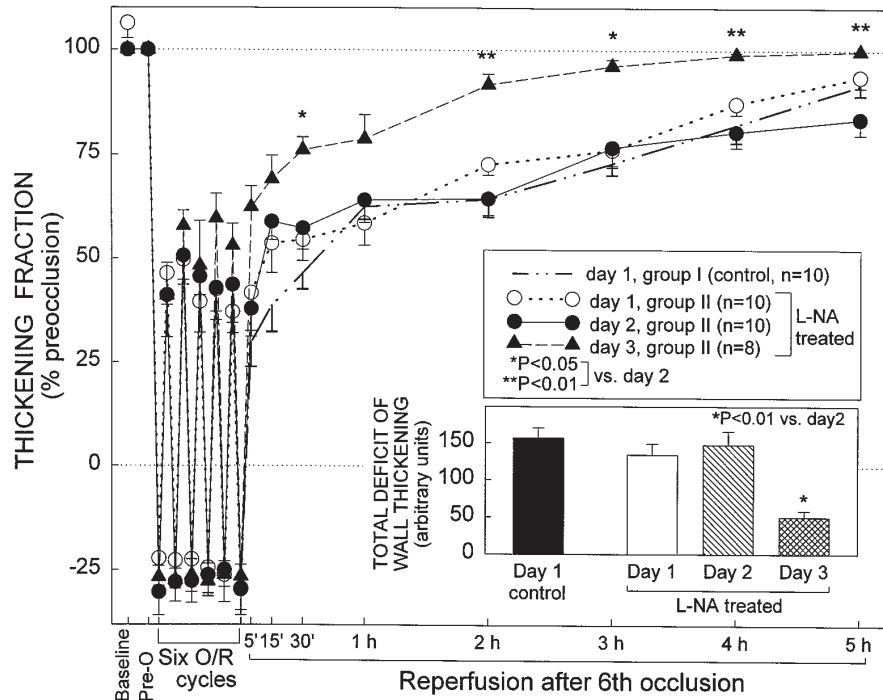


Fig. 2 Systolic thickening fraction in the ischemic-reperfused region in L-NA treated rabbits before administration of L-NA (baseline), 9 min after the end of the infusion of L-NA (immediately before the first occlusion) (preocclusion (Pre-O)), 3 min into each coronary occlusion (O), 3 min into each reperfusion (R), and at selected times during the 5 h reperfusion interval following the sixth occlusion. Conscious rabbits underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles for three consecutive days (day 1, 2, and 3). L-NA was given on day 1 prior to the first occlusion/reperfusion cycle. ○ indicates measurements taken on day 1; ●, measurements taken on day 2; and ▲, measurements taken on day 3. To facilitate comparisons, the data pertaining to day 1 of the control group are also shown (thick interrupted line without symbols). Thickening fraction is expressed as a percentage of preocclusion values. Data are mean ± SEM. In contrast to control rabbits, in which the recovery of wall thickening was markedly enhanced on day 2 compared with day 1 (data not shown in this figure), in L-NA-treated rabbits the recovery of wall thickening was similar on days 1 and 2, indicating that administration of L-NA on day 1 abrogated the development of late PC against stunning on day 2. The expected late PC effect became apparent on day 3, as a result of the ischemic PC stimulus applied on day 2 (when rabbits were not treated with L-NA). **Inset.** Total deficit of wall thickening during the 5 h reperfusion period following the 6th reperfusion. The total deficit of wall thickening is an integrated measure of the overall severity of myocardial stunning. Notice that the total deficit was similar on day 1 and day 2, indicating that L-NA prevented late PC against stunning. The deficit of wall thickening decreased on day 3, indicating that the ischemic stimulus on day 2 induced a late PC effect on day 3. (Reproduced with permission of the American Heart Association from Bolli et al. (1997) *Circ Res* 81: 42-52.)

prevented the development of late PC against myocardial stunning on day 2 (35), indicating that the generation of ROS during the PC ischemia plays an essential role in triggering this cardioprotective response. In subsequent studies in conscious rabbits, the development of late PC against stunning was found to be blocked by MPG alone, but not by SOD alone or catalase alone, implicating MPG-sensitive oxidants (peroxynitrite [ONOO⁻] and/or hydroxyl radical [•OH]) in the initiation of late PC (42). Conversely, intracoronary administration of an ROS-generating solution in the absence of ischemia was found to induce a late PC effect against stunning that was equivalent to that observed after ischemic PC (36). Taken together, these data indicate an important role of ROS in triggering late PC against stunning. But what is the mechanism that leads to the formation of these species?

We postulated that one possible source of the ROS that trigger late PC is NO. NO is produced by the oxidation of L-arginine by a family of isoenzymes (nitric oxide synthases NOS) that includes two constitutive isoforms, viz., endothelial NOS (eNOS) and neuronal (or brain) NOS (nNOS), and an inducible isoform (iNOS) (15, 19). eNOS produces NO via a complex reaction that is stimulated by calcium and requires NADPH, among other co-factors (15). Reperfusion following transient ischemia could stimulate rapid NO synthesis by providing the oxygen needed to produce NO, since calcium and NADPH have already been made available by the ischemic insult. At the same time, production of superoxide anion (•O₂⁻) is also accelerated in the early phase of reperfusion (45). •O₂⁻ and NO react rapidly to form the peroxynitrite anion

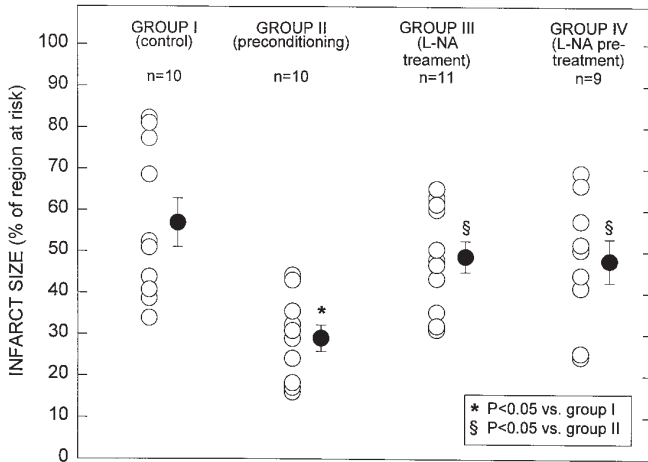


Fig. 3 Effect of the NOS inhibitor L-NA on late PC against infarction in conscious rabbits. All rabbits underwent a 30-min coronary occlusion followed by 3 d of reperfusion. Rabbits in group I received neither ischemic PC nor drug treatment. Rabbits in group II were preconditioned 24 h earlier with a sequence of six 4-min coronary occlusion/4-min reperfusion cycles. Rabbits in group III underwent the same protocol as group II, except that they received L-NA prior to the six 4-min occlusion/reperfusion cycles. Rabbits in group IV received L-NA without ischemic PC 24 h before the 30-min occlusion. In group III, administration of L-NA prior to the PC ischemia abrogated the infarct-sparing effect of late PC, indicating that the development of late PC against infarction is triggered by NO. In group IV (administration of L-NA without PC), infarct size did not differ from that observed in controls, demonstrating that administration of L-NA in itself did not exert a delayed deleterious effect on myocardial infarction. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent means \pm SEM. * $P < 0.05$ vs. group I (controls); § $P < 0.05$ vs. group II. (Reproduced with permission of the American Physiological Society from Qiu et al. (1997) Am J Physiol 273: H2931–36.)

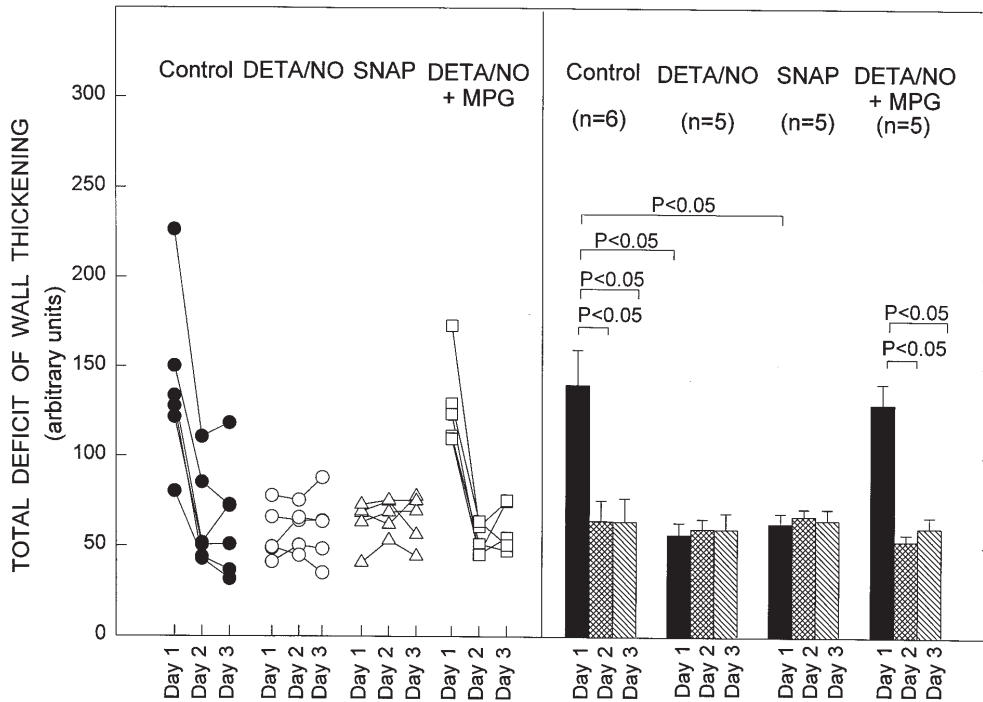


Fig. 4 Total deficit of wall thickening (WTh) after the 6th reperfusion on days 1, 2, and 3 in the control (n = 6), DETA/NO (n = 5), SNAP (n = 5), and DETA/NO+MPG groups (groups I, II, III, and IV, respectively). Conscious rabbits underwent a sequence of six 4-min coronary occlusion/4-min reperfusion cycles for three consecutive days (day 1, 2, and 3). Twenty-four hours before the first sequence of occlusion/reperfusion, rabbits received either no treatment (control), the NO donor DETA/NO, the NO donor SNAP, or DETA/NO in conjunction with the antioxidant MPG. Pretreatment with either DETA/NO or SNAP resulted in an atten-

uation of the total deficit of WTh on day 1 equivalent to that induced by ischemic PC on day 2 in control rabbits. This effect was abrogated by MPG, indicating that DETA/NO-induced late PC against stunning is mediated by MPG-sensitive oxidants (e.g., ONOO⁻ and/or [•]OH). The values of total deficit of WTh in individual rabbits are illustrated in the left panel; the mean \pm SEM values of total deficit of WTh are depicted in the right panel. The total deficit of WTh was measured in arbitrary units, as described in the text. (Reproduced with permission of the American Heart Association from Takano et al. (1998) Circ Res 83: 73–84.)

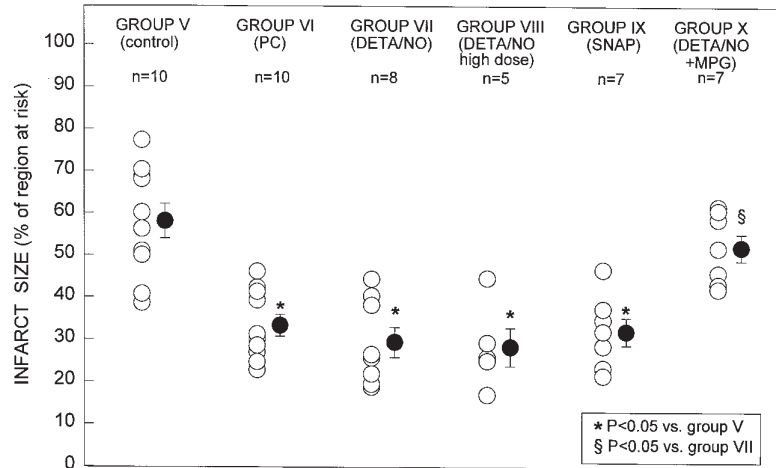


Fig. 5 Myocardial infarct size in groups V (n = 10, control group), VI (n = 10, ischemic PC group), VII (n = 8, DETA/NO group), VIII (n = 5, DETA/NO high dose group), IX (n = 7, SNAP group), and X (n = 7, DETA/NO+MPG group). Conscious rabbits underwent a 30-min coronary occlusion and three days of reperfusion. Twenty-four hours before the 30-min occlusion, they received either ischemic PC (six 4-min occlusion/reperfusion cycles), DETA/NO (at two doses), SNAP, or DETA/NO in conjunction with MPG. Pretreatment with either DETA/NO or SNAP resulted in a reduction of infarct size equivalent to that induced by ischemic PC in group VI. This effect was abrogated by MPG, indicating that NO-induced late PC against infarction is mediated by MPG-sensitive oxidants. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent means \pm SEM. *P < 0.05 vs. group V (control group); §P < 0.05 vs. group VII (DETA/NO group). (Reproduced with permission of the American Heart Association from Takano et al. (1998) *Circ Res* 83: 73–84.)

ischemic PC is triggered by NO, it should be possible to reproduce this cardioprotective phenomenon by administering NO in the absence of ischemia. Indeed, pretreatment with two structurally-unrelated NO donors, diethylenetriamine/NO (DETA/NO) and S-nitroso N-penicillamine (SNAP), induces a delayed protective effect against both myocardial stunning (Fig. 4) and infarction (Fig. 5) that is indistinguishable from that observed during the late phase of ischemic PC (39). Similar results have been obtained with nitroglycerin (2). Taken together, these studies (2, 7, 30, 39) indicate that the generation of NO during the PC ischemia serves as the trigger not only for late PC against stunning, but also for late PC against infarction.

(ONOO⁻), which then protonates and decomposes to generate the hydroxyl radical ([•]OH) or some other potent oxidant with similar reactivity (5). Both ONOO⁻ and [•]OH are effectively scavenged by MPG (5, 10), which could explain the ability of MPG to block late PC (35, 42).

The source of increased NO formation during the PC ischemia is likely to be eNOS, which has been identified not only in endothelial cells but also in cardiac myocytes (15, 19). This concept is supported by the finding that the development of late PC against stunning in conscious rabbits is blocked by the administration of the nonselective NOS antagonist L-NA, but not of the relatively selective iNOS antagonists aminoguanidine (AG) and S-methylisothiourea (SMT), prior to the initial ischemic challenge (day 1) (8). Ischemic PC could stimulate constitutively-expressed eNOS by at least three mechanisms: ischemia-induced increases in intracellular calcium and NADPH, increased shear stress during the repeated reactive hyperemias, and/or release of bradykinin with activation of endothelial B₂ receptors (23). However, this latter mechanism seems implausible because in recent unpublished studies we have found that late PC against stunning is not blocked by pretreatment with HOE 140, a bradykinin B₂ receptor antagonist, at doses that completely block the hypotensive effects of 0.45 μ g/kg of bradykinin.

On the basis of these considerations, we hypothesized that generation of NO during the first ischemic stress triggers the development of late PC against myocardial stunning 24 h later (7). To test this hypothesis, the effect of N^ω-nitro-L-arginine (L-NA), a nonselective inhibitor of all three NOS isoforms, was examined in conscious rabbits undergoing a sequence of six 4-min occlusion/4-min reperfusion cycles on three consecutive days (7). Administration of L-NA on day 1 completely blocked the development of late PC against myocardial stunning on day 2 (Fig. 2), demonstrating that this cardioprotective phenomenon is triggered by NO generated during the initial PC ischemia. A subsequent study found that administration of L-NA before the PC ischemia also blocked the development of late PC against myocardial infarction 24 h later in conscious rabbits (Fig. 3) (30). If the late phase of

Although several mechanisms are possible, the available evidence suggests that NO triggers late PC via the formation of ONOO⁻ and/or secondary ROS. This concept is strongly supported by the fact that the late PC effect of NO donors

(DETA/NO and SNAP) is completely abrogated by the co-administration of MPG (39), implying that MPG-sensitive oxidants (ONOO⁻ and/or [•]OH) serve as second messengers of NO-dependent signaling in late PC. This concept is also consistent with the finding that the development of late PC is abrogated by administration of antioxidants in both conscious pigs (35) and in conscious rabbits (42), implicating ROS as an essential factor in the genesis of this phenomenon.

In summary, in the conscious rabbit, NO (presumably generated by eNOS) triggers the development of late PC both against myocardial stunning and against myocardial infarction. NO appears to act by forming secondary oxidant species, such as ONOO⁻ and/or [•]OH.

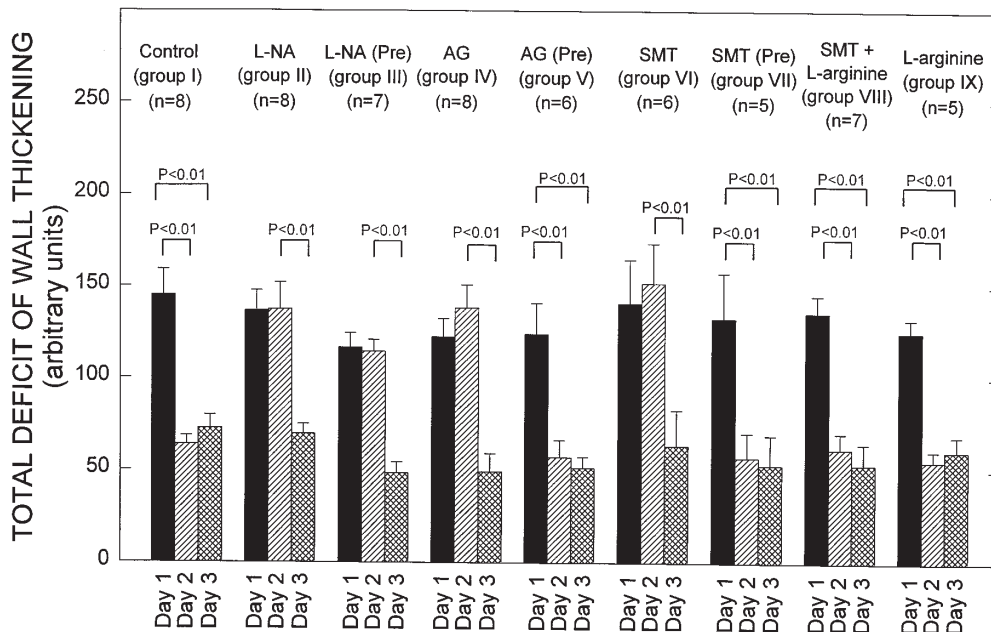
Role of NO as the mediator of late PC

Perhaps the most important unresolved issue pertaining to late PC is the identity of the mediator(s) that confers resistance to myocardial ischemia 24 h after the initial PC stimulus. The fact that the protective effect requires 12–24 h to develop and lasts for 3–4 days (4, 40) is consistent with the synthesis and degradation of cardioprotective protein(s). Rizvi et al. (32) have demonstrated in conscious rabbits that the same protocol that triggers late PC against stunning and infarction (six 4-min occlusion/4-min reperfusion cycles) causes a rapid increase in the rate of myocardial protein synthesis and that blocking such an increase with cycloheximide blocks the development of late PC. Thus, late PC requires increased synthesis of new proteins,

not simply activation of preexisting proteins. However, the nature of the protein(s) that is responsible for the protective effects of late PC remains unclear.

It has been postulated that late PC protects by upregulating one or more antioxidant enzymes. However, Tang et al. (41) found in conscious pigs that there was no increase in Mn SOD, Cu-Zn SOD, catalase, glutathione peroxidase, or glutathione reductase activity 24 h after ischemic PC. We have recently obtained similar findings in conscious rabbits. Therefore, it seems unlikely that the cardioprotection afforded by late PC is mediated by increased endogenous antioxidant defenses.

Fig. 6 Total deficit of systolic wall thickening after the sixth reperfusion on days 1, 2 and 3 in nine groups of conscious rabbits. All groups underwent a sequence of six 4-min occlusion/4-min reperfusion cycles for three consecutive days (days 1, 2, and 3). Rabbits in group I received no treatment. Rabbits in groups II (L-NA), IV (AG), and VI (SMT) received L-NA, AG, and SMT, respectively, before the first coronary occlusion on day 2. Rabbits in groups III (L-NA-pre), V (AG-pre), and VII (SMT-pre) received the same doses of L-NA, AG, and SMT, respectively, before the first coronary occlusion on day 1. Rabbits in group VIII received the same dose of SMT in conjunction with L-arginine before the first coronary occlusion on day 2. Rabbits in group IX received L-arginine alone before the first coronary occlusion on day 2. Data are mean SEM. Pre indicates treatment on day 1. Administration of either a nonselective (L-NA) or an iNOS-selective (AG and SMT) NOS inhibitor on day 2 completely abrogated late PC against myocardial stunning (groups II, IV, and VI, respectively), indicating that NOS (specifically, iNOS) is the mediator of this cardioprotective phenomenon. The abrogation of late PC by SMT was completely reversed by L-arginine (group VIII), indicating that SMT acted specifically by inhibiting NOS activity. (Reproduced with permission of the American Heart Association from Bolli et al. (1997) *Circ Res* 81: 1094–1107.)



NO, or its second messenger, cGMP, has been shown to exert a number of actions that would be expected to be beneficial during myocardial ischemia, including antagonism of the effects of beta-adrenergic stimulation, inhibition of calcium influx into myocytes, decrease in myocardial contractility, reduction in myocardial oxygen consumption, and opening of K_{ATP} channels (15, 19). Accordingly, we hypothesized that the protective effects of late PC are mediated by augmented NO formation secondary to ischemia-induced alterations of NOS gene expression.

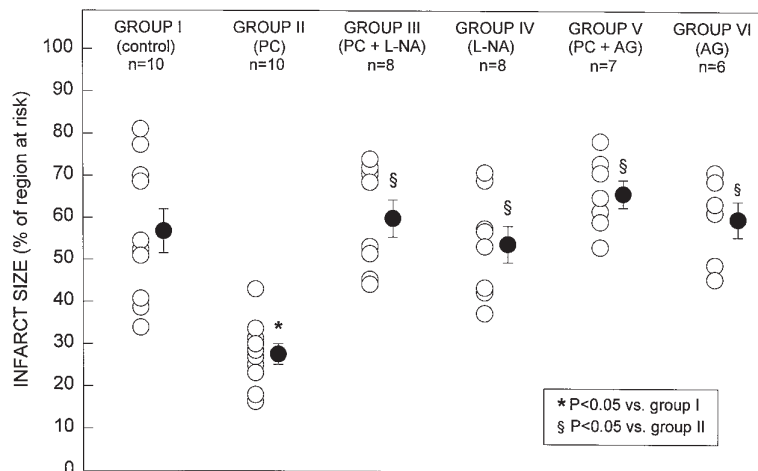
Pharmacological evidence that NO mediates late PC

To test this hypothesis, we examined the effects on late PC of NOS antagonists administered on day 2 (i.e., after the PC effect has already been triggered by the first ischemic challenge but prior to the second ischemic challenge) (8, 37). Administration of the nonselective NOS inhibitor L-NA completely abrogated late PC against stunning in conscious rabbits (Fig. 6) (8), indicating that the protection afforded by late PC is a NOS-dependent phenomenon. Administration of two structurally-unrelated iNOS inhibitors, AG and SMT, on day 2 also abrogated late PC against myocardial stunning (Fig. 6) (8), suggesting that the specific isoform involved in mediating the protective effects of late PC is iNOS. The abrogation of late PC by the competitive iNOS inhibitor SMT was blocked by the concomitant administration of L-arginine, confirming that SMT acted specifically by inhibiting NOS activity (8). Using a conscious rabbit model of late PC against infarction, it was subsequently found that administration of L-NA prior to a 30-min coronary occlusion on day 2 completely blocked the infarct-sparing effects of late PC (Fig. 7), indicating that the activity of NOS is necessary to mediate not only the attenuation of myocardial stunning, but also the limitation of infarct

size by late PC (37). The same abrogation of the infarct-sparing effects of late PC was produced by administration of AG prior to the 30-min occlusion on day 2 (Fig. 7), again suggesting that iNOS is the isoform involved in the protection against cell death (37). None of the NOS inhibitors had any appreciable effect on either myocardial stunning or myocardial infarction in nonpreconditioned hearts (8, 37). Taken together, these results (8, 37) indicate that the cardioprotective effects of late PC are mediated by the activity of NOS, and strongly implicate iNOS as the isoform involved.

If the activity of NOS on day 2 is essential for the protective effects of late PC, inhibition of tetrahydrobiopterin (BH_4) synthesis should block the protection. BH_4 is an essential cofactor for the formation of NO by NOS (15, 19). Since the activity of iNOS is thought to be independent of calcium or other stimuli, upregulation of iNOS should result in continu-

Fig. 7 Effect of the nonselective NOS inhibitor L-NA and the relatively-selective iNOS inhibitor aminoguanidine (AG) on late PC against infarction in conscious rabbits. All groups underwent a 30-min coronary occlusion followed by 3 d of reperfusion. Group II was preconditioned 24 h earlier with six 4-min occlusion/reperfusion cycles. Group III underwent the same protocol, except that L-NA was given prior to the 30-min occlusion. Group IV received L-NA as group III, but was not preconditioned 24 h earlier. Group V underwent the same protocol as group II, except that AG was given before the 30-min occlusion. Group VI received AG without undergoing ischemic PC 24 h earlier. Administration of either L-NA or AG completely abrogated the infarct-sparing effect of late PC, indicating that NOS (specifically, iNOS) is the mediator of late PC against infarction. Infarct size in groups IV and VI was not different from controls, indicating that L-NA and AG did not exert a delayed deleterious effect on infarct size independent of PC. Infarct size is expressed as a percentage of the region at risk of infarction. Open circles represent individual rabbits, whereas solid circles represent means \pm SEM. * $P < 0.05$ vs. group I (controls); § $P < 0.05$ vs. group II (PC group). (Reproduced with permission of the American Physiological Society from Takano et al. (1998) *Circulation* 98: 441–449.)



ous NO generation, which would require continuous resynthesis of BH₄ to support the enzymatic activity. The role of BH₄ in late PC was explored by Takano et al. (38) in conscious rabbits using *N*-acetylserotonin (NAS), an inhibitor of sepiapterin reductase, an enzyme necessary for BH₄ synthesis. Administration of NAS prior to the second ischemic challenge on day 2 completely abrogated the protective effects of late PC against myocardial stunning, indicating that intact BH₄ biosynthesis is necessary for the protection of late PC to become manifest. This finding strongly supports the hypothesis that NOS is the mediator of late PC. Interestingly, the same dose of NAS given on day 1 prior to the first ischemic challenge had no effect on late PC (38). This is consistent with the concept that the isoform involved in triggering late PC is eNOS. In contrast to iNOS, the activity of eNOS is thought to be pulsatile rather than continuous (15, 19). Apparently, the preexisting tissue stores of BH₄ are sufficient to support a burst of eNOS activity on day 1, so that the development of late PC is unaffected by inhibition of BH₄ synthesis on day 1.

Molecular genetic evidence that iNOS mediates late PC

Although the pharmacologic studies summarized above (8, 37) strongly implicate iNOS as the specific isoform responsible for mediating the protective effects of the late PC, these results are inherently limited by two factors: first, the selectivity of AG and SMT for iNOS vs. eNOS is only relative, with an IC₅₀ ratio of approximately 30:1 (33). Since the concentrations of AG and SMT attained *in vivo* are unknown, the possibility that administration of these iNOS inhibitors to intact rabbits may result in inhibition of eNOS as well cannot be ruled out. Second, AG is known to exert a number of nonspecific actions besides inhibition of NOS, including increasing angiotensin II-mediated release of prostacyclins, inhibition of oxidative modification of low density lipoproteins, inhibition of formation of glycosylation end-products in diabetes, and inhibition of histamine metabolism, polyamine catabolism, aldose reductase, catalase, and other iron- and copper-containing enzymes (33). Although SMT is devoid of these actions, it could still exert other nonspecific effects. Inhibitors of iNOS with greater selectivity and specificity are not available at the present time. Thus, conclusive evidence that late PC is mediated by iNOS cannot be provided by pharmacological studies alone.

Accordingly, the effect of targeted disruption of the iNOS gene on late PC was examined by Guo et al. in a murine model of late PC (16). Mice homozygous for a null iNOS allele and wild-type mice of the same genetic background were subjected to a 30-min coronary occlusion and 24 h of reperfusion with or without prior PC with six 4-min coronary occlusion/4-min reperfusion cycles 24 h earlier (Fig. 8). In wild-type mice, a robust late PC effect was evident, with infarct size

averaging 57.6 ± 3.2 % of the region at risk in the absence of prior PC and 22.5 ± 3.3 % of the region at risk following prior PC. In iNOS knockout mice, infarct size (63.5 ± 2.6 % of the region at risk) was similar to that in wild-type mice, indicating that iNOS activity does not affect infarct size in the absence of PC (Fig. 8). This is not unexpected, since iNOS is induced in response to stresses, such as ischemia. However, when iNOS knockout mice were subjected to ischemic PC 24 h earlier, infarct size (55.0 ± 4.1 % of the region at risk) was not decreased compared with nonpreconditioned iNOS knockout mice, indicating that the late PC effect was completely abrogated (Fig. 8). Interestingly, disruption of the iNOS gene had no effect on the early phase of ischemic PC (30-min occlusion performed 10 min after the six 4-min occlusion/reperfusion cycles) (Fig. 8) (16). These results provide unequivocal molecular genetic evidence that iNOS is necessary for the protection of late PC (but not early PC) to occur.

In further studies in the same murine model, administration of the NO donor DETA/NO induced an infarct-sparing effect 24 h later equivalent to that observed in mice preconditioned 24 h earlier with ischemia (group III) (infarct size in DETA/NO-pretreated mice averaged 22.2 ± 5.7 % of risk region), demonstrating that NO can mimic the late phase of ischemic PC in this model, in analogy with the results previously obtained in conscious rabbits (39). Importantly, DETA/NO failed to induce a late PC effect in iNOS knockout mice (infarct size: 51.3 ± 6 % of risk region), demonstrating that iNOS serves as an obligatory mediator not only for late

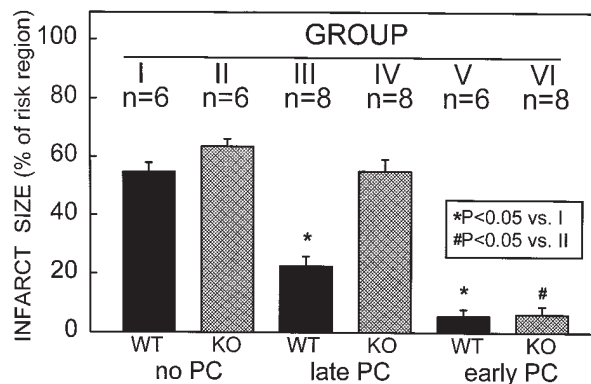


Fig. 8 Myocardial infarct size in wild-type (WT) and iNOS knockout (KO) mice. Mice were subjected to 30 min of coronary occlusion and 24 h of reperfusion, in the absence of prior ischemic PC (groups I and II), 24 h after ischemic PC (six 4-min occlusion/4-min reperfusion cycles) (groups III and IV (late PC groups)), or 10 min after the same ischemic PC protocol (groups V and VI (early PC groups)). Infarct size is expressed as a percentage of the region at risk of infarction. Note that late PC produced a significant reduction in infarct size in wild-type mice but not in iNOS knockout mice, whereas early PC produced marked infarct size reduction in both. *P < 0.05 vs. Group I; #P < 0.05 vs. Group II.

PC induced by ischemia, but also for late PC induced pharmacologically by NO donor pretreatment.

In conclusion, NO plays a dual role in the pathophysiology of the late phase of ischemic PC, acting initially as the trigger and subsequently as the mediator of the protection.

Regulation of iNOS transcript levels by ischemic PC

It is well known that iNOS is regulated by multiple mechanisms, including transcriptional upregulation, transcript stability, translational efficiency, and post-translational modification (25, 43). To elucidate the molecular basis for the increased iNOS activity on day 2, the steady-state levels of iNOS mRNA were measured in conscious rabbits by quantitative RNA dot blot hybridization (18). A robust increase in steady-state iNOS mRNA levels was found in the ischemic-reperfused region 3 h after six 4-min coronary occlusion/4-min reperfusion cycles ($+287 \pm 8\%$ vs. nonischemic region), indicating that ischemic PC has a profound influence on the *iNOS* transcript level. Interestingly, the increase in iNOS mRNA was completely abolished when rabbits were treated with L-NA prior to the six occlusion/reperfusion cycles, indicating that the increase in *iNOS* transcript levels is triggered by increased NO formation on day 1. These results support a complex paradigm in which NO generated during ischemic PC causes increased *iNOS* transcription or transcript stability, which results in cardioprotection 24 h later. Thus, it appears that two isoforms of NOS are activated sequentially, with initial activation of eNOS causing the subsequent activation of iNOS.

Direct evidence for the NO hypothesis of late PC

To obtain direct evidence for increased NOS activity in late PC, Xuan et al. (44) measured calcium-dependent (constitutive NOS (cNOS), i.e., eNOS and/or nNOS) and calcium-independent (iNOS) activity in myocardial samples obtained from conscious rabbits subjected to ischemic PC with six 4-min coronary occlusion/4-min reperfusion cycles. NOS activity was measured by the conversion of L-arginine to L-citrulline. On day 1, a marked increase in cNOS activity was observed at the end of the six occlusion/reperfusion cycles ($+290 \pm 47\%$ vs. nonischemic tissue ($p < 0.05$)), persisting for 30 min thereafter. iNOS activity was unchanged on day 1. Twenty-four hours after ischemic PC, however, there was a marked increase in iNOS activity in the ischemic/reperfused region ($+131 \pm 21\%$ vs. the nonischemic region ($p < 0.05$)), with no change in cNOS activity. These results were paralleled by the results obtained with measurements of myocardial levels of NO_2 and NO_3 (NO_x), the stable byproducts of NO. Tissue levels of NO_2/NO_3 (assessed by the Griess reaction)

increased by $34 \pm 8\%$ vs. nonischemic tissue ($p < 0.05$) at 60 min after PC on day 1 and by $36 \pm 7\%$ vs. nonischemic tissue ($p < 0.05$) on day 2, confirming increased NO generation. Administration of the iNOS inhibitors AG and SMT 1 h before euthanasia on day 2 blocked the increase in iNOS activity and NO_x . Importantly, both the increase in iNOS activity and NO_2/NO_3 levels at 24 h after ischemic PC were abrogated by the administration on day 1 of the NOS inhibitor L-NA, the ROS scavenger MPG, the PKC inhibitor chelerythrine, and the tyrosine kinase inhibitor lavendustin A (44). These results provide direct evidence that ischemic PC results in increased iNOS activity 24 h later concomitant with increased NO_x production, strongly supporting the hypothesis that iNOS is the mediator of late PC. Furthermore, these results provide direct evidence that the upregulation of iNOS on day 2 is the result of a sequence of events occurring on day 1, which is triggered by the generation of NO and involves the formation of ROS, the activation of PKC, and the activation of tyrosine kinases.

The signaling pathway of late PC

The NO hypothesis of late PC predicts that NO triggers a cascade of signaling events that culminates in increased iNOS activity resulting in cardioprotection. While the full sequence of such events remains to be elucidated, recent data point to PKC and NF- κ B as critical components of this pathway.

Protein kinase C

The role of PKC in ischemic PC has been controversial, since it seems to be supported by some studies but not by others and since there is no evidence that ischemic PC translocates PKC *in vivo* (20). To directly test the PKC hypothesis of ischemic PC, Ping et al. performed a series of studies in which the expression and subcellular distribution of all eleven PKC isoforms were systematically analyzed in conscious rabbits subjected to various ischemic PC protocols in the presence and absence of PKC inhibitors (26, 31). Ischemic PC was found to induce isoform-selective translocation of the ϵ and η isozymes of PKC with no significant changes in the subcellular distribution of the remaining nine isozymes and with no significant changes in total myocardial PKC activity or in its subcellular distribution (26). A subsequent study demonstrated that chelerythrine (a specific PKC inhibitor), at a dose that blocked the translocation of both PKC ϵ and η (5 mg/kg), also blocked the development of late PC against myocardial stunning (31). In contrast a ten-fold lower dose (0.5 mg/kg), which blocks the translocation of PKC η but not that of PKC ϵ , failed to block late PC against myocardial stunning, indicating that the translocation of PKC ϵ is necessary for late PC to occur

whereas that of PKC η is not (31). Taken together, these results (26, 31) indicate that PKC plays an essential role in the late phase of ischemic PC and specifically implicate a novel isoform (the ε isoform) in the development of this phenomenon.

Thus, if NO is the trigger of late PC, it must somehow modulate PKC activity. In recent unpublished studies in our laboratory, Ping et al. have demonstrated in conscious rabbits that the translocation of PKC ε induced by ischemic PC is blocked by the administration of L-NA, indicating that NO generated during the initial ischemic stress serves as the stimulus that causes mobilization of the ε isozyme (particulate fraction of PKC ε : $35 \pm 2\%$ of total PKC ε in control rabbits, $60 \pm 1\%$ after ischemic PC ($P < 0.05$ vs. control), and $32 \pm 1\%$ in L-NA-treated rabbits). Furthermore, a translocation of PKC ε quantitatively similar to that induced by ischemic PC could be reproduced in the absence of ischemia by the administration of two structurally-unrelated NO donors (DETA/NO and SNAP), at doses that induced a delayed protective effect equivalent to that induced by ischemic PC (particulate fraction of PKC ε : $52 \pm 2\%$ of total in DETA/NO-treated and $54 \pm 2\%$ in SNAP-treated rabbits ($P < 0.05$ vs. controls)). Co-administration of chelerythrine completely blocked the DETA/NO-induced translocation of PKC ε on day 1 (particulate fraction of PKC ε : $38 \pm 4\%$ of total ($P < 0.05$ vs. DETA/NO-treated rabbits)) and the DETA/NO-induced late PC effect on day 2, demonstrating that mobilization of the ε isotype of PKC plays an obligatory role in the delayed cardioprotective effect observed after pharmacologic PC with NO releasing agents. Taken together, these studies indicate that PKC ε serves as a critical signaling factor in the development of NO dependent late PC, both after an ischemic stimulus (endogenous NO) and after NO donor treatment (exogenous NO). Thus, NO induces late PC by activating PKC, and ε appears to be the specific isoform involved.

The exact mechanism whereby NO activates PKC appears to involve generation of NO-derived ROS. This hypothesis is supported by recent studies in our laboratory in which Ping et al. found that the antioxidant MPG, given prior to the ischemic PC protocol, blocked the activation of PKC ε , indicating an essential role of ROS in this phenomenon. We propose that NO reacts with $\cdot\text{O}_2^-$ to form ONOO $^-$, which in turn generates secondary ROS that activate PKC, either by direct oxidative modification (14) or via activation of phospholipase D (24).

The downstream targets of PKC phosphorylation remain unknown. We have recently found in conscious rabbits that ischemic PC activates the p44/p42 mitogen-activated protein kinases (MAPKs) and MEK1 and MEK2, the direct activators of p44/p42 MAPKs (27). Analysis of the subcellular distribution of these kinases in response to the ischemic stimulus revealed that p44/p42 MAPKs are activated in the cytosolic fraction and subsequently translocated to the nuclear fraction, so that the phosphorylation activity increases in the nuclear compartment but remains unchanged in the cytosol. This

selective nuclear activation of p44/p42 MAPKs is blocked by chelerythrine, indicating that it is mediated by, and downstream of, PKC (27). Using PKC ε transgenic mouse lines, we have obtained additional evidence that the ε isoform of PKC plays a critical role in modulating the activity of p44/p42 MAPKs *in vivo* (28). Whether p44/p42 MAPKs play a role in ischemic PC, however, remains to be elucidated. Furthermore, we have found that ischemic PC selectively activates two members of the Src family of tyrosine kinases (Src and Lck) and that this activation is blocked by chelerythrine, indicating that these tyrosine kinases are downstream of PKC in the signaling cascade of ischemic PC (29). A critical role of tyrosine kinases in the development of late PC was demonstrated by Imagawa et al. (17) in a rabbit model of late PC against infarction and by Dawn et al. (11), who found that the specific protein tyrosine kinase inhibitor, lavendustin A (LD-A), given to conscious rabbits prior to the ischemic PC protocol, completely abrogated the development of late PC against myocardial stunning on day 2. Taken together, these findings are compatible with the hypothesis that the PKC-dependent signaling that underlies ischemic PC involves tyrosine kinases.

Role of NF- κ B in late PC

The oxidant-sensitive transcription factor NF- κ B is known to be a major modulator of iNOS gene expression (1). The involvement of NF- κ B in late PC was explored by Xuan et al. in a series of studies conducted in our laboratory in the conscious rabbit model (Fig. 9). Using electrophoretic mobility shift assays (EMSA), a marked increase in NF- κ B DNA-binding activity was found at 30 min (4.1-fold), 1 h (1.6-fold), and 2 h (1.7-fold) after six 4-min coronary occlusion/4-min reperfusion cycles ($p < 0.05$ at all time-points), with a return to baseline by 4 h. This indicates NF- κ B activation early after ischemic PC. This increase in DNA-binding activity was accompanied by a redistribution of p65 (one of the subunits of NF- κ B) to the nuclear fraction and by a parallel decrease in the cytosolic fraction. The specificity of the NF- κ B-DNA complex was confirmed by competitive assays and supershift gel assays. The administration of the NF- κ B inhibitor diethyl-dithiocarbamate (DDTC) resulted in inhibition of increased NF- κ B DNA-binding activity (Fig. 9) and redistribution of p65 to the nuclear compartment and, at the same time, inhibition of the protective effects of late PC. The increase in the nuclear NF- κ B DNA-binding activity and the redistribution of p65 to the nuclear compartment were inhibited by a number of interventions that were previously found to abrogate late PC, including L-NA, MPG, chelerythrine, and lavendustin A (Fig. 9), indicating that the activation of NF- κ B after the initial PC ischemia is the result of NO and ROS generation and involves PKC and tyrosine kinase activation. Thus, the same signaling factors involved in the development of the protective effects

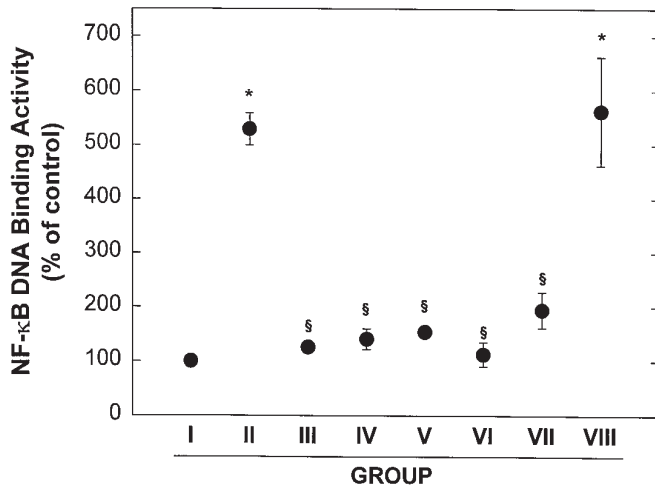


Fig. 9 Measurements of NF- κ B DNA binding activity (electrophoretic mobility shift assay) in the ischemic/reperfused left ventricular region of conscious rabbits undergoing ischemic PC. All values are expressed as a percentage of the values in the control group (group I), which did not undergo ischemia. A marked rise in the DNA binding activity was noted 30 min after the ischemic PC protocol (six 4-min coronary occlusion/4-min reperfusion cycles) (group II). This increase in DNA binding activity was completely abrogated when DDTC (an inhibitor of NF- κ B, group III), MPG (a scavenger of \cdot OH and ONOO $^-$, group IV), L-NA (a NOS inhibitor, group V), chelerythrine (an inhibitor of PKC, group VI), or LD-A (an inhibitor of tyrosine kinases, group VII) were administered before the ischemic PC protocol. Interestingly, when DETA/NO was administered in the absence of PC ischemia (group VIII), there was a marked increase in NF- κ B DNA binding activity similar to that in preconditioned rabbit hearts (group II). * $P < 0.05$ vs. group I; § $P < 0.05$ vs. group II ($n = 4$ in all groups).

of late PC and in the upregulation of iNOS activity on day 2 are also necessary for the early activation of NF- κ B after the ischemic stimulus on day 1. Taken together, these results support the concept that NF- κ B is a key signaling step in the cascade of events whereby brief ischemia results in the upregulation of iNOS activity and in the development of late PC.

Role of tyrosine kinases on day 2

It is known that the activity of iNOS can be modulated at the post-translational level by tyrosine phosphorylation (25). To test this hypothesis, Dawn et al. (12) administered the tyrosine kinase inhibitor LD-A on day 2 prior to the second ischemic challenge. It was found that LD-A completely abrogated the protective effects of late PC, both against myocardial stunning and against myocardial infarction, but had no effect in the absence of ischemic PC. Therefore, a tyrosine kinase-dependent signaling mechanism appears to be necessary for the protection of late PC to become manifest on day 2, possibly via iNOS modulation.

Overview of the NO hypothesis of late PC

A summary of our current understanding of the pathophysiology of the late phase of ischemic PC is illustrated in Figs. 1 and 10. We propose that a brief ischemic stress causes a burst of NO production (probably via eNOS) as well as \cdot O $_2^-$ production. NO and \cdot O $_2^-$ could then react to form ONOO $^-$, which could then activate PKC, either directly or via secondary byproducts such as \cdot OH. Both NO and ROS could also stimulate PKC independently. The specific isoform involved appears to be PKC ϵ . Activation of PKC ϵ triggers a complex signal transduction cascade which involves tyrosine kinases and probably other kinases, the transcription factor NF- κ B, and most likely other as yet unknown components, leading to increased transcription of the iNOS gene, increased iNOS protein and activity, and increased generation of NO during the second ischemic challenge. Among the numerous tyrosine kinases, two elements of the Src family (Src and Lck) are likely to be involved on day 1. Tyrosine kinases also appear to be involved on day 2, possibly by modulating iNOS activity. According to this paradigm, NO plays two completely different roles in late PC: on day 1, it initiates the development of the cardioprotective mechanism, whereas on day 2, it protects against myocardial stunning. We propose that two different NOS isoforms are sequentially involved in the pathophysiological cascade of late PC, with eNOS generating the NO that initiates the development of the PC response on day 1 and iNOS then generating the NO that protects against recurrent ischemia on day 2. Previous studies have documented that NO exerts a variety of biological actions resulting in rapid but transient physiological responses (15, 19). The results summarized in this review support a novel pathophysiological paradigm in which NO acts as an intracellular signal that modulates cardiac gene expression in response to ischemia and possibly other stresses, resulting in delayed but long-lasting cellular adaptations mediated by de novo synthesis of cardioprotective protein(s). This novel, previously unrecognized function of NO could have implications not only for ischemic PC but also for many other situations that are associated with enhanced NOS activity.

Conclusions

In recent years, intense research has focused on the delayed cardioprotection that develops following a brief ischemic stress (late phase of ischemic PC). While the exact cellular and molecular mechanisms underlying this phenomenon remain to be deciphered, it is clear that NO plays a prominent role both in initiating and in mediating this cardioprotective response. NO appears to trigger late PC by activating the ϵ isoform of

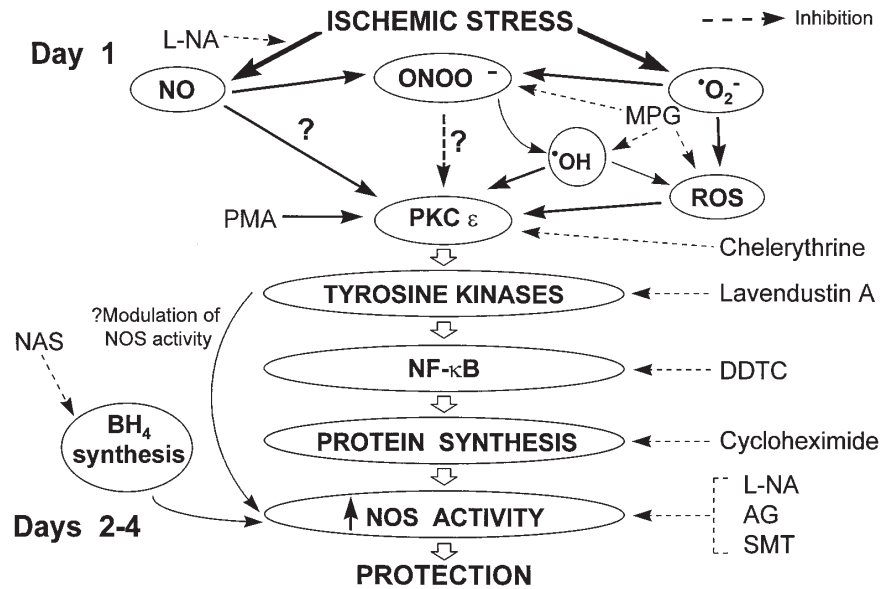


Fig. 10 Schematic representation of the cellular mechanisms involved in triggering late PC against myocardial stunning. A brief episode of myocardial ischemia/reperfusion causes increased production of NO and $\cdot\text{O}_2^-$, which then react to form ONOO^- . ONOO^- , in turn, activates the novel subgroup of PKCs (most likely PKC ϵ) either directly or via its reactive byproducts, such as $\cdot\text{OH}$. $\cdot\text{O}_2^-$ could also generate secondary ROS capable of activating PKC, and it is possible that NO could directly activate PKC. PKC activation triggers a complex signaling cascade that involves tyrosine kinases and probably other kinases, the transcription factor NF- κ B, and probably other as yet unknown components, resulting in increased synthesis of new proteins and increased NOS activity on day 2, which is responsible for the protection. Among the numerous tyrosine kinases, two elements of the Src family (Src and Lck) are likely to be involved on day 1. Tyrosine kinases also appear to be involved on day 2, possibly by modulating iNOS activity. The increased activity of iNOS on day 2 requires synthesis of tetrahydrobiopterin (BH_4) to support NO production. According to this paradigm, late PC against stunning can be abrogated by several interventions targeted at different components: by blocking increased NO synthesis following the initial stress with L-NA on day 1, by scavenging reactive species derived from NO (MPG on day 1), by inhibiting PKC, tyrosine kinases, NF- κ B or protein synthesis (with chelerythrine, lavendustin A, DDTC and cycloheximide, respectively, on day 1), by blocking the enhanced NOS activity on day 2 (L-NA, AG, or SMT on day 2), by blocking tyrosine kinases on day 2 (lavendustin A on day 2), or by blocking sepiapterin reductase (an enzyme involved in BH_4 synthesis) on day 2 (NAS on day 2). Conversely, administration of PMA on day 1 can bypass the triggering events and activate the development of late PC in the absence of ischemia.

PKC, which mobilizes a signal transduction cascade that involves tyrosine kinases, NF- κ B, and probably other elements, and eventually culminates in upregulation of iNOS. The NO hypothesis of late PC puts forth a comprehensive, testable paradigm which can explain both the initiation and the mediation of this phenomenon. Besides its pathophysiological

implications, this hypothesis has potential clinical reverberations, since NO donors (i.e., nitrates) are widely used clinically and could be a useful approach to the protection of the ischemic myocardium in patients. The concept that relatively brief treatment with NO donors can induce long-lasting cardioprotective effects raises the intriguing possibility that these agents may precondition the heart against subsequent ischemic injury occurring at a distance of hours or days, and thereby maintain the heart in a protracted preconditioned state.

Appendix (new data since submission)

We have recently obtained evidence for upregulation of iNOS in mice preconditioned with six 4-min occlusion/4-min reperfusion cycles. Twenty-four hours after ischemic PC, there was a 68 % increase in the expression of iNOS protein ($n = 12$), a 140 % increase in iNOS activity ($n = 8$), and a 23 % increase in NO_x levels ($n = 6$) in the ischemic/reperfused zone vs. the nonischemic zone ($P < .05$ for all). In contrast, no iNOS protein and no increase in iNOS activity or NO_x levels were detectable in iNOS knockout mice after ischemic PC. These results further confirm the role of iNOS as mediator of late PC in the mouse.

Acknowledgments This work was supported in part by NIH ROI grants HL-43151 and HL-55757 (Dr. Bolli), and R29 HL-58166 (Dr. Ping), by National AHA award 9750721N (Dr. Ping); by AHA Fellowship awards to Dr. Dawn (KY-97-F-24 and 9804556), Dr. Takano (9804558), Dr. Guo (9804557), and Dr. Li (9804503); and by the Medical Research Grant Program of the Jewish Hospital Foundation, Louisville, KY.

References

1. Baeuerle PA, Henkel T (1994) Function and activation of NF- κ B in the immune system. *Annu Rev Immunol* 12: 141–179
2. Banerjee S, Tang X-L, Qiu Y, Takano H, Manchikalapudi S, Dawn B, Shirk G, Bolli R (1998) Nitroglycerin induces late preconditioning against myocardial stunning via protein kinase C mediated pathway in conscious rabbits. *Circulation* (abstract, in press)
3. Baxter GF, Marber MS, Patel VC, Yellon DM (1994) Adenosine receptor involvement in a delayed phase of myocardial protection 24 hours after ischemic preconditioning. *Circulation* 90: 2993–3000
4. Baxter GF, Goma FM, Yellon DM (1997) Characterisation of the infarct-limiting effect of delayed preconditioning: Time-course and dose-dependency studies in rabbit myocardium. *Basic Res Cardiol* 92: 159–167
5. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1624
6. Bolli R (1996) The early and late phases of preconditioning against myocardial stunning and the essential role of oxyradicals in the late phase: An overview. *Basic Res Cardiol* 91: 57–63
7. Bolli R, Bhatti ZA, Tang X-L, Qiu Y, Zhang Q, Guo Y, Jadoon AK (1997) Evidence that late preconditioning against myocardial stunning in conscious rabbits is triggered by the generation of nitric oxide. *Circ Res* 81: 42–52
8. Bolli R, Manchikalapudi S, Tang X-L, Takano H, Qiu Y, Guo Y, Zhang Q, Jadoon AK (1997) The protective effect of late preconditioning against myocardial stunning in conscious rabbits is mediated by nitric oxide synthase. Evidence that nitric oxide acts both as a trigger and as a mediator of the late phase of ischemic preconditioning. *Circ Res* 81: 1094–1107
9. Bolli R, Tang X-L, Qiu Y, Park S-W (1997) The late phase of preconditioning against myocardial stunning. In: Mentzer RM, Kitakazi M, Downey JM, Hori M (eds) *Adenosine, Cardioprotection and Its Clinical Application*. Kluwer Academic Publishers, Boston: 29–35
10. Crow JP, Beckman JS (1995) Reactions between nitric oxide, superoxide, and peroxynitrite: footprints of peroxynitrite in vivo. *Adv Pharmacol* 34: 17–43
11. Dawn B, Qiu Y, Tang X-L, Takano H, Banerjee S, Bolli R (1998) Involvement of tyrosine kinases in the development of late preconditioning against myocardial stunning in conscious rabbits. *J Mol Cell Cardiol* 30: A264 (abstract)
12. Dawn B, Qiu Y, Tang X-L, Takano H, Banerjee S, Bolli R (1998) The protective effects of late preconditioning are mediated by tyrosine kinase activity in conscious rabbits. *Circulation* (abstract, in press)
13. Downey JM, Cohen MV, Ytrehus K, Liu Y (1994) Cellular mechanisms in ischemic preconditioning: The role of adenosine and protein kinase C. *Ann NY Acad Sci* 723: 82–98
14. Gopalakrishna R, Anderson WB (1989) Ca²⁺- and phospholipid-independent activation of protein kinase C by selective oxidative modification of the regulatory domain. *Proc Natl Acad Sci USA* 86: 6758–6762
15. Gross SS, Wolin MS (1995) Nitric oxide: Pathophysiological mechanisms. *Annu Rev Physiol* 57: 737–769
16. Guo Y, Jones WK, Tang X-L, Wu W-J, Qiu Y, Yang Z, Bao W, Flaherty M, Shirk G, Bolli R (1998) Targeted disruption of iNOS gene abrogates the late phase of ischemic preconditioning. *Circulation* (abstract, in press)
17. Imagawa J, Baxter GF, Yellon DM (1997) Genistein, a tyrosine kinase inhibitor, blocks the “second window of protection” 48 h after ischemic preconditioning in the rabbit. *J Mol Cell Cardiol* 29: 1885–1893
18. Jones WK, Flaherty MP, Tang X-L, Banerjee S, Qiu Y, Bolli R (1998) Nitric oxide dependent increase in steady state iNOS transcript levels in a conscious rabbit model of late preconditioning. *Circulation* (abstract, in press)
19. Kelly RA, Balligand JL, Smith TW (1996) Nitric oxide and cardiac function. *Circ Res* 79: 363–380
20. Kloner RA, Bolli R, Marban E, Reinlib L, Braunwald E (1998) Medical and cellular implications of stunning, hibernation, and preconditioning. An NHLBI workshop. *Circulation* 97: 1848–1867
21. Maldonado C, Qiu Y, Tang X-L, Cohen MV, Auchampach J, Bolli R (1997) Role of adenosine receptors in late preconditioning against myocardial stunning in conscious rabbits. *Am J Physiol* 273: H1324–1332
22. Marber MS, Yellon DM (1996) Myocardial adaptation, stress proteins, and the second window of protection. *Ann NY Acad Sci* 793: 123–141
23. Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* 329: 2002–2012
24. Natarajan V, Taher MM, Roehm B, Parinandi NL, Schmid HH, Kiss Z, Garcia JG (1993) Activation of endothelial cell phospholipase D by hydrogen peroxide and fatty acid hydroperoxide. *J Biol Chem* 268: 930–937
25. Pan J, Burgher KL, Szczepanik AM, Ringheim GE (1996) Tyrosine phosphorylation of inducible nitric oxide synthase: implications for potential posttranslational regulation. *Biochem J* 314: 889–894
26. Ping P, Zhang J, Qiu Y, Tang X-L, Manchikalapudi S, Cao X, Bolli R (1997) Ischemic preconditioning induces selective translocation of protein kinase C isoforms ϵ and η in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res* 81: 404–414
27. Ping P, Zhang J, Cao X, Kong D, Tang X-L, Qiu Y, Manchikalapudi S, Li RCX, Bolli R (1998) Brief episodes of ischemia induce activation of the MEK 1/2 – p44/p42 MAPK signaling cascade in the cytosolic fraction and subsequent nuclear translocation of p44/p42 MAPKs via a PKC-dependent pathway in conscious rabbits. *Circulation* (abstract, in press)
28. Ping P, Li RCX, Zhang J, Kong D, Jones K, Zheng Y-T, Cao X, Bolli R (1998) Identification of two distinct mechanisms for the activation of p44/p42 mitogen activated protein kinases (MAPKs) in vivo by a PKC isoform epsilon dependent signaling pathway in transgenic mice. *Circulation* (abstract, in press)
29. Ping P, Zhang J, Li RCX, Tang X-L, Qiu Y, Banerjee S, Zheng Y-T, Bolli R (1998) Ischemic preconditioning (PC) induces selective activation of Src and Lck tyrosine kinases in conscious rabbits via a PKC-dependent pathway. *Circulation* (abstract, in press)
30. Qiu Y, Rizvi A, Tang X-L, Manchikalapudi S, Takano H, Jadoon AK, Wu W-J, Bolli R (1997) Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol* 273: H2931–2936
31. Qiu Y, Ping P, Tang X-L, Manchikalapudi S, Rizvi A, Zung J, Takano H, Wu W-J, Teschner S, Bolli R (1998) Direct evidence that protein kinase C plays an essential role in the development of late preconditioning against myocardial stunning in conscious rabbits and that is the isoform involved. *J Clin Invest* 101: 2182–2198



32. Rizvi AN, Qiu Y, Tang X-L, Xuan Y-T, Takano H, Jadoon A, Bolli R (1997) Increased synthesis of proteins is necessary for the development of late preconditioning against myocardial stunning in conscious rabbits. *Circulation* 96 (Suppl I): I-256 (abstract)
33. Southan GJ, Szabo C (1996) Selective pharmacological activation of distinct nitric oxide synthase isoforms. *Biochem Pharmacol* 51: 383-394
34. Sun J-Z, Tang X-L, Knowlton AA, Park SW, Qiu Y, Bolli R (1995) Late preconditioning against myocardial stunning. An endogenous protective mechanism that confers resistance to postischemic dysfunction 24 h after brief ischemia in conscious pigs. *J Clin Invest* 95: 388-403
35. Sun J-Z, Tang X-L, Park SW, Qiu Y, Turrens JF, Bolli R (1996) Evidence for an essential role of reactive oxygen species in the genesis of late preconditioning against myocardial stunning in conscious pigs. *J Clin Invest* 97: 562-576
36. Takano H, Tang X-L, Qiu Y, Manchikalapudi S, Wu W-J, French BA, Bolli R (1997) Intracoronary administration of oxygen radicals induces late preconditioning against stunning in conscious rabbits. *Circulation* 96 (Suppl I): I-256-57 (abstract)
37. Takano H, Manchikalapudi S, Tang X-L, Qiu Y, Rizvi A, Jadoon AK, Zhang Q, Bolli R (1998) Nitric oxide synthase is the mediator of late preconditioning against myocardial infarction in conscious rabbits. *Circulation* 98: 441-449
38. Takano H, Tang X-L, Qiu Y, Banerjee S, Dawn B, Bolli R (1998) The tetrahydrobiopterin synthesis inhibitor N-acetylsertotonin abrogates late preconditioning against myocardial stunning in conscious rabbits. *Circulation* (abstract, in press)
39. Takano H, Tang X-L, Qiu Y, Guo Y, French BA, Bolli R (1998) Nitric oxide donors induce late preconditioning against myocardial stunning and infarction in conscious rabbits via an antioxidant sensitive mechanism. *Circ Res* 83: 73-84
40. Tang X-L, Qiu Y, Park SW, Sun J-Z, Kalya A, Bolli R (1996) Time-course of late preconditioning against myocardial stunning in conscious pigs. *Circ Res* 79: 424-434
41. Tang X-L, Qiu Y, Turrens JF, Sun J-Z, Bolli R (1997) Late preconditioning against stunning is not mediated by increased antioxidant defenses in conscious pigs. *Am J Physiol* 273: H1651-1657
42. Tang X-L, Rizvi AN, Qiu Y, Takano H, Zhang Q, Guo Y, Bolli R (1997) Evidence that the hydroxyl radical triggers late preconditioning against myocardial stunning in conscious rabbits. *Circulation* 96: I-255 (abstract)
43. Wang Y, Marsden PA (1995) Nitric oxide synthases: gene structure and regulation. *Adv Pharmacol* 34: 71-90
44. Xuan Y-T, Tang X-L, Qiu Y, Banerjee S, Takano H, Han H, Bolli R (1998) Direct evidence that inducible nitric oxide synthase mediates the late phase of ischemic preconditioning in conscious rabbits. *Circulation* (abstract, in press)
45. Zweier JL, Broderick R, Kuppusamy P, Thompson-Gorman S, Lutty GA (1994) Determination of the mechanism of free radical generation in human aortic endothelial cells exposed to anoxia and reoxygenation. *J Biol Chem* 269: 24156-24162

