The Late Phase of Preconditioning

Roberto Bolli

Abstract—Unlike the early phase of preconditioning (PC), which lasts 2 to 3 hours and protects against infarction but not against stunning, the late phase of PC lasts 3 to 4 days and protects against both infarction and stunning, suggesting that it may have greater clinical relevance. It is now clear that late PC is a polygenic phenomenon that requires the simultaneous activation of multiple stress-responsive genes. Chemical signals released by a sublethal ischemic stress (such as NO, reactive oxygen species, and adenosine) trigger a complex cascade of signaling events that includes the activation of protein kinase C, Src protein tyrosine kinases, and nuclear factor κB and culminates in increased synthesis of inducible NO synthase, cyclooxygenase-2, aldose reductase, Mn superoxide dismutase, and probably other cardioprotective proteins. An analogous sequence of events can be triggered by a variety of stimuli, such as heat stress, exercise, and cytokines. Thus, late PC appears to be a universal response of the heart to stress in general. Importantly, the cardioprotective effects of late PC can be reproduced pharmacologically with clinically relevant agents (eg, NO donors, adenosine receptor agonists, endotoxin derivatives, or opioid receptor agonists), suggesting that this phenomenon might be exploited for therapeutic purposes. The purpose of this review is to summarize current information regarding the pathophysiology and mechanism of late PC. (Circ Res. 2000;87:972-983.)

Key Words: myocardial ischemia & myocardial reperfusion & nitric oxide & cyclooxygenase & aldose reductase

The heart possesses a remarkable ability to adapt to stress by changing its phenotype in a manner that makes it more resistant to injury. The power of this phenotypic plasticity is perhaps illustrated most eloquently by the phenomenon of ischemic preconditioning (PC), the process whereby a sublethal ischemic stress enhances the tolerance of the myocardium to a subsequent ischemic stress. Originally described as an immediate adaptation of the heart to brief coronary occlusions, ischemic PC was subsequently found to be a biphasic phenomenon, with an early phase of protection that develops within minutes from the initial ischemic insult and lasts 2 to 3 hours and a late (or delayed) phase that becomes apparent 12 to 24 hours later and lasts 3 to 4 days. Unlike the early phase, the late phase of ischemic PC protects not only against myocardial infarction but also against myocardial stunning. Because of this, and because of its sustained duration, considerable interest has recently focused on the late phase and on its clinical relevance. If the mechanism of this adaptive metamorphosis is elucidated, then it should be possible to exploit it to protect the ischemic myocardium in patients.

The recognition that the heart shifts to a preconditioned phenotype upon exposure to stress has undoubtedly been one of the major advances in the field of myocardial ischemia. In the past few years, much has been learned regarding the intricate signaling pathways and genetic changes that underlie this defensive adaptation. The purpose of the present essay is to succinctly summarize current information regarding the
pathophysiology and mechanism of late PC. Although this review will focus solely on the heart, it should be noted that late PC has also been observed in other organs (eg, brain, intestine, and liver), suggesting that this is a universal mechanism whereby tissues protect themselves from an impending threat.

Classification of Late PC

Late PC can be categorized on the basis of the stimulus that elicits this response and of the end point examined (Table 1). Delayed protection against myocardial ischemia/reperfusion injury can be induced by a wide variety of stimuli, which can be broadly classified as nonpharmacological and pharmacological. Besides ischemia, the former include heat stress,5 rapid ventricular pacing,6 and exercise.7 The latter consist of naturally occurring—and often noxious—agents (eg, endotoxin,8 interleukin-1,9 tumor necrosis factor-α [TNF-α],10 TNF-β,11 leukemia inhibitory factor,11 reactive oxygen species [ROS]12) and of clinically applicable drugs (NO-releasing agents,13 adenosine receptor agonists,14 endotoxin derivatives such as monophosphoryl lipid A [MLA]15 and its analog RC-552,16 and opioid receptor agonists17). Most of these forms of late PC have been shown to protect against lethal ischemia/reperfusion injury (infarction), and at least some have been demonstrated to protect against reversible postischemic dysfunction (stunning).18 arrhythmias,6 and endothelial dysfunction19 (Table 1). This classification is not purely phenomenological, because recent evidence suggests the existence of mechanistic differences among the various forms of late PC outlined in Table 1 (eg, adenosine and ATP-sensitive potassium [KATP] channels play an obligatory role in ischemia-induced late PC against infarction14 but not against stunning18.24.26.27). Therefore, to avoid inappropriate generalizations, it is important to define (using the nature of the stimulus and of the protective effect) which specific form of late PC is being examined (eg, NO donor–induced late PC against stunning; ischemia-induced late PC against infarction, etc) (Table 1).

Components of the Mechanism of Late PC

Ischemia-induced late PC is the result of a complex cascade of cellular events that represents an archetypical response of the heart to diverse stressful stimuli (Figure). Conceptually, it is useful to subdivide this cascade into 3 major components: (1) the molecular species that are generated during the first ischemic challenge and are responsible for initiating the adaptation (“triggers” of late PC), (2) the molecular species that are expressed in the heart 24 to 72 hours later and are responsible for conferring protection during the second ischemic challenge (“mediators” of late PC), and (3) the signaling pathways that are activated by the triggers and culminate in the expression of the mediators (Figure). It should be noted that both triggers and signaling pathways operate soon after the PC stimulus; the distinction between them is hierarchical, with the former being upstream of the latter (Figure).

Triggers (or Initiators) of Late PC

Brief myocardial ischemia and the ensuing reperfusion are associated with major metabolic perturbations that result in the generation of a wide variety of metabolites and ligands. Among these, there is evidence that adenosine, NO, ROS, and perhaps opioid receptor agonists serve as chemical signals that trigger the development of the late phase of ischemic PC (Figure). These substances warn the myocardium that a danger is imminent, essentially acting as a cellular “alarm system” to which the heart responds by switching to a defensive phenotype.

Adenosine

The concept that adenosine released during the PC stimulus triggers the development of delayed protection was first proposed by Baxter et al14 and subsequently expanded by the

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Unlike NO, however, which is necessary for the development of ischemia-induced delayed protection against both myocardial stunning and myocardial infarction and is also sufficient to trigger both of these forms of adaptation, activation of adenosine receptors is neither necessary nor sufficient to trigger late PC against stunning. The differential role of adenosine receptors in the genesis of late PC against infarction versus late PC against stunning is but one of several pieces of evidence suggesting the existence of important differences in the mechanisms underlying these 2 phenomena (see above).

The identity of the adenosine receptor subtype(s) responsible for triggering late PC is under investigation. Because 2-chloro-N<sup>-6</sup>-cyclopentyladenosine (CCPA) is highly selective for A<sub>1</sub> receptors and because CCPA-induced late PC is blocked by selective A<sub>1</sub> receptor antagonists, it seems likely that this agonist elicits delayed cardioprotection by activating A<sub>1</sub> receptors. Recent studies indicate that delayed protection against infarction can also be triggered by selective activation of adenosine A<sub>3</sub> receptors. Thus, it appears that pharmacological stimulation of either A<sub>1</sub> or A<sub>3</sub> receptors can elicit late PC against infarction. Whether only one or both of these adenosine receptor subtypes contributes to triggering ischemia-induced late PC against infarction is unknown, because the only adenosine receptor antagonist shown to block the development of late PC after an ischemic stress is not selective for A<sub>1</sub> versus A<sub>3</sub> receptors.

Nitric Oxide

The first indication that NO triggers late PC was provided by a study in which administration of N<sup>-</sup>nitro-L-arginine (L-NA), a nonselective inhibitor of all 3 NO synthase (NOS) isoforms (neuronal [nNOS], endothelial [eNOS], and inducible [iNOS]), before the PC ischemic stimulus was found to block the development of late PC against infarction. These substances activate a complex signal transduction cascade that includes PKC (specifically, the ε isoform), PTKs (specifically, Src and/or Lck), and probably other as-yet-unknown kinases. A similar activation of PKC and downstream kinases can be elicited pharmacologically by a wide variety of agents, including naturally occurring—and often noxious—substances (eg, endotoxin, interleukin-1, TNF-α, TNF-β, leukemia inhibitor factor, or ROS), as well as clinically applicable drugs (NO donors, adenosine A<sub>1</sub>, or A<sub>3</sub> receptor agonists, endotoxin derivatives, or δ<sub>1</sub>-opioid receptor agonists). The recruitment of PKC and distal kinases leads to activation of NF-κB and almost certainly other transcription factors, resulting in increased transcription of multiple cardioprotective genes and synthesis of multiple cardioprotective proteins that serve as co mediators of protection 2 to 4 days after the PC stimulus. The mediators of late PC identified thus far include iNOS, COX-2, aldose reductase, Mn SOD. Among the products of COX-2, PGE<sub>2</sub> and/or PGI<sub>2</sub> appear to be the most likely effectors of COX-2 dependent protection. Increased synthesis of HSPs is unlikely to be a mechanism of late PC, whereas the role of posttranslational modification of preexisting HSPs remains to be determined. In addition, the occurrence of cardioprotection on days 2 to 4 requires the activity of PTKs and p38 MAPKs, potentially because iNOS and other mediators need to undergo posttranslational modification to confer protection against ischemia. Opening of K<sub>ATP</sub> channels is also essential for the protection against infarction (but not against stunning) to become manifest. The exact interrelationships among iNOS, COX-2, aldose reductase, Mn SOD, and K<sub>ATP</sub> channels are unknown, although recent evidence suggests that COX-2 may be downstream of iNOS (ie, COX-2 is activated by NO).
block the development of delayed protection against myocardial stunning,39 demonstrating that NO generation during the initial PC ischemia is necessary to trigger this cardioprotective phenomenon. A subsequent study demonstrated that NO is also necessary to trigger ischemia-induced late PC against myocardial infarction.50 Importantly, exposure to exogenous NO is sufficient to reproduce late PC, since pretreatment with NO donors in the absence of ischemia induces a delayed protective effect against both myocardial stunning and infarction that is indistinguishable from that observed during the late phase of ischemic PC.13,31–33 Administration of nitroglycerin can elicit late PC both by the intravenous31,33 and the transdermal33 route; this salubrious effect is not abrogated by the development of nitrate tolerance, indicating that different mechanisms underlie the hemodynamic and PC actions of nitrates.33 The ability of NO-releasing agents such as nitrates to faithfully mimic the late phase of ischemic PC despite nitrate tolerance supports the possibility of novel clinical applications of these drugs.

Recent studies34 have provided direct evidence of enhanced biosynthesis of NO in myocardium subjected to brief episodes of ischemia/reperfusion. The source of increased NO formation during the PC ischemia is likely to be eNOS, since the development of late PC is blocked by pretreatment with the nonsel ective NOS inhibitor L-NA, but not with the relatively selective iNOS inhibitors aminoguanidine and S-methylisothiourea.35 Interestingly, the development of late PC is not affected by pretreatment with the guanylate cyclase inhibitor ODQ36 but is completely prevented by pretreatment with the antioxidant mercaptopropionyl glycine (MPG).13,37 NO is known to react rapidly with O$_2^-$ to form the peroxynitrite anion (ONOO$^-$), which then protonates and decomposes to generate the hydroxyl radical (OH) or some other potent oxidant with similar reactivity.38,39 Because MPG scavenges both ONOO$^-$ and OH,39,40 the ability of MPG to block late PC,13,37 coupled with the failure of ODQ to do so,36 suggests that NO triggers this response via formation of ONOO$^-$ and/or secondary ROS, rather than via cGMP-dependent pathways.

**Reactive Oxygen Species**

The concept that the generation of ROS during the PC ischemia is essential to trigger delayed protection was first proposed by Sun et al.12 These investigators demonstrated in conscious pigs that the administration of a combination of antioxidants (superoxide dismutase [SOD] plus catalase plus MPG) during the initial ischemic challenge prevented the development of late PC against stunning. Similar findings were obtained in rabbits with MPG alone.37 MPG has also been found to prevent ischemia-induced late PC against infarction,41 arrhythmias,41 and coronary endothelial injury19 as well as heat stress–induced42 and exercise-induced43 late PC against infarction, thus implicating ROS as initiators of these forms of delayed protection as well. Conversely, intracoronary infusion of an ROS-generating solution in rabbits elicits a late-PC response.43 Taken together, these results12,19,37,41,42 suggest that sublethal oxidative stress plays a useful role by triggering delayed cardioprotection. Further studies will be necessary to determine the source(s) and the identity of the ROS responsible for initiating late PC and whether NO and ROS are parts of the same mechanism (ie, whether ROS are derived from the reaction of NO with O$_2^-$, as discussed above) or act in parallel as 2 independent triggers.

**Opioids**

Recent data in rats37 and mice44 indicate that pharmacological activation of δ-opioid receptors induces a delayed infarct-sparing effect 24 to 48 hours later. Whether δ-opioid receptors are also involved in triggering the late phase of ischemia-induced PC is currently unknown.

**Mediators (or Effectors) of Late PC**

Ischemic PC causes an increase in the rate of myocardial protein synthesis; if this increase is blocked with cycloheximide, the development of delayed protection is also blocked.45 Thus, unlike early PC, late PC requires increased synthesis of new proteins, not simply activation of preexisting proteins.45 The time course of the enhanced tolerance to ischemia, which requires 12 to 24 hours to develop and lasts for 3 to 4 days,46,47 is also consistent with the synthesis and degradation of cardioprotective proteins. Several proteins have been proposed as possible mediators (or effectors) of the protection afforded by late PC, including NOS, cyclooxygenase-2 (COX-2), aldose reductase, antioxidant enzymes (particularly Mn SOD), and heat stress proteins (HSPs). In addition, considerable evidence implicates K$_{ATP}$ channels as mediators of this defensive phenotype.

**Nitric Oxide Synthase**

The first demonstration that the cardioprotective effects of the late phase of ischemic PC are mediated by NOS was provided by 2 studies in conscious rabbits, in which the delayed protection against both myocardial stunning55 and myocardial infarction48 was found to be completely abrogated when preconditioned animals were given L-NA 24 hours after ischemic PC, just before the second ischemic challenge. The same effects were observed with the relatively selective iNOS inhibitors aminoguanidine and S-methylisothiourea, implicating iNOS as the specific NOS isoform involved in mediating the protective effects of late PC.35,48 These results were subsequently confirmed by others.49 Because of the limited selectivity and possible nonspecific effects of iNOS inhibitors, however, conclusive identification of the NOS isoform responsible for enhancing tolerance to ischemia during late PC cannot be attained pharmacologically. Using an in vivo murine model of myocardial infarction, Guo et al50 were the first to demonstrate that the late phase of ischemic PC is associated with upregulation of myocardial iNOS (whereas eNOS remains unchanged) and that targeted disruption of the iNOS gene completely abrogates the delayed infarct-sparing effect, providing unequivocal molecular genetic evidence for an obligatory role of iNOS in the cardioprotection afforded by the late phase of ischemic PC. Immunohistochemical and in situ hybridization studies have identified cardiac myocytes as the specific cell type that expresses iNOS during late PC.51

Thus, NO appears to play a dual role in the pathophysiology of the late phase of ischemic PC, acting initially as the trigger13,29–33 and subsequently as the mediator35,48–51 of this
adaptive response (“NO hypothesis of late PC”). In support of a dual role of NO are also direct measurements of NOS activity, which have shown a biphasic regulation of NOS by ischemic PC, with an increase in calcium-dependent NOS (cNOS [eNOS and/or nNOS]) activity immediately after the PC ischemia followed by an increase in calcium-independent NOS (iNOS) activity (with no change in cNOS activity) 24 hours later. The finding that both ischemic PC and nitroglycerin induce a rapid increase in steady-state levels of iNOS mRNA, which is abolished by administration of L-NA before the PC ischemia (53) (NO-dependent iNOS induction), is also congruent with this concept. Taken together, the studies reviewed above support a complex paradigm in which 2 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 (reviewed in Reference 52).

The quantitative aspects of the upregulation of iNOS after ischemic PC are noteworthy. In the Guo et al study, the increase in cardiac iNOS protein expression was mild, ~18-fold less than that observed after a lethal dose of lipopolysaccharide. This supports the hypothesis that induction of iNOS after ischemic PC is protective because it is relatively modest, in contrast to other situations (such as inflammation or septic shock) in which iNOS induction is massive and promotes tissue injury. The precise mechanism(s) whereby iNOS-derived NO protects against ischemia remains to be elucidated but appears to involve the activation of guanylate cyclase, given that both the alleviation of stunning and the reduction in infarct size limitation of this enzyme abrogate the infarct size limitation observed after pretreatment with endothelin derivatives (MLA and RC-552) and diazoxide. Recent reports on the role of iNOS in CCPA-induced late PC have arrived at conflicting conclusions.

Cyclooxygenase-2

An obligatory role of COX-2 in late PC was first shown by Shinmura et al. Using conscious rabbits, this study found that COX-2 protein expression was upregulated 24 hours after ischemic PC, concomitant with an increase in the myocardial levels of prostaglandin (PG) E₂, 6-keto-PGF₁α (the stable metabolite of PGI₂), and (to a lesser extent) PGE₂. Administration of 2 unrelated COX-2–selective inhibitors (NS-398 and celecoxib) 24 hours after ischemic PC abolished the increase in prostanooids and, at the same time, completely blocked the cardioprotective effects of late PC, demonstrating that COX-2 activity is necessary for this phenomenon to occur. Similar results were subsequently obtained in mice. These observations identify COX-2 as a cardioprotective protein and strongly point to PGE₂ and/or PGI₂ as the likely effectors of COX-2–dependent protection. Induction of COX-2 is generally though to be detrimental. The finding that COX-2 mediates the antistunning and antiinfarct effects of late PC impels a reassessment of current views regarding this enzyme and supports a more complex paradigm in which COX-2 can play either a beneficial or a deleterious role depending on various factors (eg, the intensity of its induction, the pathophysiological setting, and the ability of specific cell types to metabolize PGH₂ produced by COX-2 into cytoprotective prostanooids).

The recognition that iNOS and COX-2 are co-induced and serve as co-mediators of late PC logically leads to the question of whether there is an interaction between these 2 proteins or they act as independent effectors of cytoprotection. Previous studies have suggested that NO can directly activate COX-2. In keeping with these data, we have recently observed in unpublished studies that inhibition of iNOS blocks the increased COX-2 activity associated with late PC, whereas inhibition of COX-2 has no effect on iNOS activity, suggesting that COX-2 is downstream of iNOS in the preconditioned heart.

Aldose Reductase

Oxidative stress, osmotic stress, cytokines, and NO are known to upregulate the expression of aldose reductase, an enzyme that catalyzes not only the metabolism of glucose to sorbitol but also the detoxification of ROS-derived lipid aldehydes. Shinmura et al have recently found that the protein expression of aldose reductase is upregulated 24 hours after ischemic PC in conscious rabbits and that inhibition of this enzyme abrogates the infarct-sparing effects observed in untreated animals. Thus, in addition to iNOS and COX-2, aldose reductase is a third necessary mediator of the cardioprotective actions of the late phase of ischemic PC. The mechanism whereby aldose reductase enhances resistance to ischemia/reperfusion remains to be determined but could involve the removal of toxic byproducts of lipid peroxidation such as 4-hydroxy-trans-nonenal.

Antioxidant Enzymes (Mn SOD)

Studies in dogs have shown that ischemic PC induces, 24 hours later, an increase in the protein expression and activity of Mn SOD (while other antioxidant enzymes are unchanged) and that the time course of Mn SOD induction parallels that of protection against lethal ischemia/reperfusion injury. A similar association between the time course of Mn SOD induction and that of delayed cardioprotection has been observed after heat stress, exercise, and administration of CCPA, although in one study heat stress did not augment Mn SOD activity. The increase in Mn SOD activity after heat stress and exercise appears to be caused by the production of ROS, TNF-α, and interleukin-1β. Importantly, in vivo administration of antisense oligodeoxynucleotides to Mn SOD has been reported to block heat stress–induced exercise-induced, and CCPA-induced late PC, indicating...
that Mn SOD upregulation is essential for these 3 forms of delayed cardioprotection. No such data are available to determine whether Mn SOD is necessary for ischemia-induced late PC in vivo (although evidence supporting this concept has been obtained in isolated neonatal myocytes).72 An increase in the activity of various antioxidant enzymes (Mn SOD, Cu-Zn SOD, catalase, and/or glutathione peroxidase) has also been reported to 24 to 72 hours after pharmacological PC with interleukin-1 and endotoxin, concomitant with increased myocardial resistance to ischemia/superfusion injury, but a cause-and-effect relationship remains to be established. Not all studies, however, have found upregulation of antioxidant defenses during late PC. In conscious pigs75 and rabbits subjected to ischemic PC, no increase in Mn SOD, Cu-Zn SOD, catalase, glutathione peroxidase, or glutathione reductase activity could be detected 24 hours after the PC stimulus, when the delayed cardioprotection was fully manifest. Thus, the role of antioxidant proteins in ischemia-induced late PC is currently unknown.

Heat Stress Proteins
Although studies in transgenic mice overexpressing HSP70 have shown that this protein confers protection against ischemia/reperfusion injury,77–79 it remains controversial whether ischemic or pharmacological PC upregulates HSPs in vivo. For example, whereas earlier investigations reported an increase in myocardial HSP70 content 24 hours after ischemic PC, subsequent studies found no HSP70 induction in rabbits preconditioned with ischemia, CC PA, or MLA. Furthermore, several studies in rats subjected to whole-body hyperthermia or to ischemic PC have shown that the changes in myocardial HSP70 and HSP27 content do not correlate with protection against infarction. In addition, induction of HSP70 by heat stress fails to confer delayed cardioprotection in mice. Recently, another member of the HSP family, HSP27, has been suggested to participate in late PC. Studies in rabbits have shown that CC PA-induced late PC is associated with a redistribution of HSP27 from the membrane to the cytosolic fraction of the homogenate and with increased phosphorylation of this protein, which is abolished by pretreatment with protein kinase C (PKC) or tyrosine kinase inhibitors. Since HSP27 is a substrate for the p38 mitogen-activated protein kinase (MAPK) pathway, which is activated 24 hours after CC PA, the hypothesis has been proposed that posttranslational modulation of HSP27 may play an important role in mediating the delayed cardioprotection afforded by CC PA. The evidence reviewed above suggests that induction of HSPs is unlikely to account for the cardioprotection afforded by late PC, because the expression of these proteins does not correlate with the presence of the late infarct-sparing effects induced by 4 different stimuli (ischemia, heat stress, adenosine A1 agonists, and MLA). It appears that increased expression of HSPs might be just a marker of the response of myocytes to stress.

KATP Channels
Pharmacological studies have provided evidence that opening of KATP channels is necessary for the infarct-sparing effects of late PC induced by MLA, heat stress, ischemia, adenosine A1 and A2a receptor agonists, and δ1-opioid receptor agonists. The diversity of the PC stimuli that converge on KATP channels suggests that the activity of these channels may be a common distal mechanism of delayed protection against cell death. In contrast to late PC against infarction, however, ischemia-induced late PC against stunning does not appear to require KATP channel activity. The differential role of KATP channels in late PC against stunning versus late PC against infarction provides further evidence that different mechanisms underlie these 2 forms of cardioprotection, as mentioned above.

Major issues that remain to be elucidated are the identity of the KATP channels involved in late PC (ie, sarcolemmal versus mitochondrial) and the mechanism whereby their opening confers protection. Given the limitations of the available pharmacological tools, it seems likely that definitive assessment of the role of mitochondrial versus sarcolemmal KATP channels will require molecular approaches (eg, gene targeting or transgenesis).

The Signaling Pathway of Late PC
The stimuli listed in Table 1 trigger late PC by activating a complex cascade of signaling events that ultimately results in increased transcription of cardioprotective genes. Among the various families of cellular kinases, there is now convincing evidence that PKC and Src protein tyrosine kinases (PTKs) play an essential role in the development of late PC.

Protein Kinase C
The notion that PKC is essential for the genesis of late PC was first proposed by Baxter et al, who found that the delayed infarct-sparing effects of ischemic PC in rabbits were abrogated by pretreatment with the PKC inhibitor chelerythrine. Conversely, administration of the PKC activator dioctanoyl-sn-glycerol induced cardioprotection 24 hours later. Subsequent studies have also implicated PKC in the development of CC PA-induced and heat stress–induced late PC against infarction. Direct evidence that PC stimuli activate PKC in vivo, however, was lacking. Furthermore, no information was available as to which PKC isoform is involved. These issues were addressed in a series of studies in conscious rabbits in which it was found that ischemic PC causes selective translocation (and activation) of PKC and PKC (2 members of the subfamily of novel PKC isoforms) but does not affect the other 8 isoforms expressed in the rabbit heart (PKC isoforms α, β, γ, δ, ε, ζ, η, θ, and µ) and does not significantly change total PKC activity. Inhibition of PKCe translocation (and activation) by chelerythrine blocked the development of late PC against myocardial stunning, whereas inhibition of PKCe translocation did not, indicating that the translocation of PKCe (but not that of PKCη) is necessary for late PC to occur. Thus, activation of PKC after ischemic PC is isoform selective and ε appears to be the specific PKC isotype responsible for the development of delayed protection. The ischemic PC–induced activation of PKCe is caused by the generation of NO during the initial ischemic stress, because it is blocked by pretreatment with L-NA. Interestingly, administration of NO donors in
The absence of ischemia induces a selective activation of PKCε quantitatively similar to that induced by ischemic PC99; this event is essential for NO donor–induced late PC, since coadministration of chelerythrine blocks both the activation of PKCε and the delayed protection elicited by the NO donors.99 Thus, the recruitment of the ε isoform of PKC appears to be a critical signaling event in the development of both ischemia-induced and NO donor–induced late PC in rabbits. The recent finding that transgenic expression of constitutively active PKCε recapitulates both the signaling events and the cardioprotective effects of late PC supports a role of PKCε in mice as well.101 The precise mechanism whereby NO activates PKCε remains to be elucidated. Because MPG blocks NO donor–induced late PC,13 it seems reasonable to postulate that NO-derived reactive species (ONOO− and/or ROS) may activate PKCε either by direct oxidative modification or via activation of phospholipases.99

Protein Tyrosine Kinases
Since >1000 different PTKs have been identified so far, assessing the contribution of this class of enzymes to late PC represents a gargantuan task. A role of PTKs in the genesis of ischemia-induced late PC was proposed on the basis of studies using genistein,102 which, however, is a broad inhibitor of most known PTKs and has modest selectivity for these kinases versus PKC and other kinases. Recent studies in conscious rabbits100,103 have focused on 2 major families of PTKs, the Src PTKs and the epidermal growth factor (EGF) receptor PTKs. It was found that ischemic PC selectively activates 2 members of the Src family of PTKs (Src and Lck) (among the 7 members expressed in the rabbit heart) and that this activation is blocked by chelerythrine, suggesting that these kinases are distal to PKC.100 Inhibition of Src and Lck activation with lavendustin A (LD-A) completely abrogates the development of late PC against myocardial stunning,103 indicating that Src and/or Lck signaling plays a causative role in this phenomenon. In contrast, EGF receptor PTKs are not activated by ischemic PC.100 Thus, Src and/or Lck (but not EGF receptor PTKs) appear to be essential components of the signaling pathway responsible for the development of ischemia-induced late PC and to be downstream targets of PKC phosphorylation in rabbits. Subsequent studies in mice have corroborated these concepts by demonstrating that Lck is a direct substrate of PKCε100 and that targeted disruption of the Lck gene abolishes ischemia-induced late PC.105 LD-A has also been found to block CCPA-induced late PC,95 implicating tyrosine kinases in the genesis of this form of adaptation as well. In contrast, genistein failed to block heat stress–induced late PC.96

Mitogen-Activated Protein Kinases
Another potential downstream target of PKC-dependent signaling during the development of late PC is the MAPK superfamily, which includes 3 major subfamilies: the p44/p42 MAPKs (or extracellular signal–regulated kinases [ERKs]), the p38 MAPKs, and the p46/p54 MAPKs (or c-jun N-terminal kinases [JNKs]). Several studies in isolated hearts have documented that brief myocardial ischemia/reperfusion is associated with activation of p44/p42 MAPKs, p38 MAPKs, and JNKs,106,107 but it is unknown whether the ischemic protocols used in those investigations elicit late PC. Recent studies in conscious rabbits have demonstrated that an ischemic PC protocol known to induce delayed cardioprotection activates all of the 3 MAPK subfamilies, although the activation of p38 MAPK is short-lived.108,109 The activation of p44/p42 MAPKs and JNKs is abolished by chelerythrine, indicating that it is downstream of, and dependent on, activation of PKC.108,109 Interestingly, selective overexpression of PKCε in adult rabbit myocytes induces activation of p44/p42 MAPKs and protects against simulated ischemia, an effect that can be abolished by p44/p42 MAPK inhibitors.108 The critical unresolved issue now is whether the recruitment of MAPKs contributes to the development of late PC or is merely an epiphenomenon. Elucidation of this issue will require studies in in vivo models of late PC in which the activity of MAPKs is inhibited either with genetic approaches (eg, gene targeting or transgenesis of dominant-negative mutants) or with pharmacological agents more specific than those (eg, SB203580) currently available.

Role of Kinases in Mediating Protection
In addition to their role in initiating the development of late PC shortly after the stimulus (day 1), there is mounting evidence that cellular kinases are also necessary for the protection to become manifest 24 hours later (day 2) (Figure). This concept is supported by the recent finding that administration of the PTK inhibitor LD-A before the second ischemic challenge (on day 2) completely abrogates the protective effects of late PC against myocardial stunning and the concomitant increase in iNOS activity.103 It appears, therefore, that PTKs play a bifunctional role in ischemia-induced late PC, contributing not only to its development shortly after the initial ischemic stress but also to the occurrence of protection 24 hours later. The mechanism whereby PTKs participate in the protection on day 2 remains to be established. Because inhibition of PTKs with LD-A on day 2 abolishes the increase in iNOS activity, it has been suggested that posttranslational modulation of iNOS proteins via tyrosine phosphorylation is necessary to activate this enzyme and to confer tolerance to ischemia/reperfusion injury.103 In addition, the recent finding that p38 MAPK activity is markedly increased 24 hours after administration of CCPA (concomitant with increased phosphorylation of HSP27) raises the possibility that this subfamily of MAPKs may also be involved in mediating the protective effects of late PC.91

Transcription Factors
The recruitment by the PC stimulus of PKC, Src PTKs, and almost certainly other as-yet-identified kinases leads to the activation of transcription factors that govern the expression of the cardioprotective genes responsible for late PC. The first transcription-regulatory element to be identified as an integral component of the late PC response was nuclear factor-κB (NF-κB),110 which is known to be a major modulator of
TABLE 2. Mechanism of Late PC

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<td>Adenosine receptor agonists</td>
<td>+ ? ? ?</td>
<td>+ + ?</td>
<td>± + + -</td>
</tr>
</tbody>
</table>

+ indicates evidence based on pharmacological studies; ++, evidence based on molecular approaches (eg, knockout mice); ±, conflicting evidence; -, studies suggest no role in this form of late PC; and ?, available information insufficient.

AR indicates aldose reductase.

*Adenosine receptors and KATP channels have been found to participate in late PC against infarction but not against stunning.

iNOS, COX-2, and aldose reductase gene expression. Using conscious rabbits, Xuan et al.110 demonstrated that ischemic PC induces rapid activation of NF-κB and that this event can be mimicked by infusing NO donors in the absence of ischemia. Inhibition of NF-κB with diethylthiocarbamamide completely abrogated the cardioprotective effects observed 24 hours later, indicating that NF-κB plays a critical role in the genesis of late PC.110 The ischemic PC-induced activation of NF-κB was blocked by pretreatment with L-NA, MPG, chelerythrine, and LD-A (all given at doses previously shown to block late PC), indicating that the cellular mechanism whereby ischemic PC activates NF-κB involves the formation of NO and ROS and the subsequent activation of PKC- and PTK-dependent signaling events.110 Thus, NF-κB appears to be a common downstream pathway though which multiple signals elicited by ischemic stress (NO, ROS, PKC, and PTKs) act to induce gene expression in the heart.

Subsequent studies have shown that ischemic PC induces both serine and tyrosine phosphorylation of IκBα (the inhibitor of NF-κB) concomitant with PKC-dependent activation of IKKα and IKKβ (the serine-threonine kinases that phosphorylate IκBα)111 suggesting that a dual mechanism accounts for the activation of NF-κB during ischemic PC, one via PKC- and IKK-dependent serine phosphorylation of IκBα and the other via IKK-independent tyrosine phosphorylation of IκBα. The Lck kinase also plays an essential role in the activation of NF-κB induced by ischemia, as this event is absent in Lck−/− mice.105 A molecular link between PKCe (the isoform implicated in the genesis of late PC in vivo17,20,22,25,26,28,83,92 at least 3 stress-responsive, inducible proteins (ie, iNOS, COX-2, aldose reductase, and Mn SOD), it seems likely that the upregulation of iNOS, COX-2, and other co-mediators after a PC stimulus involves simultaneous activation of multiple stress-responsive transcription factors acting in an additive or synergistic manner.

Polycyclic Nature of Late PC

As detailed above, there is now solid evidence that, in addition to KATP channel activity17,20,22,25,26,28,83,92 at least 3 stress-responsive, inducible proteins (ie, iNOS, COX-2, aldose reductase, and Mn SOD) are required to mediate the protection afforded by the late phase of ischemic PC, and at least 2 proteins (iNOS and Mn SOD) are required to mediate exercise-induced late PC7 and possibly adenosine-induced late PC7 (and possibly adenosine-induced late PC7 and possibly adenosine-induced late PC7) (Table 2). Thus, the paradigm of late PC has evolved from the original (and in retrospect naïve) proposal that this adaptation is mediated by one protein to the recognition that the shift of the heart to a defensive phenotype represents a complex response requiring the coordinated activation of multiple genes. This is not dissimilar from other conditions in which the heart changes its phenotype (eg, hypertrophy). As the number of newly identified mediators of late PC increases, so will the number of triggers, transcription-regulatory mechanisms (kinases/transcription factors), and posttranscriptional modulators.
involved. Unraveling the complexity of this polygenic phenotypic change will likely be a challenge for years to come.

**Late Versus Early PC**

It is useful to recapitulate here some of the fundamental differences between the early and late phases of PC. Both early and late PC limit infarct size, but the infarct-sparing effects of early PC are more robust. On the other hand, late PC mitigates myocardial stunning, whereas early PC does not. The duration of the protection conferred by the 2 phases (2 to 3 hours versus 72 to 96 hours) is vastly different, supporting the concept that the late phase may ultimately have greater clinical usefulness. The duration of the early phase cannot be extended by continuous infusion of pharmacological triggers (ie, CCPA) nor by repeated brief ischemic episodes, presumably because of desensitization of adenosine receptors. This problem should not apply to late PC, in which stimuli need to be applied only at 48- to 72-hour intervals to maintain the defensive phenotype. Indeed, Yellon’s group has demonstrated that the infarct-sparing effects of early PC can also be induced by a single dose of CCPA persist for 72 hours and that repeated administration of CCPA at 48-hour intervals results in the maintenance of continuous protection against infarction for at least 10 days with no evidence of downregulation of A1 receptor function. These results provide proof of concept that late PC can be exploited pharmacologically to maintain the heart in a chronically preconditioned state without development of tolerance.

**Conclusions**

The heart reacts to a sublethal ischemic stress by mobilizing a complex sequence of cellular events that results in a shift from a naïve (nonpreconditioned) to a defensive (preconditioned) phenotype. Although the exact cellular and molecular mechanisms underlying the phenomenon of late PC remain to be deciphered, remarkable progress has been made in the last few years in our understanding of this powerful cardioprotective adaptation. A schematic representation of the mechanism of late PC is presented in the Figure. It is now clear that this is a polygenic process that requires the synthesis of multiple proteins. Specifically, chemical signals released by the ischemic stress (such as NO, ROS, and adenosine) are transduced by a cascade of signaling elements that includes PKC, Src PTKs, and NF-κB to the nucleus where they direct the transcription of iNOS, COX-2, aldose reductase, and probably other cardioprotective genes (Figure). An analogous sequence of events (possibly involving additional genes such as Mn SOD) can be triggered by a wide variety of stimuli, including heat stress, exercise, and cytokines. Thus, late PC appears to be a universal response of the heart to stress in general. A sustained cardioprotection similar to that afforded by the late phase of ischemic PC can also be induced pharmacologically with clinically relevant agents, such as NO donors, adenosine A1 or A2 receptor agonists, endotoxin derivatives, and δ-opioid receptor agonists (“PC mimetics”), suggesting that this endogenous adaptive response might be exploited for therapeutic purposes. The extent to which the various forms of nonpharmacological and pharmacological PC share the same molecular mechanism remains to be established. Some components of the proposed paradigm (eg, PKC, PTKs, iNOS, and Mn SOD) appear to be common to several forms of late PC, but others (eg, adenosine and KATP channels) appear to differ. Deciphering the mechanism of late PC is important not only for our understanding of how the heart adapts to stress but also for its potential clinical implications. The identification of the cellular basis of this phenomenon should provide a conceptual framework for developing novel therapeutic strategies aimed at mimicking the cardioprotective effects of late PC with pharmacological agents (eg, PC-mimetic drugs) or genetic approaches (eg, transfer of cardioprotective genes) that can maintain the heart in a sustained or chronic defensive (preconditioned) state.

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The Late Phase of Preconditioning
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