

Bifunctional Role of Protein Tyrosine Kinases in Late Preconditioning Against Myocardial Stunning in Conscious Rabbits

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Abstract—Although protein tyrosine kinases (PTKs) have been implicated in late preconditioning (PC) against infarction, their role in late PC against stunning is unknown. Furthermore, it is unknown whether PTK signaling is necessary only to trigger late PC on day 1 or also to mediate it on day 2. Thus, conscious rabbits underwent a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles for 3 consecutive days (days 1, 2, and 3). In the control group (group I, n=7), the recovery of systolic wall thickening after the 6 occlusion/reperfusion cycles was markedly improved on days 2 and 3 compared with day 1, indicating the development of late PC against stunning. Administration of the PTK inhibitor lavendustin-A (LD-A, 1 mg/kg IV) before the first occlusion on day 1 (group II, n=7) completely prevented the late PC effect against stunning on day 2. Late PC against stunning was also abrogated when LD-A was given before the first occlusion on day 2 (group III, n=7); however, in these rabbits, the late PC effect became apparent on day 3, indicating that LD-A itself did not have any delayed deleterious actions on myocardial stunning. In group V (n=5), the sequence of 6 occlusion/reperfusion cycles resulted in a robust increase in the activity of inducible NO synthase (iNOS [assessed as Ca²⁺-independent L-citrulline formation]) and nitrite+nitrate (NO_x) tissue levels 24 hours later (on day 2), with no concomitant change in Ca²⁺-dependent NO synthase (endothelial NO synthase and/or neuronal NO synthase) activity. Similar results were obtained on day 3 (group VIII, n=6), indicating sustained upregulation of iNOS. Administration of LD-A either on day 1 (group VI, n=5) or on day 2 (group VII, n=6) abrogated the increase in iNOS activity and NO_x levels on day 2. LD-A had no effect on iNOS activity or NO_x levels in the absence of PC (group X, n=5). This study demonstrates that in conscious rabbits, PTK activity is necessary not only to trigger late PC against stunning on day 1 but also to mediate the protection on day 2. This investigation also provides the first direct evidence that cardiac iNOS activity is upregulated during the late phase of ischemic PC in rabbits. Furthermore, the data indicate that PTK signaling is essential for the augmentation of iNOS activity and that PTKs modulate this enzyme at two distinct levels: at an early stage on day 1 and at a late stage on day 2. This bifunctional role of PTKs in late PC has broad implications for the signaling mechanisms that underlie the response of the heart to ischemic stress and, possibly, other stresses. (*Circ Res.* 1999;85:1154-1163.)

Key Words: myocardial stunning ■ protein tyrosine kinase ■ lavendustin A ■ inducible nitric oxide synthase ■ ischemia/reperfusion injury

Ischemic preconditioning (PC) confers myocardial protection in two temporally distinct phases: an early phase, which develops immediately and lasts for 2 to 4 hours after the ischemic stimulus, and a late phase, which begins after 12 to 24 hours and lasts for 3 to 4 days.¹⁻³ Recent studies indicate that NO plays a dual role (trigger and mediator) in late PC.^{1,4-8} Specifically, the generation of NO associated with an ischemic stress initiates a cascade of events that involves the activation of several kinases and transcription factors and culminates in the upregulation of the inducible NO synthase (iNOS) gene; NO produced by iNOS then confers cardiopro-

tection during subsequent ischemic insult(s) (reviewed in Reference 8). Although reactive oxygen species^{9,10} and protein kinase C (PKC)¹¹⁻¹⁴ are known to be involved in the genesis of late PC, the exact signal transduction mechanism whereby a brief ischemic stress leads to subsequent cardioprotection remains unclear. Elucidation of the cellular basis of late PC is an essential step toward harnessing this cardioprotective phenomenon for therapeutic benefit.

Protein tyrosine kinases (PTKs), a diverse family of enzymes that transfer phosphate from ATP to tyrosine residues on specific cellular proteins, are known to mediate a

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wide variety of cellular responses.^{15–17} The nonreceptor PTKs are specifically designed for signal transduction from cell surface to intracellular enzymes and factors, usually by protein-protein interactions.¹⁶ Recent evidence indicates that PTKs play a role in the signaling mechanism underlying the early^{18–21} and the late²² phases of PC. Specifically, in isolated rat hearts, inhibition of PTKs has been found to block the PC-induced increase in the activity of phospholipase D (PLD), PKC, and mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK 2) as well as the cardioprotective effects of early PC,¹⁹ suggesting that the PTK-PLD-PKC-MAPKAPK 2 signaling pathway plays a role in the early phase of PC. Using isolated rabbit hearts, Baines et al²⁰ have demonstrated that the protection afforded by early PC against myocardial infarction is abrogated by the PTK inhibitors genistein and lavendustin A (LD-A). In addition, genistein has been reported to block the development of late PC against infarction in open-chest rabbits.²²

Although these studies^{18–22} implicate a PTK-dependent pathway in the genesis of early and late PC, a number of important issues remain to be addressed: First, while the available evidence suggests an involvement of PTKs in late PC against myocardial infarction,²² virtually nothing is known regarding the role of PTKs in the genesis of late PC against myocardial stunning. Myocardial stunning and infarction represent two very different types of injury, so that the effects of PC on one cannot be extrapolated to the other. For example, in dogs, the early phase of PC confers powerful protection against myocardial infarction^{23,24} but fails to protect against the stunning induced by a 10- or 15-minute coronary occlusion.^{25–27} Conversely, in conscious pigs, a sequence of ten 2-minute coronary occlusions elicits a late PC effect against stunning^{9,28,29} but not against infarction.³⁰ These examples of a dissociation between the effects of PC on stunning and infarction underscore the notion that, at least under certain experimental conditions, different mechanisms may be involved in the PC protection against reversible and irreversible ischemic injury.²⁵ Second, the precise role(s) of PTKs in late PC has not been fully elucidated. In this regard, it is important to distinguish the cellular mechanisms that initiate the development of late PC immediately after the first ischemic stress (day 1) from those that mediate cardioprotection 24 to 72 hours later (days 2 to 4). Although one previous study²² examined the effect of genistein on the development of late PC against infarction on day 1, the role of PTKs as mediators of cardioprotection on days 2 to 4 has not been explored. Thus, it is unknown whether PTK-dependent signaling is important only to trigger or also to mediate late PC. Third, virtually nothing is known regarding the cellular mechanisms by which PTKs contribute to late PC on day 1 or day 2. Although several studies have implicated iNOS as the mediator of late PC,^{5,7,8} direct evidence that iNOS activity is augmented in the rabbit model of late PC is still lacking, and the role of PTKs in iNOS modulation is unknown.

The present study was undertaken to address these issues. We tested the hypothesis that PTKs play a dual role in the pathophysiology of late PC against myocardial stunning, ie, that they are essential not only for the initiation of this phenomenon on day 1 but also for the manifestation of

cardioprotection on day 2. We further hypothesized that the mechanism by which PTKs contribute to late PC is the modulation of iNOS activity. Accordingly, the present study had three aims. First, we determined whether administration of the PTK inhibitor LD-A before the first ischemic stress (on day 1) blocks the development of late PC against myocardial stunning. Second, we investigated whether administration of LD-A before the second ischemic stress (on day 2) abrogates the cardioprotection afforded by late PC against stunning. Finally, we assessed whether LD-A (given on day 1 or day 2) interferes with the increase in iNOS activity that underlies the cardioprotection afforded by late PC. All studies were performed in conscious rabbits. The rationale for using a conscious animal model was to obviate potential problems resulting from factors associated with open-chest preparations, such as the exaggerated generation of reactive oxygen species after myocardial ischemia/reperfusion,³¹ which could have a major impact on the severity of myocardial stunning,^{31,32} and the trauma of a thoracotomy and the ensuing inflammatory reaction, which may lead to release of cytokines. LD-A was chosen because (1) this agent is more selective for PTKs than other inhibitors^{33,34} and (2) it has previously been shown to inhibit Src and Lck PTKs in our conscious rabbit model of late PC,³⁵ which enabled us to assess the functional significance of Src and Lck PTK activation in this cardioprotective phenomenon.

Materials and Methods

Experimental Protocol

Phase A: Studies of Myocardial Stunning

The experimental protocol consisted of 3 consecutive days (days 1, 2, and 3, respectively) of coronary artery occlusions (a sequence of six 4-minute coronary occlusion/4-minute reperfusion cycles) (Figure 1). Rabbits were assigned to 3 groups (Figure 1): group I (control) did not receive any treatment; groups II and III received an intravenous bolus of LD-A (1 mg/kg) 10 minutes before the first coronary occlusion on day 1 or day 2, respectively. Regional myocardial function was assessed as systolic thickening fraction, as previously described.^{4,5,36} At the conclusion of the study, the size of the occluded/reperfused coronary vascular bed was determined by postmortem perfusion.^{4,5}

Phase B: Studies of NOS Activity

Rabbits were assigned to 7 groups: group IV (sham control), group V (PC-day 2), group VI (PC-day 2+LD-A on day 1), group VII (PC-day 2+LD-A on day 2), group VIII (PC-day 3), group IX (PC-day 3+LD-A on day 2), and group X (LD-A without PC) (Figure 1). Rabbits in group IV did not receive coronary occlusion/reperfusion and were euthanized a minimum of 10 days after surgery. Rabbits in groups V, VI, and VII underwent a sequence of six 4-minute occlusion/4-minute reperfusion cycles as in phase A; in groups VI and VII, LD-A (1 mg/kg IV) was administered 10 minutes before the first coronary occlusion on day 1 and day 2, respectively. Rabbits in groups V and VI were euthanized 24 hours later. Rabbits in group VII were euthanized 25 minutes after the administration of LD-A (time interval corresponding to the interval elapsed between the administration of LD-A and the mid-part of the 6 occlusion/reperfusion cycles in group III). Rabbits in groups VIII and IX underwent a sequence of six 4-minute occlusion/4-minute reperfusion cycles on days 1 and 2 and were euthanized 24 hours later; in group IX, LD-A was given on day 2 as in group III (1 mg/kg IV 10 minutes before the first coronary occlusion). Rabbits in group X received the same dose of LD-A (1 mg/kg IV) and were euthanized 25 minutes later (similar to group VII). In all 7 groups, the heart was

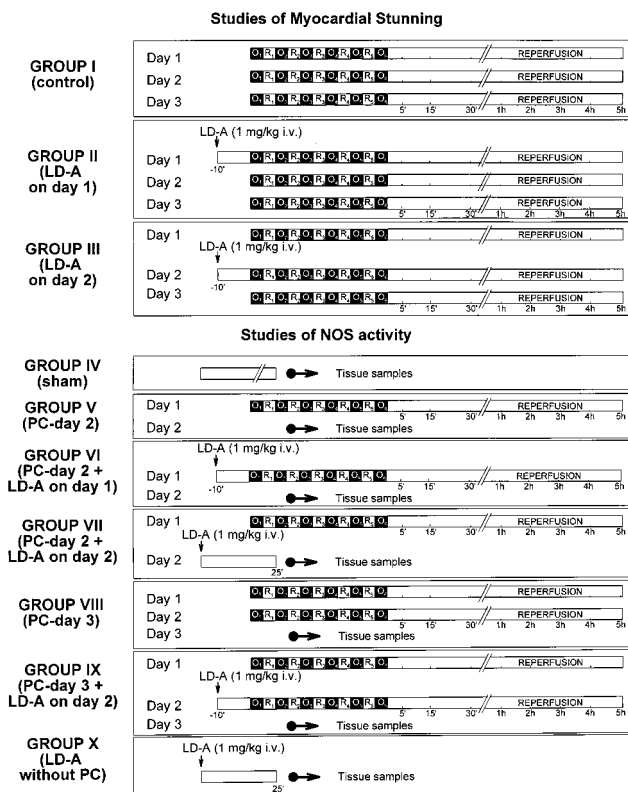


Figure 1. Experimental protocol. In phase A (studies of myocardial stunning), 3 groups of rabbits were studied. All groups underwent a sequence of six 4-minute coronary occlusion (O)/4-minute reperfusion (R) cycles followed by a 5-hour observation period on 3 consecutive days (days 1, 2, and 3). Rabbits in group I ($n=7$, control) received no treatment. On day 1, rabbits in group II ($n=7$, LD-A on day 1) received an intravenous bolus of LD-A (1 mg/kg) 10 minutes before the first coronary occlusion. Rabbits in group III ($n=7$, LD-A on day 2) received the same dose of LD-A 10 minutes before the first coronary occlusion on day 2. In phase B (studies of NOS activity), 7 groups of rabbits were studied. Rabbits in group IV ($n=5$, sham) underwent surgical instrumentation but did not receive O/R; the rabbits were euthanized a minimum of 10 days after surgery. Groups V, VI, and VII underwent 6 cycles of 4-minute O/4-minute R on day 1. Rabbits in group V ($n=5$, PC-day 2) received no treatment and were euthanized 24 hours later. Rabbits in group VI ($n=5$, PC-day 2+LD-A on day 1) received LD-A (1 mg/kg IV) 10 minutes before the first coronary occlusion on day 1 and were euthanized 24 hours later. Rabbits in group VII ($n=6$, PC-day 2+LD-A on day 2) were euthanized 25 minutes after receiving the same dose of LD-A (1 mg/kg IV) on day 2. Rabbits in groups VIII (PC-day 3) and IX (PC-day 3+LD-A on day 2) underwent a sequence of six 4-minute O/4-minute R cycles on days 1 and 2 and were euthanized 24 hours later; in group IX, LD-A was given on day 2 as in group III (1 mg/kg IV 10 minutes before the first coronary occlusion). Rabbits in group X ($n=5$, LD-A without PC) received the same dose of LD-A (1 mg/kg IV) without undergoing ischemic PC and were euthanized 25 minutes later.

excised, and myocardial samples (≈ 0.5 g) were rapidly removed from the ischemic/reperfused and the nonischemic region and frozen in liquid nitrogen.

Measurement of NOS Activity

Tissue samples were homogenized in the appropriate buffer, and cytosolic and membrane fractions were isolated. Ca^{2+} -dependent (cNOS) and Ca^{2+} -independent (iNOS) NOS activities were determined by measuring the conversion of [^{14}C]L-arginine to [^{14}C]-L-

citruilline using a modification of the procedure of Bredt and Snyder.³⁷

Measurement of Nitrite and Nitrate

Tissue nitrite was assayed by using the Griess reaction as modified by Gilliam et al.³⁸ Tissue nitrate was determined after conversion of nitrate to nitrite with *Aspergillus* nitrate reductase.³⁸ All assays were performed in duplicate.

Statistical Analysis

Data are reported as mean \pm SEM. One-way or two-way repeated-measures ANOVA followed by Student's *t* tests for paired or unpaired data with the Bonferroni correction was used, as appropriate.^{39,40}

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

Results

A total of 63 conscious rabbits were used in the present study (2 for the pilot studies, 23 for phase A, and 38 for phase B).

Pilot Studies

Pilot studies were conducted in 2 rabbits to identify a dose of LD-A that has no effect on heart rate, arterial blood pressure, and systolic wall thickening (WTH). The concern was that hemodynamic perturbations caused by LD-A (eg, a fall in blood pressure or an increase in heart rate) could nonspecifically induce a late PC effect unrelated to the ischemic stimulus. Arterial pressure was measured by cannulating the dorsal ear artery with a 22-gauge angiocatheter under local anesthesia (benzocaine), as previously described.^{4,5,12} The IC_{50} of LD-A for receptor PTKs (eg, epidermal growth factor receptor) is $0.011 \mu\text{mol/L}$, while the IC_{50} for nonreceptor PTKs (pp60^{c-src}) is $0.5 \mu\text{mol/L}$.^{33,34} We initially tested a dose of 0.45 mg/kg IV , which was calculated to produce plasma concentrations ≈ 5 times the IC_{50} of LD-A against nonreceptor PTKs. This dose did not have any hemodynamic effect when administered on day 1 (before the PC occlusion/reperfusion protocol) or (in another rabbit) on day 2, before the second ischemic challenge; however, this dose failed to block late PC against stunning. Therefore, we increased the dose to 1 mg/kg (≈ 10 times the IC_{50} for nonreceptor PTKs); this dose did not have any significant effect on blood pressure or heart rate but did block late PC when given on day 1 or day 2. Doses higher than 1 mg/kg would have been prohibitively expensive. Consequently, a dose of 1 mg/kg was chosen for the present experiments.

Phase A: Studies of Myocardial Stunning

Exclusions and Postmortem Analysis

Of the 23 rabbits instrumented for phase A, 7 were assigned to group I (control group), 9 to group II (LD-A on day 1), and 7 to group III (LD-A on day 2). All animals assigned to the control group completed the protocol on days 1, 2, and 3. Of the 9 rabbits assigned to group II, 2 were excluded because of persistent dyskinesia after the sixth reperfusion on day 1 (triphenyltetrazolium chloride staining demonstrated myocardial infarction that was most likely due to malfunction of the occluder), and 1 died on day 3 because of ventricular fibrillation during the fourth occlusion. Therefore, 6 rabbits in group II completed days 1, 2, and 3, whereas 1 rabbit

Heart Rate During Coronary Occlusion and Reperfusion in Phase A

	Heart Rate, bpm										
	Baseline	Preocclusion	First Occlusion	Sixth Occlusion	Reperfusion						
					30 Minutes	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours	
Group I (control)											
Day 1	267±8	260±9	261±11	249±14	258±10	254±13	240±10	237±5	234±5	237±4	
Day 2	264±8	265±9	275±12	256±8	250±10	245±5	252±6	238±7	239±8	235±6	
Day 3	257±5	257±5	268±7	257±10	259±10	248±9	254±5	252±12	243±9	240±8	
Group II (LD-A on day 1)											
Day 1	261±9	254±7	253±6	251±6	263±16	253±7	260±15	255±13	252±16	258±15	
Day 2	242±7	242±7	253±11	234±7	241±11	242±8	237±12	230±8	229±9	219±7	
Day 3	246±16	246±16	246±14	233±13	247±11	231±9	240±9	236±9	241±8	235±10	
Group III (LD-A on day 2)											
Day 1	241±12	241±12	239±8	227±4	218±7	223±7	217±7	220±9	219±6	225±8	
Day 2	243±9	254±12	253±11	242±10	234±7	231±8	231±8	231±12	236±12	227±12	
Day 3	254±12	254±12	251±13	229±9	237±10	249±9	227±11	227±3	228±12	225±14	

Values are mean±SEM. Rabbits were subjected to a sequence of 6 cycles of 4-minute coronary occlusion/4-minute coronary reperfusion followed by a 5-hour observation period. On day 1, rabbits in group II (LD-A on day 1, n=7) received an intravenous bolus of LD-A (1 mg/kg, total volume 1 mL/kg) 10 minutes before the first occlusion. On day 2, rabbits in group III received an intravenous bolus of LD-A (same dose and volume as on day 1 in group II) 10 minutes before the first occlusion. Heart rate was measured before treatment (baseline), 9 minutes after treatment (preocclusion), at 3 minutes into the first coronary occlusion, at 3 minutes into the sixth coronary occlusion, and at selected times after the sixth reperfusion.

completed only days 1 and 2. Of the 7 rabbits assigned to group III, 1 died of ventricular fibrillation on day 3 during the fifth occlusion. Therefore, 6 rabbits in group III completed days 1, 2, and 3.

Postmortem analysis showed that the size of the occluded/reperfused vascular bed was similar in the 3 groups: 0.79±0.13 g (17.5±2.6% of left ventricular [LV] weight) in group I, 0.83±0.09 g (16.1±1.5% of LV weight) in group II, and 0.84±0.07 g (15.8±1.6% of LV weight) in group III. Tissue staining with triphenyltetrazolium chloride confirmed the absence of infarction in all animals included in the final analysis. In all rabbits, the ultrasonic crystal was found to be at least 3 mm from the boundaries of the ischemic/reperfused region.

Regional Myocardial Function

As shown in the Table, there were no appreciable differences in heart rate among the 3 groups, either during the sequence of coronary occlusion/reperfusion cycles or during the 5-hour reperfusion period. These results are in agreement with our pilot studies and confirm that the dose of LD-A selected in this study has no effect on hemodynamic variables in conscious rabbits. Baseline systolic thickening fraction in the region to be rendered ischemic averaged 35.0±4.9%, 33.8±4.0%, and 34.4±4.2% on days 1, 2, and 3, respectively, in group I; 41.0±3.2%, 40.2±3.8%, and 39.7±4.1% in group II; and 38.5±3.8%, 37.8±3.4%, and 37.7±4.3% in group III (Figures 2, 3, and 4). There were no significant differences among the 3 groups on the same day or among different days within the same group. In group II, thickening fraction on day 1 was 41.0±4.2% at baseline and 39.4±3.4% after administration of LD-A (preocclusion) (P=NS, Figure 3), indicating that this agent had no significant effect on regional myocardial function. This conclusion is further corroborated by the results in group III, in which thickening fraction on day 2 was

37.8±3.4% at baseline and 37.7±3.6% after administration of LD-A (P=NS, Figure 4).

Group I (Control)

On day 1, thickening fraction remained significantly (P<0.05) depressed for 3 hours after the sixth reperfusion and recovered by 5 hours (Figure 2), indicating that the sequence of six 4-minute occlusion/4-minute reperfusion

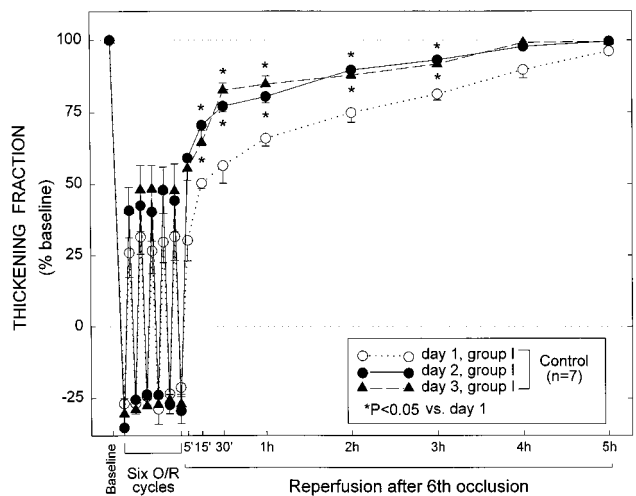


Figure 2. Systolic thickening fraction in the ischemic/reperfused region in the control group (group I) at baseline, 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. Measurements taken on day 1 are represented by the dashed line with open circles, measurements taken on day 2 are represented by the continuous line with solid circles, and measurements taken on day 3 are represented by the interrupted line with solid triangles (n=7 for all 3 days). Thickening fraction is expressed as a percentage of baseline values. Data are mean±SEM.

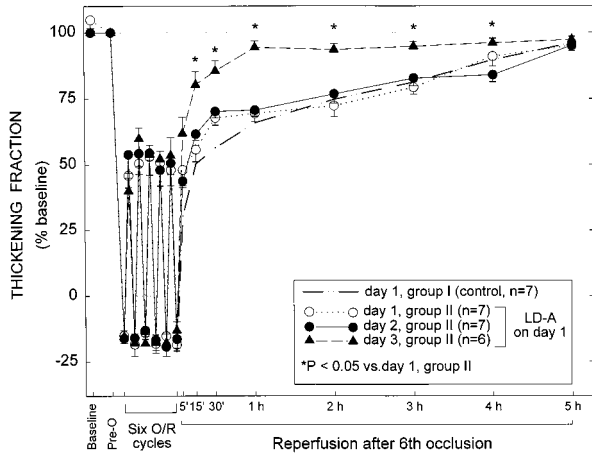


Figure 3. Systolic thickening fraction in the ischemic/reperfused region in group II (LD-A on day 1) before administration of LD-A (baseline), 9 minutes after LD-A (immediately before the first occlusion, preocclusion [Pre-O]), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. Measurements taken on day 1 are represented by the dashed line with open circles (n=7), measurements taken on day 2 are represented by the continuous line with solid circles (n=7), and measurements taken on day 3 are represented by the interrupted line with solid triangles (n=6). To facilitate comparisons, the data pertaining to day 1 of group I (control group) are also shown (thick interrupted line without symbols, n=7). Thickening fraction is expressed as a percentage of Pre-O values. Data are mean±SEM.

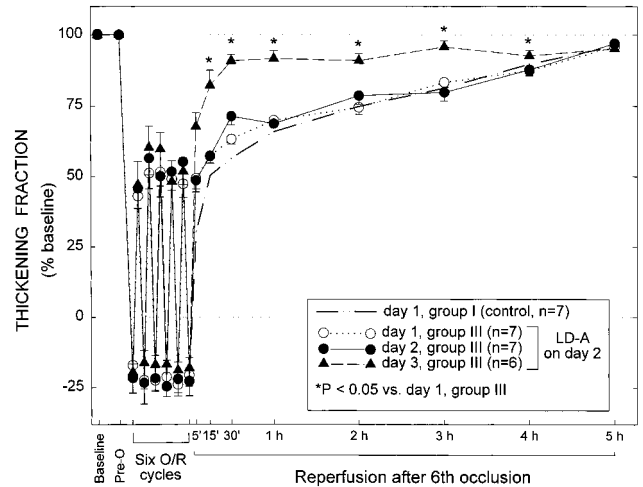


Figure 4. Systolic thickening fraction in the ischemic/reperfused region in group III (LD-A on day 2) at baseline, 9 minutes after LD-A (immediately before the first occlusion, Pre-O), 3 minutes into each coronary occlusion (O), 3 minutes into each reperfusion (R), and at selected times during the 5-hour reperfusion interval after the sixth occlusion. Measurements taken on day 1 are represented by the dashed line with open circles (n=7), measurements taken on day 2 are represented by the dashed line with solid circles (n=7), and measurements taken on day 3 are represented by the interrupted line with solid triangles (n=6). To facilitate comparisons, the data pertaining to day 1 of group I (control group) are also shown (thick interrupted line without symbols, n=7). Thickening fraction is expressed as a percentage of Pre-O values. Data are mean±SEM.

cycles resulted in severe myocardial stunning that lasted, on average, 4 hours. On days 2 and 3, however, the recovery of WTh was markedly improved after the 6 occlusion/reperfusion cycles compared with day 1 (Figure 2). The total deficit of WTh after the sixth reperfusion was 52% and 53% less on days 2 and 3, respectively, compared with day 1 ($P < 0.01$) (Figure 5). Thus, as expected,^{4,5,12,41} myocardial stunning was attenuated markedly, and to a similar extent, on days 2 and 3 compared with day 1.

Group II (LD-A on Day 1)

On day 1, the recovery of WTh (Figure 3) and the total deficit of WTh (Figure 5) were similar to values observed in the

control group, indicating that LD-A had no appreciable effect on the severity of myocardial stunning in nonpreconditioned myocardium. On day 2, however, the recovery of WTh during the 5-hour period after the sixth reperfusion was not improved compared with day 1 (Figure 3), and the total deficit of WTh on day 2 was not significantly different from that observed on day 1 (Figure 5). The total deficit of WTh on day 2 was 90% greater than the corresponding value in control rabbits ($P < 0.01$) and similar to that observed in control rabbits on day 1 (Figure 5). On day 3, the recovery of WTh in LD-A-treated rabbits was markedly improved compared with day 2 (Figure 3) and was similar to that noted on day 2

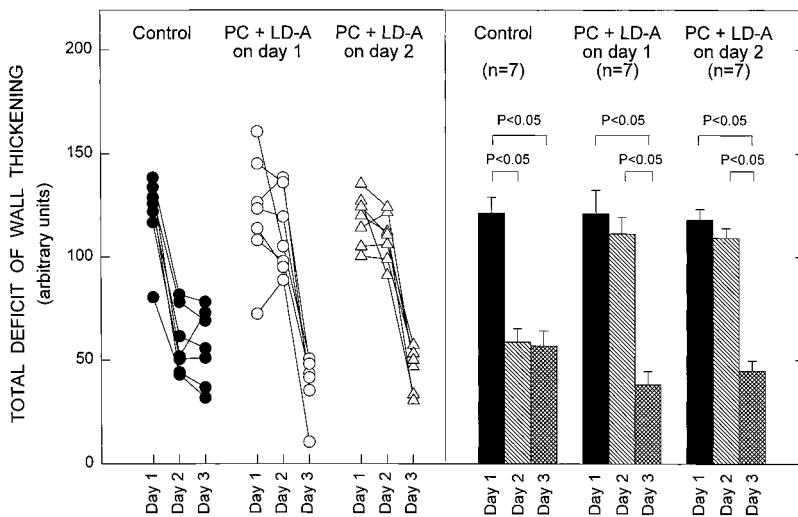


Figure 5. Total deficit of WTh after the sixth reperfusion on days 1, 2, and 3 in groups I, II, and III (control [n=7], LD-A on day 1 [n=7], and LD-A on day 2 [n=7] groups, respectively). In groups II and III, only 6 of the 7 rabbits were studied on day 3 (see text). The values of total deficit of WTh in individual rabbits are illustrated in the left panel; the mean±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.

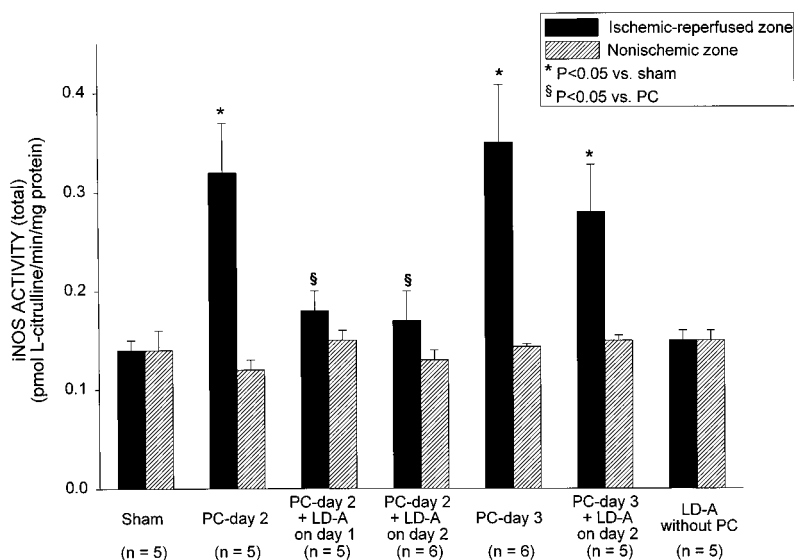


Figure 6. Ca^{2+} -independent NOS (iNOS) activity in the homogenate (cytosolic+membranous fractions) of myocardial samples obtained from rabbits that underwent surgical instrumentation but did not receive coronary occlusion/reperfusion (sham controls [group IV]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute coronary occlusion/reperfusion cycles (PC-day 2 [group V]), from rabbits subjected 24 hours earlier to six 4-minute coronary occlusion/reperfusion cycles after pretreatment with 1 mg/kg IV LD-A (PC-day 2+LD-A on day 1 [group VI]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute coronary occlusion/reperfusion cycles and given the same dose of LD-A on the second day 25 minutes before euthanasia (PC-day 2+LD-A on day 2 [group VII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2 and euthanized on day 3 (PC-day 3 [group VIII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2, given LD-A on day 2 (same dose as group VII), and euthanized on day 3 (PC-day 3+LD-A on day 2 [group IX]), and from rabbits

subjected to surgical instrumentation but not to coronary occlusion/reperfusion and given the same dose of LD-A 25 minutes before euthanasia (LD-A without PC [group X]). iNOS activity was measured as L-NAME-inhibitable L-citrulline production in the absence of Ca^{2+} , as described in Materials and Methods. Ischemic PC resulted in a robust increase in iNOS activity in the ischemic/reperfused region on day 2, which was suppressed by administration of LD-A either on day 1 or on day 2. Administration of LD-A on day 2 had no effect on the increase in iNOS activity on day 3. Data are mean \pm SEM.

in the control group (Figure 2). The total deficit of WTh was 66% less than that noted on day 2 in the same animals ($P < 0.01$) and was comparable to that noted on day 2 in control rabbits (Figure 5). Thus, the sequence of 6 coronary occlusions and reperfusion performed on day 1 after the administration of LD-A failed to induce late PC against stunning on day 2, but the same sequence performed on day 2 did precondition against stunning on day 3.

Group III (LD-A on Day 2)

On day 1, the recovery of regional function was similar to that observed in the control group (Figures 4 and 5). On day 2, however, both the recovery of WTh (Figure 4) and the total deficit of WTh (Figure 5) were similar to values observed on day 1. The total deficit of WTh on day 2 was 86% greater than the corresponding value in control rabbits ($P < 0.01$) and was similar to that observed in control rabbits on day 1 (Figure 5). Thus, administration of LD-A on day 2 completely abrogated the protective effects of late PC. On day 3, the recovery of WTh was markedly improved compared with day 2 (Figure 4) and was similar to that observed on day 3 in the control group (Figure 2). The total deficit of WTh was 59% less than that noted on day 2 in the same animals ($P < 0.01$) and was comparable to that noted on day 3 in control rabbits (Figure 5). Thus, administration of LD-A before the sequence of 6 coronary occlusions and reperfusion on day 2 completely abolished the late PC effect against stunning that was induced by the ischemic stimulus on day 1. The significant improvement in the recovery of WTh noted on day 3 in these rabbits indicates that LD-A in itself did not have any delayed deleterious effects on myocardial stunning.

Phase B: Studies of NOS Activity

Having observed in phase A that late PC is blocked by the administration of LD-A on day 1 as well as on day 2, in phase

B we investigated whether PTKs participate in late PC by modulating iNOS activity.

Exclusions

Of the 38 rabbits instrumented for phase B, 5 were assigned to groups IV, V, VI, IX, and X, 6 to group VIII, and 7 to group VII. One rabbit in group VII was excluded from analysis because of probable infarction.

Effect of Ischemic PC on NOS Activity and Myocardial Nitrite+Nitrate (NO_x) Levels

As detailed in Materials and Methods, NOS activity was measured as N^G -nitro-L-arginine methyl ester (L-NAME)-inhibitable L-citrulline production. In group V (ischemic PC-day 2), the sequence of 6 cycles of 4-minute occlusion/4-minute reperfusion on day 1 resulted in a robust induction of iNOS activity 24 hours later (on day 2). In these rabbits, total (cytosolic+membranous fractions) Ca^{2+} -independent NOS activity (iNOS activity) in the ischemic/reperfused region was 167% higher than the activity in the nonischemic region ($P < 0.05$) and 119% higher than the activity in the anterior LV wall in sham control rabbits (group IV) ($P < 0.05$) (Figure 6). The increase in iNOS activity was more pronounced in the cytosolic fraction (+233%, $P < 0.05$) than in the membranous fraction (+100%, $P < 0.05$). No significant changes in iNOS activity were observed in the nonischemic region (Figure 6). In contrast to iNOS activity, Ca^{2+} -dependent NOS (endothelial NOS and/or neuronal NOS) activity in the ischemic/reperfused region did not change appreciably 24 hours after ischemic PC (on day 2), either in the total homogenate (cytosolic+membranous fractions) (Figure 7) or in the individual (cytosolic and membranous) tissue fractions (data not shown). The changes in iNOS activity were associated with directionally concordant changes in the tissue levels of nitrite+nitrate (NO_x), the stable oxidation products of NO: as illustrated in Figure 8, in group V total (cytosolic+membranous fractions) myocardial NO_x levels in the ischemic/reperfused region were significantly increased 24 hours after ischemic PC (day 2)

(+62% versus the nonischemic region [$P<0.05$] and +43% versus the anterior LV wall in group IV [sham controls] [$P<0.05$]).

In group VIII (ischemic PC-day 3), rabbits were subjected to a sequence of six 4-minute occlusion/reperfusion cycles on day 1 and to another sequence on day 2; 24 hours later (on day 3), the increases in iNOS activity (Figure 6) and NO_x levels (Figure 8) were similar to those measured on day 2 in group V (ischemic PC-day 2). No change in cNOS activity was noted on day 3 (Figure 7). Thus, myocardial iNOS activity and NO_x levels were augmented both on day 2 and on day 3, indicating sustained upregulation of iNOS by late PC.

Effect of LD-A on NOS Activity and Myocardial NO_x

In the absence of ischemic PC (group X), LD-A did not have any appreciable effect on iNOS activity (Figure 6), cNOS activity (Figure 7), or NO_x levels (Figure 8). When LD-A was administered before the PC ischemia on day 1 (group VI), iNOS activity and NO_x levels in the ischemic/reperfused region 24 hours later (day 2) were not significantly different from those measured in the nonischemic region in the same group and in the anterior LV wall of sham control rabbits (group IV) (Figures 6 and 8). Both iNOS activity and NO_x levels were significantly less in preconditioned rabbits treated with LD-A on day 1 (group VI) compared with untreated preconditioned rabbits (group V) (Figures 6 and 8). Thus, pretreatment with LD-A completely abrogated the ischemic PC-induced increase in iNOS activity and NO_x levels observed 24 hours later (day 2) in group V.

When LD-A was administered on day 2 (group VII), iNOS activity and NO_x levels in the ischemic/reperfused region 25 minutes after LD-A did not differ significantly from those measured in the anterior LV wall in sham control rabbits (group IV) and were significantly less ($P<0.05$) than those measured in rabbits subjected to ischemic PC (group V) (Figures 6 and 8). Thus, administration of LD-A 24 hours after the initial PC ischemia (day 2) markedly suppressed the ischemic PC-induced increase in iNOS activity and abrogated the ischemic PC-induced increase in myocardial NO_x levels on day 2. In contrast, administration of LD-A on day 2 had no significant effect on the ischemic PC-induced increase in iNOS activity and NO_x levels observed 24 hours later (on day 3) (group IX).

Administration of LD-A either on day 1 or on day 2 had no discernible effect on cNOS activity (Figure 7).

Discussion

Although previous studies have identified the initial components of the signal transduction pathway that underlies late PC, including NO,⁴⁻⁷ reactive oxygen species,^{9,10} and PKC,¹¹⁻¹⁴ the mechanisms downstream from these molecules are poorly understood. Recent studies have shown that ischemic PC activates Src and Lck PTKs,²³ but the functional significance of this observation remains unknown. In particular, no information is available regarding whether PTKs are causally involved in late PC against myocardial stunning and, if so, whether they participate in the development of this adaptive phenomenon on day 1 and/or in the manifestation of its cardioprotective effects on day 2. Furthermore, no infor-

mation is available regarding the mechanism whereby PTKs contribute to late PC.

Salient Findings

The present investigation provides significant new insights regarding these issues. Using conscious rabbits, we found that administration of LD-A before the ischemic PC stimulus (on day 1) completely blocked the development of protection against myocardial stunning 24 hours later, indicating that PTK activity is necessary to trigger this mechanism. The protective effects of late PC against stunning were also abrogated when LD-A was administered before ischemia/reperfusion on day 2, indicating that PTK activity is also necessary to mediate this phenomenon. To our knowledge, this is the first indication that PTKs are involved in the manifestation of protection during the late phase of ischemic PC (on day 2). Finally, administration of LD-A either on day 1 or on day 2 abrogated the increase in myocardial iNOS activity and NO_x levels on day 2, indicating that the upregulation of iNOS (which mediates the cardioprotective effects of late PC^{1,5,7,8}) occurs via at least two PTK-dependent pathways, one that is operative on day 1 and one that is active on day 2.

Previous studies have implicated PTKs in early¹⁸⁻²¹ and late²² PC against myocardial infarction. To our knowledge, this is the first study to demonstrate that PTKs play an obligatory role in the development of late PC against myocardial stunning. This is also the first study (1) to identify two distinct functions for PTKs in late PC against stunning (ie, PTKs are required both to trigger this phenomenon on day 1 and to mediate it on day 2), (2) to directly demonstrate that iNOS activity is upregulated during the late phase of ischemic PC in rabbits, (3) to indicate that this upregulation of iNOS activity requires PTK activity, and (4) to examine the effect of PTK inhibitors on ischemic PC in conscious animals. The finding that cardiac iNOS upregulation is PTK dependent reveals a new signaling mechanism and a new function for PTKs, which has significant implications for many pathophysiological processes besides ischemic PC.

Rationale for Selecting LD-A

There were several reasons for selecting LD-A instead of genistein as a PTK inhibitor. First, the selectivity of LD-A for PTKs versus other kinases is superior to that of genistein. The IC₅₀ ratio of genistein for nonreceptor PTKs versus protein kinase A/PKC has been found to be 1:10 to 1:14,^{34,42} whereas LD-A is a highly selective inhibitor of PTKs, with an IC₅₀ of 0.011, 0.5, and >100 μmol/L for receptor PTKs, nonreceptor PTKs, and serine-threonine kinases (including PKC, protein kinase A, and calmodulin-dependent kinases), respectively.^{33,34} Thus, the IC₅₀ ratio for nonreceptor PTKs versus PKC is <1:200 for LD-A³⁴ compared with <1:10 for genistein. The dose of LD-A selected for the present study (1 mg/kg) was calculated to result in peak plasma concentrations of 4.5 μmol/L, ≈10 times higher than the IC₅₀ for Src PTKs (0.5 μmol/L).^{33,34} Because in intact organisms LD-A is redistributed after intravenous injection, the plasma and tissue levels present during the 6 occlusion/reperfusion cycles 10 to 54 minutes after the intravenous bolus of LD-A should have been considerably lower, making it very

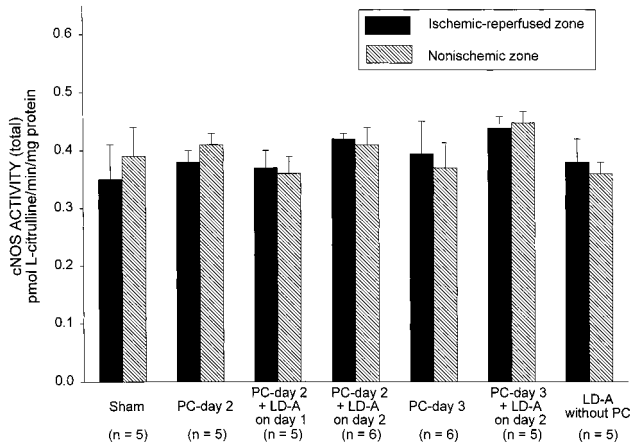


Figure 7. Ca^{2+} -dependent NOS (cNOS [endothelial NOS and/or neuronal NOS]) activity in the homogenate (cytosolic + membranous fractions) of myocardial samples obtained from rabbits that underwent surgical instrumentation but did not receive coronary occlusion/reperfusion (sham controls [group IV]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute coronary occlusion/reperfusion cycles (PC-day 2 [group V]), from rabbits subjected 24 hours earlier to six 4-minute coronary occlusion/reperfusion cycles after pretreatment with 1 mg/kg IV LD-A (PC-day 2+LD-A on day 1 [group VI]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute occlusion/reperfusion cycles and given the same dose of LD-A on the second day 25 minutes before euthanasia (PC-day 2+LD-A on day 2 [group VII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2 and euthanized on day 3 (PC-day 3 [group VIII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2, given LD-A on day 2 (same dose as group VII), and euthanized on day 3 (PC-day 3+LD-A on day 2 [group IX]), and from rabbits subjected to surgical instrumentation but not to coronary occlusion/reperfusion and given the same dose of LD-A 25 minutes before euthanasia (LD-A without PC [group X]). cNOS activity was measured as L-NAME-inhibitable L-citrulline production in the presence of Ca^{2+} and calmodulin, as described in Materials and Methods. Neither ischemic PC nor LD-A had any effect on cNOS activity. Data are mean \pm SEM.

unlikely that the observed inhibition of late PC could be ascribed to inhibition of PKC or other serine-threonine kinases.

The second advantage of LD-A over genistein is that it is much more selective for the Src family of PTKs. Genistein and other flavonoids are general inhibitors of PTKs with little specificity for individual enzymes.⁴³ In contrast, LD-A preferentially inhibits two families of PTKs, epidermal growth factor receptor kinases and Src PTKs.^{33,34} In view of the fact that previous studies in our conscious rabbit model have shown that ischemic PC activates Src PTKs but has no effect on epidermal growth factor receptor PTKs,³⁵ LD-A would appear to be a useful tool for interrogating the Src family of PTKs. The third reason for selecting LD-A was that this agent (at the same dose used in the present study) has been documented to block Src PTK activation during ischemic PC.³⁵ In view of these facts, the actions of LD-A documented in the present study suggest a critical role of the Src family of PTKs both in the development of late PC on day 1 and in the manifestation of its cardioprotective effects on day 2.

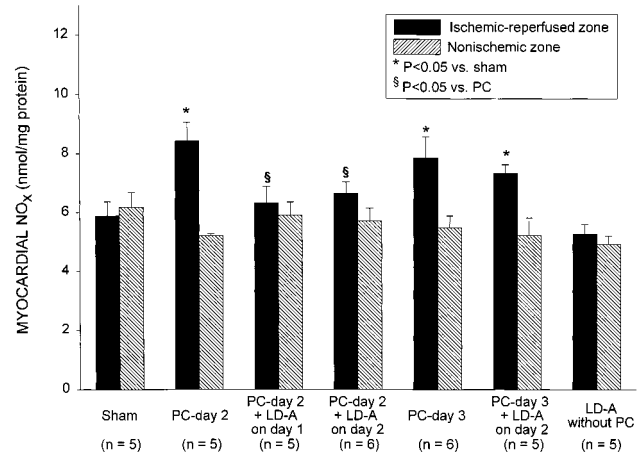


Figure 8. Total (cytosolic+membranous fractions) myocardial content of NO_x in myocardial samples obtained from rabbits that underwent surgical instrumentation but did not receive coronary occlusion/reperfusion (sham controls [group IV]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute coronary occlusion/reperfusion cycles (PC-day 2 [group V]), from rabbits subjected 24 hours earlier to six 4-minute coronary occlusion/reperfusion cycles after pretreatment with 1 mg/kg IV LD-A (PC-day 2+LD-A on day 1 [group VI]), from rabbits subjected 24 hours earlier (day 1) to six 4-minute occlusion/reperfusion cycles and given the same dose of LD-A on the second day 25 minutes before euthanasia (PC-day 2+LD-A on day 2 [group VII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2 and euthanized on day 3 (PC-day 3 [group VIII]), from rabbits subjected to six 4-minute occlusion/reperfusion cycles on day 1 and on day 2, given LD-A on day 2 (same dose as group VII), and euthanized on day 3 (PC-day 3+LD-A on day 2 [group IX]), and from rabbits subjected to surgical instrumentation but not to coronary occlusion/reperfusion and given the same dose of LD-A 25 minutes before euthanasia (LD-A without PC [group X]). Nitrite was measured by using the Griess reaction; nitrate was determined after conversion to nitrite as described in Materials and Methods. Ischemic PC resulted in a significant increase in myocardial NO_x levels, which was abrogated when LD-A was administered before ischemic PC (day 1) or 24 hours later (day 2). Administration of LD-A on day 2 had no effect on the increase in NO_x levels on day 3. Data are mean \pm SEM.

Role of PTKs in the Development of Late PC (Day 1)

Recent studies in conscious rabbits have identified a pivotal role of the transcription regulatory protein nuclear factor (NF)- κ B in the development of late PC on day 1.⁴¹ These studies have also found that the activation of NF- κ B by ischemic PC was prevented by pretreatment with LD-A (given at the same dose that was used in the present investigation), indicating that the recruitment of this transcription factor requires PTK signaling.⁴¹ However, the mechanism whereby PTK-dependent recruitment of NF- κ B produces delayed cardioprotection remained unknown. The present findings that pretreatment with LD-A on day 1 abrogated the increase in iNOS activity and tissue NO_x levels 24 hours later (Figures 6 and 8) indicate that PTK signaling on day 1 is required for the upregulation of iNOS on day 2. NF- κ B is known to be a central mechanism controlling iNOS induction,^{44,45} and mounting evidence indicates that the late phase of PC is mediated by iNOS activity.^{5,7,8} Thus, based on the fact that the mobilization of PTKs shortly after the initial PC stimulus (on day 1) is essential not only for the activation of NF- κ B, as shown previously,⁴¹ but also for the increase in iNOS

activity and for the acquisition of cardioprotection 24 hours later, as shown in the present study (Figures 5, 6, and 8), we propose that PTKs participate in the genesis of late PC by promoting iNOS transcription via an NF- κ B-dependent pathway.

This paradigm is consistent with emerging evidence in non-cardiac cells, in which tyrosine phosphorylation has been found to be necessary for NF- κ B mobilization. For example, the PTK inhibitors herbimycin A and genistein can block hypoxia-induced phosphorylation of inhibitory κ B α (I κ B α) on tyrosine residues and the consequent activation of NF- κ B in Jurkat T cells.⁴⁶ Recently, it has been shown that tyrosine phosphorylation of I κ B α induced by the phosphatase inhibitor pervanadate promotes NF- κ B DNA-binding activity without degradation of I κ B α .⁴⁷ In that study, a PTK of the Src family (Lck) was proven essential for pervanadate-induced I κ B α tyrosine phosphorylation and NF- κ B activation.⁴⁷ Interestingly, Lck is activated by ischemic PC in conscious rabbits, and the activation is blocked by LD-A, given at the same dose as in the present study,³⁵ raising the possibility that this specific kinase may be involved in the ischemic PC-induced recruitment of NF- κ B.

In the present study, iNOS activity was augmented not only on day 2 (group V) but also on day 3 (group VIII), demonstrating sustained upregulation of this enzyme during late PC. The mechanism for the increased iNOS activity of day 3 appears to be different from that on day 2, in view of the fact that the administration of LD-A on day 2 had no significant effect on myocardial stunning (Figures 4 and 5), iNOS activity (Figure 6), and NO_x levels (Figure 8) 24 hours later (on day 3) (group III and IX). Thus, although the protection against stunning and the concomitant increase in iNOS activity observed on day 2 require PTK signaling on day 1 (groups II and VI), it appears that neither the protection against stunning nor the concomitant upregulation of iNOS activity observed on day 3 is dependent on PTK signaling on day 2 (groups III and IX). These results are congruent with the finding that, in conscious rabbits, a single sequence of six 4-minute occlusion/reperfusion cycles elicits a state of PC against stunning that lasts for 72 hours even when no additional ischemic PC stimuli are applied.⁴⁸ Together with this previous study,⁴⁸ the present results suggest that both the preconditioned state and the rise in iNOS activity seen on day 3 are induced by the initial PC stimulus on day 1 and, therefore, do not require any signaling events on day 2.

Role of PTKs in the Mediation of Late PC (Day 2)

A novel finding of the present study was that LD-A abolished the cardioprotective effects of late PC when given on day 2, after the preconditioned state had developed, revealing a role of PTK signaling 24 hours after the initial ischemic stress. To our knowledge, this is the first indication that the activity of a cellular kinase is involved in the mediation (as opposed to the triggering) of delayed protection after a PC stimulus. This finding impels a reassessment of current paradigms, because it implies that the augmented iNOS activity that underlies the late phase of PC does not result exclusively from increased iNOS transcription⁴⁹ but must also involve other mechanisms. Specifically, the fact that both the increase in myocardial iNOS activity (Figure 6) and the concomitant increase in NO_x levels (Figure 8) were abrogated by the administration of LD-A on day 2 suggests that in addition to the synthesis of

new iNOS proteins,⁵⁰ posttranslational modulation of iNOS proteins via tyrosine phosphorylation is also critical in activating this enzyme and conferring enhanced tolerance to myocardial ischemia/reperfusion injury. Thus, a complex scenario emerges from the present observations, in which PTK signaling elicited by brief ischemia upregulates iNOS both via increased transcription (day 1) and via posttranslational modulation (day 2).

The concept that tyrosine phosphorylation is required for stress-induced iNOS proteins to be protective reveals a new function of PTKs in cardiac pathophysiology. This concept is congruent with a previous study⁵¹ in which tyrosine phosphorylation of iNOS in murine macrophages was found to be associated with increased iNOS activity. Further studies will be needed to elucidate the mechanism whereby PTK activity is enhanced on day 2 and to identify the specific kinase(s) involved.

Conclusions

The present study provides new insights into the role of PTKs in cardioprotection. The results of phase A demonstrate that, in conscious rabbits, PTKs perform two distinct functions within the signal transduction pathway that underlies late PC against myocardial stunning: on day 1, they are essential for the development of the late PC effect, whereas on day 2, they are essential for its manifestation. The results of phase B are the first direct evidence that iNOS activity is enhanced during late PC in rabbits. Furthermore, phase B provides a mechanistic basis for the results of phase A, in that it indicates that PTKs are involved in the modulation of cardioprotective iNOS activity at two distinct levels: at an early stage on day 1 and at a late stage on day 2. This heretofore unrecognized bifunctional role of PTKs suggests a new pathophysiological paradigm for the late phase of ischemic PC and has broad implications for our understanding of the signaling mechanisms that underlie the response of the heart to ischemic stress and, possibly, other stresses. The present findings support the concept that tyrosine phosphorylation serves as a critical regulatory mechanism for stress-activated gene upregulation in the heart.

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