

## Demonstration of Selective Protein Kinase C-Dependent Activation of Src and Lck Tyrosine Kinases During Ischemic Preconditioning in Conscious Rabbits

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**Abstract**—Src tyrosine kinases have been shown to mediate cellular responses to stress in noncardiac cells. However, the effect of myocardial ischemia on Src tyrosine kinases is unknown. Furthermore, the identity of the tyrosine kinase(s) involved in the genesis of ischemic preconditioning (PC) remains obscure. Here, we present the first evidence that ischemic PC (6 cycles of 4-minute coronary occlusion and 4-minute reperfusion) induces selective activation of 2 members of the Src family of tyrosine kinases, Src and Lck, in the heart of conscious rabbits. The activation of Src in the particulate fraction was not evident at 5 minutes after ischemic PC but became apparent at 30 minutes (+119% versus control), whereas the activation of Lck in the particulate fraction was apparent both at 5 minutes (+103% versus control) and at 30 minutes (+89%) after ischemic PC. The activity of the other 5 members of the Src tyrosine kinases expressed in the rabbit heart (Fyn, Fgr, Yes, Lyn, and Blk) was not affected by ischemic PC. Ischemic PC had no effect on the activity of epidermal growth factor receptor kinases, either at 5 or at 30 minutes. The activation of Src and Lck was completely abrogated by the tyrosine kinase inhibitor lavendustin A, given at doses that have previously been shown to block the protective effect of ischemic PC in this same conscious rabbit model, suggesting that Src and Lck kinases are essential for the development of ischemic PC. The activity of the  $\epsilon$  isoform of protein kinase C (PKC) in the particulate fraction increased at 5 minutes (+72%) and at 30 minutes (+67%) after ischemic PC. Pretreatment with lavendustin A had no effect on the activation of PKC $\epsilon$ , whereas pretreatment with the PKC inhibitor chelerythrine (given at doses that have previously been shown to block ischemic PC) blocked not only the activation of PKC $\epsilon$  but also that of Src and Lck, indicating that Src and Lck are downstream of PKC $\epsilon$  in the signaling cascade of ischemic PC. This study identifies a new component of the signaling mechanism of ischemic PC. The results support the concept that, in conscious rabbits, 2 specific members of the Src family of tyrosine kinases, Src and Lck, play an important role in the genesis of late PC by serving as downstream elements of PKC-mediated signal transduction. (*Circ Res.* 1999;85:542-550.)

**Key Words:** PKC $\epsilon$  ■ cardiac signaling ■ chelerythrine ■ lavendustin A ■ myocardial stunning ■ myocardial infarction

Although it is well established that ischemic preconditioning (PC) exerts powerful cardioprotective effects,<sup>1-17</sup> the signaling mechanisms that underlie this phenomenon are not fully understood. Considerable evidence indicates that activation of protein kinase C (PKC) plays an important role in the development of ischemic PC<sup>9,17-24</sup> and that the  $\epsilon$  isoform of PKC is specifically involved as an upstream signaling element.<sup>19,21-23</sup> Nevertheless, the downstream effectors of the signaling transduction pathway in which PKC operates remain poorly defined. Recent studies have demonstrated that genistein, a general tyrosine kinase inhibitor, blocks the development of ischemic PC, which implicates the involvement of tyrosine kinase(s).<sup>1,7,12,25</sup> However, >1000 different tyrosine kinases have been identified so far, which have

different subcellular localizations, different substrates, and most importantly, different biological functions.<sup>26,27</sup> It is unknown which specific family of tyrosine kinases and which specific kinase(s) is involved in ischemic PC. In noncardiac cells, the Src family of tyrosine kinases has been shown to serve as a downstream signaling target for PKC.<sup>28-33</sup> We therefore postulated that Src tyrosine kinases might be likely candidates for the mediation of PKC-dependent signaling during ischemic PC. This hypothesis is supported by recent reports<sup>1,34</sup> demonstrating that ischemic PC is blocked by lavendustin A (LD-A), a more selective tyrosine kinase inhibitor with high affinity for the Src family of tyrosine kinases.<sup>35-37</sup> At present, however, there is virtually no information regarding the role of the Src family of tyrosine kinases

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either in myocardial ischemia/reperfusion or in the development of ischemic PC.

The overall goal of the present study was to test the hypothesis that Src tyrosine kinases participate in the PKC-dependent signaling events that underlie the development of ischemic PC in vivo. Using conscious, chronically instrumented rabbits, we specifically sought to determine the following: (1) whether ischemic PC is associated with activation of the Src family of tyrosine kinases, (2) which of the 9 members of this superfamily (Fyn, Yrk, Fgr, Yes, Src, Lyn, Hck, Lck, and Blk) is/are activated during ischemic PC, (3) whether the activation of Src kinase(s) is blocked by the selective PKC inhibitor chelerythrine (CHE) (given at the same doses that block the activation of PKC $\epsilon$  and the development of the late phase of ischemic PC<sup>21,23</sup> in this model), and (4) whether the same dose of LD-A that blocks late PC in this model<sup>34</sup> also blocks the activation of Src tyrosine kinase(s). The role of Src tyrosine kinases was systematically interrogated by measuring the kinase-specific phosphorylation activity of each individual member of this family. Because LD-A also inhibits the activity of the epidermal growth factor (EGF) receptor tyrosine kinases,<sup>35,36,38–41</sup> we examined the effect of ischemic PC on the activity of these kinases as well. All studies were conducted in conscious animals in an effort to obviate any potential interference of factors associated with open-chest preparations.<sup>42–45</sup>

## Materials and Methods

The present study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and with the *Guide for the Care and Use of Laboratory Animals* (Department of Health and Human Services, Publication No. [NIH] 86-23).

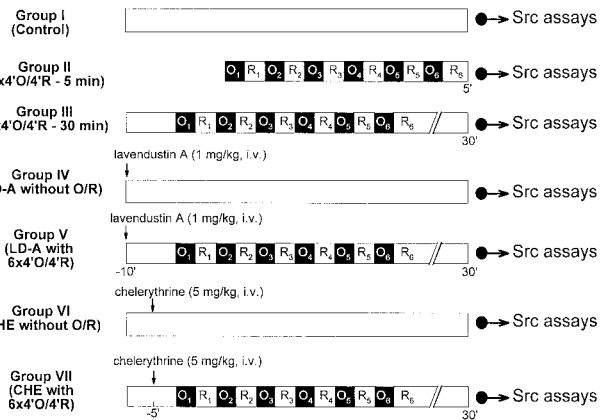
### Studies in Conscious Rabbits

#### Experimental Preparation

The conscious rabbit model of ischemic PC has been described in detail previously.<sup>4,5,13,14,16,21,23</sup> Briefly, New Zealand White male rabbits (2.0 to 2.5 kg) were instrumented under sterile conditions with a balloon occluder around a major branch of the left coronary artery, a 10-MHz pulsed ultrasonic crystal in the region to be rendered ischemic, and ECG leads on the chest wall. The chest wound was closed in layers, and a small tube was left in the thorax for 3 days to aspirate air and fluids. Gentamicin was administered before surgery and on the first and second postoperative days (0.7 mg/kg IM each day). The animals were allowed to recover for a minimum of 10 days after surgery. Throughout the experiments, the rabbits were kept in a cage in a quiet, dimly lit room. Left ventricular systolic wall thickening, the range gate depth, and the ECG were continuously recorded on a thermal array chart recorder (Gould TA6000). Coronary artery occlusion was produced by inflating the balloon occluder. The performance of successful occlusions was verified by observing the appearance of ST-segment elevation and the widening of the QRS complex on the ECG and the development of paradoxical systolic wall thinning on the ultrasonic crystal recordings. Successful reperfusion was documented by the normalization of the ECG and by the resumption of active systolic wall thickening. No sedative or antiarrhythmic agents were given at any time.

#### Experimental Protocol

Rabbits were assigned to 7 groups (Figure 1). Group I (control) did not undergo coronary occlusion. At 10 to 14 days after surgery (time



**Figure 1.** Diagram of the experimental protocols. Tissue samples were collected for Src tyrosine kinase and EGF receptor kinase assays. O indicates coronary occlusion; R, coronary reperfusion.

corresponding to the interval elapsed between instrumentation and euthanasia in the other groups), the rabbits were given heparin (1000 units IV), after which they were anesthetized with sodium pentobarbital (50 mg/kg IV) and euthanized with a bolus of KCl. The heart was immediately excised, and myocardial samples ( $\approx 0.5$  g) were rapidly removed from the anterior left ventricular wall and stored in liquid nitrogen until used. To study the role of Src tyrosine kinases in ischemic PC, rabbits in groups II and III underwent an ischemic PC protocol consisting of 6 cycles of 4-minute coronary occlusion separated by 4 minutes of reperfusion and were euthanized at the following 2 time points: 5 minutes after the last reperfusion (when activation of PKC<sup>21,22</sup> and mitogen-activated protein kinases<sup>46</sup> has previously been found in this model; group II) and 30 minutes after the last reperfusion (when activation of nuclear factor- $\kappa$ B<sup>16</sup> has previously been found in this model; group III). Myocardial samples were rapidly removed from the ischemic-reperfused region (the boundaries of which had been marked with sutures at the time of instrumentation) and stored in liquid nitrogen. Group IV received the tyrosine kinase inhibitor LD-A (1 mg/kg IV) without ischemia/reperfusion, whereas group V received LD-A (1 mg/kg IV 10 minutes before the first occlusion) and then underwent the sequence of 6 cycles of 4-minute occlusion/4-minute reperfusion. This dose of LD-A has been shown to block late PC against myocardial stunning in this conscious rabbit model.<sup>34</sup> Group VI received the PKC inhibitor chelerythrine without ischemia/reperfusion (5 mg/kg IV), whereas group VII received chelerythrine (5 mg/kg IV 5 minutes before the first occlusion) and then underwent the sequence of 6 cycles of 4-minute occlusion/4-minute reperfusion. This dose of chelerythrine has previously been shown to effectively block the translocation of PKC $\epsilon$  and the protection of late PC in conscious rabbits.<sup>23</sup> In groups V and VII, the rabbits were euthanized 30 minutes after the last reperfusion, and tissue samples were obtained as described above. In groups IV and VI, the rabbits were euthanized 79 minutes after the administration of LD-A or chelerythrine, respectively (this time interval corresponded to the interval elapsed between treatment and euthanasia in groups V and VII).

#### Tissue Sample Preparation

Tissue samples were processed for determination of the protein expression and phosphorylation activity of PKC $\epsilon$  and the expression of all 9 kinases in the Src family, including Fyn, Yrk, Fgr, Yes, Src, Lyn, Hck, Lck, and Blk. Frozen myocardial tissue samples were powdered in a prechilled stainless steel mortar and pestle. Total cellular proteins were obtained by glass-glass homogenization of the powdered tissue in sample buffer containing (in mmol/L) 50 Tris-HCl (pH 7.5), 5 EDTA, 10 EGTA, 10 benzamide, and 1 sodium orthovanadate; (in  $\mu$ g/mL) 50 PMSF, 10 aprotinin, 10 leupeptin, and 10 pepstatin A; and 0.3%  $\beta$ -mercaptoethanol. The cytosolic and particulate fractions were prepared as previously described.<sup>22,23,46</sup>

Protein concentration was determined using the method of Bradford (Bio-Rad). The yields of total cellular proteins, cytosolic proteins, and particulate proteins were carefully recorded for each tissue sample tested. Total myocardial proteins were calculated as the sum of the proteins from the cytosolic and the particulate fractions.<sup>22</sup> To ensure the most accurate assessment of Src tyrosine kinase protein expression and to avoid any decay in the kinase phosphorylation activity, samples were processed for Western immunoblotting and phosphorylation assays immediately after tissue sample preparation.

#### **PKC $\epsilon$ Isoform-Selective Phosphorylation Activity Assay**

The phosphorylation activity of the  $\epsilon$  isoform of PKC was determined in both the cytosolic and the particulate fractions as previously described.<sup>21,46,47</sup> Briefly, 50  $\mu$ g of proteins from either the cytosolic or the particulate fraction were immunoprecipitated overnight with PKC $\epsilon$  isoform antibodies (Transduction Laboratories). The immunoprecipitates were then subjected to a phosphorylation assay using a PKC $\epsilon$ -selective substrate (ERMRRKRQGSVRRRV).

#### **EGF Receptor Activity Assay**

The activity of EGF receptor tyrosine kinases was determined using a standard ELISA assay kit (Upstate Biotechnology). This assay system provided quantification of the phosphotyrosine residues on activated EGF receptors in the tissue sample homogenates. Briefly, 200  $\mu$ g of proteins from the particulate fraction were immunocoupled to 0.5  $\mu$ g of monoclonal antibodies to EGF receptor. Horseradish peroxidase-conjugated anti-phosphotyrosine antibodies were then used to detect activated (tyrosine-phosphorylated) EGF receptors. The ELISA reaction was quantified by the Multiskan MCC/340 microplate reader II (MTX Laboratory Systems, Inc). EGF-stimulated A431 cell lysates were used as positive controls, whereas nonstimulated A431 cell lysates were used as negative controls (basal level). Each reaction was performed in triplicate.

#### **Western Immunoblotting for Src Tyrosine Kinases**

The expression of all 9 tyrosine kinases in the Src family was determined using standard Western immunoblotting techniques as previously described.<sup>22</sup> Src tyrosine kinases and the sources of their respective antibodies were as follows: Src (Upstate, anti-mouse), Fyn (Transduction Laboratories, anti-mouse), Yes (Transduction Laboratories, anti-mouse), Lyn (Santa Cruz Biotechnology, anti-mouse), Lck (Upstate Biotechnology, anti-mouse), Hck (Transduction Laboratories, anti-mouse), Blk (Santa Cruz Biotechnology, anti-rabbit), Fgr (Santa Cruz Biotechnology, anti-rabbit), and Yrk (a gift from Dr Marius Sudol, Mount Sinai Medical School<sup>48</sup>). The expression of 7 Src tyrosine kinases was detected in the rabbit myocardium.

#### **Kinase-Specific Activity Assays for Src Tyrosine Kinases**

The phosphorylation activity of all 7 members of the Src family that are expressed in the rabbit myocardium was determined by immunoprecipitation followed by substrate-specific phosphorylation assay.<sup>49,50</sup> The amount of proteins applied in each assay was chosen on the basis of the optimal sensitivity of the enzyme, which was derived from sample protein-versus-enzymatic activity dose-response curves. Specific enzymatic activity was calculated by subtracting the nonspecific activity (basal background activity) from the total activity.

#### **Immunoprecipitation**

Briefly, 50  $\mu$ g of myocardial tissue protein was immunoprecipitated overnight with 5  $\mu$ g of antibodies (antibody source given above) against the corresponding kinase and 10  $\mu$ L of protein A/G agarose beads (Santa Cruz). The mixture of tissue, antibodies, and beads was incubated overnight in RIPA buffer containing (in mmol/L) 150 NaCl, 50 Tris (pH 7.4), 1% NP-40, 1 EDTA, 1 EGTA, 1 sodium orthovanadate, and 1 PMSF, and (in  $\mu$ g/mL) 16 benzamidine-HCl, 10 phenanthroline, 10 aprotinin, 10 leupeptin, and 10 pepstatin A.

#### **Src Kinase Assay**

The kinase-specific activity of the Src kinases was then determined by subjecting the immunoprecipitates to a phosphorylation assay

using an assay kit purchased from Upstate Biotechnology. Briefly, the immunoprecipitates were incubated with 10  $\mu$ g of substrate peptide (KVEKIGEGTYGVVYK) in reaction buffer (total volume, of 35  $\mu$ L) containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP and with (in mmol/L) 0.125 ATP, 18.75 MnCl<sub>2</sub>, 100 Tris-HCl (pH 7.0), 2 EGTA, 0.25 sodium orthovanadate, and 2 DTT for 15 minutes at 30°C. The reaction was terminated by the addition of 20  $\mu$ L of 40% trichloroacetic acid. The phosphorylated substrates were transferred to P81 binding paper (Upstate Biotechnology) prewet with 0.75% phosphoric acid. The P81 binding papers were washed 3 times in 0.75% phosphoric acid and once in acetone, and the radioactivity was measured using a  $\beta$ -scintillation counter. The Src kinase-specific activity was calculated from the specific counts (total counts minus nonspecific counts). The nonspecific counts were determined by performing parallel assays in the absence of tissue immunoprecipitates.

#### **Statistical Analysis**

Data are reported as mean  $\pm$  SEM. To facilitate comparisons, measurements of kinase activity and protein expression in each individual rabbit heart were expressed as a percentage of the average value for the control group. Differences among the 7 experimental groups were analyzed using a 1-way ANOVA. If the ANOVA showed an overall difference, post hoc contrasts were performed with Student *t* tests for unpaired data using the Bonferroni correction.<sup>51</sup>

## **Results**

#### **Exclusions**

Of the 35 rabbits instrumented for this study, 5 were assigned to group I (control group), 5 to group II (5 minutes after ischemic PC), 5 to group III (30 minutes after ischemic PC), 5 to group IV (LD-A without ischemic PC), 5 to group V (LD-A with ischemic PC), 5 to group VI (chelerythrine without ischemic PC), and 5 to group VII (chelerythrine with ischemic PC) (Figure 1). All rabbits completed the protocol successfully.

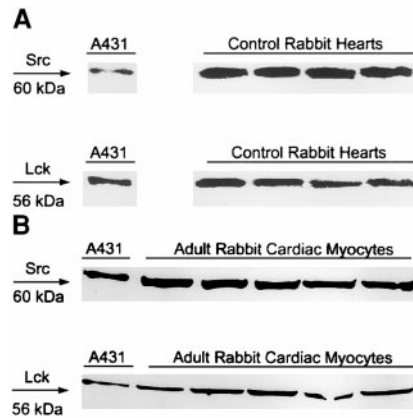
#### **Seven Src Tyrosine Kinases Are Expressed in the Heart of Conscious Rabbits**

A total of 9 members of the Src tyrosine kinase family have been identified in noncardiac cells.<sup>32,33,39,40</sup> However, it is unknown which member(s) of this family is/are expressed in the heart and in which subcellular compartment. We found that the adult rabbit heart expresses the following 7 members in the Src family of tyrosine kinases: Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk. The expression of Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk was also detected in isolated adult rabbit cardiac myocytes (which were isolated as previously described<sup>46</sup>), excluding the possibility that these kinases may be expressed solely in noncardiomyocytes. Examples of immunoblots for Src and Lck are shown in Figures 2A and 2B. We were unable to detect the expression of Yrk and Hck kinases using currently available antibodies.

Using immunoprecipitation followed by kinase-specific assay, we detected kinase-specific phosphorylation activity of Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk both in the cytosolic and in the particulate fraction. However, the particulate fraction contained the majority of the phosphorylation activity for Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk (Table 1).

#### **Ischemic PC Induces Selective Activation of Src and Lck**

To examine the effect of ischemic PC on Src tyrosine kinases, a PC protocol (6 cycles of 4-minute coronary occlusion/4-



**Figure 2.** Western immunoblots performed to identify the expression of Src and Lck kinases in the rabbit heart (A) and in isolated adult cardiac myocytes (B). A431 cell lysates were used as positive control for the Src kinase (60 kDa), whereas Jurkat cell lysates were used as positive control for the Lck kinase (56 kDa).

minute reperfusion) that has previously been shown to induce late PC against myocardial stunning<sup>4,5,16,21,23</sup> and infarction<sup>13,14,16,21</sup> was used. Tissue samples were obtained both at 5 minutes (group II) and at 30 minutes (group III) after the sixth reperfusion (Figures 3 and 4). The 5-minute time point was selected because this is the interval at which significant activation of PKC $\epsilon$  has been documented in this model.<sup>22</sup> Because the activation of Src noted at 5 minutes was not consistent (see below) and because we hypothesized that Src tyrosine kinases may be downstream of PKC, we also examined tissue samples obtained at 30 minutes after ischemic PC.

At 5 minutes after ischemic PC (group II), there was a significant increase in the particulate Lck activity (Figure 4) but only a borderline increase in the particulate Src activity (Figure 3) (2 of 5 rabbits exhibited an increase, whereas the other 3 did not). At 30 minutes after ischemic PC (group III), the activity of both Src and Lck was significantly increased compared with control rabbits (group I) (Figures 3 and 4). Src activity increased by  $119.0 \pm 23.9\%$  and  $34.4 \pm 7.9\%$  in the particulate and cytosolic fractions, respectively (Figure 3), whereas Lck activity increased by  $103.1 \pm 11.9\%$  and  $89.4 \pm 21.0\%$ , respectively ( $P < 0.05$ ) (Figure 4). In contrast, ischemic PC had no significant effect on the phosphorylation activity of Fyn, Fgr, Yes, Lyn, and Blk tyrosine kinases, either at 5 minutes or at 30 minutes after ischemic PC (Table 2). Thus, ischemic PC induced selective activation of Src and Lck with no significant change in the activity of the other members of this family of tyrosine kinases. The activation of Lck was apparent both at 5 and 30 minutes after ischemic PC, whereas that of Src became apparent only at 30 minutes.

### Ischemic PC Does Not Trigger Activation of EGF Receptor Kinases in the Rabbit Heart

In contrast to the marked activation of Src and Lck kinases after ischemic PC (group III), the activity of EGF receptor tyrosine kinases remained unaltered both at 5 minutes after ischemic PC (group II;  $115.1 \pm 8.9\%$  of control) and at 30 minutes after ischemic PC (group III;  $95.2 \pm 18.1\%$  of con-

**TABLE 1. Kinase-Specific Activity of Src Tyrosine Kinases in the Heart of Conscious Rabbits**

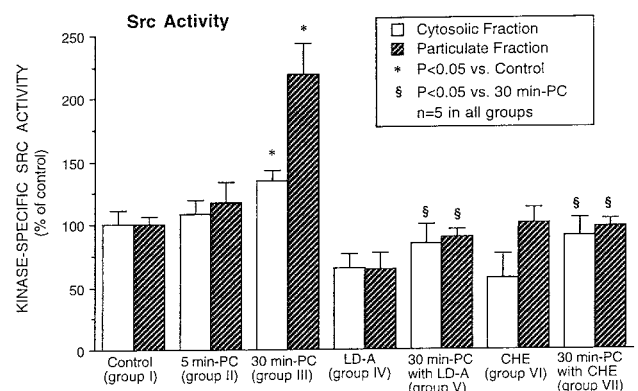
Kinase	Total Activity (Units)	Particulate Fraction (Units)	Cytosolic Fraction (Units)
Fyn	$6.65 \pm 0.46$	$4.22 \pm 0.51$	$2.62 \pm 0.15$
Fgr	$6.74 \pm 0.63$	$4.62 \pm 0.26$	$2.12 \pm 0.23$
Yes	$8.44 \pm 0.55$	$6.25 \pm 0.28$	$3.71 \pm 0.65$
Src	$5.23 \pm 0.42$	$3.44 \pm 0.48$	$1.09 \pm 0.16$
Lyn	$1.91 \pm 0.29$	$1.16 \pm 0.20$	$0.75 \pm 0.11$
Lck	$5.99 \pm 0.89$	$4.09 \pm 0.37$	$1.85 \pm 0.14$
Blk	$7.25 \pm 1.36$	$4.53 \pm 0.51$	$2.73 \pm 0.17$

The tyrosine kinase-specific phosphorylation activity of all 7 Src kinases expressed in hearts of control rabbits (group I,  $n=5$ ) are shown. One unit of phosphorylation activity is defined as the amount that catalyzed the incorporation of 1 pmol phosphate into Src substrate peptide per minute per milligram of protein. Note that for all 7 Src kinases, most of the phosphorylation activity resides in the particulate fraction. Data are mean  $\pm$  SEM.

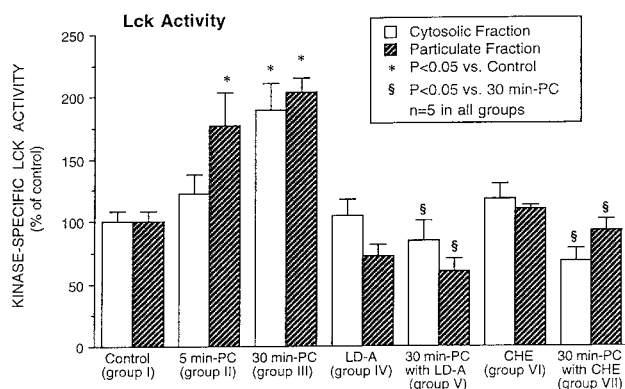
rol). The EGF receptor assays in groups I, II, and III were run in parallel to the positive control samples (A431 cells). The EGF-stimulated A431 cell lysates exhibited a positive activation signal that was  $381.6 \pm 11.8\%$  of the nonstimulated A431 cell lysates (basal level), excluding the possibility that the ELISA assay was insensitive to the activity of EGF receptor kinases during ischemic PC.

### LD-A Blocks Ischemic PC-Induced Activation of Src and Lck Without Affecting the Activity of PKC $\epsilon$

Previous studies in conscious rabbits have shown that the particulate PKC $\epsilon$  is activated 5 minutes after ischemic PC.<sup>21,22</sup> The results obtained in this study confirmed that significant activation of PKC $\epsilon$  in the particulate fraction occurs 5 minutes after ischemic PC (group II,  $172.3 \pm 8.8\%$  of control [group I]) and demonstrated that this activation of PKC $\epsilon$  persists at 30 minutes (group III,  $167.4 \pm 4.2\%$  of



**Figure 3.** Particulate and cytosolic phosphorylation activity of the Src kinase, as determined by kinase-specific assay, in the 7 experimental groups ( $n=5$  in each group). Compared with the control group (group I), both the particulate and the cytosolic Src activities were significantly increased 30 minutes after ischemic PC (6 cycles of 4-minute occlusion/4-minute reperfusion) (group III). Ischemic PC-induced activation of Src kinase was blocked by both the tyrosine kinase inhibitor LD-A (group V) and the PKC inhibitor CHE (group VII). Data are mean  $\pm$  SEM.



**Figure 4.** Particulate and cytosolic phosphorylation activity of the Lck kinase, as determined by kinase-specific assay, in the 7 experimental groups (n=5 in each group). Compared with the control group (group I), the particulate Lck activity increased 5 minutes after ischemic PC (6 cycles of 4-minute occlusion/4-minute reperfusion) (group II) and remained elevated at 30 minutes (group III), whereas the cytosolic Lck activity increased significantly only 30 minutes after ischemic PC (group III). Ischemic PC-induced activation of Lck kinase was blocked by both the tyrosine kinase inhibitor LD-A (group V) and the PKC inhibitor CHE (group VII). Data are mean±SEM.

control) (Figure 5), which is congruous with the robust activation of the Src and Lck kinases observed at the same time point (Figures 3 and 4). The position of Src and Lck with respect to PKC $\epsilon$  is unclear (ie, it is unclear whether these kinases are proximal, distal, or parallel to PKC $\epsilon$ ). In group V, we found that LD-A completely inhibited the ischemic PC-induced activation of Src and Lck (Figures 3 and 4) but had no effect on the particulate activity of PKC $\epsilon$  (Figure 5), indicating that Src and Lck are not upstream of PKC $\epsilon$ . The ability of this dose of LD-A to block ischemic PC-induced activation of Src and Lck in vivo without interfering with the

activation of PKC $\epsilon$  indicates that LD-A is a useful tool to explore the role of Src and Lck in ischemic PC.

### Activation of Src and Lck During Ischemic PC Is PKC Dependent

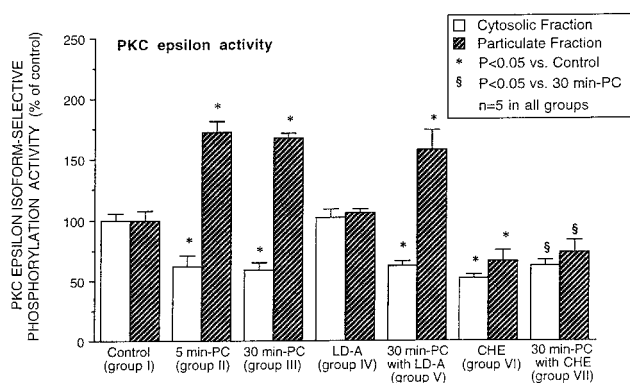
The results obtained with LD-A (group V) indicate that activation of PKC $\epsilon$  is not dependent on activation of Src or Lck, because it persisted when both of these kinases were inhibited (Figure 5). Two possibilities remain, as follows<sup>1</sup>: Src and/or Lck might be in parallel with PKC $\epsilon$  (ie, PKC $\epsilon$  and Src/Lck would both be necessary for late PC but would act independently), and<sup>2</sup> Src and/or Lck are downstream of PKC $\epsilon$  (ie, activation of PKC $\epsilon$  is necessary to activate Src and/or Lck). To distinguish between these 2 possibilities and to determine whether both Src and Lck occupy the same position relative to PKC $\epsilon$ , we measured Src and Lck activity in rabbits undergoing the ischemic PC protocol (6 cycles of 4-minute occlusion/4-minute reperfusion) after pretreatment with 5 mg/kg of chelerythrine (group VII). Previous studies in this conscious rabbit model have documented that this dose of chelerythrine completely abrogates both the ischemic PC-induced translocation of PKC $\epsilon$  and the cardioprotective effects of late PC against stunning and infarction.<sup>23</sup> Tissue samples were obtained 30 minutes after ischemic PC. We found that chelerythrine abrogated the ischemic PC-induced activation of PKC $\epsilon$  (Figure 5) and, at the same time, completely blocked the ischemic PC-induced activation of both Src and Lck kinases (Figures 3 and 4). In the absence of ischemic PC (group VI), chelerythrine had no significant effect on Src or Lck (Figures 3 and 4). These results indicate that, in the adult rabbit heart, the Src and Lck kinases are located downstream of PKC $\epsilon$  and that their activation during ischemic PC occurs via a PKC $\epsilon$ -dependent pathway.

**TABLE 2. Effect of Ischemic PC on the Activity of Src Tyrosine Kinases in the Particulate and Cytosolic Fractions**

Kinase	Cytosolic Fraction			Particulate Fraction		
	Control (Group I)	5 Minutes After Ischemic PC (Group II), % of Control	30 Minutes After Ischemic PC (Group III), % of Control	Control (Group I)	5 Minutes After Ischemic PC (Group II), % of Control	30 Minutes After Ischemic PC (Group III), % of Control
Fyn	100±7.2%	105.7±9.8%	97.4±9.6%	100±4.1%	107.7±10.5%	105.2±6.4%
Fgr	100±10.1%	110.7±10.8%	115.9±19.5%	100±14.7%	103.1±7.5%	96.4±5.8%
Yes	100±6.6%	97.1±7.3%	98.9±3.0%	100±3.7%	109.1±13.8%	94.4±9.4%
Src	100±11.3%	108.3±11.4%	134.4±7.9%*	100±6.1%	117.6±16.5%	219.0±23.9%*
Lyn	100±14.2%	90.7±4.0%	97.1±14.4%	100±8.5%	106.6±12.6%	88.7±6.0%
Lck	100±8.3%	122.9±14.6%	189.4±21.0%*	100±8.3%	176.9±26.2%*	203.1±11.9%*
Blk	100±12.1%	102.4±11.0%	108.5±10.2%	100±15.4%	93.7±8.1%	94.0±9.9%

The kinase-specific phosphorylation activities of Src tyrosine kinases in experimental groups I through III are shown. Group I did not undergo coronary occlusion or reperfusion and served as sham control. Groups II and III underwent an ischemic PC protocol consisting of 6 cycles of 4-minute coronary occlusion separated by 4-minute reperfusion. In group II, myocardial tissue samples were obtained 5 minutes after the last reperfusion, whereas in group III, myocardial tissue samples were obtained 30 minutes after the last reperfusion. Compared with control (group I), ischemic PC had no significant effect on the phosphorylation activity of Fyn, Fgr, Yes, Lyn, and Blk kinases (groups II and III). In contrast, a marked increase in the Lck phosphorylation activity was observed at both 5 minutes (group II) and 30 minutes (group III) after ischemic PC. Similarly, the phosphorylation activity of Src kinase was significantly elevated 30 minutes after ischemic PC (group III). Data are expressed as percentage of control (group I). Values are mean±SEM.

\* $P<0.05$  vs group I.



**Figure 5.** PKC $\epsilon$  isoform-selective phosphorylation activity, measured after immunoprecipitation, in the 7 experimental groups (n=5 in each group). Compared with the control group (group I), the activity of PKC $\epsilon$  in the particulate fraction was significantly increased 5 minutes after ischemic PC (6 cycles of 4-minute occlusion/4-minute reperfusion) (group II) and remained elevated 30 minutes after ischemic PC (group III). The ischemic PC-induced activation of PKC $\epsilon$  was not affected by the tyrosine kinase inhibitor LD-A (group V). In contrast, the ischemic PC-induced activation of PKC $\epsilon$  was completely abolished by the PKC inhibitor CHE (group VII). Data are mean  $\pm$  SEM.

## Discussion

Although Src tyrosine kinases have been found to mediate cellular responses to stress in noncardiac cells,<sup>32,33,39–41</sup> the role of this family of tyrosine kinases in myocardial adaptation to ischemic stress is unknown. Furthermore, there is no direct evidence that any tyrosine kinase is activated after ischemic PC. In this study, we have directly measured the activity of 2 major tyrosine kinase families, the Src and the EGF receptor families, in the heart of conscious rabbits. Our results demonstrate that ischemic PC induces selective activation of Src tyrosine kinases, ie, it activates 2 members of this family (Src and Lck) without affecting the other 5 members (Fyn, Fgr, Yes, Lyn, and Blk). Thus, a brief ischemic stimulus causes rapid recruitment of Src tyrosine kinase signaling in the heart, a stress adaptation mechanism reminiscent of that which occurs in noncardiac cells.<sup>32,33,39–41</sup> In contrast, EGF receptor kinases were not activated by ischemic PC. The activation of Src and Lck by ischemic PC appears to be distal to PKC $\epsilon$  because (1) the activation of PKC $\epsilon$  preceded the activation of Src and paralleled that of Lck; (2) LD-A, given at a dose that has previously been shown to block the protective effects of ischemic PC,<sup>34</sup> blocked the increase in Src and Lck activity but had no effect on the increase in PKC $\epsilon$  activity; and (3) chelerythrine, given at a dose that has previously been shown to block ischemic PC,<sup>23</sup> blocked both the activation of PKC $\epsilon$  and that of Src and Lck. To our knowledge, this is the first evidence that the signaling mechanism underlying ischemic PC involves specifically the recruitment of Src and Lck and that this activation is downstream of, and dependent on, activation of PKC $\epsilon$ . Taken together with previous studies demonstrating that both LD-A<sup>34</sup> and chelerythrine<sup>23</sup> abrogate the delayed cardioprotection observed in this model, the present results indicate that the activation of Src and Lck is a critical step in the development of the late phase (second window) of ischemic PC in the conscious animal and therefore identify a new

signaling step responsible for the adaptation of the heart to ischemic stress.

## Expression of Src Tyrosine Kinases in the Rabbit Heart

A total of 9 members of the Src tyrosine kinase family have been described in various tissues.<sup>32,33,39,40</sup> Although c-Src has been identified in cardiac fibroblasts<sup>52</sup> and neonatal cardiomyocytes,<sup>53</sup> the complete expression profile of this family of tyrosine kinases in the adult mammalian myocardium has never been characterized. The present results demonstrate that the adult rabbit heart expresses the following 7 of the 9 known members of the Src tyrosine kinase family: Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk. The presence of these proteins cannot be ascribed to noncardiomyocytes, because our *in vitro* data show that the same 7 kinases (Fyn, Fgr, Yes, Src, Lyn, Lck, and Blk) are robustly expressed in isolated adult rabbit cardiac myocytes (see Results). This is the first documentation that cardiac myocytes express multiple Src tyrosine kinases, a finding that has important implications for future studies focusing on the role of this family of enzymes in cardiac pathophysiology. In noncardiac cells, it has been established that, despite a certain level of redundancy, each member of the Src tyrosine kinase family possesses a distinct biological function.<sup>32,33,40,41</sup> Our finding that myocardial ischemia activates only 2 of the 7 members of the Src tyrosine kinase family is consistent with this general concept. The identification of Src and Lck as the 2 Src tyrosine kinases involved in ischemic PC now provides a rationale for focusing on these kinases and investigating the cellular signaling mechanisms responsible for their regulation as well as their targeted substrates in the adult mammalian heart.

## Role of Src Tyrosine Kinases in Ischemic PC

Previous studies have implicated tyrosine kinases in the early<sup>1,11,12,25</sup> and late<sup>7,34</sup> phases of ischemic PC. Baines et al<sup>1</sup> have reported that either genistein or LD-A blocked the ischemia- and phorbol 12-myristate 13-acetate-induced early protection against myocardial infarction in the isolated rabbit heart, and Imagawa et al<sup>7</sup> have demonstrated that genistein blocks the second window (late phase) of protection in open-chest rabbits. However, >1000 tyrosine kinases have been discovered so far.<sup>26,27</sup> Because of the lack of selective tyrosine kinase inhibitors, the identity of the tyrosine kinases involved in ischemic PC remains unknown. Genistein is a broad inhibitor of most known tyrosine kinases<sup>1</sup> and therefore does not provide insights into which tyrosine kinase(s) is involved in ischemic PC. For this reason, we elected to use LD-A, which preferentially inhibits the Src and EGF receptor tyrosine kinase families over other tyrosine kinases.<sup>35–38</sup>

The use of LD-A also provided greater selectivity for Src tyrosine kinases versus other kinases. Besides having an inhibitory effect on tyrosine kinases, genistein also blocks a number of serine/threonine kinases, including PKC and protein kinase A (PKA).<sup>35</sup> Indeed, the selectivity of genistein for Src tyrosine kinases compared with other kinases is rather modest.<sup>35</sup> For example, genistein has an IC<sub>50</sub> of 18  $\mu$ mol/L for Src tyrosine kinases,  $\approx$ 185  $\mu$ mol/L for PKC, and  $\approx$ 500  $\mu$ mol/L for PKA<sup>35</sup> and thus is only  $\approx$ 10-fold selective

for Src tyrosine kinases versus PKC. In contrast, LD-A has an  $IC_{50}$  of 0.5  $\mu\text{mol/L}$  for Src tyrosine kinases<sup>36,37</sup> and exhibits a wider range of specificity, being at least 200-fold less effective at inhibiting PKC and PKA versus Src tyrosine kinases.<sup>35-37</sup> Therefore, LD-A can be regarded as a more specific Src tyrosine kinase inhibitor compared with genistein. We used the same dose of LD-A that has been shown to block the cardioprotective effects of late PC against myocardial stunning in the same rabbit model.<sup>34</sup> LD-A completely abolished the ischemic PC-induced activation of both Src and Lck (Figures 3 and 4), suggesting that the recruitment of these 2 kinases is an important signaling event in the genesis of delayed cardioprotection. However, because LD-A is also a potent inhibitor ( $IC_{50}=11$  nmol/L) of the EGF receptor tyrosine kinases,<sup>36,38</sup> it is theoretically possible that it may block late PC by interfering with these kinases. To rule out this possibility, we assessed the activity of EGF receptor tyrosine kinases in ischemic PC. The significance of using LD-A as an effective tool to assess the role of Src tyrosine kinases in ischemic PC is substantiated by the fact that, in contrast to the activation of Src and Lck, the activity of the EGF receptor tyrosine kinases remained unchanged. Therefore, it is unlikely that EGF receptor tyrosine kinases participate in the signaling events that lead to ischemic PC.

Taken together with our previous studies of cardioprotection,<sup>23,34</sup> these results support the concept that the Src family of tyrosine kinases, and specifically Src and Lck kinases, but not the EGF receptor kinases, play a critical role in the signal transduction pathway that underlies the development of myocardial adaptations to ischemic stress.

### PKC-Dependent Activation of Src Tyrosine Kinases

In noncardiac cell types, evidence has been reported that tyrosine kinases can be downstream of,<sup>29-31</sup> parallel to,<sup>54</sup> or upstream of<sup>55,56</sup> PKC. In principle, it is possible that during PC, PKC $\epsilon$  and Src tyrosine kinases may function in a parallel fashion, ie, they may be both necessary for PC to occur but may act through independent signaling cascades.<sup>32,33,39</sup> In an effort to further elucidate the mechanism by which PKC $\epsilon$  and Src/Lck mediate cardioprotection during ischemic PC, we investigated whether Src and Lck are downstream signaling elements of PKC. Our results demonstrate that the activation of PKC $\epsilon$  in the particulate fraction was fully manifest immediately after the ischemic PC protocol (5 minutes after the last reperfusion; group II) and persisted 30 minutes after ischemic PC (group III; Figure 5). On the other hand, the activation of Src and Lck in the particulate fraction became fully manifest at 30 minutes after ischemic PC (group III; Figures 3 and 4). Thus, the changes in the isoform-specific activity of PKC $\epsilon$  preceded the changes in the kinase-specific activity of Src and paralleled those of Lck (Figures 3, 4, and 5), a pattern consistent with the notion that the recruitment of Src and Lck is a distal event that follows the activation of PKC $\epsilon$ .

Further evidence that Src and Lck are downstream of PKC $\epsilon$  is provided by the results of studies in which these signaling elements were manipulated pharmacologically with the selective tyrosine kinase inhibitor LD-A (see

above) and with chelerythrine, a potent and selective PKC inhibitor ( $IC_{50}$  for PKC, 0.7  $\mu\text{mol/L}$ ;  $IC_{50}$  for tyrosine kinases, 100  $\mu\text{mol/L}$ <sup>57</sup>). When Src and Lck activation was inhibited by LD-A (group V), the activation of PKC $\epsilon$  was essentially unaffected (Figure 5), indicating that Src and Lck cannot be upstream of PKC $\epsilon$ . On the other hand, when the activation of PKC $\epsilon$  was inhibited by chelerythrine (group VII), the activation of Src and Lck was completely abrogated (Figures 3 and 4), indicating that PKC $\epsilon$  is upstream of (and not parallel to) Src and Lck. The finding that Src and Lck are downstream of PKC has significant implications not only for the late phase but also for the early phase of ischemic PC, given that tyrosine kinases have been shown to play an important role in this phase as well.<sup>1,11,12,25</sup>

In contrast to the particulate fraction, the PKC $\epsilon$  activity in the cytosolic fraction was reduced after ischemic PC (Figure 5), probably because translocation of the PKC $\epsilon$  protein to the particulate fraction.<sup>21-23</sup> Additionally, our results show that the activity of Src and Lck in the cytosolic fraction was elevated 30 minutes after ischemic PC (Figures 3 and 4), and thus was not coupled to the decreased cytosolic PKC $\epsilon$  activity (Figure 5). This could reflect the differential regulation of Src tyrosine kinases in various cellular compartments. It is well established that the cytosolic Src tyrosine kinases are under tonic inhibition by the C-terminal Src kinases,<sup>58</sup> whereas the particulate Src tyrosine kinases are not.<sup>40,41</sup> Because the inhibitory effect of C-terminal Src kinases present *in vivo* is lost when Src kinase activity is determined by *in vitro* kinase assays, measurements of Src tyrosine kinase activity in the cytosolic fraction may not reflect the function of these enzymes in intact cells.<sup>40,58</sup> Therefore, the increased cytosolic activity of Src and Lck activity observed 30 minutes after ischemic PC (Figures 3 and 4) may not correlate with the *in vivo* situation.

Although our results support the concept that tyrosine kinases are distal to PKC in the rabbit heart, it is possible that the hierarchical order of kinase activation in PC may vary in different species. Recent studies in rats by Fryer et al<sup>59</sup> indicate that tyrosine kinase inhibitors have only a partial effect on ischemic PC and that combined inhibition of both PKC and tyrosine kinases is required to completely abolish the protection.<sup>60</sup> Similarly, Vahlhaus et al<sup>61</sup> have found that, in pigs, ischemic PC is prevented only by combined inhibition of PKC and tyrosine kinases. These studies<sup>59,60,61</sup> suggest that, in rats and pigs, PKC and tyrosine kinases may function in parallel rather than in series. In addition, investigations in dogs<sup>62</sup> and pigs<sup>63,64</sup> have questioned the role of PKC in the development of ischemic PC. It is conceivable that the apparent discrepancy between these previous studies<sup>59-64</sup> and the present results reflects species differences in the cellular signaling pathways underlying ischemic PC. Future investigations will be necessary to address this issue.

### Conclusions

We have identified a new component of the signal transduction cascade of ischemic PC, ie, the PKC $\epsilon$ -Src/Lck signaling pathway. By measuring kinase-selective activity, this study provides the first direct documentation of the recruitment of Src tyrosine kinases after a brief ischemic stress in the heart.

Taken in conjunction with previous functional studies,<sup>23,34</sup> the results reported herein demonstrate that PKC-dependent activation of 2 members of the Src family, Src and Lck, is an essential signaling event underlying the development of the late phase of ischemic PC in conscious rabbits. Evidence is mounting that Src tyrosine kinases are mobilized by diverse stimuli in noncardiac cells.<sup>32,33</sup> The present observations in the setting of myocardial ischemia are consistent with this notion and therefore suggest a role of Src tyrosine kinases as general mediators of cellular responses to stress. It is becoming increasingly apparent that the signaling mechanisms responsible for the cardiac adaptations to ischemia are extremely complex and incompletely understood. The identification of 2 specific kinases significantly expands our understanding of this complex process and should stimulate further research into the Src tyrosine kinase-dependent phosphorylation events that lead to enhanced myocardial tolerance to ischemic injury.

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