protein tyrosine kinase is not involved in the infarct size–limiting effect of ischemic preconditioning in canine hearts

Masafumi Kitakaze, Koichi Node, Hiroshi Asanuma, Seiji Takashima, Yasuhiko Sakata, Masanori Asakura, Shoji Sanada, Yoshiro Shinozaki, Hidezo Mori, Tsunehiko Kuzuya, Masatsugu Hori

Abstract—Protein kinase C (PKC) plays an important role in ischemic preconditioning (IP). Because (1) tyrosine kinase is located at the downstream of PKC for IP in the rabbit hearts and (2) we have reported that ecto-5'-nucleotidase is the substrate for PKC and plays a crucial role for the infarct size–limiting effect, we tested whether tyrosine kinase activation contributes to either activation of ecto-5'-nucleotidase or the infarct size–limiting effect of the early phase of IP in the canine heart. In dogs, the IP procedure (4 cycles of 5-minute occlusion of coronary artery) and exposure to 12,13-phorbol myristate acetate (PMA) each activated myocardial ecto-5'-nucleotidase and Lck tyrosine kinase. Genistein (10, 30, and 100 μg · kg⁻¹ · min⁻¹ IC), an inhibitor of tyrosine kinase, attenuated the activation of Lck tyrosine kinase but did not attenuate the activation of ecto-5'-nucleotidase due to either IP or PMA. In the other canine hearts, IP attenuated infarct size (49±5 versus 11±3 or 16±3%, P<0.01) due to 90 minutes of coronary occlusion followed by 6 hours of reperfusion, which was not blunted by 3 or 2 (30 and 100 μg · kg⁻¹ · min⁻¹ ) doses of genistein (infarct sizes, 15±4, 13±4, and 13±3%, respectively, and 17±3 and 15±4%, respectively) or lavendustin A. Tyrosine kinase does not activate ecto-5'-nucleotidase or trigger the infarct size–limiting effect of the early phase of IP in canine hearts. (Circ Res. 2000;87:303-308.)

Key Words: ischemic preconditioning ■ protein kinase C ■ genistein ■ lavendustin A

Brief periods of ischemia that precede sustained ischemia limit infarct size markedly, a phenomenon known as ischemic preconditioning (IP).1–3 The mechanisms underlying this phenomenon have been studied extensively,4–8 and several lines of evidence support the idea that activation of protein kinase C plays an essential role in IP.9–12 We have previously reported that IP increases ecto-5'-nucleotidase activity,13,14 which seems to be consistent with the results of Liu et al,6 because adenosine production in the ischemic myocardium is attributable to ecto-5'-nucleotidase.5,15 The adenosine production via ecto-5'-nucleotidase may enhance the triggering mechanisms of IP or may contribute as an intermediate mediator to activate the final mediators such as the opening of ATP-sensitive K⁺ (KₐTP) channels. Interestingly, protein tyrosine kinase is reported to be located downstream of activation of protein kinase C in the rat and rabbit hearts.17–22 Indeed, protein kinase C activation phosphorylates and activates protein tyrosine kinase.21–23 Interestingly, Ping et al24 reported that myocardial Lck and Src tyrosine kinases are phosphorylated and activated 5 and 30 minutes after the IP procedure, which may play an important role for the cardioprotection. However, it is not clarified what subtypes of Src protein tyrosine kinases are activated and whether the activated tyrosine kinase is involved in the infarct size–limiting effect of IP in canine hearts.

To clarify the role of Src tyrosine kinases and the linkage between tyrosine kinase and protein kinase C, we measured myocardial tyrosine kinase activity and infarct size in the control and preconditioned myocardium with or without administration of several doses of genistein or lavendustin A, each of which is an inhibitor of tyrosine kinase.

Materials and Methods

The procedure of the operation on the mongrel dogs (12 to 25 kg) for the experiments was described previously.9–11 All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Experimental Protocols

Protocol 1: Effect of Either Genistein or Lavendustin A on IP

Both coronary perfusion pressure and blood flow (CBF) were measured. Four cycles of 5 minutes of occlusion of the left anterior descending (LAD) coronary artery and a subsequent 5 minutes of reperfusion were performed (n=7; IP group). As a control, after 45
minutes of hemodynamic stabilization, the LAD artery was occluded for 90 minutes and reperfused for 6 hours (n=7; control group).

In 31 dogs, either genistein (10, 30, and 100 μg·kg⁻¹·min⁻¹ [n=7 each]) or lavendustin A (2 and 20 μg·kg⁻¹·min⁻¹ [n=5 each]) was administered into the LAD artery 5 minutes before and during IP. Genistein was dissolved with saline, and lavendustin A was dissolved in 0.1% DMSO. Neither saline nor DMSO affected infarct size in the preliminary study. In 12 dogs, genistein (100 μg·kg⁻¹·min⁻¹, n=7) or lavendustin A (20 μg·kg⁻¹·min⁻¹, n=5) was infused into the LAD artery for 45 minutes before ischemia without IP.

Protocol II: Effect of Either Genistein or Lavendustin A on Pharmacological Preconditioning

Four cycles of administration of 12,13-phorbol myristate acetate (PMA, 0.5 pg·kg⁻¹·min⁻¹) for 5 minutes with 5-minute intervals (n=7; PMA group) were performed. PMA was dissolved with 0.1% DMSO. In 19 dogs, either genistein (30 and 100 μg·kg⁻¹·min⁻¹ IC [n=7 each]) or lavendustin A (20 μg·kg⁻¹·min⁻¹ IC [n=5 each]) was administered 5 minutes before and during PMA administration.

Protocol III: Activities of Myocardial Tyrosine Kinase and Ecto-5'-Nucleotidase

With (n=10) and without (n=10) IP, or with (n=10) and without (n=10) PMA preconditioning, we measured tyrosine kinase and 5'-nucleotidase activities of the endocardial myocardium. In each condition, 5 dogs received 30 μg·kg⁻¹·min⁻¹ of genistein, and the remaining 5 dogs received saline.

Measurements of the Physiological and Biochemical Parameters

We measured infarct size, risk area, collateral flow, and endocardial ecto-5'-nucleotidase activity, as described previously. Tyrosine kinase activity was assessed by the enzymatic assay technique using the AUSA tyrosine kinase assay kit. We also performed the kinase-specific activity assays. Five Src tyrosine kinases (Src, Fyn, Lyn, Lck, and Blk) were expressed using reverse transcriptase–polymerase chain reaction, and the expression of 4 in 5 tyrosine kinases was detected by immunoblotting in the canine myocardium (Figure 1).

The phosphorylation activity of all 4 members of the Src family was determined by immunoprecipitation followed by substrate-specific phosphorylation assay.

Statistical Analysis

Statistical analyses were performed using paired and unpaired t tests and were adjusted by a modified Bonferroni method. Analysis of covariance was used to account for the effect of collateral blood flow on infarct size. Each value was expressed as mean±SEM, with P<0.05 considered significant.

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

Results

Effects of Either Genistein or Lavendustin A in the Infarct Size–Limiting Effect of IP or PMA Preconditioning

Systolic (~138 mm Hg) and diastolic (~82 mm Hg) blood pressures and heart rate (~139 bpm) before, during, and after 90 minutes of myocardial ischemia were not significantly
Figure 4. Infarct size in the following groups: PMA (n=7), PMA with 30 µg \cdot kg^{-1} \cdot min^{-1} IC genistein (n=7; PMA+30Gen), PMA with 100 µg \cdot kg^{-1} \cdot min^{-1} IC genistein (n=7; PMA+100Gen), and PMA with 20 µg \cdot kg^{-1} \cdot min^{-1} IC lavendustin A (n=5; PMA+20Lav). Infarct size was markedly decreased in the PMA group, and this effect was not blunted by either genistein or lavendustin A. Data from the control and IP groups, the 100 Gen group, and the 20 Lav group were the same as in Figure 2, and we did not test the differences between these 4 groups and the data from protocol II.

Figure 5. Plot of infarct size expressed as percentage of the risk area and regional collateral flow during ischemia. In all groups, there were inverse relations between normalized infarct area and collateral flow. Infarct size in PMA-treated hearts was smaller (P<0.001) with respect to any given value in collateral blood flow than that in control hearts. Infarct size was not affected in the 3 doses of genistein with PMA groups compared with the PMA group. Data from the control and IP groups, the 100 µg \cdot kg^{-1} \cdot min^{-1} IC genistein group (n=7; 100Gen), and the 20 µg \cdot kg^{-1} \cdot min^{-1} IC lavendustin A group (n=5; 20Lav) were the same as in Figure 3, and we did not test the differences between these 4 groups and the data from protocol II. Abbreviations are as in Figures 2 and 4.

Discussion
Infarct Size–Limiting Effect of IP: Role of Tyrosine Kinase
In the present study, we showed that neither genistein nor lavendustin A blunts the infarct size–limiting effect of IP and that genistein does not blunt the activation of ecto-5’-nucleotidase in the canine hearts. This result may contradict
The previous studies showing that either genistein or another inhibitor of protein tyrosine kinase blunts the infarct size-limiting effect of IP in the rat or rabbit hearts.17–21 There may be several possibilities to explain this disparity. The first possibility is the differences in the activation of Src tyrosine kinases in the dogs and rabbit hearts. IP phosphorylates and activates Src and Lck tyrosine kinases in the rabbit hearts,24 but IP phosphorylates and activates Lck tyrosine kinases in the canine hearts in the present study, which may explain the differences. However, Src activation occurs 30 minutes after the IP procedure in the rabbit hearts,24 suggesting that the activation of Src may not account for the cardioprotection of the early phase of IP. Therefore, Lck activation soon after the IP procedure is common in the rabbit and dog, and the differences in the role of tyrosine kinases in the previous17–21 and present studies may not be attributable to different activation of the subtypes of Src tyrosine kinases. The second idea is that there are several pathways to cause the cardioprotection due to IP, and the pathway to elicit cardioprotection may be different among the cycles of the IP procedure, the length of the sustained ischemia, and species. In rats, cardioprotection due to a single cycle of IP requires the activation of both protein kinase C and tyrosine kinase,30 on the other hand, in the pig hearts, synergistic activation of protein kinase C and tyrosine kinase is necessary to provoke full cardioprotection, proposed by the group of Vahlhaus et al,31 and the ratio of the role of

<table>
<thead>
<tr>
<th>Particulate fraction</th>
<th>Src</th>
<th>Fyn</th>
<th>Lyn</th>
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Values are mean±SEM. n=5 in each group. Data are compared with the control or genistein values. *P<0.01 and †P<0.05 vs control or genistein group.

Figure 6. Comparison of canine ecto-5'-nucleotidase activity after 45 minutes of steady-state observation (control group; n=5), IP (n=5), IP with 3 doses of genistein (n=5 each), and IP with genistein (n=5).

Figure 7. Comparison of canine ecto-5'-nucleotidase activity after exposure to PMA (n=5), PMA with 3 doses of genistein (n=5 each), and genistein (n=5). PMA increased ecto-5'-nucleotidase activity, which was not blunted by genistein. Data from the control and IP groups were the same as in Figure 6.
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Cellular Mechanisms of the Infarct Size–Limiting Effect of IP in Canine Hearts

If protein tyrosine kinase is not involved in the infarct size–limiting effect of IP, what is the trigger for IP in the canine hearts? In the present and previous studies, we have observed that IP translocates protein kinase C from cytosolic fractions to membrane fractions, indicating that IP activates protein kinase C in the canine myocardium.11 There are several reports of the activation of the isoforms of protein kinase C,37–38 suggesting that total activity of protein kinase C may not be necessarily increased.

There are several ways to activate protein kinase C during IP. First, because adenosine is reported to trigger the infarct size–limiting effect of IP in the rabbit heart, adenosine may activate protein kinase C through G proteins. Second, in the rabbit heart, Goto et al39 indicated that bradykinin is involved in the infarct size–limiting effect of IP through activation of protein kinase C. Furthermore, Schulz et al40 reported that the combination of adenosine and bradykinin is important to cause IP by 10 minutes of ischemia in the swine. The third possibility is endogenous norepinephrine. We have previously reported that endogenous norepinephrine during the IP procedure is responsible for activation of ecto–5′-nucleotidase and mediates the infarct size–limiting effect.9 These observations indicate that endogenous norepinephrine can also trigger the infarct size–limiting effect of IP in the canine heart.

Taken together, the involvement of either protein tyrosine kinase or protein kinase C in IP may be important for cardioprotection, but its importance may vary among species.

Acknowledgments

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References


Limitations of the Present Study

We need to be careful of the doses and characteristics of the inhibitors of tyrosine kinase. We used 10 to 100 μg · kg⁻¹ · min⁻¹ of genistein, which corresponds to 10 to 100 μL/L or 37 to 370 μmol/L. Because the Ki value of genistein for deactivation of protein tyrosine kinase is 10 to 20 μmol/L,35 the dose of 30 μg · kg⁻¹ · min⁻¹ of genistein seems to be enough to inhibit tyrosine kinase. We observed that genistein attenuates the activation of protein tyrosine kinase. Furthermore, 2 and 20 μg · kg⁻¹ · min⁻¹ of lavendustin A, which corresponds to 2 and 20 mg/L or 5 to 50 μmol/L, also seems to be enough to inhibit Src tyrosine kinases. The failure of the attenuation of the infarct size–limiting effect of IP using these 2 different tyrosine kinase inhibitors further supports the conclusion of this study.

Because we infused genistein or lavendustin A during the IP procedure into the coronary artery, genistein or lavendustin A was assumed to be washed out and should not have existed during the sustained ischemia and subsequent reperfusion. However, an effective amount of either genistein or lavendustin A infused into the coronary artery was spilled over and was diluted in the systemic blood, which may have affected the cardioprotection of IP. However, the recirculated amount of either genistein or lavendustin A may be less than the effective dose of genistein administered into the systemic vein. Even if either genistein or lavendustin A exists during sustained ischemia and reperfusion, the cardioprotection caused by IP is not blunted by the administration of either genistein or lavendustin A in the present study, suggesting the minimal role of tyrosine kinase as a trigger or even a mediator of IP in the canine hearts. In the rat or rabbit, genistein was infused into the systemic vein, and genistein may largely affect infarct size due to sustained ischemia and reperfusion. This difference may explain the different results using the inhibitors of tyrosine kinase in cardioprotection due to IP. We cannot deny this possibility. However, in the rabbit heart, Weinbrenner et al38 infused genistein into the coronary artery during the IP procedure in the Langendorff preparation and found that genistein inhibits the infarct size–limiting effect, suggesting that activation of protein tyrosine kinase can trigger the infarct size–limiting effect of IP in the rabbit hearts.

There is a possibility that the activation of Lck tyrosine kinase is involved in the late phase of IP.36 Indeed, it is reported that tyrosine kinase activation is involved in the development of the late phase of IP in the rabbits. We need to test this issue in canine hearts.

these enzymes may be different among the species. To fully activate the end effector(s), different sites of phosphorylations of proteins or enzymes may be necessary via protein kinase C and tyrosine kinase. Furthermore, if KATP channels constitute the end effector of IP, the dual control of the activation of KATP channels via protein kinase C and tyrosine kinase may also be likely. This is because sarcoplasmic KATP channels are activated by adenosine receptor activation32 or protein kinase C,33 and mitochondrial KATP channels are activated by protein kinase C.34
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