Tongxinluo reduces myocardial no-reflow and ischemia-reperfusion injury by stimulating the phosphorylation of eNOS via the PKA pathway

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Li XD, Yang YJ, Geng YJ, Jin C, Hu FH, Zhao JL, Zhang HT, Cheng YT, Qian HY, Wang LL, Zhang BJ, Wu YL. Tongxinluo reduces myocardial no-reflow and ischemia-reperfusion injury by stimulating the phosphorylation of eNOS via the PKA pathway. Am J Physiol Heart Circ Physiol 299: H1255–H1261, 2010. First published August 6, 2010; doi:10.1152/ajpheart.00459.2010.—The objective of the present study was to investigate whether pretreatment with single low loading dose of tongxinluo (TXL), a traditional Chinese medicine, 1 h before myocardial ischemia could attenuate no-reflow and ischemia-reperfusion injury by regulating endothelial nitric oxide synthase (eNOS) via the PKA pathway. In a 90-min ischemia and 3-h reperfusion model, minipigs were randomly assigned to the following groups: sham, control, TXL (0.05 g/kg, gavaged 1 h before ischemia), TXL + H-89 (a PKA inhibitor, intravenously infused at a dose of 1.0 μg·kg⁻¹·min⁻¹ 30 min before ischemia), and TXL + L-NNA (an eNOS inhibitor, intravenously administered at a dose of 10 mg/kg 30 min before ischemia). TXL decreased creatine kinase (CK) activity (P < 0.05) and reduced the no-reflow area from 48.6% to 9.5% and infarct size from 78.5% to 59.2% (reversed by L-NNA. TXL elevated PKA activity and the expression of these effects of TXL were partially abolished by H-89 and completely abolished by H-89 (a PKA inhibitor, intravenously infused at a dose of 1.0 μg·kg⁻¹·min⁻¹ 30 min before ischemia), and TXL + L-NNA counteracted the phosphorylation of eNOS at Ser1179 and Ser635, and this effect is partially mediated by the PKA pathway.

myocardial infarction; reperfusion therapy; Chinese herbal drugs; cardioprotection

TONGXINLUO (TXL), a compound of traditional Chinese medicine, is composed of Radix ginseng, Buthus martensi, Hirudo, Eupolyphaga seu steleophaga, Scolopendra subsinipes, Periostracum cicadae, Radix paeoniae rubra, Semen ziziphi spino-

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MATERIALS AND METHODS

Animal care. All animals used in this study received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experimental procedures were approved by the Care of Experimental Animals Committee of Fuwai Hospital, Peking Union Medical College, and Chinese Academy of Medical Sciences.

Experimental protocol. As previously described (8), 6-mo-old Chinese minipigs weighing 20–30 kg were anesthetized with a mixture of ketamine hydrochloride (700 mg) and diazepam (30 mg) intramuscularly and were continuously infused with the mixture (2
were collected at baseline, at the end of ischemia, and after 180 min
gravimetrically. AN/AAR), respectively, with the mass of each area being determined
AN were expressed as a percentage of the AAR (ANR/AAR and
expressed as a percentage of LV mass (AAR/LV), and the ANR and
and incubated in a 1% solution of buffered (pH 7.4) triphenyltetrazo-
area, area of reflow, and ANR were homogenized on ice in PKA
administered, H-89 (Alexis), a PKA inhibitor, was intravenously and
and constantly infused at the dose of 1.0 µg·kg⁻¹·min⁻¹ throughout the
procedure (27) (TXL + H-89 group); 4) 30 min after TXL was
administered, N⁰-nitro-1-arginine (l-NNA; Aldrich), an eNOS inhibitor,
was intravenously infused at a dose of 10 mg/kg to inhibit eNOS activity
(TXL + l-NNA group); and 5) the LAD was encircled by a suture but not occluded (sham group).

**Hemodynamic experiments.** Hemodynamic data, including heart rate (HR), mean aortic pressure, left ventricular (LV) end-diastolic pressure (LV dP/dt max and LV dP/dt min, respectively), and mean coronary blood flow were recorded on a polygraph (MP-150, Biopac Systems) at baseline, after 90 min of ischemia, and after 3 h of reperfusion and then analyzed with software (Acqknowledge version 3.8.1).

**Measurement of the area at risk, area of no reflow, and infarct size.** Measurements of the myocardial area at risk (AAR), area of no reflow (ANR), and area of necrosis (AN) were performed according to previously described methods (8). In brief, the area of impaired perfusion was delineated by a bolus injection of 4% fluorescent dye thioflavion S (1 ml/kg, Sigma) saline solution into the left atrium and LV at the same time. Approximately 30 s later, the LAD was religated at the original site, and the AAR was outlined by fusion with a bolus of 2% Evans blue dye (1 ml/kg) saline solution into the left atrium. The heart was excised, the extra-LV tissue was removed, and the LV tissue was then sliced transversely into six to seven slices in parallel to the atrioventricular groove. The AAR, the area unstained by Evans blue dye, was traced in visible light. The ANR, the area not perfused by thioflavion S, was photographed under ultraviolet light (wavelength: 365 nm), and the area between the AAR and ANR was the area of reflow. Tissue samples were collected from the ANR, area of reflow, and nonischemic area and were immediately placed in liquid nitrogen for the next examination. Tissue slices were weighed and incubated in a 1% solution of buffered (pH 7.4) triphenyltetrazolium chloride at 37°C for 15 min to identify the AN. The AAR was expressed as a percentage of LV mass (AAR/LV), and the ANR and AN were expressed as a percentage of the AAR (ANR/AAR and AN/AAR), respectively, with the mass of each area being determined gravimetrically.

**Plasma creatine kinase activity.** Venous blood samples (0.3 ml) were collected at baseline, at the end of ischemia, and after 180 min of reperfusion and then centrifuged at 2,500 g for 10 min at 4°C. Plasma creatine kinase (CK) activity (in IU/ml) was analyzed spectrophotometrically according to the manufacturer’s instructions (Nanjing JianCheng).

**Tissue PKA activity assay.** PKA activity was measured according to previously described methods using a nonradioactive PKA assay kit (Promega) (24). Myocardial tissue samples from the nonischemic area, area of reflow, and ANR were homogenized on ice in PKA extraction buffer, which contained 25 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin, and 1 µg/ml aprotinin. The homogenate was centrifuged at 20,000 g for 5 min at 4°C, and the supernatant was assayed according to the manufacturer’s instructions. The reaction products were separated on a 0.8% agarose gel at 100 V for 15 min. The phosphorylated species migrated toward the positive electrode, whereas the nonphosphorylated substrate migrated toward the negative electrode. The fluorescence intensity of the phosphorylated peptides, which reflects PKA activity, was quantified by spectrophotometry at 570 nm. One unit of kinase activity is defined as the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.

**Western blot analysis.** Expression of PKA, Thr⁰⁰⁸ phosphorylated (p-PKA), eNOS, Ser¹⁷⁷⁰ p-eNOS, and Ser⁶₅⁵ p-eNOS was detected by a Western blotting assay as previously described (26). Rabbit polyclonal PKA-α/β/γ (H-56; sc-98551, 1:100 dilution, Santa Cruz Biotechnology), rabbit polyclonal p-PKA-α/β/γ (Thr⁰⁸⁹; sc-32968, 1:100 dilution, Santa Cruz Biotechnology), rabbit polyclonal eNOS (no. 9572, 1: 250 dilution, Cell Signaling), rabbit monoclonal p-eNOS (Ser¹⁷⁷⁰; no. 369100, 1: 250 dilution, Invitrogen), rabbit polyclonal p-eNOS (Ser⁶₅⁵; no. 07-562, 1:200 dilution, Upstate), or mouse monoclonal β-actin (no. 60008-1, 1:10,000 dilution, Proteintech) antibodies were applied. Finally, immunoreactive bands were visualized by a chemiluminescence reagent, and the results are presented as the percent intensity of β-actin.

**Criteria for exclusion.** To ensure that all of the animals included in the data analysis were healthy and exposed to a similar extent of ischemia, the exclusion criteria previously described (28) regarding hemodynamicism, excessive collateral flow, and lethal arrhythmia were used. Data were excluded from further analysis if 1) the AAR was <15% of the LV mass and 2) more than six attempts at electrical cardioversion were made.

**Statistical analysis.** All data are expressed as means ± SE. Data from all stages, such as hemodynamic data and other time-dependent determinations, were compared by repeated-measures ANOVA followed by post hoc analysis with Student-Newman-Keuls multiple comparisons. Differences in a single variable, such as ANR, AN, and CK activity, were compared among groups by one-way ANOVA followed by Duncan’s post hoc test. P values of <0.05 were considered statistically significant.

**RESULTS**

**Hemodynamic data.** Hemodynamic data from the five groups are shown in Tables 1 and 2. There were no significant differences between the five groups at baseline (P > 0.05). Compared with baseline, HR, mean aortic pressure, and the rate-pressure product were lowered by TXL during myocardial ischemia and reperfusion (P < 0.05). LVEDP was elevated in the control and TXL + H-89 groups after ischemia and reperfusion (P < 0.05). dP/dt max decreased in the control, TXL, and TXL + H-89 groups before reopening of the LAD (P < 0.05) and also declined in the TXL group at the end of the experiment (P < 0.01). dP/dt min was augmented in the control, TXL, TXL + H-89, and TXL + l-NNA groups after ischemia and reperfusion (P < 0.05). LAD mean blood flow decreased in the control, TXL + H-89, and TXL + l-NNA groups after reperfusion (P < 0.05) but increased in the TXL group after 5 min of reperfusion (P < 0.01). Compared with the control group, HR decreased in the TXL, TXL + H-89, and TXL + l-NNA groups before reperfusion (P < 0.01), and LAD mean blood flow increased in the TXL group after 5 min of reperfusion (P < 0.01).

**AAR, ANR, and infarct size.** The AAR (AAR/LV) was comparable among the control, TXL, TXL + H-89, and TXL + l-NNA groups (P > 0.05), averaging between 25.56% and 30.59% (Fig. 1). The ANR (ANR/AAR) was significantly smaller in the TXL and TXL + H-89 groups, representing a 38.9% and 19.38% reduction, respectively, from the control group (P < 0.05) and was also smaller in the TXL group than in the TXL + H-89 and TXL + l-NNA groups (P < 0.05). The AN (AN/AAR) was significantly reduced in the TXL group com-
pared with the control, TXL + H-89, and TXL + l-NNA groups (< 0.05).

Plasma CK activity. Plasma CK activity at baseline was comparable among the five groups (P > 0.05; Table 3). After 90 min of ischemia, plasma CK activity significantly increased in the control and TXL + l-NNA groups compared with the sham group (P < 0.01) but decreased in the TXL and TXL + H-89 groups relative to the control group (P < 0.05). After 3 h of reperfusion, plasma CK activity was greatly augmented in the control, TXL, TXL + H-89, and TXL + l-NNA groups compared with the sham group (P < 0.01) but was significantly reduced in the TXL group compared with the control, TXL + H-89, and TXL + l-NNA groups (< 0.01).

Myocardial PKA activity. PKA activity in the nonischemic, reflow, and no-reflow myocardium is shown in Fig. 2. There were no statistical differences of tissue PKA activity among the five groups in the nonischemic area (P > 0.05). However, PKA activity in the areas of reflow and no-reflow significantly increased in the control, TXL, TXL + H-89, and TXL + l-NNA groups compared with the sham group (P < 0.01). PKA activity in the reflow area was higher in the TXL group compared with the control, TXL + H-89, and TXL + l-NNA groups (P < 0.05). No statistical differences were observed in the no-reflow area among the control, TXL, TXL + H-89, and TXL + l-NNA groups (P > 0.05).

Expression of PKA and eNOS in the nonischemic, reflow, and no-reflow myocardium. Western blot analysis was performed to investigate the expression of PKA, Thr$^{198}$ p-PKA, eNOS, and p-eNOS (Ser$^{1172}$ and Ser$^{1435}$) in different areas of myocardium; the results are shown in Fig. 3. In the nonischemic area (Fig. 3A), the expression of PKA increased after ischemia-reperfusion injury compared with the sham group (P < 0.01), with no significant differences existing among the control, TXL, TXL + H-89, and TXL + l-NNA groups (P > 0.05). Expression of eNOS increased in the control and TXL groups compared with the sham group (P < 0.01) but decreased in the TXL + H-89 group compared with the control group (P < 0.05). Phosphorylation of eNOS at Ser$^{635}$ was higher in the control and TXL + l-NNA groups relative to the sham group (P < 0.01) but was lower in the TXL and TXL + H-89 groups than in the control group (P < 0.01). No great differences were found in the expression of Thr$^{198}$ p-PKA or Ser$^{1172}$ p-eNOS among the five groups (P > 0.05).

In the reflow area (Fig. 3B), the expression of PKA decreased in the TXL + H-89 group compared with the sham, control, and TXL groups (P < 0.01). Phosphorylation of PKA at Thr$^{198}$ increased in the control group compared with the sham group (P < 0.05). Expression of eNOS significantly increased after ischemia and reperfusion compared with the sham group (P < 0.01), and no statistical differences were

Table 1. Hemodynamic data at baseline, at the end of ischemia, and at the end of reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Ischemia (90 min)</th>
<th>Reperfusion (180 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart Rate, beats/min</td>
<td>Mean Aortic Pressure, mmHg</td>
<td>Rate-Pressure Product, mmHg</td>
</tr>
<tr>
<td>Sham group</td>
<td>102.6 ± 10.34</td>
<td>84.56 ± 10.21</td>
<td>3,677.25 ± 1,063.47</td>
</tr>
<tr>
<td>Control group</td>
<td>115.46 ± 10.12</td>
<td>90 ± 7.97</td>
<td>10,103.48 ± 783.04</td>
</tr>
<tr>
<td>TXL group</td>
<td>86.25 ± 6</td>
<td>88.28 ± 5.93</td>
<td>7,619.68 ± 741.33</td>
</tr>
<tr>
<td>TXL + l-NNA group</td>
<td>112 ± 8.64</td>
<td>83.42 ± 5.36</td>
<td>9,826.06 ± 820.74</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 animals/group. TXL, tongxinluo; l-NNA, N$^\text{N}$-nitro-l-arginine. *P < 0.05 vs. baseline within the same group; †P < 0.05 vs. the control group at the same time point.

Table 2. Comparison of coronary blood flow among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>5 min</th>
<th>30 min</th>
<th>60 min</th>
<th>120 min</th>
<th>180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>25.03 ± 5.01</td>
<td>17.49 ± 3.44</td>
<td>16.38 ± 3.27</td>
<td>25.95 ± 6.39</td>
<td>16.91 ± 3.04</td>
<td>18.1 ± 4.37</td>
</tr>
<tr>
<td>Control group</td>
<td>27.58 ± 4.6</td>
<td>23.97 ± 3.72</td>
<td>18.09 ± 1.63</td>
<td>26.16 ± 5.66</td>
<td>14.88 ± 1.27*</td>
<td>8.65 ± 1.71*</td>
</tr>
<tr>
<td>TXL group</td>
<td>17.13 ± 2.79</td>
<td>37.13 ± 6.39</td>
<td>19.02 ± 1.6</td>
<td>17.55 ± 1.97</td>
<td>12.33 ± 1.52</td>
<td>8.64 ± 0.53</td>
</tr>
<tr>
<td>TXL + H-89 group</td>
<td>31.24 ± 6.89</td>
<td>27.59 ± 3.98</td>
<td>16.18 ± 2.44*</td>
<td>18.57 ± 2.43*</td>
<td>19.81 ± 2.94*</td>
<td>14.56 ± 2.03*</td>
</tr>
<tr>
<td>TXL + l-NNA group</td>
<td>20.19 ± 2.32</td>
<td>24.56 ± 3.33</td>
<td>18.47 ± 1.87</td>
<td>16.7 ± 2.1</td>
<td>11.26 ± 1.73*</td>
<td>7.72 ± 0.97*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in ml/min); n = 8 animals/group. *P < 0.05 vs. baseline within the same group; †P < 0.05 vs. the control group at the same time point.
observed among the control, TXL, TXL + H-89, and TXL + L-NNA groups ($P > 0.05$). Phosphorylation of eNOS at Ser$^{1179}$ was greatly augmented in the TXL and TXL + H-89 groups compared with the sham group ($P < 0.01$) but was decreased in the TXL + L-NNA group compared with the TXL group ($P < 0.01$). Phosphorylation of eNOS at Ser$^{635}$ was elevated after ischemia and reperfusion relative to the sham group ($P < 0.05$) and was increased in the TXL + H-89 group compared with the control and TXL groups ($P < 0.01$).

In the no-reflow area (Fig. 3C), the expression of PKA and Thr$^{198}$ p-PKA was higher in the TXL and TXL + H-89 groups than in the control group ($P < 0.05$). There were no significant differences in eNOS expression among the five groups ($P > 0.05$). Phosphorylation of eNOS at Ser$^{1179}$ was higher in the control, TXL, and TXL + H-89 groups relative to the sham group ($P < 0.05$) but was decreased in the TXL + L-NNA group compared with the control and TXL groups ($P < 0.01$). Phosphorylation of eNOS at Ser$^{635}$ was greatly increased in the TXL group compared with the sham, control, TXL + H-89, and TXL + L-NNA groups ($P < 0.01$).

### Discussion

This study showed that 1) acute pretreatment with a single low loading dose of TXL preserved cardiac function and coronary blood flow and reduced CK activity and infarct size and partially suppressed the reduction of the no-reflow area; 2) the protective effects of TXL were completely suppressed by inhibition of eNOS with L-NNA, whereas inhibition of PKA by H-89 completely abolished the effects of TXL on hemodynamic function and infarct size and partially suppressed the reduction of the no-reflow area; 3) TXL increased PKA activity and the expression of PKA, Thr$^{198}$ p-PKA, Ser$^{1179}$ p-eNOS, and Ser$^{635}$ p-eNOS in the ischemic myocardium; and 4) H-89 reversed the TXL-induced enhancement of PKA activity and increased the expression of Ser$^{635}$ p-eNOS in the reflow area, whereas L-NNA counteracted the phosphorylation of eNOS at Ser$^{1179}$ and Ser$^{635}$ without an apparent influence on PKA activity.

### PKA is involved in TXL-induced cardioprotection by regulating of eNOS.

Our results suggest that pretreatment with a low loading dose of TXL just 1 h before ischemia can attenuate myocardial no-reflow and ischemia-reperfusion injury in a PKA-dependent manner. The results in the present study are consistent with those of previous studies (8, 31, 32) in which pretreatment with TXL at low to high doses (0.05–0.5 g·kg$^{-1}$·day$^{-1}$) for 3 days or with a high loading dose (0.4 g/kg) 3 h before ischemia effectively reduces the area of no reflow and infarction.
reduced myocardial no reflow and infarction. It appears that the effects of TXL on the size of the no-reflow and infarcted areas can be attributed primarily to the effects on myocardial O2 demand and microcirculation, as evidenced by the significant reduction in the rate-pressure product and dP/dt max during ischemia and reperfusion as well as the improvement of LAD mean blood flow during reperfusion, which were reversed back to control levels after treatment with H-89 and L-NNA.

The cardioprotective effects of TXL in the present study mainly depend on the activation of eNOS because the protective effects of TXL were completely canceled by the eNOS inhibitor in this study and our previous study (8). The results of present study provide substantial evidence for the crucial role of eNOS in the cardioprotection of TXL, as supported by the fact that inhibition of eNOS by L-NNA completely attenuated the TXL-induced upregulation of eNOS at Ser635 and Ser1179 in the ischemic myocardium.

Fig. 3. Expression of PKA, Thr198 phosphorylated (p-)PKA, endothelial nitric oxide synthase (eNOS), Ser1179 p-eNOS, and Ser635 p-eNOS in the ischemic myocardium. A: in the NA, expression of PKA, eNOS, and Ser635 p-eNOS was upregulated after ischemia and reperfusion compared with the sham group. B: in the AR, the expression of PKA was decreased in the TXL + H-89 group compared with the sham, control, and TXL groups. TXL pretreatment induced the upregulation of Ser1179 p-eNOS, which was inhibited by 1-NNa. Phosphorylation of eNOS at Ser635 was elevated after ischemia-reperfusion injury relative to the sham group and was further increased in the TXL + H-89 group compared with the control and TXL groups. C: in the ANR, the expression of PKA, Thr198 p-PKA, Ser1179 p-eNOS, and Ser635 p-eNOS was increased by TXL pretreatment, whereas coadministration of H-89 partially abolished the elevation of these proteins, and 1-NNa reversed the upregulation of Ser1179 p-eNOS and Ser635 p-eNOS. Data are expressed as means ± SE.
Ser\textsuperscript{1179} p-eNOS in the reflow myocardium and Ser\textsuperscript{635} p-eNOS in the no-reflow area.

The importance of eNOS in cardioprotection after ischemia-reperfusion injury has been well demonstrated, and its regulation is mainly the result of the PI3K/Akt pathway (12–14, 17–19). In this study, we found that PKA is another mediator in the TXL-regulated phosphorylation of eNOS in myocardial ischemia-reperfusion injury. PKA physically interacts with the phosphorylated form of Akt in early and late preconditioning in endothelial cells, suggesting that PKA is required for the phosphorylation of Akt and that there is cross-talk between PKA and Akt (3). However, Zhang et al. (35) recently reported that preconditioning with ethanol-extracted TXL (100 µg/ml) reduced palmitic acid-induced endothelial apoptosis and intracellular oxidative stress by the activation of the 5'-AMP-activated protein kinase (AMPK) and p38 MAPK stress pathways and by the inhibition of the PKA and PI3K/Akt/PKC pathways (35). The contradiction of TXL regulating PKA activity between Zhang et al.’s study and the present study is probably because of the different stress stimuli and variance in vitro and in vivo experiments.

**Mechanism of PKA-mediated TXL regulation of eNOS.** Numerous evidence has demonstrated that eNOS activity can be regulated by PKA-mediated phosphorylation (4, 5, 20, 21), and Ser\textsuperscript{1179}, Ser\textsuperscript{635}, and Ser\textsuperscript{617} have been identified as target sites of PKA in bovine eNOS (in humans: Ser\textsuperscript{1177}, Ser\textsuperscript{633}, and Ser\textsuperscript{617}, respectively) (1, 33, 36). In present study, we demonstrated that PKA-mediated phosphorylation of eNOS at Ser\textsuperscript{1179} and Ser\textsuperscript{635} played a critical role in TXL-induced cardioprotection.

Previous studies have found that the phosphorylation of Ser\textsuperscript{1177/1179}, regulated by the PKA and PI3K/Akt pathways, is pivotal for eNOS activation in response to shear stress, adiponectin, and statins (6, 10, 11), whereas the phosphorylation of Ser\textsuperscript{633/635}, mainly activated by PKA and AMPK (2, 4, 7), is the most potent in stimulating NO production among the four phosphorylating sites (including Ser\textsuperscript{1179}, Ser\textsuperscript{635}, Ser\textsuperscript{617}, and Ser\textsuperscript{116}) and plays a central role in maintaining eNOS activity after the initial activation sparked by Ca\textsuperscript{2+} flux and Ser\textsuperscript{1177/1179} phosphorylation (22). This was confirmed by our finding that, at the end of the experiment, the phosphorylation of Ser\textsuperscript{635} p-eNOS in the no-reflow area was still at a high level but that there were no significant differences in the phosphorylation of Ser\textsuperscript{1179} p-eNOS observed between the control and TXL groups.

The present study showed that TXL enhanced PKA activity and the expression of PKA, Thr\textsuperscript{198} p-PKA, Ser\textsuperscript{1179} p-eNOS, and Ser\textsuperscript{635} p-eNOS in the no-reflow area but that H-89 reversed the elevation of PKA activity and the expression of Ser\textsuperscript{635} p-eNOS in the no-reflow area. This suggests that PKA-induced phosphorylation of Ser\textsuperscript{635} p-eNOS plays a key role in the TXL-induced attenuation of myocardial no-reflow and ischemia-reperfusion injury and may be a common mechanism in maintaining eNOS activity after prolonged ischemia. The exact mechanism that TXL activates eNOS phosphorylation via the PKA pathway is not yet clear. It has been demonstrated that PKA plays a role in ischemic preconditioning by inhibiting the activity of Rho A and Rho kinase (ROCK) (15, 27), which negatively regulates mRNA stability and expression of eNOS (25), indicating that the PKA-mediated mechanism of TXL that regulates eNOS activity is probably through the G protein/adenylate cyclase/ROCK pathway (11, 27).

In the present study, the beneficial effects of TXL could be completely eliminated by L-NNA but only partially canceled by H-89, suggesting that other mechanisms compensated for the inhibition of PKA. It has been demonstrated that there is cross-talk between the PKA and PKG pathways in NO-induced cerebral vascular relaxation (23) and cross-talk also exists between PKA and Akt in preconditioning (3). Therefore, it is plausible to postulate that the decrease in cGMP concentration after ischemia and reperfusion results in the activation of PKA and, in turn, sensitizes the phosphorylation of eNOS to activate guanylyl cyclase and thereby subsequently elevates the concentration of cGMP and eventually activates PKG (23, 29), whereas the inhibition of PKA probably leads to the activation of the Akt/PKG pathway.

It is interesting to note that TXL increased the expression of Ser\textsuperscript{635} p-eNOS in the no-reflow area but decreased it in the nonischemic area, indicating that TXL pretreatment effected a new balance in the expression of Ser\textsuperscript{635} p-eNOS between the nonischemic and ischemic myocardium. This may be the most important mechanism for TXL that protects hearts from no-reflow and ischemia-reperfusion injury. Furthermore, inhibition of PKA by H-89 diminished the TXL-induced enhancement of PKA activity and the phosphorylation of eNOS at Ser\textsuperscript{635} p-eNOS in the no-reflow area but increased the phosphorylation of eNOS at Ser\textsuperscript{635} in the reflow area, suggesting that the phosphorylation of eNOS at Ser\textsuperscript{635} could be regulated by different mechanisms in the areas of reflow and no reflow and that the inhibition of one way may lead to the activation of the phosphorylated eNOS at Ser\textsuperscript{635}. Previous studies (7, 9) have reported that AMPK, a downstream activator of PKA, also catalyzes the phosphorylation of eNOS at Ser\textsuperscript{635}, but the exact mechanism needs further study.

**Conclusions.** In summary, the present study showed that a single low loading dose of TXL just 1 h before ischemia reduced no-reflow and ischemia-reperfusion injury and that this effect partially depended on the PKA pathway by increasing the phosphorylation of eNOS at Ser\textsuperscript{1179} and Ser\textsuperscript{635}. Phosphorylation of eNOS at Ser\textsuperscript{1179} and Ser\textsuperscript{635}, by potentiating eNOS activity, is crucial for TXL to protect against ischemia-reperfusion injury at the early period, whereas Ser\textsuperscript{635} p-eNOS is more important in the later protection mechanism of TXL. As TXL augmented the expression of Ser\textsuperscript{635} p-eNOS in the no-reflow area but not in the nonischemic area, we suppose that pretreatment with TXL rebalances the phosphorylation of eNOS between the nonischemic and ischemic myocardium. The facts that all of the cardioprotective effects of TXL could be extinguished by H-89 except for the reduction of no-reflow size suggests that the PKA pathway is important in the no-reflow area by upregulating Ser\textsuperscript{635} p-eNOS. Traditional Chinese medicine has been of great benefit to Asian people for centuries; therefore, understanding the mechanisms involved in TXL-induced cardioprotection may help explain the improved outcomes after the immediate administration of TXL to patients with acute coronary syndrome (16) and may lead to the development of a new area of therapeutic use.

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DISCLOSURES

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