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 + N⁰-nitro-L-arginine (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% (P < 0.05), whereas these effects of TXL were partially abolished by H-89 and completely reversed by L-NNA. TXL elevated PKA activity and expression of PKA, Thr⁶⁸ phosphorylated PKA, Ser¹¹⁷⁹ phosphorylated eNOS, and Ser²³⁵ phosphorylated eNOS in the ischemic myocardium. H-89 repressed the TXL-induced enhancement of PKA activity and phosphorylation of eNOS at Ser²³⁵, and L-NNA counteracted the phosphorylation of eNOS at Ser¹¹⁷⁹ and Ser²³⁵ without an apparent influence on PKA activity. In conclusion, pretreatment with a single low loading dose of TXL 1 h before ischemia reduces myocardial no-reflow and ischemia-reperfusion injury by upregulating the phosphorylation of eNOS at Ser¹¹⁷⁹ and Ser²³⁵, 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 teleophaga, Scolopendra subspinipes, Periostocicum cicaeae, Radix paeoniae rubra, Senior tzigphi spino-
sae, Lignum dalbergiae odoriferae, Lignum santali albi, and Borneolum syntheticum (30). Because of its definite effects in improving symptoms and reducing infarct size and complications with few adverse effects except for slight gastrointestinal discomfort in a clinical trial (8), TXL has been widely used in China to treat patients with acute coronary syndrome (16, 30, 34).

Our previous studies (8, 31, 32) have found that pretreatment with TXL at low to high doses for 3 days or with a high loading dose 3 h before ischemia can reduce myocardial no-reflow and infarction in swine. However, it is not clinically practical to pretreat patients undergoing acute postinfarct percutaneous coronary intervention with high-dose of TXL several hours or days before the operation. Therefore, whether a low loading dose of TXL just before acute percutaneous coronary intervention can also ameliorate no-reflow and ischemia-reperfusion injury needs to be clarified in terms of clinical benefits.

The enhancement of endothelial nitric oxide (NO) synthase (eNOS) activity and NO bioavailability, whose regulation is mainly the result of the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (Akt) signaling cascade (12–14, 17–19), is the fundamental mechanism of TXL against myocardial no-reflow and ischemia-reperfusion injury (8). Recent studies (11, 15, 27) have shown that PKA is also involved in the activation of eNOS induced by ischemic preconditioning and statins. However, it is unclear whether the cardioprotective effects of TXL are mediated by the cAMP/PKA pathway. In this study, we hypothesized that a single pharmacological loading dose of TXL just 1 h before ischemia could protect hearts from no-reflow and ischemia-reperfusion injury by enhancing eNOS activity in a PKA-dependant manner.

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

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were collected at baseline, at the end of ischemia, and after 180 min of reflow. AN were expressed as a percentage of the AAR (ANR/AAR) and LV mass (AAR/LV), and the ANR and LV mass were traced in visible light. The AAR and LV mass were outlined by perfusion with a 0.8% agarose gel at 100 V for 15 min. Tissue slices were weighed at the original site, and the AAR was outlined by perfusion with a bolus of 4% fluorescent dye (wavelength: 365 nm), and the area between the AAR and ANR was photographed under ultraviolet light. The ANR, the area not perfused by thioflavin S, was traced in visible light. The AN, the area not perfused by Evans blue dye, was assessed by spectrophotometry at 570 nm. One unit of kinase activity was defined as the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.

Western blot analysis. Expression of PKA, Thr\(^{198}\) phosphorylated (p)-PKA, eNOS, Ser\(^{1179}\) p-eNOS, and Ser\(^{635}\) p-eNOS was detected by Western blotting as previously described (26). Rabbit polyclonal PKA-α/β/γ (H-56; sc-98951, 1:100 dilution, Santa Cruz Biotechnology), rabbit polyclonal p-PKA-α/β/γ (Thr\(^{198}\); sc-39685, 1:100 dilution, Santa Cruz Biotechnology), rabbit polyclonal eNOS (no. 9572, 1: 250 dilution, Cell Signaling), rabbit monoclonal p-eNOS (Ser\(^{1179}\); no. 369100, 1: 250 dilution, Invitrogen), rabbit polyclonal p-eNOS (Ser\(^{635}\); 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 were 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 hemodynamics, 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.

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\(_{\text{max}}\) 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\(_{\text{min}}\) was augmented in the control, TXL, TXL + H-89, and TXL + l-NNA groups after ischemia and 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 after reperfusion (P < 0.05) but increased in the TXL group after 5 min of reperfusion (P < 0.01).

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 was defined as the number of nanomoles of phosphate transferred to a substrate per minute per milliliter.
pared with the control, TXL + H-89, and TXL + L-NNA groups (P < 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 (P < 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, Thr198 p-PKA, eNOS, and p-eNOS (Ser1177 and Ser1435) 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 Ser635 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 Thr198 p-PKA or Ser1435 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 Thr198 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
In the TXL/H11001-P compared with the sham group (myocardial ischemia and reperfusion Table 3. Variation of plasma creatine kinase activity during myocardial ischemia and reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatine Kinase Activity (IU/ml)</th>
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<tbody>
<tr>
<td>Sham</td>
<td>Control 0.07 ± 0.09, TXL 0.07 ± 0.11, TXL + L-NNA 0.13 ± 0.16</td>
</tr>
<tr>
<td>Control</td>
<td>1.47 ± 0.26, TXL 1.07 ± 0.11, TXL + H-89 1.38 ± 0.19, TXL + L-NNA 1.31 ± 0.16</td>
</tr>
<tr>
<td>TXL</td>
<td>2.81 ± 0.44*, TXL 3.62 ± 0.16**, TXL + H-89 1.88 ± 0.33†, TXL + L-NNA 1.27 ± 0.33*</td>
</tr>
<tr>
<td>TXL + H-89</td>
<td>4.83 ± 0.13*, TXL 3.62 ± 0.29**, TXL + H-89 4.46 ± 0.26**, TXL + L-NNA 4.8 ± 0.44*</td>
</tr>
<tr>
<td>TXL + L-NNA</td>
<td>1.40 ± 0.19, TXL 3.62 ± 0.16**, TXL + H-89 4.46 ± 0.26**, TXL + L-NNA 4.8 ± 0.44*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in IU/ml); n = 8 animals/group. *P < 0.05 vs. the sham group; †P < 0.05 vs. the control group; ‡P < 0.05 vs. TXL + L-NNA group.

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 Thr198 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 Ser1179 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 Ser635 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 the size of the no-reflow and infarcted areas; 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, Thr198 p-PKA, Ser1179 p-eNOS, and Ser635 p-eNOS in the ischemic myocardium; and 4) H-89 reversed the TXL-induced enhancement of PKA activity and increased the expression of Ser635 p-eNOS in the reflow area, whereas L-NNA counteracted the phosphorylation of eNOS at Ser1179 and Ser635 without an apparent influence on PKA activity.

PKA is involved in TXL-induced cardioprotection by regulating 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...
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.

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 + H11001 H-89 group compared with the sham, control, and TXL groups. TXL pretreatment induced the upregulation of Ser1179 p-eNOS, which was inhibited by L-NNA. Phosphorylation of eNOS at Ser635 was elevated after ischemia-reperfusion injury relative to the sham group and was further increased in the TXL + H89 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 H89 partially abolished the elevation of these proteins, and L-NNA reversed the upregulation of Ser1179 p-eNOS and Ser635 p-eNOS. Data are expressed as means ± SE.
Ser^{1179} p-eNOS in the reflow myocardium and Ser^{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^{1179}, Ser^{635}, and Ser^{617} have been identified as target sites of PKA in bovine eNOS (in humans: Ser^{1177}, Ser^{633}, and Ser^{617}, respectively) (1, 33, 36). In present study, we demonstrated that PKA-mediated phosphorylation of eNOS at Ser^{1179} and Ser^{635} played a critical role in TXL-induced cardioprotection.

Previous studies have found that the phosphorylation of Ser^{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^{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^{1179}, Ser^{635}, Ser^{617}, and Ser^{116}) and plays a central role in maintaining eNOS activity after the initial activation sparked by Ca^{2+} flux and Ser^{1177/1179} phosphorylation (22). This was confirmed by our finding that, at the end of the experiment, the phosphorylation of Ser^{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^{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^{198} p-PKA, Ser^{1179} p-eNOS, and Ser^{635} p-eNOS in the no-reflow area but that H-89 reversed the elevation of PKA activity and the expression of Ser^{635} p-eNOS in the no-reflow area. This suggests that PKA-induced phosphorylation of Ser^{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^{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^{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^{635} in the no-reflow area but increased the phosphorylation of eNOS at Ser^{635} in the reflow area, suggesting that the phosphorylation of eNOS at Ser^{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 another way to phosphorylate eNOS at Ser^{635}. Previous studies (7, 9) have reported that AMPK, a downstream activator of PKA, also catalyzes the phosphorylation of eNOS at Ser^{635}, but the exact mechanism need 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^{1179} and Ser^{635}, Phosphorylation of eNOS at Ser^{1179} and Ser^{635}, by potentiating eNOS activity, is crucial for TXL to protect against ischemia-reperfusion injury at the early period, whereas Ser^{635} p-eNOS is more important in the later protection mechanism of TXL. As TXL augmented the expression of Ser^{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^{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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Tongxinluo reduces the area of no-reflow via the PKA pathway

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