Letter to the Editor:

Ligustrazine Attenuates Myocardial Ischemia Reperfusion Injury in Rats by Activating the Phosphatidylinositol 3-kinase/Akt Pathway

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To the Editor: Coronary revascularization has been well-established as the most effective treatment of limiting the eventual infarct size for coronary artery diseases. However, reperfusion can elicit a number of adverse reactions that may limit its beneficial actions. Although reduction of ischemia reperfusion (IR) injury through the use of antioxidant therapy, calcium-channel blockers, sodium-hydrogen exchange inhibitors, and antiinflammatory drugs has previously been attempted in many studies, few agents are clinically available for protecting the heart from IR injury [1].

Activation of the pro-survival kinase-signaling cascade phosphatidylinositol 3-kinase (PI3K) /Akt at the time of reperfusion promotes cell survival and recruits the anti-apoptotic pathway during reperfusion [2]. Experimental studies have indicated that the intervention and ischemia preconditioning of some pharmacological agents can recruit the PI3K/Akt pathway and confer powerful cardioprotection [3]. It has been proposed that pharmacological targeting of the Akt pathway may potentially diminish IR injury [4].

In a recent study [5], we reported that ligustrazine (2,3,5,6-tetramethylpyrazine,TMP), an alkaloid extracted from *Ligusticum wallichii* Franchat (Apiaceae), has cardioprotective effects against myocardial IR injury through limitation of infarct size and reduction of apoptosis. In this article, we further investigated whether the cardioprotective effect of TMP was dose-dependent through observing the ultrastructure, enzyme level, and oxidative stress of the myocardium and whether the PI3K/Akt signal pathway was involved in the cardioprotective effect of TMP using Western blot analysis.

Materials and Methods

Sprague-Dawley rats (n=68, weighing 250-280g) were divided into sham (n=8), IR control (n=10), 3 TMP pretreated (5, 10, 30mg/kg, n=10, respectively), TMP+wortmannin (a PI3K inhibitor, n=10) and wortmannin group (n=10). *In vivo* rat model of myocardial IR was built by the left anterior descending (LAD) artery ligation and reperfusion. Rats were subjected to a sustained coronary occlusion of 35 min and reperfusion of 120 min. TMP (National Institute for the Control of Pharmaceutical and Biological Products, China) of different dose was given by intravenous injection 5 min before ligation, and wortmannin (Sigma-Aldrich, Milwaukee, WI, USA) was given 16ug/kg by intravenous injection 15 min before reperfusion.

After the experiment, the rats were killed and tissue samples of 1mm³ were taken 4mm from the apex. The samples were fixed in 2.5% glutaraldehyde in 0.1 mol/l phosphate buffer at 4°C for 4 h and post-fixed in 1% buffered osmium tetroxide at 4°C for 2 h. The tissue blocks were dehydrated in graded ethanols and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed under a Joel JEM-1011 transmission electron microscope (Joel Ltd, Japan).

To assess the extent of myocardial tissue injury, blood samples were obtained from the right atrium at the end of reperfusion. Serum was isolated from blood by centrifugation (3000g, 5 min), and the activity of creatine kinase MB fraction (CK-MB) was measured by an automatic biochemistry...
Figure 1. Representative examples of electron micrograph.

Compared with the sham group (A), the ultrastructure in the IR control group revealed evidence of widespread mitochondrial damage (B), consisting of severe disturbance in the mitochondrial crista arrangement, loss of mitochondrial matrix substance, presence of intramitochondrial vacuoles, and areas of disruption of the mitochondrial membrane. In contrast, the ultrastructure of myocardial cells of TMP-pretreated rats (C-E) was preserved, although some cells were slightly damaged. However, when wortmannin was jointly used with TMP, the electron microscopy findings still showed substantial damages in mitochondria in which many ruptured cristae were observed (F). The ultrastructure in the wortmannin group was similar to that in the IR control group (G). Wort, wortmannin.
Analyzer (Hitachi model 7600, Japan). The results were expressed in international units per liter.

Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were used as indices of oxygen free radical and lipid superoxide levels. Tissue samples from risk zones were rinsed, homogenized in deionized water (1:10, wt/vol), and centrifuged at 16,000 × g for 10 min. The content of MDA in tissue samples was measured by the formation of thiobarbituric acid reactive substances (TBARS), and the activity of SOD was determined by the xanthine oxidase method, using detection kits (JianCheng Bioengineering Institute, Nanjing, China) with an ultraviolet-visible spectrophotometer (No.3 Analytical Instrument Factory, Shanghai, China).

Expression of phosphorylated Akt and endothelial nitric oxide synthase (eNOS) was observed by Western blot. Myocardial tissue was homogenized on ice in lysis buffer with a protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Phosphorylation states of Akt (phospho-Akt, Ser473), eNOS (phospho-eNOS, Ser1177) and total levels of Akt and eNOS proteins were analyzed by SDS-PAGE immunoelectrophoresis using antibodies obtained from Cell Signaling Technology (Beverly, MA, USA). Levels of phosphorylated proteins were normalized to their total protein levels.

All values are presented as mean ± SD. Differences between groups were analyzed using one-way ANOVA followed by Student-Neuman-Keuls t-test. Statistical significance was defined as p < 0.05.

**Results**

**Electron microscopy findings.** In the sham group (Figure 1A), the ultrastructure of the myocardium was normal, with regularly arranged myofibrils. Mitochondria possessed complete structure and well-developed cristae. In the IR control group (Figure 1B), the normal ultrastructure of myocardial cells was destroyed, with swollen mitochondria in which cristae became fuzzy or ruptured and vacuole degeneration was noted. In contrast, reduced damage to mitochondria was found in the TMP pretreatment groups (Figures 1C-E), particularly when a high dose TMP pretreatment was adopted. However, when wortmannin was jointly used with TMP, the electron microscopy findings still showed substantial damages in mitochondria in which many ruptured cristae were observed (Figure 1F). Pretreatment of wortmannin alone had little effect on the widespread mitochondrial damage caused by IR, with fuzzy cristae and vacuole degeneration observed (Figure 1G).

**Serological estimation of infarct size from CK-MB.** The activity of CK-MB in serum was used to monitor the damage of myocardial tissue. Compared with the sham group, the activity of

Table 1. The levels of enzymatic dynamics and oxidative stress of myocardium

<table>
<thead>
<tr>
<th>Group</th>
<th>CK-MB (U/L)</th>
<th>MDA (nmol/mgpro)</th>
<th>SOD (U/mgpro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>885.4±160.3*Δ</td>
<td>0.223±0.092*Δ</td>
<td>55.1±5.9*</td>
</tr>
<tr>
<td>IR</td>
<td>7355.8±870.5Δ</td>
<td>0.693±0.053Δ</td>
<td>35.6±4.3Δ</td>
</tr>
<tr>
<td>TMP-L</td>
<td>6042.8±541.1*</td>
<td>0.508±0.082*</td>
<td>43.8±4.8</td>
</tr>
<tr>
<td>TMP-M</td>
<td>5839.2±413.0*</td>
<td>0.417±0.069*</td>
<td>50.2±3.2*</td>
</tr>
<tr>
<td>TMP-H</td>
<td>4702.0±796.3*Δ</td>
<td>0.331±0.023*</td>
<td>51.2±6.4*</td>
</tr>
<tr>
<td>TMP+wort</td>
<td>7215.6±530.0Δ</td>
<td>0.458±0.061*</td>
<td>48.4±3.5*</td>
</tr>
<tr>
<td>Wort</td>
<td>7004.4±906.0Δ</td>
<td>0.632±0.065Δ</td>
<td>39.6±3.7Δ</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (n=6). *p < 0.05 compared with the IR control group; Δp < 0.05 compared with the TMP-M group. Wort, wortmannin.
CK-MB in serum of the IR control group markedly increased after reperfusion. Treatment of TMP in the TMP-L, -M, and -H groups significantly reduced the effect of IR on the CK-MB activity. However, the effect of TMP was largely abrogated when wortmannin was used (Table 1).

**MDA content and SOD activity.** Oxidative stress was evaluated by detecting MDA content and SOD activity of the supernatant of tissue samples after homogenization from all groups. The results showed a decrease in the MDA content and an increase in the SOD activity in TMP-L, -M, and -H groups compared with the IR control group ($p<0.05$), except for the SOD activity in the TMP-L group ($p>0.05$). The addition of wortmannin attenuated the changes brought by TMP to MDA content and SOD activity on oxidative stress (Table 1).

**TMP activates Akt and eNOS in cardiac tissue at time of reperfusion.** TMP induced phosphorylation of Akt Ser 473 (in arbitrary units; 1.61±0.18 in TMP-M group and 1.65±0.18 in TMP-H group vs. 0.79±0.10 in IR control group, $p<0.05$) and phosphorylation of eNOS Ser1177 (1.69±0.41 in TMP-L group, 1.87±0.33 in TMP-M group and 3.21±0.62 in TMP-H group vs. 0.94±0.22 in IR control group, $p<0.05$). When wortmannin, a PI3K inhibitor, was given 15 min before reperfusion, the TMP-induced phosphorylation was ceased: Akt with wortmannin (0.54±0.15, $p<0.05$ vs. IR control group) and eNOS with wortmannin (0.53±0.14, $p>0.05$ vs. IR control group).

**Discussion**

In the current study, we demonstrated that treatment with ligustrazine significantly improved the ultrastructure of the myocardium and reduced the activity of CK-MB and oxidative stress in our model of IR injury in a dose-dependent manner. The protective effect of TMP was associated with increased phosphorylation of the survival kinase Akt at Ser473 and its downstream target eNOS following IR, while inhibition of Akt by wortmannin decreased phosphorylated Akt and phosphorylated eNOS levels and abolished TMP’s protective effect. These results suggest that TMP protects the myocardium from IR injury, at least in part, by activating of Akt and its downstream target eNOS.

As a traditional Chinese medicine for treating many ischemia disorders, TMP has been reported to increase coronary blood flow and systemic circulation, relieve stasis, and inhibit endothelial cell damage and vascular smooth muscle cell proliferation [6]. In this paper, we examined the effects of TMP on IR injury rats through evaluation of electron microscopic, myocardial enzyme and tissue oxidant parameters.

Ultrastructural alterations of myocardium in the IR control group characterized massive mitochondrial swelling and sarcolemmal disruption. Pretreatment with TMP attenuated mitochondrial injury as noted by lessened swelling of the matrix and cristae. The finding that the myocyte ultrastructure was largely preserved in TMP pretreated groups helps explain the protective effect of TMP.

Myocardial enzymes can be released from the injured myocytes induced by IR. Therefore, enzyme analysis of CK-MB, which is myocardium specific, has proved valuable in evaluating cardiac injury. The data from this study showed that the activity of CK-MB in serum of TMP-treated hearts was significantly lower in a dose-dependent manner than those of control animals, suggesting that TMP can protect myocardial cells.

SOD and MDA are effective indices reflecting oxidative stress. Consistent with previous studies [7, 8], we found that SOD activity was significantly increased with a remarkable reduction in the level of MDA after pretreatment with TMP in the IR myocardial tissue of rats, especially in the medium and high dose TMP-pretreated groups. This indicates that TMP can reduce oxidative stress.

To further explore the mechanism underlining TMP’s protective effect on IR rats, the phosphorylation and expression of Akt were investigated through Western blot analysis. In the previous study [5], the infarct size-reducing effect of TMP was abolished when jointly used with wortmannin, a PI3K inhibitor. The current study, through evaluation of the ultrastructure and oxidative stress of the myocardium and the CK-MB activity in serum, also confirmed that wortmannin abolished the cardioprotective effect of TMP, suggesting TMP acted...
through activation of PI3K. PI3K is a family of evolutionary conserved lipid kinases that mediate many cellular responses in both physiologic and pathophysiologic states [9]. PI3K/Akt is one of the pathways in the reperfusion injury salvage kinase (RISK) pathway group, which confer powerful cardioprotection when activated specifically at the time of reperfusion. Previous studies have proposed that pharmacological agents that target the RISK pathways imitate the efficacy of interventions such as ischemia preconditioning and postconditioning [2, 3]. Activation of PI3K/Akt has been associated with cell cycle progression, survival, metabolism and migration through phosphorylation of many physiological substrates. In the present study, the expression of phosphorylated Akt was significantly up-regulated in TMP-M and TMP-H groups, but this up-regulation was abolished by the co-treatment with wortmannin, suggesting that TMP activates the PI3K/Akt pathway.

Since phosphorylation of eNOS at Ser1177 is a downstream target of the serine kinase Akt, we further examined the involvement of eNOS in the heart. Similarly, phosphorylated eNOS expression was also significantly up-regulated in TMP groups, and this effect too was blocked by the co-treatment with wortmannin, suggesting that phosphorylation of eNOS was through PI3K/Akt. Therefore, the cardioprotective effect of TMP appeared to be mediated through the activation of PI3K/Akt-eNOS pathway.

In summary, administration of ligustrazine at the onset of ischemia followed by reperfusion induces phosphorylation of Akt Ser473 and eNOS Ser1177 and protects the myocardium from IR in a dose-dependent manner. Pharmacological blockade of PI3K abrogated the cardioprotective effect of ligustrazine. These observations suggest that the cardioprotective effect of ligustrazine is mediated through activating the PI3K/Akt-eNOS pathway.

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References