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Anti-ischemic Effect of Polygala Tenuifolia in Isolated Rat Heart

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Polygala tenuifolia **(PT) is one of the most well-known traditional herbal medicines in Korea which is commonly used for the treatment of cardiovascular symptoms. The anti-ischemic effects of PT in isolated rat heart was investigated by analyzing changes in blood pressure, aortic flow, coronary flow, and cardiac output. And, its underlying mechanism was examined by quantitating intracellular calcium content in rat neonatal cardiomyocytes. Rats were divided into two groups: an ischemia-induced group without any treatment, and an ischemia-induced group treated with PT. Ischemia of isolated heart was induced by stopping the supply of oxygen and buffer for 10 min. The isolated heart was exposed to PT for the first 5 min of 10 min ischemia. PT treatment significantly prevented the decreases of perfusion pressure, aortic flow, coronary flow, and cardiac output under ischemic conditions. In addition, hemodynamics (except heart rate) of the PT-treated group was significantly recovered 60 min after reperfusion compared to the control group (systolic aortic pressure: 83.3% vs. 64.9%, aortic flow volume: 69.5% vs. 48.7%, coronary flow volume: 77.7% vs. 58.4%, and cardiac output: 71.6% vs. 51.2%, $p < 0.01$). As for the underlying mechanism, PT significantly prevented intracellular calcium increase which was induced by isoproterenol ($p < 0.01$), suggesting that the anti-ischemic effect of PT is mediated by inhibition of intracellular calcium increase.**

Key Words: Polygala tenuifolia, Isolated rat heart, Anti-ischemia effect

INTRODUCTION

Cardiac ischemia leads to coronary heart disease, angina pectoris, myocardial infarction, heart failure and ultimately heart attack (Shirai, 2004). Aspirin, beta-blockers, angiotensin-converting enzyme inhibitors, and lipid-lowering agents are currently the backbone of pharmacologic therapy (Mehta et al, 2000). Because of the adverse effects associated with these anti-ischemia drugs, many trials have recently been attempted to find and develop new anti-ischemic drugs from herbal medicines that may have minimum side effects. Numerous animal and clinical studies with various herbal medicines have been carried out, and some studies reported significant improvements in controlling ischemic symptom without any noticeable adverse effect (Sun et al, 2002). In traditional Chinese medicine, Polygalae Radix (Japanese name: Onji), the root of *Polygala tenuifolia* (Polygalaceae; PT), has been prescribed for amnesia, neurasthenia, palpitation, noctural emission and insomnia. The root of PT, a traditional oriental medicine, is known to have sedative, antipsychotic, cognitive improving/neuroprotective, and anti-inflammatory therapeutic effects on the central nervous system (Ikeya et al, 2004). There have also been numerous studies on the reputed memory-enhancing potential of the roots of PT: DX-9368, which is composed of four herbs (*Panax ginseng*, PT, *Acorus gramineus* and *Poria cocos*), ameliorates ethanol- and scopolamine-induced memory impairment in mice (Park et al, 2002), the water-extract of these plants up-regulates choline acetyltransferase (ChAT) activity and increases NGF secretion *in vitro* (Yabe et al, 2003), and the water-extract of plants improved the scopolamine- induced impairment of passive avoidance response and enhanced oxotremorine-induced tremors in mice (Egashira et al, 2003). IKeya et al (2004) showed that PT has a cerebral protective effect on potassium cyanide (KCN)-induced anoxia and an ameliorative effect on the scopolamine-induced impairment of passive avoidance response in rats. It is well known that a decrease of oxygen supply to the brain (hypoxia) depresses cerebral function in experimental animals and humans (Yamamoto et al, 1987; Sakurai et al, 1990) and that memory and learning are impaired by hypoxia in animals and humans (Allweis et al, 1984; Schaffler & Klausnitzer, 1988). Similar phenomena observed in the hypoxic brain have also been found in the heart ischemia (Asano et al, 2003). Such cerebral protective effect of PT on KCN-induced anoxia of PT suggests that PT can serve as an effective anti-heart ischemia agent to improve deficient oxygen supply and ameliorate dysfunction of heart induced by ischemia. However, anti-heart ischemia effect of PT has not yet been reported. Therefore, in the present study, we

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ABBREVIATIONS: PT, Polygala tenuifolia; ChAT, acetyltransferase; NGF, nerve growth factors; KH buffer, Krebs-Henseleit bicarbonate buffer; HPLC, high performance liquid chromatography; LA, left atrium; DMEM, Dulbecco's Modified Eagle Medium; ISO, isoproterenol; NMDA, N-Methyl-D-aspartate; DRG, dorsal root ganglion; NE, norepinephrine; OH, hydroxyl radical.

investigated the anti-ischemia effects of PT using an *ex vivo* Langendorff system and *in vitro* calcium imaging from neonatal cardiomyocytes.

METHODS

Drugs

All chemicals were from Sigma Chemical Co (St. Louis, MO), except Fluo-2AM that was purchased from Serva (Heidelberg, Germany). The spray-dried extracts of PT used in this study were purchased from Sun Ten Pharmaceutical Company (Taipei, Taiwan). PT were dissolved in perfusate. Namely, PT powder was dissolved in Krebs-Henseleit (KH) buffer and the suspension was centrifuged at 15,000 rpm for 10 min. The supernatant was transferred to another tube and filtered through a 0.2 μm syringe filter. Fluo-3AM was dissolved in dimethyl sulfoxide plus pluronic acid. All substances were diluted in distilled water immediately before the experiments.

Animals

All rats received humane care in accordance with the "Guide for the Care and Use of Laboratory Animals" of Chonbuk National University (Jeonju, Korea). In addition, the experimental protocol met with approval of the Laboratory Care and Animal Use Committee at the Armed Forces Institute of Pathology. Sprague-Dawley male rats, weighing 250~300 g, were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA). Upon arrival to our Institute, animals were quarantined for a minimum of 1 week. All rats were kept in a constant temperature and humidity controlled environment [room temperature: 23±1°C, relative humidity: 50±10%, and light cycle (06 : 00~18 : 00 h)]. Animals were fed water and a standard rodent chow diet (Ralston Purina) *ad libitum,* and were acclimatized to their environment for 2 weeks before commencement of the experiments.

The animals were divided into four groups: N/C, normal control group for no ischemic conditions; (+) PT, N/C group treated with *Polygala tenuifolia* alone; (+) PT + Isch., the ischemic group treated with *Polygala Tenuifolia*; and Isch., ischemia-induced group, but not treated with *Polygala tenuifolia.*

Preparation of Polygala tenuifolia

We obtained the spray-dried PT extract from Sunten Pharmaceutical Co. (Taipei, Taiwan, lot number: 295810). PT was deposited at the Department of Physiology, College of Oriental Medicine, Kyung Hee University. One gram of PT powder contained 0.68 g of PT extract and 0.32 g of starch. We dissolved 3 mg of PT powder in 0.68 ml of KH buffer, and the suspension was centrifuged at 15,000 rpm for 10 min. The suspernatant was transferred to an another tube and filtered through a 0.2 μm syringe filter. Then, 0.96 mg of starch was removed from the PT preparation, and the final PT concentration was 3 mg/ml. The resultant solution was administered into the aortic line for 5 min to observe the effects of AF on an ischemia-induced heart with a 65 mmHg perfusion pressure to examine its anti-ischemic effects.

HPLC analysis of standard PT material

One g of the commercial spray-dried water extract, which contained 32 % starch, was accurately weighed, placed in test tubes, and dissolved in 10 ml of chloroform and acetonitrile [(50 : 50 (v/v) solution)] (HPLC reagent, J.T. Baker Co. Ltd, Malinckrodt Baker, Inc. Philipsburg, NJ 08865, USA). This was filtered through a 0.45 um syringe filter (PVDF, Waters, USA). The marker compound (standard material) used for quantitative analysis was harmane. Ten milligrams of each standard material was dissolved in a solution, and this solution was then diluted at 0.1, 0.5, 1.0, 1.5, and 2.0 mg/ml. In order to obtain a standard HPLC chromatogram, each standard solution was again diluted at 0.1, 0.5, 1.0, 1.5, and 2.0 mg/ml. In HPLC analysis, column of XTerra^{TM} RP18 5 μm (4.6×150 mm, ODS, Waters, USA), mobile phase of MeOH: [(65 : 35 (v/v)] containing 0.01 M SDS UV detector (280 nm), flow rate of 1.0 ml/min and column temperature of 25°C were used. The relationship between the concentration and the peak-area was measured by the method of least sum of squares (R^2 value). The quantity of standard material solution added to each herbal extract was calculated using the following formula: amount (mg) of standard materials = [quantitative amount (mg) of standard materials×AT/AS]/n (n=3), where AT is the peak-area of the test samples containing the standard materials and AS is the peak-area of standard materials. From the standard calibration curve, the R^2 values of all marker substances were found to range between 0.991 and 0.999. The standard material used for the quantitative analysis of PT was harmane, and its content in PT preparation was 0.006±0.0001 mg/g (0.0006 ±0.00001%).

Ischemia induction of isolated-perfused rat heart

Sprague-Dawley male rats were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). In order to recover heart function, hearts were retrograde perfused for 15 min according to the Langendorff method described previously (Li et al, 1996). Brifely, hearts were rapidly excised and mounted on a Langendorff apparatus (IPH-W, Labo Support, Osaka, Japan) via the aorta, and then perfused at a constant pressure of 65 mmHg with KH buffer. The heart was maintained at 37°C by a circulating water jacket. The buffer was gassed with 95% O_2/5% CO_2 at pH 7.4. The anti-ischemic effect of PT was measured by the comparing functional differences in perfusion pressure, aortic flow, coronary flow and cardiac output between the ischemia-induced control group and ischemia-induced PT treated group. To measure the left ventricular pressure, a pressure transducer was connected to the aortic cannula. Heart rate was monitored from the left ventricular pressure. Coronary flow was measured by coronary flow volume (ml/min). After stabilization (non-working system) at 100 cm H_2O (100 mmHg) for 15 min via the aortic cannula, the perfusion pressure was reduced to 20 cm H_2O (20 mmHg) for 20 min at the LA cannula (working system), and then ischemia was induced for 10 min accompanied by PT injection for 5 min. Global ischemia was achieved by clamping both the aortic and atrial lines for 10 min. When ischemia was started, PT extract (50 ml of 3 mg/ml PT) dissolved in KH buffer was injected into the aortic line for 5 min, and the effects of PT on the isolated heart were observed with a 65-mmHg

perfusion pressure. Ischemic conditions were maintained for additional 5 min. In the control group, equal volume of KH buffer was injected into the aortic line for 5 min. Then, the heart was perfused again through the working heart system for 60 min. To observe the anti-ischemia effect of PT, the functional recovery rates were compared between the ischemia-induced group and PT treated-ischemia group by measuring changes in perfusion pressure, aortic flow, coronary flow, and cardiac output.

Preparation of cardiomyocytes from neonatal rats

The hearts from 20~30 neonatal (1~3 days) Sprague-Dawley rats were minced in Krebs-Ringer buffer by using sharp scissors and aseptic method. The tissue was subjected to five cycles of digestion in a collagenase type II/pancreatin mixture (80 U/ml and 0.6 mg/ml, respectively). The cell suspension was then incubated with DNase (0.01 mg/ml) for 10 min, and filtered through a nylon mesh. The cell suspension was centrifuged at 350×g for 40 min in a discontinuous Ficoll gradient to obtain a cardiomyocyte-enriched fraction. The fraction was then washed twice with DMEM by centrifugation at 200×g for 10 min. The cells were plated in a Petri dish for 1 h to allow remaining fibroblasts to attach. Unattached cardiomyocytes were then plated in 96-well plates (15,000 per well) covered with gelatin in a culture medium (DMEM:M 199=4:1) supplemented with 10% horse serum, 5% fetal calf serum, antibiotics (50 mg/ml penicillin and 50 mg/ml streptomycin) and 10 mg/ml cytosine-1-β-D-arabinofuranoside. The cultures of rat neonatal cardiomyocytes were prepared just before each experiment, and experiments were performed 48 h after confirming spontaneous contraction of cardiomyocytes. Then, the growth medium was replaced every 3 days.

Intracellular Ca^{2+} imaging

Cardiomyocytes from neonatal Sprague-Dawley rats were incubated for 40~60 min at room temperature with 5 μM fura-2/AM (Molecular Probes) and 0.001% pluronic F-127 in Hepes-buffered solution composed of (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, and 10 glucose, and pH adjusted to 7.4 with NaOH. Cells were illuminated using a xenon arc lamp, and excitation wavelengths (340 and 380 nm) were selected by a computer-controlled filter wheel (Sutter Instruments, CA). Emitter fluorescence was reflected through a 515 nm long-pass filter to a frame transfer cooled CCD camera, and the ratios of emitted fluorescence were then calculated using a digital fluorescence analyzer and converted to intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). All imaging data were collected and analyzed using Universal Imaging software (West Chester, PA). Inhibitory effects of PT were expressed as a percentage of the response to a maximal value of intracellular Ca^{2+} induced by 1 nM isoproterenol (ISO) which was initially administered to each cell. In all cases, each experiment was repeated 4 to 5 times.

Statistical analysis

The results are presented as mean±SEM. Statistical significance was compared between the treatment and control groups using unpaired Student's *t*-test. Results with a $p<0.05$ were considered statistically significant.

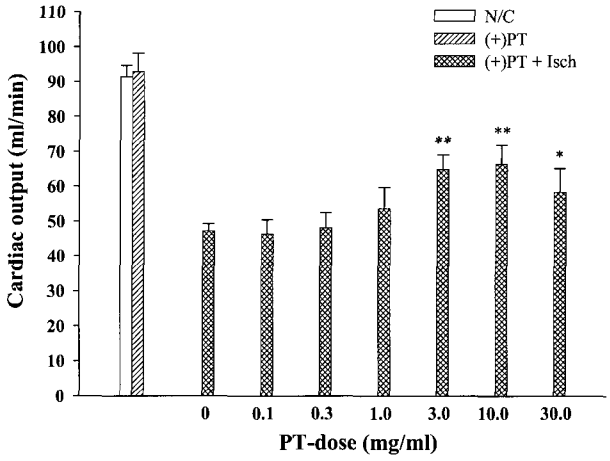

Fig. 1. Determination of maximum PT dose to exert maximum anti-ischemic effect. In order to determine PT dose (0~30 mg/ml) to exert maximal anti-ischemia effect, cardiac output was measured throughout the working heart model perfusion periods in the aortic outflow line with a hemodynamic monitoring system in both groups. White histograms represent mean±SEM from 10 rats per control group without any treatment under pre ischemia conditions. Striped histograms represent mean±SEM from 10 rats per PT treatment under pre ischemia conditions. Web histograms represent mean±SEM from 10 rats per PT treatment group under post-ischemia conditions according to PT dose (0~30 mg/ml). *$p<0.05$ and **$p<0.01$, compared with the 0 mg/ml PT-dose under ischemic conditions. N/C: normal control, (+)PT: PT alone treatment.

RESULTS

Maximal effective dose of PT

As seen in Fig. 1, under pre-ischemic conditions there was no difference between the groups treated with and without 3 mg/ml of PT [92.9±5.4% (101.6%) vs. 91.4±3.3% (100%)], suggesting that PT itself does not influence cardiac output under normal conditions. The maximum protective effect on cardiac output after ischemia was obtained with 10.0 mg/ml of PT. The protective effect of PT on cardiac output after ischemia decreased with doses over 10.0 mg/ml (66.4±5.6 ml/min with 10 mg/ml and 58.4±6.8 ml/min with 30 mg/ml). However, 10 mg/ml of PT, which is the maximum protective concentration on cardiac output, is a very high concentration to apply. Furthermore, the protection of cardiac output after ischemia was not significantly different between 3 mg/ml and 10.0 mg/ml PT (64.9±4.3 ml/min with 3 mg/ml and 66.4±5.6 ml/min with 10 mg/ml). Also, as shown in Fig. 1, cardiac output at the doses of 3.0, 10 and 30 mg/ml PT was statistically significant. Thus, 3.0 mg/ml PT was selected to be an appropriate dose to optimize the anti-ischemic effect in isolated rat heart under ischemic condition.

Heart rate in ischemia-induced isolated rat heart

It is well-known that heart rate does not significantly change under ischemic conditions (Yu et al, 2001; Galagudza et al, 2004), therefore, the heart rate of isolated rat heart under ischemic condition was assessed. As shown in Table 1, the heart rate after 60 min under ischemic condi-

Table 1. Heart rate in ischemia-induced isolated rat heart

Groups	Pre-ischemia (beats/min)	Post-ischemia (beats/min)		
	15 min	10 min	30 min	60 min
Control	291.5±16.4 (100%)	288.5±18.2 (98.86%)	285.8±15.3 (97.24%)	279.6±19.8 (96.90%)
PT treatment	297.3±19.6 (100%)	291.5±22.5 (97.97%)	283.9±18.1 (95.28%)	281.7±23.7 (94.61%)

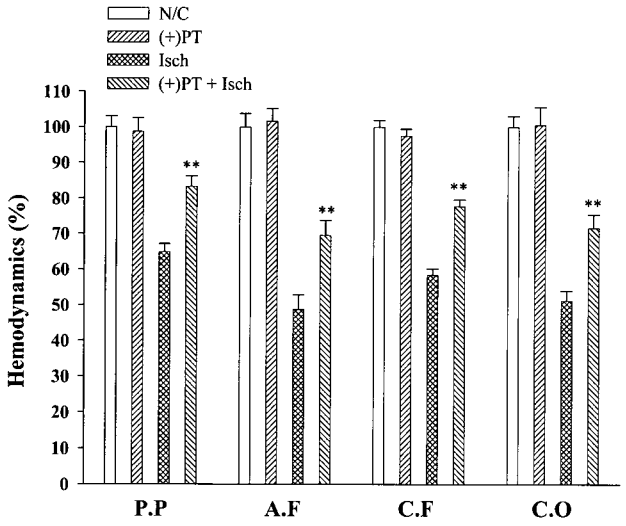

Fig. 2. Average anti-ischemic effects for 60 min in isolated heart. Perfusion pressure (PP), aortic flow (AF), coronary flow (CF), and cardiac output (CO) were measured by timed collection of perfusate from the aortic and pulmonary trunk cannula of both groups to detect an anti-ischemia effect. Each histogram represents mean± SEM from 10 rats per group, the control group without any treatment (first histogram, N/C), the PT treatment group [second histogram, (+) PT] under normal conditions, the control group without any treatment (third histogram, Isch) and the PT treatment group [fourth histogram, (+) PT+Isch] under ischemic conditions. **Significantly different from control group ($p<0.01$) based on Student's *t*-test.

tion was not significantly different between pre-ischemic and post-ischemic conditions [291.5±16.4% (100%) vs. 279.6±19.8% (96.9%)]. Also, the heart rate under post-ischemic conditions was not significantly different between the control and PT treated groups [279.6±19.8% (96.9%) vs. 281.7±23.7% (94.6%)]. Table 1, also shown that the heart rate did not show statistically significant difference after 10 or 30 min compared to pre-ischemic condition ischemia, thus indicating that the heart rate does not change in the isolated heart regardless of PT treatment.

Average anti-ischemic effects of PT for 60 min

The degree of ischemic injury was assessed by measuring the extent of perfusion pressure, aortic flow, coronary flow, and cardiac output, all of which are standard basic assessments of cardiac function. As shown in Fig. 2, all four parameters were substantially decreased to an average of

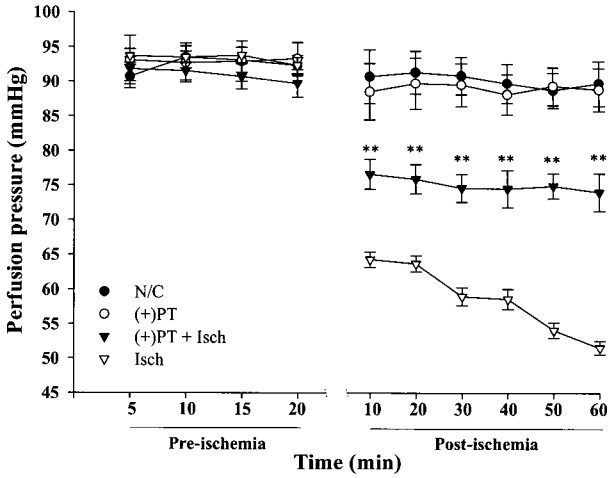

Fig. 3. Recovery of decreased perfusion pressure (PP) of ischemia-induced isolated rat heart by PT. Perfusion pressure was measured throughout the working heart model perfusion periods in the aortic outflow line with a hemodynamic monitoring system in the control and PT treatment groups to detect an anti-ischemia effect. Each symbol represents mean±SEM from 10 rats per group with (●) denoting the control group without any treatment, (○) the PT treatment group under normal conditions, (▽) the control group for ischemia conditions, and (▼) the PT treatment group under ischemic conditions. **Significantly different from the control group without any treatment under ischemic conditions ($p<0.01$) compared to the PT treatment group under ischemic conditions based on Student's *t*-test.

64.9±2.3%, 48.7±4.2%, 58.4±1.8%, and 51.2±2.8%, respectively, by ischemia (100% being pre-ischemic values). However, PT treatment reduced the decreases to 83.3± 3.0%, 69.5±4.3%, 77.7±1.9%, and 71.6±3.9%, respectively, compared to pre-ischemic conditions ($p<0.01$, Fig. 2). These protective rates correspond to average increases of 28% (perfusion pressure), 43% (aortic flow), 33% (coronary flow), and 40% (cardiac output), compared to the control under post-ischemic conditions ($p<0.01$, Fig. 2). These results indicate that PT treatment significantly restored heart dysfunction under ischemic condition.

Recovery of decreased perfusion pressure and aortic flow of ischemia-induced isolated rat heart by PT

Perfusion pressure was substantially decreased by ischemia induction to 64.9±2.3% of the control under pre-ischemic conditions (Fig. 2). However, these decreases were lessened by PT treatment to 83.3±3.0% of the control before ischemia was induced ($p<0.01$, Fig. 2). These anti-ischemic effects of PT on perfusion pressure (mmHg) were consistently observed for 10 to 60 min during the post-ischemic period (Fig. 3). However, any effects of PT on perfusion pressure under normal conditions were not observed for 5 to 20 min during the pre-ischemic period and 10 to 60 min during the post-ischemic period (control vs. PT, $p>0.05$, Fig. 3). Taken together, these results suggest that PT does not influence perfusion pressure, and that it does recover specifically the decreased perfusion pressure induced by ischemia.

Similarly, PT treatment protected successfully the aortic flow reduced by ischemia to 69.5±4.3% of the control value

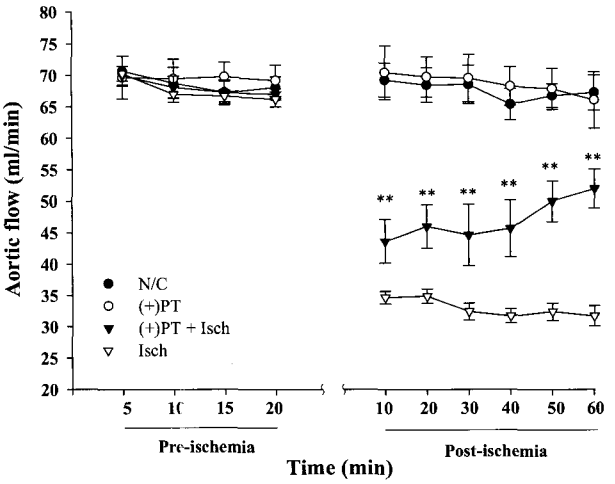

Fig. 4. Recovery of decreased aortic flow (AF) of ischemia-induced isolated rat heart by PT. AF was measured by timed collection of perfusate from the aortic and pulmonary trunk cannula in the control and PT treatment groups to detect an anti-ischemia effect. Each symbol represents mean±SEM from 10 rats per group with (●) denoting the control group without any treatment, (○) the PT treatment group under normal conditions, (▽) the control group for ischemia conditions, and (▼) the PT treatment group under ischemic conditions. **Significantly different from the control group without any treatment under ischemic conditions ($p<0.01$) compared to the PT treatment group under ischemic conditions based on Student's *t*-test.

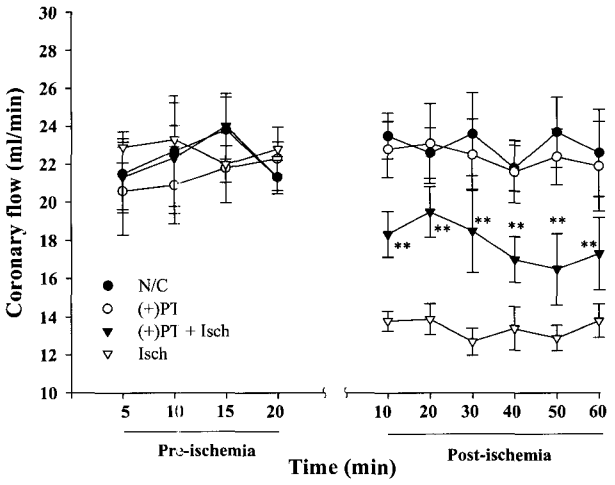

Fig. 5. Recovery of decreased coronary flow (CF) of ischemia-induced isolated rat heart by PT. CF was measured by timed collection of perfusate from the aortic and pulmonary trunk cannula in the control and PT treatment groups to detect an anti-ischemia effect. Each symbol represents mean±SEM from 10 rats per group with (●) denoting the control group without any treatment, (○) the PT treatment group under normal conditions, (▽) the control group for ischemia conditions, and (▼) the PT treatment group under ischemic conditions. **Significantly different from the control group without any treatment under ischemic conditions ($p<0.01$) compared to the PT treatment group under ischemic conditions based on Student's *t*-test.

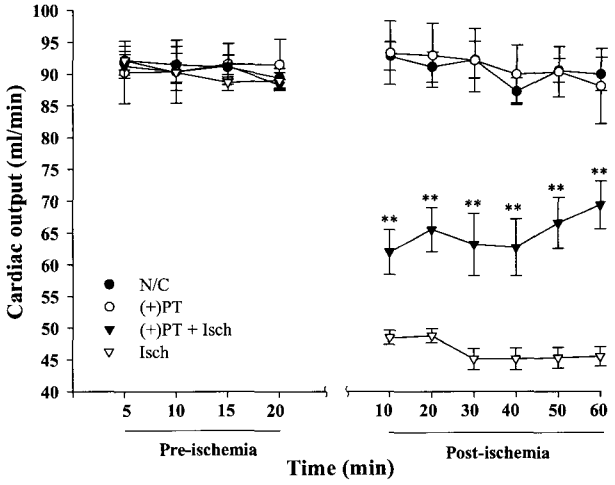

Fig. 6. Recovery of decreased cardiac output (CO) of ischemia-induced isolated rat heart by PT. CO was calculated by summing the aortic and coronary flows (CO = CF+AF). Each symbol represents mean±SEM from 10 rats per group with (●) denoting the control group without any treatment, (○) the PT treatment group under normal conditions, (▽) the control group for ischemia conditions, and (▼) the PT treatment group under ischemic conditions. **Significantly different from the control group without any treatment under ischemic conditions ($p<0.01$) compared to the PT treatment group under ischemic conditions based on Student's *t*-test.

($p<0.01$, Fig. 2). In the working heart model, PT treatment continuously inhibited decreases of aortic flow for 10 to 60 min after ischemia was induced (Fig. 4).

Recovery of decreased coronary flow in ischemia-induced isolated rat heart by PT

Induction of ischemia substantially decreased the coronary flow up to 58.4±1.8% of the control (Fig. 2). However, PT treatment dramatically counteracted it to 77.7 ±1.9% of the control under pre-ischemic conditions ($p<0.01$, Fig. 2). Such protection in the working heart model continued for 10 min to 60 min after ischemia was induced (Fig. 5).

Recovery of decreased cardiac output in ischemia-induced isolated rat heart by PT

Cardiac output was substantially decreased by ischemia to 51.2±2.8% of the control (see Fig. 2). However, such decreases become less by PT treatment to 71.6±3.9% of the control under pre-ischemic conditions ($p<0.01$, see Fig. 2). Also, such decreases were significantly increased by PT treatment to 40% of the control under post-ischemic conditions ($p<0.01$, see Fig. 2). In the working heart model, PT treatment significantly inhibited the decrease of cardiac output during the post-ischemic period (Fig. 6).

Effects of PT on intracellular Ca^{2+} overloads induced by ISO in rat neonatal cardiomyocytes

Effects of PT on intracellular Ca^{2+} overloads induced by ISO, a representative β-agonist (Meng et al, 2005), were

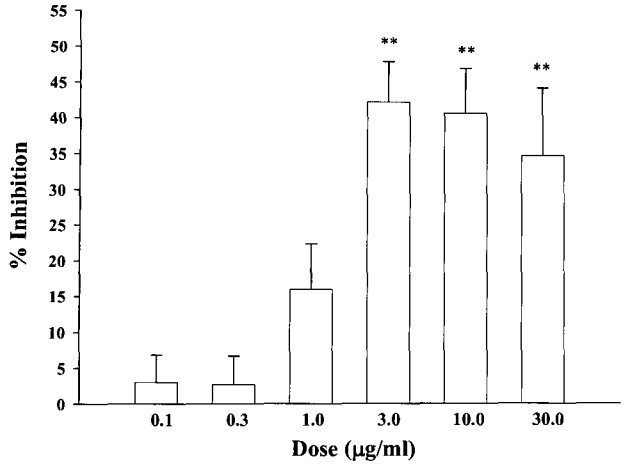

Fig. 7. Inhibitory effect of PT on isoproterenol (ISO)-mediated $[Ca^{2+}]_i$ increase in rat neonatal cardiomyocytes. Responses evoked by 1 nM ISO were quantified according to PT dose (0~30 mg/ml) to detect the maximal anti-calcium effect. Data are expressed as percentage inhibition change compared with the control response elicited by 1 nM ISO as 100%. Each bar presents mean±SEM from 4 or 5 cells per group with the PT treatment group (0~30 mg/ml) under ISO-induced intracellular calcium increase. **Significantly different from the control group under ISO-induced intracellular calcium increase ($p<0.01$) compared to the PT treatment group (0~30 mg/ml) under ISO-induced intracellular calcium increase, based on Student's *t*-test.

assessed to explore underlysing mechanism of the anti-ischemic effects of PT. As seen in Fig. 7, 1 nM ISO-induced intracellular Ca^{2+} increase was significantly ameliorated by PT treatment (3 to 30 mg/ml PT), compared to the control (42.1±5.7% inhibition with 3 mg/ml, 40.5±6.3% inhibition with 10 mg/ml and 34.6±9.4% inhibition with 30 mg/ml vs. 100% in control, $p<0.001$). This result suggests that PT may exert its anti-ischemic effect by blocking Ca^{2+}. Fura-2AM was dissolved in dimethyl sulfoxide plus pluronic acid, since dimethyl sulfoxide at this concentration does not affect Ca^{2+} levels nor cell length (Jovanovic et al, 1997). Neverthless, the treatment of PT alone could not change intracellular Ca^{2+} (data not shown).

DISCUSSION

Under ischemic conditions, myocardial oxidative metabolism is suppressed, and glycolysis becomes an important source of ATP. The increased glycolytic rate in the face of impaired glucose oxidation leads to uncoupling of the two pathways and buildup of lactate and H^+, a process that may continue during reperfusion. This accumulation of protons leads to downstream activation of pathways (Na^+/H^+ exchanger, Na^+/Ca^{2+} exchanger), resulting in Ca^{2+} overload, impaired contractile function, and/or cell death (Asano et al, 2003). It has recently been reported that PT inhibits *N*-methyl-D-aspartate (NMDA)-induced neuronal cell death and NMDA-induced elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). However, its inhibition of NMDA-induced elevation of $[Ca^{2+}]_i$ was not attributable to its competitive binding to NMDA receptor as an antagonist. Thus, it is suggested that PT has an antagonist for Ca^{2+} intake or release (Lee et al, 2004). It is known that PT contains several chemicals and, among these components, harman and euxanthone have most widely been recognized as having anti-ischemic effects that could potentially prevent Ca^{2+} overload or intake by the cell (Splettstoesser et al, 2005; Fang et al, 2006). In more detail, it has been reported that harmane reduced $I_{Ca(V)}$ voltage-activated Ca^{2+} channel currents in rat DRG neurones over a wide voltage range and in a dose-dependent manner (Sun et al, 2002), and that euxanthone relaxed isolated rat thoracic aorta partly through inhibition of Ca^{2+} influx, whereas the inhibition of $[Ca^{2+}]_i$ release partly contributed to the inhibitory effect of euxanthone on NE-induced phasic contraction (Fang et al, 2006). Also, PT is capable of reducing reactive oxygen species and inhibiting lipid peroxidation by anti-oxidant effect (Park et al, 2006). Reactive oxygen species and metabolites are known to play important roles in the pathogenesis of ischemia/perfusion and anoxia/reoxygenation injury. The reduction of O_2 results in the production of superoxides as well as hydrogen peroxide (H_2O_2). H_2O_2 is highly diffusible, induces cell damage, and appears to affect not only lipids but also transmembrane proteins. The hydroxyl radical (OH) also participates in lipid hyperoxidation (Asano et al, 2003). However, anti-oxidant effect of any components in PT has not yet been reported. Therefore, harman and euxanthone in PT have been suggested to work mainly as anti-ischemic agents. Because of this assumption, we investigated the effect of PT on intracellular Ca^{2+} overload, which was induced by ISO in cultured cardiomyocytes from neonatal rats. Our data showed that PT prevented ISO-induced increase of $[Ca^{2+}]_i$ (see Fig. 7). These results demonstrate that PT may have distinct anti-ischemic effects, and prevention of Ca^{2+} overload in cardiomyocytes may be one action mechanism of PT. However, the molecular mechanism of PT with respect to its anti-ischemic effects should further be studied before firm conclusions are could be drawn.

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