Introduction

In cardiac ischemia–reperfusion injury, reactive oxygen species (ROS) are well known to be largely generated during reperfusion and induce myocardial damage (1 – 3). Antioxidant therapy after reperfusion is considered to be inadequate since ROS produced upon reperfusion are thought to have already reacted with biomolecules in the myocardium before ROS are scavenged by antioxidants judging from the high reactivity of ROS. Indeed, numerous reports including our own studies show that administering antioxidants before induction of ischemia are effective in reducing cardiac reperfusion injury, but the same treatments after reperfusion have almost no effect (4 – 9). In this regard, agents that inhibit ROS production during reperfusion are expected to be of great benefit in attenuating cardiac reperfusion injury.

A wide variety of potential ROS sources have been reported in cardiac ischemia–reperfusion studies, including NADPH oxidase (10, 11), xanthine oxidase (12), cyclooxygenase (13), lipoxygenase (13), nitric oxide synthases (14, 15), the mitochondria respiratory chain (16), and cytochrome P450 (CYP) (17, 18). We previously reported that intravenous administration of a potent CYP inhibitor, sulfaphenazole (SPZ), at the time of reperfusion reduced myocardial infarct size and improved cardiac function in a rat model of ischemia–reperfusion (17). Furthermore, we revealed that the same treatment inhibited cardiac CYP and decreased ROS generation during reperfusion (17). Taking into consideration that

Effects of Sulfaphenazole Derivatives on Cardiac Ischemia–Reperfusion Injury: Association of Cytochrome P450 Activity and Infarct Size

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Abstract. Cardiac ischemia–reperfusion injury is evoked by reactive oxygen species (ROS). We previously reported that sulfaphenazole (SPZ) attenuated cardiac ROS levels and ischemia–reperfusion injury in rats. SPZ has distinct two actions: a) elimination of ROS and b) inhibition of cytochrome P450 (CYP) that is responsible for ROS production. The aim of this study is to determine which action contributes to the attenuation of cardiac ischemia–reperfusion injury using SPZ and its derivatives [acetyl-SPZ (Ac-SPZ) and dichloro-SPZ (2Cl-SPZ)]. Administration of 2Cl-SPZ or SPZ prior to ischemia significantly reduced myocardial infarct size, myocardial lipid peroxides, and ROS levels. In addition, they inhibited rat cardiac CYP activity. However, Ac-SPZ neither reduced infarct size nor inhibited cardiac CYP activity. The three compounds had similar effects on ROS scavenging activity in that they scarcely scavenged hydrogen peroxide and superoxide anions but reduced hydroxyl radicals with the same efficacy. The serum concentration of each compound was almost the same until 24 h after reperfusion. Collectively, our findings indicate that the suppressive effects of SPZ and 2Cl-SPZ on ischemia–reperfusion injury are associated with the reduction of ROS levels, which is primarily due to a decrease in ROS production via inhibition of cardiac CYP, not via ROS scavenging activity.

Keywords: heart ischemia–reperfusion, reactive oxygen species, sulfaphenazole, cytochrome P450
SPZ is well known to inhibit human CYP2C9 (19) and that CYP is one of major sources of ROS, we concluded that the effect of SPZ on ameliorating cardiac reperfusion injury is ascribed to a decrease in ROS levels, probably due to inhibition of CYPs in vivo. However, Khan et al. have recently shown that SPZ can directly scavenge the superoxide anions, hydroxyl radical and peroxyl radical, indicating that the SPZ-induced reduction of reperfusion injury arises from the radical scavenging ability of SPZ (20). Thus, the mechanism by which SPZ reduces reperfusion injury remains to be fully understood.

SPZ is reported to lose its ability to inhibit CYP2C9 upon acetylation (Ac-SPZ), while dichlorinated SPZ (2Cl-SPZ) enhances the inhibitory effects of SPZ on CYP2C9 in microsomes from yeast-expressing human CYP2C9 (21). However, to date, the effects of Ac-SPZ and 2Cl-SPZ on CYP activity in the myocardium and ROS scavenging ability of derivatives have not been investigated. The purpose of this study is to elucidate whether the beneficial suppressive effects of SPZ on cardiac ischemia–reperfusion injury are due to cardiac CYP inhibition or ROS scavenging activity by administering Ac-SPZ and 2Cl-SPZ in a rat model of ischemia–reperfusion injury.

Materials and Methods

Materials

SPZ and 4-androstene-3,17-dione were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac and testosterone were purchased from Wako Pure Industries Limited (Osaka). 4-Hydroxyl diclofenac was obtained from Merck (Darmstadt, Germany). Dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR, USA). All other chemicals were obtained from Sigma-Aldrich, Wako, or Tokyo Chemical Industry (Tokyo) and were of reagent grade.

Synthesis of SPZ derivatives

Ac-SPZ [5-(p-acetamidobenzenesulfonamido)-1-phenylpyrazole] and 2Cl-SPZ [5-(p-aminobenzenesulfonamido)-1-(m,p-dichlorophenyl)-pyrazole] (chemical structures shown in Fig. 1) were synthesized according to the methods described by Mancy et al. (21) and identified by H1-NMR and HPLC. The purities of Ac-SPZ and 2Cl-SPZ were 98% and 99%, respectively, enabling direct use without further purifying steps.

Animals

All procedures performed on animals were in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology, Japan and the Animal Care and Use Committee of Tokushima Bunri University, Kagawa, Japan.

Male Wistar rats (250 – 350 g) obtained from Nippon CLEA (Osaka) were maintained in a temperature-controlled animal house with 12-h light–dark cycles. Rats had free access to food and water. Rats underwent experimentation only after acclimatization of 7 – 14 days at the animal facility.

Myocardial ischemia–reperfusion protocol and evaluation of infarct size

The production of myocardial ischemia and reperfusion were performed as previously reported (17). Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). Under artificial ventilation, a left thoracotomy was performed and the left anterior descending coronary artery (LAD) was occluded for 1 h by tightening a snare of thread. The LAD was reperfused by untying the thread. The chest was then closed and the rats were monitored in the animal facility for 24 h.

After the 24-h reperfusion period, the LAD was re-occluded at the same position and the heart was excised. Evans Blue (1%) was injected into the aorta to visualize the non-ischemic region. Viable tissue was stained with 1% triphenyltetrazolium for 10 min at 37°C. Each stained section was weighted to determine the area at risk and infarct size.

Experimental protocol

The LAD was occluded for 1 h and then reperfused for 24 h. Time “0” was defined as the beginning of reperfusion. SPZ and its derivatives were administered intraperitoneally 2 h before LAD occlusion. Blood was collected at 0, 3, and 24 h, and ROS levels were measured 10 min after reperfusion was started. Infarct size and lipid peroxidation were evaluated at 24 h.

Fig. 1. Chemical structures of SPZ derivatives used in the current study.
**Determination of ROS and lipid peroxidation**

Determination of ROS levels in vivo was performed as previously described (17). Briefly, after reperfusion, 1 ml of 2 mM DHE solution was administered into the left ventricle at the apex and then the heart was excised. Hearts were frozen and sectioned, with each section examined under a confocal fluorescence microscope at 488 nm excitation, with a 560 – 660 nm band-pass filter.

The content of thiobarbituric acid–reactive substance (TBARS) was used as an index of lipid peroxidation and determined according to the methods used in our previous report (17). The cardiac tissue suspension was prepared by adding KCl solution to the frozen, ground heart tissue. The suspension solution was mixed with sodium dodecyl sulfate, thiobarbituric acid, butylhydroxytoluene, and acetic acid buffer. The mixture was boiled for 1 h and extracted with 1-butanol-pyridine. The absorbance of this extract at 532 nm was measured. 1,1,3,3-tetramethoxypropane was used as a standard.

**Quantification of hydrogen peroxide**

Hydrogen peroxide concentration was determined using xylenol orange according to previously described methods (22). A known concentration of hydrogen peroxide was used as a standard.

**Quantification of superoxide anion**

Measurement of superoxide anion production induced by the ROS-generating system of purine and xanthine oxidase was performed by previously reported methods (22, 23). Briefly, the assay was carried out at 25°C in 50 mM potassium phosphate buffer containing 10 mM KCl, 100 μM purine, 60 μM acetyl cytochrome c, and 100 units/ml catalase (final pH 7.4). The reaction was started by the addition of 1 unit/ml xanthine oxidase and then the difference in absorbance between 550 and 557 nm was continuously monitored for 10 min. The amount of superoxide anion produced was calculated using the absorbance coefficient of reduced acetyl cytochrome c (ε = 21.0 mM⁻¹·cm⁻¹).

**Detection of hydroxyl radical by electron paramagnetic resonance (EPR)**

Hydroxyl radicals, generated by decomposition of hydrogen peroxide by ferrous ions (Fenton reaction), were detected by EPR spectroscopy using a DMPO spin trap (24, 25). The reaction mixture was prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 500 μM DMPO, 100 μM hydrogen peroxide, and 75 μM ammonium iron (II) sulphate. Exactly 2 min after ammonium iron (II) sulphate had been added, EPR spectra of DMPO-OH was detected. Experiments were performed in glass capillaries (inner diameter of 1 mm) on an E500 CW-EPR spectrometer (Bruker BioSpin K.K., Kanagawa) at room temperature.

**Measurement of CYP2C9 activity**

Formation of 4′-hydroxy diclofenac was used as an index of CYP2C9 activity, based on previous reports (26, 27). Briefly, a mixture of Supersomes™ expressing CYP2C9 (BD Biosciences, Woburn, MA, USA) with 100 μM diclofenac was incubated in the presence of 1 mM NADPH for 30 min at 37°C. The reaction was terminated by the addition of acetonitrile. The aliquot was analyzed by HPLC under the following conditions: column, Inertsil ODS-3 (3.0 × 250 mm, 4 μm) (GL Science Inc., Tokyo); mobile phase, acetonitrile:water:acetic acid (100:900:1) (solvent A) and acetonitrile:water:acetic acid (900:100:1) (solvent B); flow rate of 0.4 ml/min; time program of solvent B, 10% (0 – 10 min), 10% – 90% (10 – 40 min), 90% (40 – 50 min); and UV detection at 280 nm.

**Isolation of cardiac microsomes and assay for cardiac CYP activity**

Rat heart microsomes were prepared by the multistep centrifugation method (23). Cardiac CYP activity in rat cardiac microsomes was determined by the metabolism of testosterone to androstenedione (17). Briefly, rat heart microsomes were incubated at 37°C for 3 h with NADPH and testosterone. Androstenedione was extracted using ethyl acetate and then the extracts were dried and redissolved in 25% methanol. The concentration of androstenedione was determined by HPLC using a known concentration of androstenedione as a standard.

**Measurement of SPZ derivatives in serum**

Concentrations of SPZ and its derivatives in serum were determined by HPLC. Collected blood was centrifuged to separate the serum. Serum was added in equal amounts to acetonitrile and the mixture was centrifuged. An aliquot was injected into the HPLC apparatus and analyzed by the following settings: column, Inertsil ODS-3 (3.0 × 250 mm, 4 μm) (GL Science Inc.); mobile phase, acetonitrile:water:acetic acid (100:900:1) (solvent A) and acetonitrile:water:acetic acid (900:100:1) (solvent B); flow rate of 0.4 ml/min; time program of solvent B, 10% (0 – 10 min), 10% – 90% (10 – 40 min), 90% (40 – 50 min); and UV detection at 261 nm.

**Statistical analyses**

All data are expressed as the mean ± standard error (S.E.M.). Data obtained from three or more groups were compared using Dunnett’s test with Bonferroni’s correction for multiple comparisons. Probability (P) values of less than 0.05 were considered statistically significant.
Results

Effects of SPZ derivatives on myocardial infarct size

Accomplishment of myocardial ischemia by LAD occlusion was ascertained by ST-segment elevation on an electrocardiograph (data not shown). The area at risk did not differ between groups (Table 1). There was no difference in blood pressure, heart rate, and rate-pressure product among the vehicle, SPZ-, Ac-SPZ–, and 2Cl-SPZ–treated groups just before ischemia (data not shown), indicating that SPZ and SPZ derivatives do not affect hemodynamics.

Myocardial infarction was not detected in sham-operated rats (data not shown), indicating the surgical procedure itself did not cause myocardial damage. After 24 h of reperfusion, infarct size was 50.4 ± 2.2% of the area at risk (Fig. 2). Intraperitoneal administration of SPZ and 2Cl-SPZ at 10, 30, and 100 mg/kg 2 h before LAD occlusion reduced infarct size in a dose-dependent manner: SPZ: 31.9 ± 3.5%, 22.3 ± 3.8%, and 13.5 ± 0.7% and 2Cl-SPZ: 32.2 ± 5.3%, 17.4 ± 2.6%, and 10.6 ± 1.9%, respectively (Fig. 2). Ac-SPZ, given intraperitoneally, showed almost no effect on infarct size at 10, 30, or 100 mg/kg (46.3 ± 4.6%, 45.0 ± 1.3%, and 38.8 ± 4.5%, respectively), although the tendency to reduce infarct size was noted.

Effects of SPZ derivatives on ROS and TBARS levels

We next examined the effects of SPZ, Ac-SPZ, and 2Cl-SPZ on levels of ROS and lipid peroxides during reperfusion. Ethidium-derived fluorescence produced by the reaction between superoxide anions and DHE was barely detectable in the area at risk of vehicle-treated rats (Fig. 3A). After 10 min of reperfusion, marked fluorescence was detected in the nuclei of cells within the area at risk, consistent with our previous report (Fig. 3A) (17). Administration of SPZ or 2Cl-SPZ prior to ischemia almost completely abolished all fluorescence in the nuclei of cells within the area at risk, while administration of Ac-SPZ had little effect on inhibiting nuclear fluorescence (Fig. 3A).

TBARS levels within the area at risk after 24 h of reperfusion was measured to indicate the level of lipid peroxidation. TBARS levels in the area at risk after 24 h of reperfusion was approximately four times higher compared to cardiac tissue of control animals (106.3 ± 6.4 vs. 25.9 ± 2.6 nmol/g tissue) (Fig. 3B). Administration of 30 mg/kg SPZ or 2Cl-SPZ significantly attenuated the reperfusion-induced increase in lipid peroxidation to 47.7 ± 4.4 and 35.5 ± 7.4 nmol/g tissue, respectively (Fig. 3B). Ac-SPZ had almost no effect on lipid peroxidation during ischemia–reperfusion (Fig. 3B).

ROS scavenging ability of SPZ derivatives

We next evaluated the scavenging activity of SPZ, 2Cl-SPZ, and Ac-SPZ upon hydrogen peroxide, superoxide anions, and hydroxyl radicals. All three compounds at 100 μM displayed almost no capacity to scavenge hydrogen peroxide or superoxide anions (Fig. 4: A and B). Catalase (100 units/mL) and superoxide dismutase (100 units/mL) were used as positive controls for scav-

Table 1. Area at risk following ischemia–reperfusion in rats

<table>
<thead>
<tr>
<th>Conc. (mg/kg, i.p.)</th>
<th>Area at risk (%)</th>
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<tr>
<td></td>
<td>Vehicle</td>
</tr>
<tr>
<td>0</td>
<td>40.9 ± 3.8</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
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<tr>
<td>100</td>
<td>-</td>
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The left ventricle and ischemic region were weighed 24 h after reperfusion, and the area at risk (%) was calculated. Values each represent the mean ± S.E.M., n = 5 for each group.
Mechanism of Cardioprotection by SPZ

enging hydrogen peroxide and superoxide anions, respectively, reducing hydrogen peroxide by 95% and superoxide anion by 85%, respectively. Addition of 100 μM SPZ, 2Cl-SPZ, or Ac-SPZ significantly decreased DMPO-OH signal intensity (Fig. 4C), indicating all three compounds have the similar potency to scavenge hydroxyl radicals.

CYP inhibition by SPZ derivatives

SPZ is well known to be a potent inhibitor of human CYP2C9 activity (19). We first determined the IC_{50} of SPZ, Ac-SPZ, and 2Cl-SPZ on CYP2C9 activity. SPZ and 2Cl-SPZ concentration-dependently inhibited recombinant CYP2C9 activity with IC_{50} values of 1.1 ± 0.4 and 0.32 ± 0.12 μM, respectively; Ac-SPZ showed little inhibitory effect on CYP2C9 (Fig. 5A, Table 2). We next evaluated the inhibitory effects of SPZ, Ac-SPZ, and 2Cl-SPZ on rat cardiac CYP activity. Although CYP subfamilies were reported to be expressed in the rat heart (28, 29), substrates of cardiac CYPs in rats are rarely identified. In this regard, we evaluated the inhibitory effects of SPZ, Ac-SPZ, and 2Cl-SPZ on the metabolism of testosterone to androstenedione in rat cardiac mi-
crosomes, since testosterone metabolism can be used as an index of cardiac CYP activity (17). SPZ and 2Cl-SPZ dose-dependently inhibited the metabolism of testosterone to androstenedione with IC\(_{50}\) values of 33.9 ± 8.3 and 10.4 ± 3.2 \(\mu\)M, respectively (Fig. 5B, Table 2), revealing the inhibitory effects of 2Cl-SPZ to be approximately 3-fold that of SPZ on cardiac CYP activity. In contrast, Ac-SPZ had scarce inhibitive effects on testosterone metabolism, indicating incapacity to inhibit cardiac CYP activity (Fig. 5B).

**Changes in blood concentrations of SPZ derivatives**

Serum was collected at 0, 3, and 24 h of reperfusion to determine the circulating concentrations of SPZ, Ac-SPZ, or 2Cl-SPZ. Serum concentrations of all three compounds at 0 h were almost identical and each followed the same pattern with changes over time (Fig. 6). Serum levels of SPZ, Ac-SPZ, and 2Cl-SPZ 24 h after reperfusion were 58.1 ± 11.5, 54.3 ± 5.9, and 34.3 ± 7.9 \(\mu\)M, respectively (Fig. 6).

**Discussion**

Cardiac ischemia–reperfusion injury was dose-dependently suppressed by administration of SPZ or 2Cl-SPZ, but not by treatment with Ac-SPZ. Similarly, TBARS and ROS levels were reduced by treatment with SPZ and 2Cl-SPZ, but not Ac-SPZ. SPZ and 2Cl-SPZ inhibited CYP2C9 and cardiac CYP activity while Ac-SPZ did not. All three compounds were found to circulate at the same concentrations over the 24-h reperfusion period and no difference in ROS scavenging capacity was found between each compound.

Occlusion of the LAD for 1 h followed by reperfusion for 24 h caused significant increases in myocardial ROS and TBARS levels and elicited reperfusion injury as exemplified by myocardial infarction. TBARS levels are a generally accepted marker of lipid peroxidation. The findings that administration of SPZ and 2Cl-SPZ reduced the increment in infarct size as well as levels of ROS and

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**Table 2.** Comparison of the inhibitory effects of SPZ derivatives on CYP2C9 and rat cardiac CYPs

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC(_{50}) ((\mu)M)</th>
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<tr>
<td></td>
<td>CYP2C9</td>
</tr>
<tr>
<td>SPZ</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Ac-SPZ</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2Cl-SPZ</td>
<td>0.32 ± 0.12</td>
</tr>
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IC\(_{50}\) values of SPZ and its derivatives were calculated based on the data described in Fig. 5. Values each represent the mean ± S.E.M., n = 5 for CYP2C9 and n = 4 for cardiac CYPs.
TBARS indicate that infarction was caused by oxidative damage to cardiomyocytes resulting from increases in ROS levels. This is supported by the finding that Ac-SPZ had no effect on cardiac ischemia–reperfusion injury as well as levels of TBARS and ROS. Taken together, our results strongly suggest that the decrease in ROS levels is responsible for attenuating effects of SPZ and 2Cl-SPZ on cardiac reperfusion injury.

The sources of ROS generation in cardiac ischemia–reperfusion studies are reported to be numerous, including NADPH oxidase (10, 11), xanthine oxidase (12), cyclooxygenase (13), lipoxygenase (13), nitric oxide synthase (14, 15), mitochondria respiratory chain (16), and CYP (17, 18). Of these sources, CYP was chosen as one of the plausible candidates based on our previous studies (17). The current study evaluated the inhibitory effects of SPZ and its derivatives on the metabolism of testosterone to androstenedione in cardiac microsomes as a correlative index of the inhibition of cardiac CYP activity. Because CYP2C6 (Y. Ishihara et al., unpublished data), CYP2C11 (30), and CYP2C13 (30) all metabolize testosterone to hydroxyl metabolites (2α-hydroxyl testosterone, 6β-hydroxy testosterone, and 16α-hydroxy testosterone) and androstenedione, we consider that testosterone metabolism is promoted by the CYP2C family; however, the substrates for the family of rat cardiac CYP2C compounds remain unidentified. Using this index we determined that SPZ and 2Cl-SPZ concentration-dependently inhibited CYP activity in cardiac microsomes, while Ac-SPZ had no effect, suggesting that SPZ and 2Cl-SPZ, but not Ac-SPZ, reduce the generation of ROS derived from cardiac CYPs.

SPZ, 2Cl-SPZ, and Ac-SPZ rarely scavenged hydrogen peroxide and superoxide anions, even at high doses of 100 μM. However, the three compounds scavenged hydroxyl radicals with the same efficacy. Considering that SPZ and 2Cl-SPZ, but not Ac-SPZ, significantly reduced cardiac ROS levels, the above findings indicate that the reduction of ROS levels in the myocardium by SPZ and 2Cl-SPZ was not due to their scavenging effects on ROS. Furthermore, the serum concentrations of SPZ, Ac-SPZ, and 2Cl-SPZ were tightly correlated throughout the 24 h reperfusion period following a bolus of 30 mg/kg, i.e., suggesting the pharmacokinetics of the three compounds to be very similar. This premise suggests that the in vitro findings of the three compounds made here may be directly applicable to the in vivo setting, but further investigations are required. Collectively, the alleviating effects of SPZ and 2Cl-SPZ on ischemia–reperfusion injury are due to a decrease in ROS levels, resulting from the suppression of ROS generation by inhibiting cardiac CYP activity, not by their ROS scavenging actions. This is further supported by the finding that Ac-SPZ displayed ROS scavenging activity but lacked the ability to inhibit CYP and as a result, Ac-SPZ had no effect on reducing ischemia–reperfusion injury.

We have previously reported that SPZ is highly effective in reducing cardiac infarct size by bolus intravenous injection. In addition, decreases in left ventricular systolic pressure, left ventricle (dP/dt max)/P, and rate-pressure product and increase in left ventricular end-diastolic pressure accompanied with ischemia–reperfusion were also attenuated by administration of SPZ. Furthermore, reduction of infarct size is correlated with the attenuation of derangements in cardiac function (17). In this study, administration of 2Cl-SPZ largely decreased infarct size induced by ischemia–reperfusion, while Ac-SPZ showed little effect on infarct size. In this regard, 2Cl-SPZ but not Ac-SPZ might be effective in reducing exacerbation of cardiac function accompanied with ischemia–reperfusion. Further experiments are required to verify the relationship between infarct size and cardiac function using SPZ derivatives.

This study revealed that the inhibitory effects of SPZ derivatives on cardiac CYP activity were altered dependent upon chemical modification, further stimulating the potential for the development of CYP-targeted drugs. Moreover, CYP inhibitors for specific CYP subtypes responsible for ROS generation during reperfusion developed by chemical modification may avoid side effects accompanied with the inhibition of other CYPs that are not involved in ischemia–reperfusion injury. Identification of cardiac CYP subtypes in the heart is crucial for the development of new drugs to alleviate ischemia–reperfusion injury.

In conclusion, the suppressive effects of SPZ and 2Cl-SPZ on cardiac ischemia–reperfusion injury are related to the reduction of ROS levels, which is mainly due to the ability of SPZ and 2Cl-SPZ to inhibit cardiac CYPs, not due to the ROS-scavenging effects of SPZ and 2Cl-SPZ. Therefore, drugs targeting a specific cardiac CYP subtype may have potential for therapeutic intervention to attenuate cardiac ischemia–reperfusion injury.

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