Cardioprotection by Sulfaphenazole, a Cytochrome P450 Inhibitor: Mitigation of Ischemia-reperfusion Injury by Scavenging of Reactive Oxygen Species

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Abbreviations

AAPH  - 2,2-Azobis-2-amidonopropane dihydrochloride
DAF-2A  - 4, 5-Diaminofluorescein diacetate
DEPMPO - 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide
DTPA  - Diethylenetriaminepentaacetate
EDHF  - Endothelium-dependent hyperpolarizing factor
EPR  - Electron paramagnetic resonance
L-NAME - N^6^-nitro-L-arginine methylester
PTIO  - 2-Phenyl-4, 4, 5, 5-tetramethylimidazolin-1-oxyl-3-oxide
Tempol - 4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl
Abstract

Cytochrome P450 (CYP) enzymes play a significant role in promoting myocardial ischemia-reperfusion (I/R) injury. CYP 2C9, an isoform of CYP, is known to generate superoxide radicals in the reperfused heart. Sulfaphenazole (SPZ), a CYP 2C9 inhibitor, has been shown to decrease I/R injury; however, the mechanism of cardioprotection by SPZ is not well elucidated. The objective of this study was to test whether SPZ mitigates myocardial I/R injury by scavenging reactive oxygen species (ROS). Isolated rat hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. Hearts were perfused with SPZ and/or L-NAME. Coronary flow (CF), left-ventricular developed pressure (LVDP), and rate-pressure product (RPP) were monitored. Superoxide and nitric oxide (NO) generation in the reperfused tissue was determined using fluorescence methods. Myocardial infarct size was measured using TTC staining. The SPZ-treated group showed a significant recovery of cardiac function as compared to the untreated I/R group (CF, 53% vs 45%; LVDP, 48% vs 22%; RPP, 51% vs 20%). The infarct size was significantly reduced in the SPZ-treated group (15%) compared to I/R control (42%). Co-administration of L-NAME with SPZ significantly attenuated the beneficial effects of SPZ. Also, SPZ treatment showed significantly decreased superoxide levels and enhanced NO bioavailability in the reperfused heart. In conclusion, the protective effect of SPZ against I/R-mediated myocardial damage appears to be due to a reduction in the superoxide level caused by its inhibition of CYP 2C9, as well as scavenging of oxygen free radicals generated in the reperfused heart.
**Introduction**

Ischemic heart disease leading to myocardial infarction (MI) is a major clinical concern. The pathogenesis of myocardial ischemia-reperfusion (I/R) injury involves the interplay of multiple mechanisms. Reactive oxygen species (ROS) have long been recognized to cause oxidative protein modification and to act as the major mediator of ischemia/reperfusion injury (Dart and Sanders, 1988). There is also substantial evidence that ROS are generated during myocardial ischemia-reperfusion (Kloner et al., 1989; Kaul et al., 1993; Ferrari et al., 1998). The harmful effects of ROS on cardiac tissue can be blocked by antioxidant enzymes such as superoxide dismutase and catalase (Ambrosio et al., 1987; Zweier et al., 1987; Zweier et al., 1989; Ambrosio et al., 1993; Maulik et al., 1996; Dhall et al., 2000). However, myocardial reperfusion injury cannot be explained exclusively by oxidative stress, because multiple enzymatic and non-enzymatic pathways exist that can effectively reduce the damage (Willerson, 1997). Recent studies have suggested that nitric oxide (NO)-derived reactive nitrogen species (RNS) may also contribute to tissue injury by nitrative protein modification (Brookes and Darley-Usmar, 2002; Ischiropoulos and Beckman, 2003). One of the most toxic RNS is peroxynitrite (ONOO⁻), which is formed by the reaction of NO and superoxide (O₂⁻) at near diffusion-limited rate. Considerable evidence now exists that peroxynitrite plays a causative role in post-ischemic myocardial apoptosis and necrosis (Wang and Zweier, 1996; Beckman, 1999; Levrand et al., 2006).

Cytochrome P450 (CYP) enzymes are membrane-bound, heme-containing terminal oxidases that are found in organisms ranging from archaeabacteria to humans. These enzymes are responsible for the metabolic activation or inactivation of most types of drugs, as well as toxins. CYP enzymes oxidize, peroxidize, and/or reduce steroids, arachidonic acid (AA),
vitamins, and other foreign substances (for example, drugs) in an oxygen- and NADPH-dependent manner (Schwartzman et al., 1985). The majority of CYP isoforms are monooxygenases that catalyze the incorporation of a single atom of oxygen into a substrate. CYPs are also known to generate ROS during their reaction cycle, as electrons are transferred from the central heme iron to the activated, bound oxygen molecule (Fleming, 2001). In fact, CYPs make a significant contribution to the cellular production of ROS, including superoxide, hydrogen peroxide, and hydroxyl radicals and hence may also contribute to vascular homeostasis (Busse and Fleming, 1998).

Recently, Granville et al reported that inhibition of CYP 2C9, a human isozyme, by chloramphenicol (CAP), cimetidine, or sulfaphenazole (SPZ) significantly attenuated the tissue damage in an I/R model of MI in isolated rat hearts (Granville et al., 2004). The inhibition of CYP 2C9 by SPZ was also associated with increased endothelium-dependent vasodilation in human patients with coronary artery disease (Fichtlscherer et al., 2004). This effect was attributed to a decrease in CYP 2C9-mediated ROS production leading to increased NO bioavailability and NO-mediated vasodilation. In the study by Granville et al (Granville et al., 2004), we observed three striking features on the effect of SPZ: (i) SPZ was able to reduce infarct size when administered only at the time of reperfusion; (ii) SPZ inhibited CYP 2C9 (IC$_{50}$: 0.6 µM) at a dose of 10 µM, but the protection at this dose did not reach maximum attenuation, instead, a dose of 300 µM showed a significantly better protection than 10 µM; and (iii) although the IC$_{50}$ values are comparable for CAP and SPZ inhibition of CYP 2C9, SPZ demonstrated enhanced cardioprotection when compared to CAP. The above observations suggested that SPZ may have a different mechanism, for
example ROS scavenging, in addition to the inhibition of superoxide production by CYP 2C9.

The potentially greater protective efficacy of SPZ over other CYP 2C9 inhibitors against I/R injury could stem from its unique antioxidant activity. Therefore, we hypothesized that SPZ may be a potent scavenger of ROS, particularly oxygen free radicals (OFR). Based on the study by Granville et al (Granville et al., 2004), it became evident that the 10-µM SPZ was enough to block CYP 2C9-induced superoxide generation, but the further reduction in infarction by 300-µM SPZ could be due to its antioxidant action. Therefore the goal of the present study was to investigate the antioxidant and cardioprotective properties of SPZ in an isolated rat heart model of I/R injury. The results showed substantial ability of SPZ to scavenge OFR, suggesting that SPZ prevents I/R-mediated myocardial injury not only by inhibiting superoxide generation by CYP 2C9, but also by scavenging of the superoxide radicals generated in the reperfused hearts.

Materials and Methods

Chemicals

Sulfaphenazole (SPZ, 4-Amino-N-(1-phenyl-1H-pyrazol-5-yl)), chloramphenicol (CAP, 2,2-dichloro-N-[(αR,βR)-β-hydroxy-α-hydroxymethyl-4-nitrophenylethyl]acetamide), Nω-nitro-L-arginine methylester (L-NAME), dihydroethidium (DHE), xanthine (X), xanthine oxidase (XO), and superoxide dismutase (SOD) were obtained from Sigma Chemicals (St. Louis, MO). 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) was obtained from Radical Vision (Marseille, France). 4, 5-Diaminofluorescein diacetate (DAF-2A) was
purchased from Calbiochem (San Diego, CA). 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) was purchased from Oxis International (Portland, OR).

**Isolated heart preparation**

The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee of Ohio State University and conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 86–23). Male Sprague-Dawley rats (350–400 g) were anesthetized intraperitoneally with 60 mg/kg Nembutal® sodium pentobarbital and heparinized with 500 IU/kg heparin. Access to the heart was gained surgically via bilateral midaxial thoracotomy. Hearts were rapidly excised, removed from the thoracic cavity and placed into ice-cold Krebs-Henseleit buffer (KHB) to arrest residual contractions. The aorta was subsequently cannulated to the perfusion apparatus. The hearts were retrogradely perfused through the aorta with a modified KHB containing NaCl (120 mM), NaHCO₃ (25 mM), MgSO₄ (1.2 mM), KH₂PO₄ (1.2 mM), CaCl₂ (1.2 mM), and glucose (11 mM). The perfusate buffer was saturated with a 95% O₂ and 5% CO₂ gas mixture and maintained at 37°C. A latex balloon was inserted in the left ventricle via the left atrium and inflated with water to produce an end diastolic pressure of 8-12 mmHg. The contractile and hemodynamic functions of the heart were continuously recorded with a computerized data acquisition system (PC PowerLab with Chart 5 software, ADI Instruments, Colorado Springs, CO). The following parameters were monitored: coronary flow (CF), left-ventricular systolic pressure (LVSP), left-ventricular end-diastolic pressure (LVEDP), and heart rate (HR). The left-ventricular developed pressure (LVDP) was calculated as the difference between LVSP and LVEDP. The rate-pressure product (RPP),
which is an index of myocardial work, was calculated as LVDP x HR. The coronary flow rate was measured using a flowmeter with an in-line probe (Transonic Systems, Ithaca, NY).

**Ischemia/-reperfusion protocol**

Hearts were perfused for 15 min to stabilize the hemodynamic functions and then were subjected to 30 min of no-flow global ischemia at 37°C, followed by 45 min of reperfusion. Test agents were added to perfusion buffer and perfused continuously before ischemia and during reperfusion. The hemodynamic measurements, biochemical assays, and infarct-size measurements were done on the same hearts, whereas HE and DAF fluorescence measurements were done on separate groups of hearts (n=6 per group). Since the IC$_{50}$ value of SPZ is 0.6 µM (Ha-Duong et al., 2001), we reasoned that 10-µM dose would be sufficient to block CYP 2C9 and inhibit superoxide radical production. However, the scavenging of radicals is a chemical process and hence we used a higher dose (300 µM) in our experiments to study the drug’s radical-scavenging property.

**LDH and CK assays**

Myocardial injury was assessed by determining the amount of lactate dehydrogenase (LDH) and creatine kinase (CK) in the coronary effluents collected both before ischemia and during reperfusion. The level (activity) of LDH and CK in the coronary effluents was determined using commercially available kits: LDH (Sigma Diagnostics, St. Louis, MO); CK (Catachem, Bridgeport, CT). The enzyme activity was determined by measuring the rate of change in absorbance at 340 nm for 5 min using a Varian Cary 50 spectrophotometer.
Evaluation of infarct size

Measurements of the area at risk and infarct size were performed using triphenyltetrazolium chloride (TTC)-staining. TTC stains all living tissue brick red, while leaving the infarct area unstained (white). Experimental hearts were frozen, stored at -20°C for 10 min, and then sliced perpendicular to the long axis from apex to base in 2-mm sections. The sections were then incubated for 20 min at 37°C with 1% TTC in PBS (pH 7.4), fixed in 10% formalin for 20 min, and digitally imaged using a Nikon microscope. The infarct (TTC-negative) and viable (TTC-positive) areas were determined using MetaVue software. The infarct size was expressed as a percentage of the total left-ventricular area.

Measurement of superoxide generation in the tissue

Superoxide generation in the heart tissue subjected to I/R was determined using dihydroethidium (DHE) fluorescence (Miller et al., 1998). The cell-permeable DHE is oxidized to fluorescent hydroxyethidium (HE) by superoxide, which is then intercalated into DNA. Since it has been reported that the superoxide generation in hearts subjected to I/R occurs during the first 15 min of reperfusion, we measured the HE fluorescence at this time period. Transverse heart sections were placed in ice-cold PBS buffer and then embedded in OCT for cryosectioning. The frozen heart segments were cut into 6-µm thick sections and then placed on glass slides. DHE (10 µM, 0.5 ml) was topically applied to each tissue section. The slides were then incubated for 30 min at 37°C in a light-protected chamber, washed with PBS to remove any unbound DHE, fixed with mounting media, and imaged using a fluorescence microscope (Nikon) with a rhodamine filter. The fluorescence intensity was quantified using MetaMorph software.
**Measurement of NO generation in the tissue**

The NO produced in hearts subjected to I/R was determined using diaminofluorescein-2 diacetate (DAF-2A) fluorescence (Miller et al., 1998). The cell-permeable DAF-2A binds with NO resulting in irreversible fluorescence. DAF-2A (3 µM) was infused along with the test agents, through the side arm 1 min before the onset of ischemia. Hearts, after 30 min of ischemia, or 15 min of reperfusion, were placed in an ice-cold PBS buffer and embedded in OCT (optimal cutting temperature) for cryosectioning. The frozen segments were cut into 6-µm thick sections. The images of the tissue sections were obtained using a fluorescence microscope (Nikon) with a fluorescein isothiocyanate filter. The fluorescence intensity was quantitatively determined using MetaMorph software.

**Measurement of scavenging of superoxide, hydroxyl and alkylperoxyl radicals by SPZ using EPR spectroscopy**

The superoxide, hydroxyl and alkylperoxyl radical-scavenging property of SPZ was evaluated by using EPR spectroscopy. A mixture of xanthine (0.2 mM) and xanthine oxidase (0.02 U/ml) in PBS, pH 7.4, was used to generate superoxide radicals. Hydroxyl radicals were generated by reacting ferrous ammonium sulphate (0.1 mM) with hydrogen peroxide (0.1 mM) in PBS. Thermolytic fission of 2,2-azobis-2-amidonopropane dihydrochloride (AAPH, 25 mM) in aerobic PBS solution at 37°C was used to generate alkylperoxyl radicals. The EPR measurements were performed in PBS (pH 7.4) containing diethylenetriaminepentaacetate (DTPA, 0.1 mM) and DEPMPO (1 mM) in the presence or absence of 1 mM SPZ. The superoxide, hydroxyl and peroxyl radicals were detected as DEPMPO-OOH, DEPMPO-OH and DEPMPO-OOR adducts, respectively, by EPR.
spectroscopy. The spectra were quantified by double-integration and expressed as percent of untreated (-SPZ) control.

**Electrochemical measurements of the effect of SPZ on NO and H$_2$O$_2$**

NO and H$_2$O$_2$ were measured using an Apollo 4000 multichannel analyzer (WPI, Sarasota, FL) with Clark-type NO (ISO-NO, WPI) and H$_2$O$_2$ (ISO-HPO-100, WPI) electrodes in a closed-chamber of 2-ml volume with magnetic stirring at 37°C. The reaction mixture (2 ml) consisted of NO (300 µM) or H$_2$O$_2$ (300 µM) along with DTPA (0.1 mM) and Tris-HCl (50 mM, pH 7.4). After 5-min of stabilization, SPZ was introduced using a Hamilton syringe and the measurements were continued for 10 min. The results were expressed as percent of untreated (-SPZ) control.

**Data analysis**

The statistical significance of the results was evaluated using ANOVA and a Student’s t-test. The values were expressed as mean ± SD. A p value <0.05 was considered significant.

**Results**

**SPZ scavenges superoxide, hydroxyl and peroxyl radicals**

To determine whether SPZ is capable of scavenging superoxide, hydroxyl and/or peroxyl radicals *in vitro*, we used spin-trapping EPR spectroscopy. DEPMPO (1 mM) was used for direct detection of exogenously generated superoxide, hydroxyl and peroxyl radicals as DEPMPO-OOH, DEPMPO-OH and DEPMPO-OOR adducts, respectively. SPZ, added to the reaction medium, would be expected to compete with DEPMPO for the radicals and hence decrease the intensity of the DEPMPO adduct signal. As shown in Figure 1, one-mM SPZ, used against 1-mM DEPMPO, decreased the intensity of the DEPMPO-OOH spectrum.
(A1) by more than 60% (A2). Challenging of 1-mM DEPMPO with 5-mM SPZ completely abolished the signal (A3). Addition of SOD completely inhibited the EPR signal (A4) suggesting that the DEPMPO-OOH adduct formed was indeed from superoxide radicals. Similarly, the hydroxyl radical adduct (DEPMPO-OH), generated using the Fenton reaction in the presence of 1 mM DEPMPO, was inhibited by more than 80% 1-mM SPZ (B1 & B2). More importantly, SPZ (1 mM) completely abolished the peroxyl radical adduct (DEPMPO-OOR), generated by thermal decomposition of AAPH in aerated PBS (C1 & C2). The mean±SD values obtained from 3-4 independent measurements clearly demonstrated the scavenging of the reactive oxygen radicals by SPZ (Figure 2A). We further performed electrochemical measurements to determine whether SPZ was capable of scavenging nitric oxide (NO) and H₂O₂ in solution. The results, shown as a separate panel in Figure 2A, revealed that SPZ had no effect on NO or H₂O₂ under the conditions used.

We next wanted to evaluate the radical-scavenging effect of SPZ at a lower concentration (300 µM), which we used in the subsequent heart experiments. As shown in Figure 2B, challenging of 300-µM SPZ with 300-µM DEPMPO demonstrated almost similar efficacies as observed at 1 mM concentration. We also determined the radical-scavenging ability of chloramphenicol (CAP), another known inhibitor of CYP 2C9, under similar conditions. The results showed that CAP had no significant effect on superoxide radicals; however, it was effective against hydroxyl and peroxyl radicals (Figure 2B). Overall, the in vitro results provided direct evidence that SPZ, but not CAP, was a potent scavenger of SOD-inhibitable superoxide radicals at the concentrations used.
SPZ improves functional recovery in post-ischemic hearts

Hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. Coronary flow (CF), left-ventricular developed pressure (LVDP), and rate-pressure product (RPP) were continuously measured prior to the start of global ischemia and during reperfusion. The data were expressed as a percentage of their pre-ischemic baseline values, which were as follows: CF, 15±2 ml/min; LVDP, 115±14 mmHg; HR, 292±20 bpm. Hearts perfused with SPZ (300 µM) for 20 min did not show any significant chronotropic or inotropic effects (data not shown). The effect of SPZ (300 µM) on the recovery of hearts subjected to 30 min of global ischemia at 37°C followed by 45 min of reperfusion was studied. The untreated control hearts subjected to 30 min of global ischemia followed by 45 min of reperfusion showed a significant decrease in CF (45±4%), LVDP (22±4%), and RPP (20±6%), when compared to pre-ischemic baseline values (Figure 3). Hearts treated with SPZ (300 µM) showed a significant recovery of CF (53±5%), LVDP (48±6), and RPP (51±6%), when compared to the untreated I/R hearts. While infusion of L-NAME (100 µM) had no significant effect on the recovery, co-infusion of L-NAME with SPZ significantly attenuated the recovery of CF, LVDP and RPP when compared to SPZ alone.

SPZ decreases LDH and CK release upon reperfusion

In untreated hearts subjected to I/R, the LDH activity increased with a maximum activity at 10-15 min of reperfusion. The LDH activity was significantly decreased in hearts treated with SPZ as compared to the control group (Figure 4A). Similarly, the effluents from hearts treated with SPZ showed significantly less CK activity as compared to the untreated group (Figure 4B). L-NAME showed a significant decrease in both LDH and CK levels, when
compared control. However, co-infusion of L-NAME with SPZ significantly attenuated the protective effect of SPZ alone.

**SPZ inhibits myocardial infarction**

TTC-staining of I/R control hearts subjected to 30 min of ischemia followed by 120 min of reperfusion showed an infarction of 42.0±5.0% of risk area (Figure 4C). On the other hand, the infarction in hearts treated with SPZ was significantly reduced (15.0±3.2%) compared to I/R group. However, there was no significant difference in the myocardial infarct size in L-NAME (37±5.4%) alone or L-NAME+SPZ (32.0±4.5%) groups as compared to I/R control group, suggesting that co-infusion of L-NAME with SPZ significantly attenuated the protective effect of SPZ alone.

**Protective effect of SPZ in comparison with CAP**

In order to assess whether the protective effect of SPZ was due to inhibition of superoxide production by CYP 2C9 and/or scavenging of superoxide and downstream radicals generated up on reperfusion, we repeated the experiments using a lower dose of SPZ. We also compared the results with CAP, another known inhibitor of CYP 2C9. Since the IC50 values of SPZ and CAP for CYP 2C9 are ~1 µM, we used a dose of 10 µM, which was sufficient to block the enzyme. As shown in Figure 5A, SPZ or CAP at 10-µM concentration did not show any significant difference in the recovery of LVDP when compared to control I/R group. On the other hand, SPZ or CAP at 300-µM concentration demonstrated a significant (p<0.01) enhancement of LVDP recovery. A similar trend was observed on the reduction of infarct size (Figure 5B), except that 10-µM SPZ, but not CAP, showed a significant (p<0.05) reduction in infarct size when compared to control I/R hearts. In order to further delineate the
inhibitory versus scavenging role of SPZ, we pretreated hearts with 10-µM CAP for 10 min followed by treatment with 300-µM SPZ up on reperfusion. Both LVDP recovery (Figure 5A) and infarct reduction (Figure 5B) were not significantly different from that treated with SPZ alone, suggesting that the beneficial effect of SPZ at this concentration could be largely mediated by its radical scavenging activity.

SPZ attenuates superoxide levels in the reperfused heart

The superoxide generation in hearts subjected to I/R was measured by hydroethidium (HE) fluorescence as described in the Methods section. The HE fluorescence intensity was significantly higher in untreated I/R (control) hearts subjected to 30 min ischemia followed by 15 min of reperfusion (Figure 6). Hearts treated with SPZ showed a dose-dependent decrease in HE fluorescence intensity as compared to control I/R group (Figure 6B). While 10-µM CAP did not show any significant effect (data not shown), 300-µM CAP showed a significant attenuation of intensity; however, the effect was significantly less compared to that of 300-µM SPZ. The figure also shows a significant decrease in HE fluorescence intensity in hearts treated with Tempol, a known superoxide radical scavenger.

SPZ enhances NO levels in the reperfused heart

The nitric oxide levels produced in hearts subjected to I/R were determined using DAF fluorescence as described in the Methods section. Control hearts subjected to 30-min ischemia followed by 15-min reperfusion showed intense fluorescence suggestive of NO generation (Figure 7A). The NO generation was significantly (p<0.05) higher in hearts treated with 300-µM SPZ (Figure 7B). In contrast, 10-µM SPZ did not show any significant effect on NO generation. Infusion of L-NAME alone or L-NAME with SPZ significantly inhibited the NO generation, suggesting that the increase in NO level upon SPZ treatment
was largely from L-NAME-inhibitable source. Co-treatment of SPZ (300 µM) with PTIO (100 µM), a known scavenger of NO, abolished the NO fluorescence, below the level of I/R group. Overall, the results indicate that SPZ increases the bioavailability NO in the reperfused heart.

Discussion

The present study clearly established the potential antioxidant property of SPZ against reactive oxygen radicals including superoxide, hydroxyl and peroxyl radicals generated under in vitro conditions. In the reperfused myocardium, SPZ significantly attenuated the superoxide levels with a concomitant increase of nitric oxide bioavailability. The scavenging of the deleterious free radicals generated during the early minutes of reperfusion is observed to have a significant impact on the recovery of hemodynamic and contractile functions, decreased LDH and CK levels, and reduced infarct size. This beneficial action of SPZ may be secondary to the inhibition of superoxide generation by CYP 2C9 in the reperfused heart (Granville et al., 2004). Thus, the results, for the first time, imply a complementary role for SPZ, namely, scavenging of superoxide and related radicals in the reperfused heart.

The EPR spectroscopic studies provided direct evidence for the oxygen free radical scavenging property of SPZ. Competitive inhibition of the reaction between DEPMPO and superoxide by equimolar concentrations of SPZ demonstrated a dose-dependent scavenging of SOD-inhibitable superoxide. Based on the bimolecular rate constant of the reaction between DEPMPO and superoxide, 7x10^1 M^-1sec^-1, the apparent second order rate constant of SPZ with superoxide is estimated to be 4x10^1 M^-1sec^-1. The EPR results also indicate that SPZ is a more effective scavenger of hydroxyl and peroxyl radicals when compared superoxide, while it has no effect on NO and H2O2 under similar conditions. On the contrary,
CAP, which is structurally not related to SPZ, has no effect on superoxide, while it showed comparable reactivity towards hydroxyl and peroxyl radicals. Thus, CAP could still be a protector of reperfused heart by scavenging the down-stream radicals of superoxide. This is indicated by the fact that while 10-µM CAP had no effect on the recovery of LVDP or infarct size, 300-µM CAP showed significant recovery which was comparable to SPZ (Figure 5). On the other hand, at 10 µM concentration, although the IC50 values of SPZ and CAP are comparable, SPZ seems to have a better efficacy over CAP, possible due to the superoxide-scavenging capability of SPZ.

Sulfaphenazole is a selective inhibitor of CYP 2C9 with an IC50 of 0.6 µM (Ha-Duong et al., 2001). Thus, at 10-µM concentration, SPZ is expected to almost completely inhibit CYP 2C9. If inhibition of CYP 2C9 were the only mechanism of reduction of ischemia and reperfusion-induced myocardial damage by SPZ, then 300-µM SPZ could not have produced substantially higher further protection as measured by infarct size and CK release (Granville et al., 2004). Our results (Figure 5) also indicated a two-fold improvement in LVDP and infarct size in hearts treated with 300-µM SPZ when compared to 10-µM SPZ. Furthermore, at 300 µM concentration, SPZ and CAP+SPZ exhibited almost similar protective effect suggesting the dominant nature of the antioxidant nature of the protection over inhibition of CYP 2C9 at this dose. A comparison of the tissue levels of superoxide in SPZ (10 µM) and CAP (300 µM)-treated hearts, as determined by HE fluorescence (Figure 6), also points to the fact that SPZ is an inhibitor/scavenger of superoxide, while CAP is an inhibitor only. Overall, the results indicate that while inhibition of CYP 2C9 and hence superoxide radical generation may be important at lower concentrations of SPZ or CAP, at higher concentrations there seems to be an additional benefit of scavenging of reactive oxygen.
radicals produced during reperfusion. This effect is particularly significant, as there are multiple pathways, other than CYP 2C9, that are known to generate superoxide in the reperfused myocardium.

The most frequently reported sources of ROS generation in cells are the NADPH oxidase, xanthine oxidase, cyclooxygenase, and eNOS (Kojda and Harrison, 1999). It has been shown that while diphenyleneiodonium (DPI) or superoxide dismutase (SOD) completely abolished, SPZ did not inhibit the NADPH oxidase-mediated ROS generation in PMA-stimulated human leukocytes (Fleming et al., 2001). Similarly, SPZ also did not inhibit the superoxide production by xanthine oxidase (Fleming et al., 2001). At present it is not known whether SPZ has any inhibitory effect on cyclooxygenase- or eNOS-mediated superoxide generation. It is also not known whether how much of the CYP 2C9-mediated generation of superoxide is contributing to the I/R-induced myocardial damage. Hence, the superoxide-scavenging effect of SPZ may be of significance in counteracting the reactive oxidants generated via CYP 2C9-independent pathways in the reperfused heart.

The literature supporting NO as a potent inducer of cardioprotection is overwhelming, regardless of the model system used. Several studies have consistently implicated NO as a required, if not sufficient, factor in the cardioprotective actions of various pharmacologic, surgical, and physiologic interventions (Bolli, 2001). Based upon this extensive literature, it seems logical to posit that chronic augmentation of NO bioavailability should be useful, for it would confer long-term protection against myocardial ischemia–reperfusion injury. A recent study showed that SPZ improved endothelium-dependent, NO-mediated vasodilation in patients with coronary artery disease (Fichtlscherer et al., 2004). The vasodilatory effect was attributed to increase in NO bioavailability after administration of SPZ (Fichtlscherer et al.,
This substantiates the fact that although the EETs could increase vasodilation, the inhibition of CYP 2C9 and simultaneous generation of superoxide suppressed the beneficial effects of EETs. Studies have also evaluated the effect of CYP inhibitors CAP, cimetidine and SPZ in ischemia-reperfusion model of myocardial infarction (Granville et al., 2004). It was also observed that SPZ reduced superoxide generation and restored endothelium-dependent, NO-mediated vasodilation (Granville et al., 2004), whereas endothelium-independent vasodilation was not affected (Hunter et al., 2005).

Nitric oxide plays a crucial role in myocardial preservation during ischemic arrest. Enhancement of NO production is associated with the preservation of the myocardium during ischemic arrest while inhibition of NO synthesis enhances myocardial I/R injury (Maulik et al., 1995). In the present study, we have observed that SPZ scavenges superoxide radicals in vitro and decreases superoxide levels in the heart, thereby restoring NO bioavailability during reperfusion. This effect could be in part responsible for the improvement in coronary flow observed at reperfusion and marked increase in the recovery of contractile function (Figure 3). The increase in DAF fluorescence in SPZ-treated hearts indicates that NO is involved in cardioprotection. In contrast, pretreatment of hearts with L-NAME, either alone or with SPZ, abolished the NO bioavailability up on reperfusion.

Apart from restoring the availability of NO in the reperfused myocardium, the scavenging of superoxide and downstream radicals by SPZ has important implications on myocardial protection. Among the oxygen free radicals, the hydroxyl radicals are the most detrimental to cells. Although organic peroxyl radicals possess a relatively lower oxidizing ability in comparison to the hydroxyl radicals, they are however capable of exerting considerable damage to tissues in biological systems (Chance et al., 1979). Furthermore,
peroxyl radicals are formed *in vivo* in membranes and lipoproteins as intermediate products of lipid peroxidation, which is associated with ischemia reperfusion injury. Resveratrol, a major component in red wine scavenges the peroxyl radicals and prevents myocardial ischemia-reperfusion injury by reducing the oxidative stress (Ray et al., 1999). Thus, the observation in the present study that SPZ could also scavenge OFR, particularly the fact that it completely abolished the peroxyl radicals generated *in vitro*, seems to suggest that SPZ could function as an antioxidant as well.

In conclusion, SPZ prevents I/R-induced myocardial injury by attenuating superoxide accumulation in the tissue, thereby enhancing the bioavailability of nitric oxide. The effect is attributed, in addition to its inhibitory effect on CYP 2C9, to the substantial ability of SPZ in scavenging oxygen free radicals.
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Footnotes

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Legends for Figure

Figure 1. EPR spectra showing the effect of SPZ on superoxide, hydroxyl, and peroxyl radicals, *in vitro*. The radicals were generated as described in the Methods section and detected by EPR spectroscopy using DEPMPO spin-trap (1 mM). SPZ was used to compete with DEPMPO for the radicals. The EPR spectra of DEPMPO adducts of superoxide (A1-A4), hydroxyl (B1, B2), and alkylperoxyl (C1, C2) were measured after a 5-min incubation. The DEPMPO-superoxide spectra were obtained from untreated control (A1) or in the presence of 1-mM SPZ (A2), 5-mM SPZ (A3), or 500-U/ml SOD (A4). Addition of 1-mM SPZ decreased the signal intensity to ~40% of control, while 5-mM SPZ or SOD completely abolished the DEPMPO-superoxide adduct signal. The DEPMPO-hydroxyl spectra, obtained from untreated control (B1) or in the presence of 1-mM SPZ (B2), show that 1-mM SPZ decreased the signal intensity to ~20% of control. On the other hand, the DEPMPO-peroxyl adduct in the absence of SPZ (C1) was completely inhibited by 1-mM SPZ (C2). Over all, the competitive inhibition study suggests that SPZ is capable of scavenging SOD-inhibitable superoxide, hydroxyl, and peroxyl radicals in solution.

Figure 2. Scavenging of reactive oxygen species (ROS) by SPZ in solution. The superoxide (O$_2^-$), hydroxyl (HO$^-$), and alkylperoxyl (ROO$^-$) radicals were determined by EPR spectroscopy using DEPMPO, as in Figure 1. Hydrogen peroxide (H$_2$O$_2$, 300 µM) and nitric oxide (NO, 300 µM) levels were monitored by electrochemical methods as described in the Methods section. Data represent mean±SD values from 3-4 independent experiments and expressed as percent of respective untreated controls. (A) Effect of 1-mM SPZ against 1-mM DEPMPO on the formation of DEPMPO spin
adducts of superoxide, hydroxyl and peroxyl radicals, or on H$_2$O$_2$ and NO. *p<0.01 or 
#p<0.001 versus to respective controls. (B) Effect of 300-µM SPZ or 300-µM CAP 
against 300-µM DEPMPO on the formation of spin adducts of superoxide, hydroxyl 
and peroxyl radicals. *p<0.01; #p<0.001 compared to respective controls.

Figure 3. Attenuation of I/R-induced cardiac dysfunction by SPZ treatment. Data show the 
recovery of CF (A), LVDP (B), and RPP (C), at the end of 45 min of reperfusion.

Figure 4. Pretreatment with SPZ attenuated I/R-induced LDH/CK release and myocardial 
infarct size. The plot shows the activity of LDH (A) and CK (B), released in the 
coronary effluents collected from hearts at 15 min of reperfusion. The treatment 
protocol was the same as shown in Figure 3. (C) Attenuation of I/R-induced 
myocardial infarction. The treatment protocol was the same as shown in Figure 3 
except that the reperfusion time was 120 min. Myocardial infarction was determined 
by TTC staining. Representative images of TTC-stained slices are displayed on the 
bar. Values are expressed as mean±SD obtained from 4-5 independent measurements 
(hearts). *p<0.01 versus control (I/R), **p<0.05 versus control (I/R), #p<0.05 versus 
SPZ alone.

Figure 5. Effect of SPZ and CAP on the recovery of left-ventricular developed pressure 
(LVDP) and myocardial infarction. (A) LVDP recovery at 45 min of reperfusion. (B)
Infarct size in hearts treated with SPZ (10/300 µM), CAP (10/300 µM), or CAP (10 µM) for 10 min followed by SPZ (300 µM). Data represent mean±SD obtained from 4-5 independent measurements (hearts). *p<0.05 versus I/R control group. #p<0.05 versus respective 10-µM data.

Figure 6. Effect of SPZ on the I/R-induced superoxide generation. The treatment protocol was as in Figure 3, except that the reperfusion time was 15 min. Cryosections of hearts after reperfusion were incubated with dihydroethidium (DHE, 10 µM) at 37°C in dark for 30 min and the formation of hydroethidine (HE) was determined by fluorescence microscopy. (A) Representative images from triplicate experiments are shown. (B) Mean fluorescence intensity after deducting the pre-ischemic baseline values of control hearts. Tempol, a known scavenger of superoxide, was included as a positive control. Data represent mean±SD from 3 hearts. *p<0.05 versus I/R; #p<0.05 versus 10-µM SPZ group.

Figure 7. Enhancement of nitric oxide (NO) generation in rat hearts perfused with SPZ. The treatment protocol was as in Figure 3, except that the reperfusion time was 15 min. DAF-2A (3 µM) was infused 1 min before the onset of ischemia. (A) Representative images from triplicate experiments are shown. (B) Mean fluorescence intensity after deducting the baseline values of the pre-ischemic control hearts. PTIO (300 µM) was used as a positive control (scavenger) of NO. Data represent mean±SD from 3 hearts. *p<0.05 versus I/R (control); #p<0.05 versus 10-µM SPZ; **p<0.01 versus 300-µM SPZ.
Figure 1
Figure 2

A

[Graph showing concentration of various reactive oxygen species (ROS) in CONTROL and +SPZ (1 mM) conditions.]

B

[Graph showing concentration of various ROS in CONTROL, +CAP (300 µM), and +SPZ (300 µM) conditions.]

*\( \text{O}_2^- \)

*\( \text{HO}^- \)

*\( \text{ROO}^- \)

*\( \text{H}_2\text{O}_2 \)

*\( \text{NO} \)
Figure 3

A

LVDP (% recovery)

B

CF (% recovery)

C

RPP (% recovery)

I/R SPZ L-NAME L-NAME +SPZ

* ^
Figure 4

A

LDH activity (U/L)

0

50

100

150

B

CK activity (U/L)

0

50

100

150

C

Infarct Size (%)

0

10

20

30

40

50

I/R SPZ L-NAME L-NAME +SPZ

**

#
Figure 5

A

LVDP (% recovery)

0
10
20
30
40
50

Control (I/R)

SPZ

CAP

SPZ

CAP

CAP+SPZ

# # #

B

Infarct Size (%)

0
10
20
30
40
50

Control (I/R)

SPZ 10 µM

CAP 10 µM

SPZ 300 µM

CAP 300 µM

CAP+SPZ

* # # #
Figure 6

A

PRE-ISCH.  I/R  SPZ (10 µM)
SPZ (300 µM)  CAP (300 µM)  TEMPO

B

HE Fluorescence (a. u.)

I/R  SPZ (10 µM)  SPZ (300 µM)  CAP (300 µM)  TPL (300 µM)

*  *  *  #
Figure 7