Ischemic heart disease leading to myocardial infarction 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., 2003). One of the most toxic reactive nitrogen species is peroxynitrite, which is formed by the reaction of NO and superoxide at near diffusion-limited rate. Considerable evidence now exists that peroxynitrite plays a causative role in ischemia/reperfusion injury.

ABSTRACT

Cytochrome P450 (P450) enzymes play a significant role in promoting myocardial ischemia-reperfusion (I/R) injury. CYP2C9, an isoform of P450, is known to generate superoxide radicals in the reperfused heart. Sulfaphenazole (SPZ), a CYP2C9 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 Nω-nitro-ω-arginine methyl ester (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 triphenyltetrazolium chloride staining. The SPZ-treated group showed a significant recovery of cardiac function compared with the untreated I/R group (CF, 53 versus 45%; LVDP, 48 versus 22%; RPP, 51 versus 20%). The infarct size was significantly reduced in the SPZ-treated group (15%) compared with the I/R control (42%). Co-administration of L-NAME with SPZ significantly attenuated the beneficial effects of SPZ. In addition, 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 CYP2C9, as well as scavenging of oxygen free radicals generated in the reperfused heart.
role in posts ischemic myocardial apoptosis and necrosis (Wang and Zweier, 1996; Beckman, 1999; Levrard et al., 2006).

Cytochrome P450 (P450) enzymes are membrane-bound, heme-containing terminal oxidases that are found in organisms ranging from Archaea bacteria to humans. These enzymes are responsible for the metabolic activation or inactivation of most types of drugs, as well as toxins. P450 enzymes oxidize, peroxidize, and/or reduce steroids, arachidonic acid, vitamins, and other foreign substances (for example, drugs) in an oxygen- and NADPH-dependent manner (Schwartzman et al., 1985). The majority of P450 isoforms are mono-oxygenases that catalyze the incorporation of a single atom of oxygen into a substrate. P450s are also known to generate ROS during their reaction cycle because electrons are transferred from the central heme iron to the activated, bound oxygen molecule (Fleming, 2001). In fact, P450s 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. (2004) reported that inhibition of CYP2C9, a human isozyme, by chloramphenicol (CAP), cimetidine, or sulfaphenazole (SPZ) significantly attenuated the tissue damage in an I/R model of myocardial infarction in isolated rat hearts. The inhibition of CYP2C9 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 CYP2C9-mediated ROS production, leading to increased NO bioavailability and NO-mediated vasodilation. In the study by Granville et al. (2004), we observed three striking features on the effect of SPZ: (1) SPZ was able to reduce infarct size when administered only at the time of reperfusion; (2) SPZ inhibited CYP2C9 (IC50 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 (3) although the IC50 values are comparable for CAP and SPZ inhibition of CYP2C9, SPZ demonstrated enhanced cardioprotection compared with 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 CYP2C9. The potentially greater protective efficacy of SPZ over other CYP2C9 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 (OFRs). Based on the study by Granville et al. (2004), it became evident that 10 μM SPZ was enough to block CYP2C9-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 CYP2C9 but also by scavenging of the superoxide radicals generated in the reperfused hearts.

Materials and Methods

Chemicals. SPZ, CAP, N-nitro-L-arginine methylester (L-NAME), dihydroethidium (DHE), xanthine, xanthine oxidase, 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-Phenyln-4,4,5,5-tetramethylimidazolin-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 Institute of Laboratory Animal Resources (1996). Male Sprague-Dawley rats (350–400 g) were anesthetized intraperitoneally with 60 mg/kg sodium pentobarbital (Nembutal) 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 to arrest residual contractions. The aorta was subsequently cannulated to the perfusion apparatus. The hearts were retrogradely perfused through the aorta with a modified Krebs-Henseleit buffer 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 water bolus was injected inside the left atrium and inflated with water to produce an end diastolic pressure of 8 to 12 mm Hg. 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, left-ventricular end-diastolic pressure, and heart rate (HR). The left-ventricular developed pressure (LVP) was calculated as the difference between left-ventricular systolic pressure and left-ventricular end-diastolic pressure. The rate-pressure product (RPP), which is an index of myocardial work, was calculated as LVP × 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 perfusate 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 hydroxycarbethoxymethylene blue (HE) and DAF fluorescence measurements were done on separate groups of hearts (n = 6 per group). Because the IC50 value of SPZ is 0.6 μM (Ha-Duong et al., 2001), we reasoned that the 10 μM dose would be sufficient to block CYP2C9 and inhibit superoxide radical production. However, the scavenging of radicals is a chemical process; hence, we used a higher dose (300 μM) in our experiments to study the drug’s radical-scavenging property.

Lactate Dehydrogenase and Creatine Kinase 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) and 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 (Varian, Inc., Palo Alto, CA).

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 (Nikon, Tokyo, Japan). 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 DHE fluorescence (Miller et al., 1998). The cell-permeable DHE is oxidized to fluorescent 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 optimal cutting temperature 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 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 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 sulfate (0.1 mM) with hydrogen peroxide (0.1 mM) in PBS. Thermolytic fission of 2,2-azobis-2-amidonopropane dihydrochloride (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 diethyl-dithretriaminepentaacetate (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 percentage 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 mM) or H$_2$O$_2$ (300 mM) along with diethyl-dithretriaminepentaacetate (0.1 mM) and Tris-HCl (50 mM, pH 7.4). After 5 min of stimulation, SPZ was introduced using a Hamilton syringe, and the measurements were continued for 10 min. The results were expressed as percentage 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 ± S.D. 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 Fig. 1, 1 mM SPZ, used against 1 mM DEPMPO, decreased the intensity of the DEPMPO-OOH spectrum (Fig. 1, A1) by more than 60% (Fig. 1, A2). Challenging of 1 mM DEPMPO with 5 mM SPZ completely abolished the signal (Fig. 1, A3). The addition of SOD completely inhibited the EPR signal (Fig. 1, A4), suggesting that the DEPMPO-OOH adduct formed was indeed from superoxide radicals. Likewise, the hydroxyl radical adduct (DEPMPO-OH), generated using the Fenton reaction in the presence of 1 mM DEPMPO, was inhibited by more than 80% (Fig. 1, B1 and B2). More importantly, SPZ (1 mM) completely abolished the peroxyl radical adduct (DEPMPO-OOR), generated by thermal decomposition of 2,2-azobis-2-amidonopropene dihydrochloride in aerated PBS (Fig. 1, C1 and C2). The mean ± S.D.

Fig. 1. EPR spectra showing the effect of SPZ on superoxide, hydroxyl, and peroxyl radicals in vitro. The radicals were generated as described under Materials and Methods and detected by EPR spectroscopy using the 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, whereas 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). Overall, the competitive inhibition study suggests that SPZ is capable of scavenging SOD-inhibitable superoxide, hydroxyl, and peroxyl radicals in solution.
values obtained from three to four independent measurements clearly demonstrated the scavenging of the reactive oxygen radicals by SPZ (Fig. 2A). We further performed electrochemical measurements to determine whether SPZ was capable of scavenging NO and H$_2$O$_2$ in solution. The results, shown as a separate panel in Fig. 2A, revealed that SPZ had no effect on NO or H$_2$O$_2$ 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 Fig. 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 CAP, another known inhibitor of CYP2C9, under similar conditions. The results showed that CAP had no significant effect on superoxide radicals; however, it was effective against hydroxyl and peroxyl radicals (Fig. 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 Postischemic Hearts.** Hearts were subjected to 30 min of global ischemia followed by 45 min of reperfusion. CF, LVDP, and RPP were continuously measured before the start of global ischemia and during reperfusion. The data were expressed as a percentage of their preischemic baseline values, which were as follows: CF, 15 ± 2 ml/min; LVDP, 115 ± 14 mm Hg; and 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%) compared with preischemic baseline values (Fig. 3). Hearts treated with SPZ (300 μM) showed a significant recovery of CF (53 ± 5%), LVDP (48 ± 6%), and RPP (51 ± 6%) compared with the untreated I/R hearts. Although infusion of L-NAME (100 μM) had no significant effect on the recovery, coinfusion of L-NAME with SPZ significantly attenuated the recovery of CF, LVDP, and RPP compared with SPZ alone.

**SPZ Decreases LDH and CK Release upon Reperfusion.** In untreated hearts subjected to I/R, the LDH activity

![Fig. 2. Scavenging of ROS by SPZ in solution. The superoxide ($O_2^-$), hydroxyl (HO$^-$), and alkylperoxyl (ROO$^-$) radicals were determined by EPR spectroscopy using DEPMPO, as in Fig. 1. Hydrogen peroxide (H$_2$O$_2$, 300 μM) and NO (300 μM) levels were monitored by electrochemical methods as described under Materials and Methods. Data represent mean ± S.D. values from three to four independent experiments and expressed as percentage 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 with respective controls.

![Fig. 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. SPZ (300 μM), L-NAME (100 μM), and L-NAME + SPZ were added to the perfusate buffer and perfused throughout the experiment. Hearts were subjected to 30 min of no-flow global ischemia at 37°C followed by 45 min of reperfusion. Data represent mean ± S.D. obtained from four to five independent measurements (hearts). *, $p < 0.05$ versus control (I/R); #, $p < 0.05$ versus SPZ.
increased with a maximum activity at 10 to 15 min of reperfusion. The LDH activity was significantly decreased in hearts treated with SPZ compared with the control group (Fig. 4A). Likewise, the effluents from hearts treated with SPZ showed significantly less CK activity compared with the untreated group (Fig. 4B). L-NAME showed a significant decrease in both LDH and CK levels compared with control. However, coinfusion 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 (Fig. 4C). On the other hand, the infarction in hearts treated with SPZ was significantly reduced (15.0 ± 3.2%) compared with 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 compared with the I/R control group, suggesting that coinfusion of L-NAME with SPZ significantly attenuated the protective effect of SPZ alone.

**Protective Effect of SPZ in Comparison with CAP.** To assess whether the protective effect of SPZ was due to inhibi-

![Graph A](image1)

**Graph A:** 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 Fig. 3. Attenuation of I/R-induced myocardial infarction was determined by TTC staining. Representative images of TTC-stained slices are displayed on the bar. Values are expressed as mean ± S.D. obtained from four to five independent measurements (hearts). *, p < 0.01 versus control (I/R); **, p < 0.05 versus control (I/R); #, p < 0.05 versus SPZ alone.
bition of superoxide production by CYP2C9 and/or scavenging of superoxide and downstream radicals generated upon reperfusion, we repeated the experiments using a lower dose of SPZ. We also compared the results with CAP, another known inhibitor of CYP2C9. Because the IC$_{50}$ values of SPZ and CAP for CYP2C9 are $\sim$1 $\mu$M, we used a dose of 10 $\mu$M, which was sufficient to block the enzyme. As shown in Fig. 5A, SPZ or CAP at 10 $\mu$M concentration did not show any significant difference in the recovery of LVDP compared with control I/R group. On the other hand, SPZ or CAP at 300 $\mu$M concentration demonstrated a significant ($p < 0.01$) enhancement of LVDP recovery. A similar trend was observed on the reduction of infarct size (Fig. 5B), except that 10 $\mu$M SPZ, but not CAP, showed a significant ($p < 0.05$) reduction in infarct size compared with control I/R hearts. To further delineate the inhibitory versus scavenging role of SPZ, we pretreated hearts with 10 $\mu$M CAP for 10 min followed by treatment with 300 $\mu$M SPZ upon reperfusion. Both LVDP recovery (Fig. 5A) and infarct reduction (Fig. 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 HE fluorescence as described under Materials and Methods. The HE fluorescence intensity was significantly higher in untreated I/R (control) hearts subjected to 30 min of ischemia followed by 15 min of reperfusion (Fig. 6). Hearts treated with SPZ showed a dose-dependent decrease in HE fluorescence intensity compared with control I/R group (Fig. 6B). Although 10 $\mu$M CAP did not show any significant effect (data not shown), 300 $\mu$M CAP showed a significant attenuation of intensity; however, the effect was significantly less compared with that of 300 $\mu$M 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 under Materials and Methods. Control hearts subjected to 30-min ischemia followed by 15-min reperfusion showed intense fluorescence suggestive of NO generation (Fig. 7A). The NO generation was significantly ($p < 0.05$) higher in hearts treated with 300 $\mu$M SPZ (Fig. 7B). In contrast, 10 $\mu$M 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 an L-NAME-inhibitable source. Cotreatment of SPZ (300 $\mu$M) with PTIO (100 $\mu$M), a known scavenger of NO, abolished the NO fluorescence below the level of I/R group. Overall, the results
Based on the bimolecular rate constant of the reaction between DEPMPO and superoxide, \(7 \times 10^3 \text{ M}^{-1} \text{s}^{-1}\), the apparent second order rate constant of SPZ with superoxide is estimated to be \(4 \times 10^3 \text{ M}^{-1} \text{s}^{-1}\). The EPR results also indicate that SPZ is a more effective scavenger of hydroxyl and peroxyl radicals compared with superoxide, whereas it has no effect on NO and \(\text{H}_2\text{O}_2\) under similar conditions. On the contrary, CAP, which is structurally not related to SPZ, has no effect on superoxide, whereas it showed comparable reactivity toward hydroxyl and peroxyl radicals. Thus, CAP could still be a protector of reperfused heart by scavenging the downstream radicals of superoxide. This is indicated by the fact that although 10 \(\mu\)M CAP had no effect on the recovery of LVDP or infarct size, 300 \(\mu\)M CAP showed significant recovery, which was comparable with SPZ (Fig. 5). On the other hand, at 10 \(\mu\)M concentration, although the IC\(_{50}\) 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 CYP2C9 with an IC\(_{50}\) of 0.6 \(\mu\)M (Ha-Duong et al., 2001). Thus, at 10 \(\mu\)M concentration, SPZ is expected to almost completely inhibit CYP2C9. If inhibition of CYP2C9 were the only mechanism of reduction of ischemia and reperfusion-induced myocardial damage by SPZ, then 300 \(\mu\)M SPZ could not have produced substantially further higher protection as measured by infarct size and CK release (Granville et al., 2004). Our results (Fig. 5) also indicated a 2-fold improvement in LVDP and infarct size in hearts treated with 300 \(\mu\)M SPZ compared with 10 \(\mu\)M SPZ. Furthermore, at 300 \(\mu\)M concentration, SPZ and CAP + SPZ exhibited almost similar protective effect, suggesting the dominant nature of the antioxidant nature of the protection over inhibition of CYP2C9 at this dose. A comparison of the tissue levels of superoxide in SPZ (10 \(\mu\)M)- and CAP (300 \(\mu\)M)-treated hearts, as determined by HE fluorescence (Fig. 6), also points to the fact that SPZ is an inhibitor/scavenger of superoxide, whereas CAP is an inhibitor only. Overall, the results indicate that although inhibition of CYP2C9 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 because there are multiple pathways, other than CYP2C9, 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 although diphenyleneiodonium or SOD completely abolished, SPZ did not inhibit the NADPH oxidase-mediated ROS generation in PMA-stimulated human leukocytes (Fleming et al., 2001). Likewise, 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 endothelial nitric-oxide synthase-mediated superoxide generation. It is also not known whether how much of the CYP2C9-mediated generation of superoxide is contributing to the IR-induced myocardial damage. Hence, the superoxide-scavenging effect of SPZ may be of significance in counteracting the reactive oxidants generated via CYP2C9-independent pathways 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 CYP2C9 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.
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., 2004). This substantiates the fact that although the epoxyeicosatrienoic acids could increase vasodilation, the inhibition of CYP2C9 and simultaneous generation of superoxide suppressed the beneficial effects of epoxyeicosatrienoic acids. Studies have also evaluated the effect of P450 inhibitors CAP, cimetidine, and SPZ in the 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, whereas 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 (Fig. 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 upon 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 with 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 CYP2C9, to the substantial ability of SPZ in scavenging oxygen free radicals.

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