The Radical Trap 5,5-Dimethyl-1-Pyrroline *N*-Oxide Exerts Dose-Dependent Protection against Myocardial Ischemia-Reperfusion Injury through Preservation of Mitochondrial Electron Transport

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ABSTRACT

Free radicals are important mediators of myocardial ischemia-reperfusion injury. Nitron spin traps have been shown to scavenge free radicals. The cardioprotective effect of the spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), was investigated in an isolated heart model of global ischemia and reperfusion. Rat hearts were perfused and subjected to global ischemia for 30 min followed by reperfusion with four treatment groups of varying DMPO concentration (0.5–10 mM) administered before induction of ischemia. DMPO treatment improved the recovery of left ventricular (LV) function and coronary flow over the 30-min period of reperfusion compared with untreated hearts. Enhanced recovery was observed for all doses studied but was highest with 1 mM treatment with 2.4-fold higher recovery of LV developed pressure and 37% reduction in infarct size. Superoxide was measured by tissue fluorometry using the O_2^- probe

hydroethidine. Hearts treated with 1 mM DMPO showed a significant reduction in O_2^- production compared with control hearts both over the first 5 min of ischemia and upon reperfusion after 30 min of global ischemia. Studies of mitochondrial function demonstrated that 1 mM DMPO increased the recovery of function of complexes I, II/III, and IV after 30 min of reperfusion. Immunoblotting with antibodies against complexes I, II, and IV further revealed marked up-regulation of mitochondrial proteins, suggesting that DMPO prevents their ischemic degradation via scavenging oxygen radicals generated during ischemia/reperfusion. Thus, DMPO functions as a protective agent against ischemic and postischemic injury via radical scavenging, conferring robust dose-dependent protection with salvage of mitochondrial function and redox homeostasis.

Reactive oxygen species (ROS) have been implicated in a variety of pathophysiological disorders. ROS generation during early reperfusion is a major cause of myocardial ischemia/reperfusion injury (Zweier, 1988; Zweier and Talukder, 2006). The spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) (Fig. 1), has been employed widely in the detection and identification of various free radicals such as superoxide

(O_2^-), hydroxyl ($HO^\bullet$), and carbon-centered radicals to form persistent spin adducts that are detectable by electron paramagnetic resonance spectroscopy (Villamena and Zweier, 2004). The capacity of DMPO to trap radicals suggests its potential use as an antioxidant. In fact, DMPO and α -phenyl-*tert*-butyl-nitron (PBN) (Fig. 1) have exhibited pharmacological activity, such as in the treatment of neurodegenerative disease and acute stroke (Floyd et al., 1997; Floyd and Hensley, 2000).

Several groups have evaluated the use of DMPO as a potential cardioprotective agent against myocardial ischemia/reperfusion injury (Tosaki and Braquet, 1990; Bradamante et al., 1993; Pietri et al., 1998; Maurelli et al., 1999).

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ABBREVIATIONS: ROS, reactive oxygen species; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; LV, left ventricular; NO, nitric oxide; EDP, end diastolic pressure; LVDP, left ventricular developed pressure; HR, heart rate; RPP, rate pressure product; CF, coronary flow; HE, hydroethidine; ET, ethidium; ETA, electron transfer activity; NQR, NADH-ubiquinone oxidoreductase; SCR, succinate-cytochrome *c* reductase; CcO, cytochrome *c* oxidase; TTBS, Tris-buffered saline containing 0.1% Tween 20; TTC, 2,3,5-triphenyltetrazolium chloride; eNOS, endothelial nitric-oxide synthase; METC, mitochondrial electron transport chain.

Fig. 1. Chemical structures.

A reperfusion-associated burst of O_2^- generation has been shown to occur when isolated hearts are subjected to ischemia and reperfusion (Zweier et al., 1987, 1989). However, the slow reactivity of DMPO with O_2^- and the low efficiency of spin trapping led investigators to use high-spin trap concentrations from 5 to 40 mM (Bradamante et al., 1993; Pietri et al., 1998; Maurelli et al., 1999). Administration of DMPO during the preischemic period or during reperfusion was assumed inconsequential in determining the extent of left ventricular (LV) functional recovery (Hearse and Tosaki, 1987; Bolli et al., 1989; Pietri et al., 1998). Both of these assumptions were predicated on the belief that the only cardioprotective effect of DMPO is due to its radical scavenging property during reperfusion.

Conflicting studies have been reported in regards to the mode of DMPO action although spin trapping of free radicals may be the most plausible mechanism for its cardioprotective property against reperfusion injury. Bradamante et al. (1993) showed that DMPO in millimolar concentrations did not show cardioprotection from ischemia-reperfusion injury using Langendorff rat heart preparations, but Pietri et al. (1998) and Tosaki et al. (1990) reported protection. Moreover, 1,2,2,4,5,5-hexamethyl-3-imidazoline-oxide (HMIO) (Tosaki et al., 1992) or pyrrolidine (PyH) (Pietri et al., 1998) (Fig. 1), which are structurally related compounds but not spin traps, did not exhibit improvement in cardiac function, further confirming the role of the nitron moiety in cardioprotection.

The mechanism of nitron antioxidant activity is perplexing because the reactivity of O_2^- to DMPO is slow at neutral pH; however, at mildly acidic pH, the reactivity is much faster (Finkelstein et al., 1980; Allouch et al., 2007). The high reactivity of O_2^- in acidic pH is due to the protonation of O_2^- to form hydroperoxyl radical ($HO_2^\cdot$) (pK_a for $HO_2^\cdot$ is 4.8), and $HO_2^\cdot$ is known to be a stronger oxidizing agent than O_2^- . Furthermore, the pK_a of DMPO was established to be 6.0 (Burgett et al., 2008) and that protonation of DMPO at mildly acidic pH can considerably increase its reactivity to O_2^- , comparable with the favorability of addition of $HO_2^\cdot$ to DMPO. Therefore, because the production of O_2^- is ubiquitous during ischemic events, and because acidosis occurs during ischemia, it is possible that the rate of DMPO- O_2^- formation can be enhanced and may exhibit cardioprotective effects during reperfusion (Simonis et al., 1998; Xiong et al., 2004).

It was also demonstrated that the O_2^- adduct of DMPO decomposes to yield nitric oxide (NO), and this characteristic could potentially exhibit therapeutic properties (Locigno et al., 2005). Moreover, it has been shown that spin trapping of carbonate radical anion ($CO_3^{\cdot-}$) by DMPO results in the formation of nitrite anions (Villamena et al., 2007). The potentially damaging role of $CO_3^{\cdot-}$ has been gaining some attention because inorganic anions are ubiquitous in most biological and environmental systems. Therefore, potentially efficacious cardioprotective interventions will probably require

targeting and inhibiting ROS-related cascades generated during both ischemia and reperfusion (Bolli, 2001).

We hypothesize that the free radical scavenging property of DMPO may offer cardioprotection from radical-mediated tissue injuries by salvaging key cellular enzymes that are susceptible to oxidative insult such as the mitochondrial electron transport chain. This study evaluated whether DMPO when administered only immediately before the initiation of global normothermic ischemia can confer myocardial preservation with enhanced postischemic recovery of cardiac function. The dose dependence of this cardiac protection and the mechanisms involved in this process were determined. It was observed that DMPO was highly effective in preventing postischemic myocardial injury with marked preservation of mitochondrial function and electron transport

Materials and Methods

Langendorff Heart Preparation. All procedures were in accordance with the Ohio State University Institutional Laboratory Animal Care and Use Committee. Male Sprague-Dawley rats weighing ~350 g were anesthetized with pentobarbital (~50 mg/kg i.p.) and heparinized with 0.1 ml of 1000 IU/kg. After hemithoracotomy, hearts were rapidly excised, and aorta were cannulated under retrograde coronary perfusion at 80 mm Hg with Krebs-Henseleit buffer (120.0 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 16.7 mM glucose, 25.0 mM $NaHCO_3$, 0.5 mM EDTA, bubbled with 95%/5% O_2/CO_2). An elastic balloon was inserted into the LV and adjusted to a constant volume, yielding end diastolic pressure (EDP) between 8 and 12 mm Hg over the first 10 min of a preischemic 20-min baseline period. Hearts were immersed in a buffer-filled glass chamber with a water jacket. Temperature was measured every 10 min using a Physitemp thermoprobe to ensure normothermic conditions during baseline, global ischemia, and reperfusion intervals. Perfusate and hearts were maintained at $37^\circ C \pm 0.2^\circ C$ with the water jacket chamber connected to an external circulating heat bath.

Global Ischemia/Reperfusion with LV Function and Coronary Flow Measurement. All hearts were subjected to a 20-min baseline period under constant perfusion pressure. Hearts were randomly assigned to one of five study groups including control and four DMPO treatment groups. Immediately before global ischemia, with the perfusion rate set at ~2 ml/min, these groups received 4 ml of oxygenated Krebs-Henseleit buffer with varying concentrations of DMPO (0, 0.5, 1.0, 5.0, and 10.0 mM). After 30 min of global ischemia, hearts were reperfused for an additional 30 min. LV pressures were continuously recorded using Powerlab 4/25 ADC (ADInstruments Ltd., Chalgrove, Oxfordshire, UK) and Chart software. The following derived indices of LV mechanical function were instantaneously recorded: peak systolic pressure, EDP, left ventricular developed pressure ($LVDP = \text{peak systolic pressure} - \text{EDP}$), heart rate (HR), and rate pressure product ($RPP = LVDP \times HR$). Coronary flow (CF) was continuously measured using the Transonic Systems TS410 flowmeter (Transonic Systems Inc., Ithaca, NY).

ROS Fluorescence of Perfused Rat Hearts. In another series of experiments, rat hearts were perfused as described above in a separate perfusion system designed for fluorescent measurements. Data were collected in control and 1 mM DMPO-treated hearts during a 20-min baseline, 30-min ischemic interval, and 30-min reperfusion. Light interference was minimized by enclosing the perfusion system in an internally painted black box. A single coil containing optical fibers for emission and excitation was carefully positioned directly on the LV surface of the heart for epifluorescence measurements. The hydroethidine (HE)/ethidium (ET) fluorescent probe was used to detect intracellular ROS production in isolated rat hearts. HE (Invitrogen, Carlsbad, CA) is a neutral fluorescent probe

specifically sensitive to O_2^- , but not to H_2O_2 . The concentrated HE stock was made in *N,N*-dimethylacetamide (Acros Organics, Fairlawn, NJ) (Zuo et al., 2000; Zuo and Clanton, 2002), and for infusion, this was diluted >400-fold in perfusate. The heart was infused with 4 ml of a 44 μ M HE solution for 2 min followed by 5-min washout. In response to ROS, HE is oxidized, resulting in the formation of ET. ET is positively charged and has better cellular retention and stability compared with HE. Thus, ET formation was chosen as an indicator of ROS production, which is a common method when using this probe (Nethery et al., 1999; Zuo et al., 2000; Zuo and Clanton, 2002). ROS fluorescence was measured using a tissue fluorometer (C&L Instruments, Inc., Hershey, PA). The excitation beam from a 150-W xenon lamp was focused on a 6-mm-diameter fiber optic coil, and the light passed through a filter wheel containing four specific band-pass filters. A second filter wheel with four emission filters was used to separate emission light at specific wavelengths. This light was focused on the photomultiplier tube, and the signal was imported to a computer equipped with FluorMeasure version 2.7 acquisition monitor interface via the A/D board (C&L Instruments, Inc.). The ET excitation filter was set at 515 ± 20 nm, and the ET emission was set at 590 ± 25 nm.

Assay of Enzymatic Activities of Mitochondrial ETC. At the end of the experiments, the myocardium of the left ventricle of rat hearts were excised and immediately frozen with liquid nitrogen. The tissue was homogenized in ice-cold HEPES buffer (3 mM, pH 7.2) containing sucrose (0.25 M), EGTA (0.5 mM), and protease inhibitor cocktail (1:40; Roche Diagnostics, Indianapolis, IN). The tissue homogenate was centrifuged at 600g for 20 min at 4°C. The supernatant was subjected to analysis of mitochondrial electron transfer activities in situ using a UV-visible spectrophotometer (Shimadzu, Kyoto, Japan). The electron transfer activity (ETA) of complex I [NADH-ubiquinone oxidoreductase (NQR)] was determined by following the rotenone-sensitive ubiquinone-1 (Q_1 ; Sigma-Aldrich, St. Louis, MO) stimulated NADH oxidation (Busch et al., 1996). In brief, an appropriate amount of tissue homogenate was added to an assay mixture (0.5 ml) containing potassium phosphate buffer (20 mM, pH 8.0), $NaNO_3$ (2 mM), Q_1 (0.1 mM), and NADH (0.15 mM). The complex I activity (nanomoles of NADH oxidized per minute per milligram of protein) was determined by measuring the decrease in absorbance at 340 nm, confirmed by inhibition with rotenone (20 μ M), and calculated using an extinction coefficient of 6.22 mM/cm. The ETA of succinate-cytochrome *c* reductase (SCR; complex II/III) in the tissue homogenate was assayed by measuring ferricytochrome *c* (from horse heart; Sigma-Aldrich) reduction (Busch et al., 1996; Chen et al., 2000). In brief, an appropriate amount of tissue homogenate was added to an assay mixture (0.5 ml) containing potassium phosphate buffer (50 mM, pH 7.4), EDTA (0.3 mM), KCN (100 μ M), succinate (20 mM), and ferricytochrome *c* (50 μ M). The SCR activity (nanomoles of cytochrome *c* reduced per minute per milligram of protein) was determined by measuring the increase in absorbance at 550 nm, confirmed by inhibition with antimycin A (20 μ M; Sigma-Aldrich), and calculated with a millimolar extinction coefficient of 18.5 mM/cm. The ETA of complex IV [cytochrome *c* oxidase (CcO)] was assayed by measuring ferrocytochrome *c* oxidation and was further confirmed by inhibition with KCN (Busch et al., 1996; Chen et al., 2000). In brief, an appropriate amount of tissue homogenate was added to an assay mixture (0.5 ml) containing potassium phosphate buffer (50 mM, pH 7.4) and ferrocytochrome *c* (60 μ M). The CcO activity (nanomoles of ferrocytochrome *c* oxidized per minute per milligram of protein) was determined by measuring the decrease in absorbance at 550 nm, confirmed by inhibition with KCN (50 μ M), and calculated with an extinction coefficient of 18.5 mM/cm (Chen et al., 2000).

Immunoblotting Analysis. Myocardial tissues were minced and homogenized with a Polytron homogenizer (250 W, 10 s for three times) in ice-cold HEPES buffer (3 mM, pH 7.2) containing sucrose (0.25 M), EGTA (0.5 mM), and protease-inhibitor cocktail (1:40). The supernatant of tissue homogenate was collected by centrifugation at

600g for 20 min. The reaction mixture was mixed with the Laemmli sample buffer at a ratio of 4:1 (v/v) in the presence of β -mercaptoethanol, incubated at 70°C for 10 min, and then immediately loaded onto a 4 to 20% Tris-glycine polyacrylamide gradient gel. Samples were run at room temperature for 2 h at 100 V. Protein bands were electrophoretically transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine, and 10% methanol. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% dry milk (Bio-Rad, Hercules, CA). The blots were then incubated overnight with anti-51-kDa (for complex I) polyclonal antibody or anti-70-kDa (for complex II) polyclonal antibody or anti-CoXI and anti-CoXv (for complex IV) monoclonal antibodies at 4°C. Blots were then washed three times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit/mouse IgG in TTBS at room temperature. The blots were again washed twice in TTBS and twice in Tris-buffered saline and then visualized using ECL Western Blotting Detection Reagents (GE Healthcare, Fairfield, CT). Measurements were repeated six times for each assay.

Myocardial Infarct Size Measurement. To delineate the viable and infarcted myocardium, 2,3,5-triphenyltetrazolium chloride (TTC) was used, which stains viable myocardium red, and areas of infarction appear white, as described previously (Talukder et al., 2008). Hearts were subjected to a 20-min baseline period under constant perfusion pressure and randomly assigned to control or DMPO treatment groups. Immediately before global ischemia, with the perfusion rate set at ~ 2 ml/min, these groups received 4 ml of oxygenated Krebs-Henseleit buffer with or without 1 mM DMPO. Hearts were then subjected to 30 min of global ischemia and 120 min of reflow and then immediately removed and prepared for sectioning. After freezing, the hearts were serially sectioned into 2-mm slices using a heart slicer and then incubated in 1% TTC (in phosphate-buffered saline) for 15 min. Staining was stopped by removing sections and placing them overnight in 10% neutrally buffered formaldehyde. Images were taken after 12 h using NIS Elements F 2.20 software and analyzed with MetaMorph software.

Statistical Analysis. All data were reported as group averages $\pm$ S.E.M. Statistical analyses of LV function and coronary flow were performed at the end of the baseline period and at the end of 30-min reperfusion using one-way analysis of variance followed by least significant difference multiple-comparison test. Evaluation of infarct size was performed by two-tailed Student's *t* test. A value of $p \leq 0.05$ was considered statistically significant.

Results

DMPO Protects Myocardial Function Recovery. Figure 2 shows the recovery of LVDP throughout the 30-min period of reperfusion after 30 min of global ischemia for each of the four DMPO-treated and control groups. Administering 0.5 to 10 mM DMPO immediately before the onset of global ischemia dose-dependently conferred robust cardioprotection during reperfusion with all doses studied. Compared with controls, impairment of LV function at the end of 30 min of reperfusion with DMPO treatment was greatly decreased in the 1 mM DMPO treatment group, with $43.0 \pm 5.0\%$ recovery of LVDP versus $17.6 \pm 3.6\%$ in the untreated control group ($n = 8$, $p < 0.01$). However this protection decreased as the dose of DMPO increased [$43.0 \pm 5.0\%$ (1 mM DMPO) versus $31.4 \pm 3.6\%$ (10 mM) ($n = 6$), $p < 0.05$]. Figure 3 shows that the RPP recovered to $46.2 \pm 4.5\%$ of preischemic baseline levels in the 1 mM DMPO group compared with only $13.4 \pm 2.1\%$ ($n = 8$, $p < 0.01$) in controls. This protection was seen in all DMPO-treated groups; however, the recovery decreases as the doses rise [$46.2 \pm 4.5\%$ (1 mM DMPO) versus $30.8 \pm 4.3\%$ (10 mM, $n = 6$), $p < 0.05$]. Figure 4 illustrates that LV EDP

Fig. 2. Time course of recovery of LVDP for various DMPO concentrations. The graphs show the preischemic values after 20 min baseline equilibrium (EQ 20') and the time course of reperfusion (R) in minutes. Data are presented as percentage of baseline (*, $p < 0.05$, control versus various DMPO treatments at the end of 30-min reperfusion; †, $p < 0.05$, 1 mM DMPO versus 10 mM DMPO treatments at the end of 30-min reperfusion). Data are average $\pm$ S.E.M. with $n = 8$ for 0 and 1 mM; $n = 4$ for 0.5 and 5 mM; $n = 6$ for 10 mM.

Fig. 3. Time course of recovery of RPP for various DMPO concentrations. The graphs show the preischemic values after 20-min baseline equilibrium (EQ 20') and the time course of reperfusion (R) in minutes. Data are presented as described in Fig. 1. *, $p < 0.05$, control versus various DMPO treatments at the end of 30-min reperfusion; †, $p < 0.05$, 1 mM DMPO versus 10 mM DMPO treatments at the end of 30-min reperfusion.

was also dramatically and dose-dependently improved in all treated groups compared with controls ($p < 0.05$). DMPO treatment also enhanced the recovery of coronary flow with significantly higher recovery seen with 1, 5, and 10 mM DMPO treatment (Fig. 5).

DMPO Reduces ROS Formation in the Isolated Heart. As shown in Fig. 6, measurements of ROS with HE/ET in separate groups of control-untreated or 1 mM DMPO-treated hearts revealed that DMPO significantly reduced the increase of ROS observed in untreated control hearts during global ischemia and after reperfusion ($n = 6$, $p < 0.05$). This supports the efficacy of DMPO in scavenging oxygen radicals during ischemia and reperfusion.

Fig. 4. Time course of recovery of EDP for various DMPO concentrations. Data are presented for the hearts described in Fig. 1 with measured values shown in mm Hg. *, $p < 0.05$, control versus various DMPO treatments at the end of 30-min reperfusion.

DMPO Decreases Infarct Size. Measurements of myocardial infarction were performed in untreated and DMPO-treated hearts subjected to 30 min of global ischemia followed by 120 min of reperfusion. TTC staining revealed that hearts treated with 1 mM DMPO had a reduced infarct size compared to control, with infarct sizes of 14.4 ± 2.6 and $23.0 \pm 3.0\%$, respectively ($p = 0.05$) (Fig. 7). Thus, with 1 mM DMPO treatment, decreased myocardial infarction is seen accompanying the improved recovery of LV function.

DMPO Increases the Recovery of Mitochondrial Function. Figure 8 shows the enzymatic activities of mitochondrial electron transport chain, including complex I (NQR), SCR (a supercomplex of complexes II and III), and complex IV (CcO) that were assayed in the tissue homogenate of postischemic hearts. In postischemic hearts without pretreatment of DMPO, myocardial NQR, SCR, and CcO activities were significantly decreased to 26.9 ± 6.6 , 57.9 ± 10.7 , and $50.9 \pm 8.6\%$ of those detected in the baseline control, respectively ($n = 6$, $p < 0.01$), indicating oxidative impairment of mitochondrial electron transport chain during myocardial ischemia/reperfusion injury. Nevertheless, in DMPO-treated hearts, all the enzymatic activities of myocardial NQR, SCR, and CcO activity were significantly protected and close to the baseline level at the end of reperfusion ($n = 6$, $p < 0.01$). These results show that the impairment of the mitochondrial function is markedly diminished during myocardial ischemia/reperfusion by pretreatment with 1 mM DMPO, thus confirming the protective efficacy of DMPO.

DMPO Prevents Ischemic Degradation and Increases the Levels of Mitochondrial Proteins. Tissue homogenates of postischemic hearts were further probed with a polyclonal antibody against 51-kDa FMN-binding protein (nuclear DNA encoded) of complex I, a polyclonal antibody against 70-kDa FAD-binding protein (nuclear DNA encoded) of complex II, and monoclonal antibodies against subunit I (CoXI, mitochondrial DNA encoded) and subunit Vb (CoXVb, nuclear DNA encoded) of complex IV. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control for Western blotting. As indicated in Fig. 9, protein expression of mitochondrial electron transport chain was

Fig. 5. Time course of recovery of CF for various DMPO concentrations. A, time course of CF recovery, for the hearts as described in Fig. 1. To more clearly depict the differences in CF recovery without data overlap, the bar graph in B shows the final values for each group. Data are presented as percentage recovery of basal flow. *, $p < 0.05$, control versus 1 mM DMPO treatment.

Fig. 6. Plots of peak ET fluorescence within 5 min during the onset of ischemia and reperfusion of untreated (controls) versus treated hearts (1 mM DMPO) relative to baseline. Control (I), onset of global ischemia; DMPO (I), onset of global ischemia with 1 mM DMPO treatment; control (R), onset of reperfusion; DMPO (R), onset of reperfusion with 1 mM DMPO pretreatment (**, $p < 0.05$ versus 1 mM DMPO treatment). Data are mean $\pm$ S.E.M. with $n = 6$.

Fig. 7. Comparison of infarct size in DMPO and untreated hearts. Infarct size as percentage LV was measured by TTC staining after 2 h of global ischemia. Data are mean $\pm$ S.E.M. with $n = 6$. *, $p = 0.05$ versus control.

generally down-regulated in the postischemic heart. In the presence of DMPO, protein expression in the postischemic heart was significantly up-regulated by $\sim 150\%$ for NQR (Fig. 9A, $p < 0.05$, $n = 6$), by $\sim 96\%$ for complex II (Fig. 9B, $p < 0.01$, $n = 6$), and by $\sim 400\%$ for CcO (CoXI, Fig. 9C, $p < 0.01$, $n = 6$). These results implicate that DMPO improved the recovery of mitochondrial function through inhibiting ischemic degradation and inducing marked up-regulation of the electron transport chain proteins.

Discussion

This work has demonstrated that cardioprotection of LV contractile function by the spin trap, DMPO, occurs in a dose-dependent manner (Table 1). Enhanced recovery was observed for all doses studied but was highest with 1 mM treatment, with 2.4-fold higher recovery of LV developed pressure and 3.4-fold higher recovery of rate pressure product after 30 min of reperfusion with 37% reduction in infarct size after 2 h of reperfusion. DMPO also preserved mitochondrial enzyme activities during reperfusion when administered as a single bolus immediately before the onset of global ischemia in the rat Langendorff model. The underlying mechanism for this pharmacologic action of DMPO is still unclear. The protocol in this study employed introduction of a single short bolus of DMPO as opposed to DMPO loading over the whole period of baseline equilibration. This single loading of DMPO solution would result in its further dilution because of its diffusion to the extravascular compartments. In a previous study, with 5 mM DMPO treatment, Maurelli et al. (1999) reported a large increase in ATP for 20-min reperfusion-treated hearts. A single study using a working heart model reperfused hearts for 10 min in the Langendorff mode with 100 μ M DMPO (no preischemic loading of DMPO) reported a significant increase in developed pressure over that observed in controls after 30-min ischemia and 30-min reperfusion (Tosaki et al., 1990). It is interesting that DMPO concentrations used in prior studies did not yield any therapeutic efficacy over the range from 5 to 40 mM (Bradamante et al., 1993; Maurelli et al., 1999), whereas the only reported cardioprotective effect in the perfused rat heart was observed

Fig. 8. The enzymatic activity of mitochondrial electron transport chain in the tissue homogenates of postischemic rat hearts ($n = 6$). A, NQR activity. B, SCR activity. C, CcO activity. *, $p < 0.05$ versus reperfusion (R) + 1 mM DMPO.

Fig. 9. Effect of DMPO treatment on the protein expression of mitochondrial electron transport chain in the postischemic heart. Tissue homogenates of untreated and 1 mM DMPO-treated postischemic hearts were subjected to SDS-PAGE and then immunoblotted with antibodies against mitochondrial electron transport complex (A–D). The antibodies used are: anti-51 kDa (FMN-binding subunit) polyclonal antibody for complex I, anti-70 kDa (FAD-binding subunit) polyclonal antibody for complex II, and anti-CoX I and anti-CoX Vb monoclonal antibodies for complex IV. The signals were normalized to glyceraldehyde-3-phosphate dehydrogenase as indicated. Data are presented as mean $\pm$ S.E.M. of six independent experiments. **, $p < 0.01$; *, $p < 0.05$ versus postischemic heart without DMPO treatment.

at a reperfusion-only concentration of 100 μ M (Tosaki et al., 1990). Because the DMPO reactivity with O_2^- is significantly enhanced at mildly acidic pH as demonstrated in the work of Allouch et al. (2007) and Burgett et al. (2008), it may exhibit a more robust protection during ischemia. In the current report, DMPO reduced the magnitude of increase in EDP at

all doses tested. This suggests that a part of DMPO related therapeutic potential may be due to the prevention of intracellular calcium overload. It has been shown that the reperfusion associated burst of oxygen radical generation leads to myocyte calcium loading with impaired function of sarcoplasmic reticulum Ca^{2+} -ATPase (Zweier and Talukder, 2006).

The observation that treatment with 1 mM DMPO prevented the increase of ROS observed in controls indirectly supports this hypothesis. Other factors contributing to this protection include potential inhibition of calcium entry through sarcolemmal L-type Ca^{2+} channels reducing cytosolic calcium overload and adduct formation with protonated O_2^- inhibiting deleterious downstream free radical cascades and regulating ROS related signal transduction (Anderson et al., 1993; Konorev et al., 1993).

The unimolecular decomposition of DMPO- O_2^- adduct involves subsequent release of NO via a ring opening mechanism (Locigno et al., 2005). We have shown recently that impairment of endothelial function during myocardial ischemia results from eNOS uncoupling via oxidation of tetrahydrobiopterin (Dumitrescu et al., 2007). Depletion of tetrahydrobiopterin by O_2^- can then decrease NO bioavailability and, through the formation of DMPO- O_2^- and its subsequent decomposition to form NO, can play a significant role in restoring endothelial function. Although NO is regarded as a potent vasodilator through activation of cGMP, which then can increase the respiratory substrate to mitochondria exerting other beneficial effects, NO at relatively high levels can cause cellular injury by reacting with O_2^- to form the highly oxidizing species, peroxynitrite. The peroxynitrite species is formed in the postischemic heart and has been shown to induce necrosis and apoptosis and to inhibit mitochondrial respiration (Wang and Zweier, 1996). Thus, if DMPO is metabolized to form NO, higher concentrations could exert adverse effects and toxicity. This could be one reason for our observations that at the 1 mM level, DMPO provided the maximal protection in recovery of both LVDP and RPP throughout the reperfusion period, whereas with 10 mM treatment, less protection was seen.

It has been reported that DMPO can exert direct blockage effects on Ca^{2+} channels with induction of relaxation in precontracted smooth muscles (Anderson et al., 1993; Konorev et al., 1993). Therefore, the decrease in therapeutic efficacy observed in the hearts treated with 10 mM DMPO also could be due to toxic interactions with L-type calcium channels. This may be one of the reasons why at higher doses, DMPO partially loses its cardioprotective efficacy.

The higher reactivity of DMPO to O_2^- at mildly acidic pH makes this mechanism an attractive rationale for the antioxidant activity of DMPO during ischemia (Burgett et al., 2008). DMPO may provide a protective signaling role via partial prevention of the deleterious ROS-mediated signaling cascade during ischemia while retaining beneficial signaling activation. Our study has shown that 1 mM DMPO significantly decreased ROS production during both ischemia and reperfusion. There has been much evidence that ROS contribute to many pathological processes associated with cardiac ischemia/reperfusion injury (Zweier and Talukder, 2006). The mitochondrial electron transport chain (METC) (complexes I and III in particular) is an important source of ROS production during ischemia and pathological conditions of postischemic injury (Zweier et al., 1987; Ambrosio et al., 1993; Paradies et al., 2001). Previous studies also have shown that under cardiac ischemia/reperfusion, ROS have been a major cause of mitochondria dysfunction via inactivation of the METC complexes (Paradies et al., 2001; Chen et al., 2007). Similar studies on Langendorff-perfused rat hearts showed that perfusion for 3 to 10 min with anoxic

TABLE 1
Hemodynamic and coronary flow data

	DMPO Concentrations											
	0 mM				0.5 mM				1 mM			
	Basal	End Reflow	Basal	End Reflow	Basal	End Reflow	Basal	End Reflow	Basal	End Reflow	Basal	End Reflow
LV systolic pressure (mm Hg)	171.5 ± 11.0	141.1 ± 8.4	142.5 ± 12.5	136.3 ± 2.5	162.1 ± 6.5	145.0 ± 4.3	159.2 ± 18.0	142.8 ± 1.8	156.3 ± 6.9	132.8 ± 5.0	156.3 ± 6.9	132.8 ± 5.0
LV EDP (mm Hg)	9.4 ± 0.4	117.8 ± 6.1	8.7 ± 2.1	92.8 ± 8.3	8.9 ± 0.7	79.1 ± 4.8	9.3 ± 1.2	84.5 ± 7.3	8.8 ± 0.9	87.7 ± 3.2	8.8 ± 0.9	87.7 ± 3.2
LVDP (mm Hg)	162.1 ± 11.1	23.3 ± 5.2	133.8 ± 14.4	43.5 ± 6.0	153.2 ± 6.4	65.9 ± 7.8	150.0 ± 18.8	58.3 ± 6.3	147.6 ± 7.5	45.2 ± 5.7	147.6 ± 7.5	45.2 ± 5.7
HR (beats/min)	269.3 ± 6.4	255.3 ± 37.7	297.8 ± 5.5	314.0 ± 13.5	290.6 ± 6.9	316.0 ± 6.7	297.3 ± 12.2	294.0 ± 23.4	290.0 ± 9.4	280.0 ± 13.0	290.0 ± 9.4	280.0 ± 13.0
RPP (10 ³ mm Hg/min)	43.6 ± 2.6	50.5 ± 0.8	40.0 ± 4.9	13.7 ± 2.1	44.8 ± 2.8	20.6 ± 2.1	44.1 ± 4.6	17.2 ± 2.4	42.6 ± 1.8	12.9 ± 1.9	42.6 ± 1.8	12.9 ± 1.9
CF (ml/min)	17.1 ± 0.9	9.3 ± 0.9	18.0 ± 2.4	11.8 ± 3.3	15.6 ± 0.7	11.0 ± 1.1	16.3 ± 0.8	11.4 ± 1.2	17.8 ± 0.7	12.9 ± 2.0	17.8 ± 0.7	12.9 ± 2.0

^a Data are mean ± S.E.M.

buffer before the onset of 1-h global ischemia results in significant protection of complex I against ischemia-reperfusion-induced damage (Veitch et al., 1992). In vitro studies, however, showed that inhibition of complex I promotes radical formation and subsequently can inactivate Krebs cycle enzymes such as α -ketoglutarate dehydrogenase and aconitase (Sadek et al., 2002). Mitochondrial respiration also has been shown to modulate eNOS activity, in which increased NO metabolites were observed under hyperoxic and sheared-stress conditions (Jones et al., 2008). Antioxidants targeting mitochondria such as MitoQ decreased heart dysfunction and mitochondrial damage from ischemia-reperfusion-induced injury (Adlam et al., 2005). Therefore, DMPO presumably can scavenge the oxygen free radicals overproduced by the mitochondrial electron transport chain during ischemia/reperfusion, exerting a protective effect on mitochondrial function (Fig. 8). In the presence of DMPO, the decreased levels of ROS along with the preservation of METC complexes activity imply that ROS play a major role in the initiation of oxidative stress during ischemia/reperfusion. The role of O_2^- in initiation of mitochondrial dysfunction also has been further demonstrated by electron paramagnetic resonance spin-trapping studies using 5-diethoxylphosphoryl-5-methyl-1-pyrroline *N*-oxide using isolated complex II/III and superoxide dismutase (Chen et al., 2006, 2007).

Immunoblotting analysis of myocardial tissue homogenates with antibodies (anti-51 kDa) against the FMN-binding subunit of complex I, antibodies (anti-70 kDa) against the FAD-binding subunit of complex II, and antibodies (anti-CoXI and anti-CoXVb) against the subunits I and Vb of complex IV, indicates that marked up-regulation of mitochondrial protein expression occurs in the postischemic heart pretreated with DMPO (Fig. 9, A–D). Subunit I of complex IV is encoded by the mitochondrial DNA, and others are encoded by the nuclear DNA. Therefore, pretreatment of DMPO synchronized up-regulation of mitochondrial proteins encoded by both mitochondrial and nuclear DNA. It is likely that DMPO may exert cardioprotection, in part through either inhibiting ischemic degradation or increasing biosynthesis of METC proteins that are required for bioenergetic function. This may occur through the scavenging of oxygen free radicals by DMPO, and this oxygen radical scavenging could enhance NO levels. NO has been reported to trigger mitochondrial biogenesis and METC biosynthesis (McLeod et al., 2005). eNOS-derived NO has been shown to contribute to mitochondrial biogenesis under the physiological conditions of thermogenesis (Nisoli et al., 2003). Thus, NO salvaged either by the DMPO-induced scavenging of O_2^- or alternatively decomposed from DMPO itself under ischemic conditions directly or indirectly exerts cardioprotection through increasing mitochondrial biogenesis. Furthermore, immunoblotting analysis with the antibodies (anti-CoXVb) against complex IV also revealed that the precursor of complex IV subunit Vb in the cytosol modestly accumulated in the postischemic heart (data not shown). However, subunit Vb accumulation in the cytosol was decreased in the postischemic heart pretreated with DMPO, leading to up-regulation of matured subunit Vb in the mitochondria (Fig. 9D). This result implicates that DMPO may also exert cardioprotection through enhancing the efficiency of protein transport from cytosol to mitochondria and increasing the accuracy of mitochondrial protein

processing and assembly in mitochondria, which in turn preserves mitochondrial function in the postischemic heart.

The nitron spin trap DMPO exerted strong cardioprotective effects when administered in low millimolar concentrations immediately before the onset of ischemia with marked enhancement in the recovery of cardiac contractile function and decreased infarct size. It was shown to decrease ROS levels upon reperfusion and greatly enhance the preservation and recovery of the function of the mitochondrial electron transport chain. Future studies will be needed to further characterize the precise molecular mechanisms of how DMPO exhibits myocardial and mitochondrial protection. The development and use of nitron spin traps targeted for specific cellular compartments may enhance their efficacy and minimize toxicity. Recently, it has been shown that a broad range of drugs including nitrones can be conjugated to a mitochondria-targeted lipophilic triphenylphosphonium cation (Hardy et al., 2007). Such intracellularly targeted nitrones derived from or similar to DMPO may offer even more robust pharmacological protection at lower drug levels.

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