ROS scavenging before 27°C ischemia protects hearts and reduces mitochondrial ROS, Ca²⁺ overload, and changes in redox state

Amadou K. S. Camara, †Mohammed Aldakkak, † James S. Heisner, †Samhita S. Rhodes, † Matthias L. Riess, † JiangZhong An, † André Heinen, and David F. Stowe1,2,3,4,5
Anesthesiology Research Laboratories, Departments of 1Anesthesiology and Physiology, 2Cardiovascular Research Center, 3The Medical College of Wisconsin, Milwaukee; Veterans Affairs Medical Center Research Service, 4Milwaukee; and Department of Biomedical Engineering, 5Marquette University, Milwaukee, Wisconsin

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Camara AK, Aldakkak M, Heisner JS, Rhodes SS, Riess ML, An JZ, Heinen A, Stowe DF. ROS scavenging before 27°C ischemia protects hearts and reduces mitochondrial ROS, Ca²⁺ overload, and changes in redox state. Am J Physiol Cell Physiol 292: C2021–C2031, 2007. First published February 7, 2007; doi:10.1152/ajpcell.00231.2006.—We have shown that cold perfusion of hearts generates reactive oxygen and nitrogen species (ROS/RNS). In this study, we determined 1) whether ROS scavenging only during cold perfusion before global ischemia improves mitochondrial and myocardial function, and 2) which ROS leads to compromised cardiac function during ischemia and reperfusion (I/R) injury. Using fluorescence spectrophotometry, we monitored redox balance (NADH and FAD), O₂⁻ levels and mitochondrial Ca²⁺ (mCa²⁺) at the left ventricular wall in 120 guinea pig isolated hearts divided into control (Con), MnTBAP (a superoxide dismutase 2 mimetic), MnTBAP (M) + catalase (C) + glutathione (G) (MCG), C+G (CG), and N⁵-nitro-L-arginine methyl ester (L-NAME; a nitric oxide synthase inhibitor) groups. After an initial period of warm perfusion, hearts were treated with drugs before and after at 27°C. Drugs were washed out before 2 h at 27°C ischemia and 2 h at 37°C reperfusion. We found that on reperfusion the MnTBAP group had the worst functional recovery and largest infarction with the highest mCa²⁺, most oxidized redox state and increased ROS levels. The MCG group had the best recovery, the smallest infarction, the lowest ROS level, the lowest mCa²⁺, and the most reduced redox state. CG and l-NAME groups gave results intermediate to those of the MnTBAP and MCG groups. Our results indicate that the scavenging of cold-induced O₂⁻ species to less toxic downstream products additionally protects during and after cold I/R by preserving mitochondrial function. Because MnTBAP treatment showed the worst functional return along with poor preservation of mitochondrial bioenergetics, accumulation of H₂O₂ and/or hydroxyl radicals during cold perfusion may be involved in compromised function during subsequent cold I/R injury.

hypothermic ischemia; mitochondrial Ca²⁺; reactive oxygen species

HYPOTHERMIA of the arrested, ischemic heart improves its function on reperfusion compared with the normothermic arrested ischemic heart. The strategy behind hypothermic protection against ischemia, i.e., better tissue perfusion, and improved metabolic and mechanical function on reperfusion, is the reduced mitochondrial respiration and oxidative phosphorylation during ischemia that results in better mitochondrial respiration and regeneration of ATP on reperfusion. For example, we demonstrated that NADH, mCa²⁺, and reactive O₂ species (ROS) levels were less altered during and after 30 min of ischemia at 17°C vs. 37°C (39). We reported that the more severe the hypothermia, the later is the onset of deleterious changes in mitochondrial function (1, 2, 16, 22, 31).

Although hypothermia is very protective against ischemia, hypothermic perfusion, e.g., before subsequent cardiac ischemia, may cause injury due to altered cellular ion homeostasis resulting from impaired membrane ion pumps and exchangers and/or to reduced activity of enzymes responsible for mitochondrial respiration, scavenging of ROS, and contractile activity. A well-known effect of hypothermia is hypercontracture with elevated cytosolic [Ca²⁺] (42). Another is a decreasing temperature-dependent effect to increase superoxide (O₂⁻) and peroxynitrite (ONOO⁻) levels during cold cardiac perfusion (14). Giving N⁵-nitro-arginine methyl ester (l-NAME) to block NO production protects hearts against long-term cold ischemic injury (41). Superoxide anions, hydroxyl radicals (OH), and H₂O₂ are known to contribute to both cellular protection and cellular injury during ischemia-reperfusion (I/R), but the effects of O₂⁻ and its reactants or products generated during cold perfusion on mitochondrial function during subsequent cold I/R are not known.

The major aim of this study was to test whether scavenging of ROS during cold perfusion affords protection against subsequent I/R injury, or is anti-protective because of a preconditioning effect of enhanced ROS before cold ischemia. A second primary aim was to determine whether the O₂⁻ radical per se, or downstream reactants, are responsible for protective or anti-protective effects. It is well known that the O₂⁻ radical is either dismutated to H₂O₂, or in the presence of NO forms ONOO⁻, a toxic reactant. To address these aims, we perfused the NO synthase inhibitor l-NAME, the mitochondrial superoxide dismutase (SOD2) mimetic Mn(III) tetrakis(4-benzoic acid)porphyrin (MnTBAP), catalase + glutathione (CG), or MnTBAP + CG (MCG) only during a period of cold perfusion before 2 h cold ischemia.

MATERIALS AND METHODS

Langendorff heart preparation. The investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and was approved by the animal studies committee, Medical College of Wisconsin (Milwaukee, WI). Our methods have been described previously (14, 16, 26, 38). Thirty milligrams of ketamine and 1,000 units of heparin were injected intraperitoneally into 120 guinea pigs of

Address for reprint requests and other correspondence: D. F. Stowe, Medical College of Wisconsin, M4280, 8701 Watertown Plank Rd., Milwaukee, WI 53226 (e-mail: dfstowe@mcw.edu).

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either sex (250–350 g wt). Animals were decapitated when unresponsive to noxious stimulation. After thoracotomy, the aorta was cannulated distal to the aortic valve, and the heart was immediately perfused retrograde with 4°C cold oxygenated Krebs-Ringer (KR) solution of the following composition (in mM): 138 Na+, 4.5 K+, 1.2 Mg2+, 2.5 Ca2+, 134 Cl−, 14.5 HCO3−, 1.2 H2PO4−, 11.5 glucose, 2 pyruvate, 16 mannnitol, 0.1 probenecid, 0.05 EDTA, and 5 U/l insulin. The inferior and superior venae cavae were ligated, and the heart was rapidly excised. After cannulation of the pulmonary artery to collect the coronary effluent, the heart was suspended in a water bath containing KR and perfused at 55 mmHg at 37°C. The perfuse was filtered (5 μm pore size) in-line and equilibrated with ~97% O2 and ~3% CO2 to maintain a constant pH of 7.4 ± 0.01 at 37°C.

Left ventricular pressure (LVP) was measured isovolumetrically with a saline-filled latex balloon inserted into the left ventricle through the left atrium. At the beginning of each experiment, the balloon volume was adjusted to achieve a diastolic LVP close to 0 mmHg, so that any subsequent increase in diastolic LVP reflected ventricular diastolic contracture. Spontaneous heart rate was monitored with bipolar electrodes placed in the right atrial and ventricular walls. Coronary flow was measured by an ultrasonic flowmeter (model T106X; Transonic Systems, Ithaca, NY) placed directly into the aortic inflow line. Coronary arterial inflow and coronary venous Na+/K+ was measured by an O2 Clark type electrode (model 203B; Instech, Plymouth Castle Hill, Australia) and recorded at 200 Hz (Chart & Scope, version 3.6.3; ADInstruments) on Power Macintosh Computer G5 (Apple, Cupertino, CA) for analysis using MATLAB (The MathWorks, Natick, MA) and Excel (Microsoft, Redmond, WA) software.

Online measurement of mCa2+ concentration. mCa2+ was measured by a modification of the indo 1-AM loading and ratiometric analysis technique (40). mCa2+ was measured at 405 nm and 460 nm at λem 405 nm was determined for each heart. Indo-1 AM (6 μM; Molecular Probes, Eugene, OR) was prepared and perfused for 30 min; this increased each F signal approximately tenfold. After washout of residual interstitial indo 1-AM for 20 min, cytosolic Ca2+ was quenched from cytosolic bound indo 1 by perfusing with 100 μM MnCl2 for 15 min. The remaining F originates predominantly from the mitochondrial matrix. Perfusion of MnCl2 throughout the experiment or for only 15 min results in the same quenching of the phasic cytosolic Ca2+ signal. This suggests that the MnCl2 does not enter the mitochondrial compartment over time to affect mitochondrial (Ca2+). Also, cardiac function is not altered during or after MnCl2 perfusion. After washout of MnCl2, baseline recordings were taken and the experiment begun with hypothermic perfusion ± treatments.

Although mitochondrial Ca2+ transients have been described in stimulated myocytes (45), F405/F460 measured with indo 1 in isolated hearts lacks the phasic character of cytosolic Ca2+ during the contractile cycle (40, 48). While both F405 and F460 declined over time, the F405/F460 ratio remained stable during time control studies. Since background auto F values obtained before indo 1-AM loading represent measures of NADH (15), all F signals were corrected for the corresponding temperature- and I/R-induced changes in auto F (NADH and FAD) obtained in previous experiments (1, 2). The indo 1 transient is nonlinearly proportional to m(Ca2+), which was calculated according to the following equation (39, 40, 48): m(Ca2+) = S460/Kd × [(Rm − Rmin)/(Rmax − Rm)], where S460 is the ratio of F intensities at 460 nm and zero saturated Ca2+, Kd is the dissociation constant of indo 1, Rm is the actually measured F405/F460 ratio, Rmin is the F405/F460 ratio at zero Ca2+, and Rmax is the F405/F460 ratio at saturated Ca2+. In previous experiments, S460 was measured as 2.29, Rmin as 0.57, and Rmax as 6.22 at the chosen photomultiplier settings (39, 40). Kd was calculated as 249 nm at 37°C (48) and was corrected for changes in temperature (42). All variables were averaged over the sampling period of 2 s.

Online assessment of ROS. We used the oxidation of the fluorescent dye dihydroethidium (DHE; Molecular Probes) to measure ROS formation, most likely O2− species (6, 14, 26, 39, 47), which converts DHE to a labile ethidium precursor that produces a red shift in F (51). DHE is very selective for O2− because H2O2 does not change the fluorescence while O2− generation does. In a preliminary study, we showed that 2′,7′-dichlorofluorescein diacetate (DCFDA) as the fluorescent probe, H2O2 dose-dependently (40–320 μM) increased DCFDA but not DHE fluorescence (Camara AKS, Chen Q, Stowe DF, unpublished observations). The relative selective sensitivity of the DHE and DCFDA probes for O2− and H2O2, respectively, has also been demonstrated by others (47); it was found that O2− generated by xanthine oxidase caused an increase in the DHE F signal, whereas H2O2 increased the DCFDA F signal but not the DHE F signal (47).

DHE and ethidium remain within cells with minimal leakage. Heart tissue was excised at λem 540 nm and F obtained at λem 590 nm. In preliminary experiments, background auto F was determined for each experimental group at these λs. All subsequent DHE F recordings were adjusted for these minimal changes in background auto F with changes in temperature and I/R over time. DHE (10 μM) was prepared and perfused for 20 min; this was followed by washout of all residual dye for 20 min (14, 26, 39). Tissue DHE loading increased F from 0.31 ± 0.02 arbitrary fluorescence units (afu) before loading to 2.20 ± 0.16 afu after washout, the initial baseline values.

Analog signals were digitized (PowerLab/16 SP; ADInstruments, Castle Hill, Australia) and recorded at 200 Hz (Chart & Scope, version 3.6.3; ADInstruments) on Power Macintosh Computer G5 (Apple, Cupertino, CA) for analysis using MATLAB (The MathWorks, Natick, MA) and Excel (Microsoft, Redmond, WA) software.

Protocol. The protocol was designed to assess the role of ROS formed during hypothermic perfusion on protection of mitochondrial bioenergetics and myocardial function during hypothermic ischemia and warm reperfusion. Experimental data were collected for 225 min...
beginning after a 30-min equilibration period. Hearts were randomly divided into five experimental groups: control (Con), MnTBAP (10 μM), catalase (50 U/ml) + glutathione (500 μM) (CG), MnTBAP + CG (MCG), and t-NAME (100 μM)(14). In addition to functional data, each treatment group was assessed for changes in NADH and FAD, [Ca^{2+}], or \(O_2^{-}\). For the 5 groups, there were 24 hearts/group or 8 hearts for each of the 3 fluorescence measurements. After residual dye was washed out, new baseline values were reestablished, the hearts were perfused with no drug (Con) or with MnTBAP, MCG, CG, or t-NAME for 10 min at 37°C, for 10 min during cooling to 27°C and for another 10 min during 27°C perfusion. Each treatment was discontinued during the last 5 min before the onset of global cold ischemia. After 2 h of 27°C ischemia, each heart was reperfused with KR alone to 37°C over 5 min and reperfusion was continued for 2 h. Washout of each drug before the onset of ischemia ensured that the drug was present only during the cold perfusion period and was not present during ischemia or reperfusion.

After 2-h reperfusion after ischemia, hearts were removed and the ventricles were cut into thin transverse sections. The sections were stained with 0.1% 2,3,5-triphenyltetrazolium chloride (TTC). TTC stains the noninfarcted myocardium a brick red color, which indicates the presence of a formazan precipitate that results from the reduction of TTC by dehydrogenase enzymes present in viable tissue. After storage overnight in 10% formaldehyde, infarcted and noninfarcted tissues of whole hearts were carefully separated and weighed. Infarct size was expressed as a percentage of ventricular weight.

Statistical analysis. All data, expressed as means ± SE, are presented in a continuous timeline fashion or as discrete time intervals as shown in the tables. Among-group data were compared by ANOVA to determine significance (Super ANOVA 1.11 software for Macintosh; Abacus Concepts, Berkeley, CA) at selected time points (0, 30, 150, 175, 200, 225 min). Hearts were perfused for another 60 min (225 to 285 min) so that infarct size could be determined after 120 min of reperfusion. There were no significant differences in functional data between 60 and 120 min of reperfusion so the 285 min data are not displayed. If F values were significant (\(P < 0.05\)), post hoc comparisons of means tests (Student-Newman-Keuls) were used to compare the five groups within each subset. Differences among means were considered statistically significant when \(P < 0.05\) (two tailed).

RESULTS

The functional variables of heart rate, diastolic LVP, rate pressure product, dLVP/dt_max (contractility), and dLVP/dt_min (relaxation), in Con-, MnTBAP-, CG-, MCG-, and t-NAME-treated hearts at baseline, during drug treatment at 37°C and 27°C, and on warm reperfusion at 2 min (protocol time 167 min), 20 min (protocol time 185 min), and 60 min (protocol time 225 min) were comparable in all groups under baseline conditions (Table 1). No drug treatment had a significant effect on any of these functional variables at 37°C. As expected, spontaneous heart rate was significantly reduced by cold perfusion in each group. At 20 and 60-min reperfusion, heart rate returned to baseline values in all groups, except for the MCG group where it was slightly higher than baseline.

Diastolic LVP did not change significantly during drug treatment at 37°C in CG, MCG, and t-NAME groups but became elevated at 27°C before ischemia in the Con and MnTBAP groups. Diastolic LVP increased markedly in all groups on warm reperfusion, with the greatest and least increases observed in the MnTBAP and MCG groups, respectively. The LV pressure product was significantly reduced during cold and warm reperfusion in all groups but was higher in the MCG group than in the Con group. The rates of contractility (dLVP/dt_max) and relaxation (dLVP/dt_min) were significantly depressed in all groups on reperfusion; however, the MnTBAP group had the worst return of contractility and relaxation, whereas the MCG group exhibited the best return of these indices.

Cardiac efficiency (O2 consumption per unit of developed LVP per beat) was not altered by drug treatment or by cooling to 27°C but was reduced below baseline at 60 min reperfusion in the MnTBAP group (Table 2). %O2 extraction (a measure of energetic inefficiency) was not altered by drug treatment at 37°C but tended to decline during cold treatment in all groups. At 2-min reperfusion, %O2 extraction was significantly elevated in all groups, except in the CG and MCG groups; at 20 and 60 min of reperfusion, %O2 extraction remained elevated only in the MnTBAP and t-NAME groups and was near baseline levels in the MCG group.

Developed LVP (systolic-diastolic pressure) was not different among the groups before or during treatment (Fig. 1A). On initial reperfusion, developed LVP (mmHg) was significantly depressed in all groups compared with baseline. At 60-min reperfusion (protocol time 225 min) Mg-treated hearts had the best recovery of developed LVP, followed by the t-NAME-treated group, whereas the Con, CG, and MnTBAP hearts had the worst recovery. Baseline coronary flow was not different among the groups; it was significantly reduced during cold perfusion and on warm reperfusion in all groups (Fig. 1B). At 60-min reperfusion, coronary flow was highest in the MCG and t-NAME groups and was lower in the Con, CG and MnTBAP groups.

To assess the role of cardiac mitochondria in the overall cardioprotective effects on contractile function and metabolism, mitochondrial redox state (reduced NADH and oxidized FAD), ROS levels, and [Ca^{2+}] were monitored in different subsets of hearts undergoing the same treatments at the same time as functional data were obtained. Time control (no ischemia) experiments were conducted for each fluorescent probe, and any interference of the signal due to a drug treatment (14) was subtracted. Baseline values for NADH and FAD autofluorescence were similar among the groups (Fig. 2, A and B). NADH was not altered significantly by any treatment before ischemia. At the onset of ischemia, NADH increased significantly in all groups but began to decline toward baseline at ~60 min into ischemia, except in the MCG group, in which the signal remained elevated above baseline. On initial reperfusion, NADH fell rapidly in all groups. At 60-min reperfusion, NADH was highest in the MCG group (57.79 ± 0.14 to 51.8 ± 2.01 au) and lowest in the MnTBAP group (57.78 ± 0.13 to 38.11 ± 1.82 au); it was intermediate in the Con, CG, and t-NAME groups. FAD did not change significantly during treatment or cold perfusion but declined significantly in all groups during ischemia. On reperfusion, FAD approached baseline values in the MCG group but was higher than baseline in all other groups.

Baseline \(O_2^-\) levels were similar in all groups (Fig. 3A). Treatment with MnTBAP and MCG each significantly decreased the level of \(O_2^-\), whereas CG and t-NAME treatment had no effect on \(O_2^-\) levels. During cold perfusion, \(O_2^-\) levels increased in Con, CG, and t-NAME groups but less so in the MnTBAP and MCG groups. \(O_2^-\) levels increased during the first 60 min of cold ischemia in all but the MnTBAP...
and MCG groups, and in all groups by 120 min of ischemia, but the rise was less in the MCG group. On reperfusion, $O_2^-$ levels decreased in all groups, with the greatest decline in the MCG group. Baseline $m[Ca^{2+}]$ was similar among the groups, except in the MnTBAP group in which it was higher (159.0 ± 12.7 baseline vs. 235.8 ± 30.3 nM) (Fig. 3B). During 27°C ischemia, $m[Ca^{2+}]$ increased gradually in all groups with the most significant increase in the MnTBAP group. On reperfusion, $m[Ca^{2+}]$ declined in each group but remained above baseline values; at 60 min reperfusion, the MCG group had the lowest $m[Ca^{2+}]$ (269 ± 31 nM), whereas the MnTBAP group had the highest $m[Ca^{2+}]$ (426 ± 24 nM). t-NAMe and CG

Table 2. Changes in cardiac efficiency and %O2 extraction before and during drug treatment, during 27°C perfusion before ischemia, and at selected intervals during warm reperfusion

<table>
<thead>
<tr>
<th>Cardiac efficiency, units</th>
<th>Baseline</th>
<th>37°C Treatment</th>
<th>27°C Treatment</th>
<th>2 min</th>
<th>20 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.7 ± 3.0</td>
<td>24.8 ± 3.2</td>
<td>19.4 ± 2.7</td>
<td>5.1 ± 1.7*</td>
<td>12.0 ± 2.2*</td>
<td>16.4 ± 2.9</td>
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<tr>
<td>MnTBAP</td>
<td>23.4 ± 3.8</td>
<td>20.8 ± 3.2</td>
<td>17.4 ± 3.1</td>
<td>3.8 ± 1.3*</td>
<td>7.4 ± 2.2†</td>
<td>15.3 ± 2.0*</td>
</tr>
<tr>
<td>CG</td>
<td>23.5 ± 2.5</td>
<td>23.6 ± 2.7</td>
<td>21.9 ± 2.4</td>
<td>9.1 ± 1.9*</td>
<td>17.4 ± 3.3‡</td>
<td>16.9 ± 2.0</td>
</tr>
<tr>
<td>MCG</td>
<td>23.2 ± 2.7</td>
<td>22.1 ± 1.7</td>
<td>19.2 ± 1.7</td>
<td>8.9 ± 2.2*</td>
<td>11.1 ± 2.2*</td>
<td>19.3 ± 2.0</td>
</tr>
<tr>
<td>t-NAMe</td>
<td>22.3 ± 4.7</td>
<td>23.3 ± 2.1</td>
<td>20.2 ± 2.4</td>
<td>7.7 ± 1.4*</td>
<td>10.3 ± 2.3*</td>
<td>16.8 ± 2.3</td>
</tr>
<tr>
<td>%O2 extraction</td>
<td>75.8 ± 4.0</td>
<td>76.1 ± 4.1</td>
<td>69.0 ± 2.9</td>
<td>85.3 ± 2.2*</td>
<td>79.3 ± 3.2</td>
<td>80.4 ± 3.7</td>
</tr>
<tr>
<td>Control</td>
<td>74.8 ± 6.0</td>
<td>76.3 ± 6.2</td>
<td>63.7 ± 6.5</td>
<td>89.5 ± 2.7*</td>
<td>87.1 ± 3.3*</td>
<td>92.5 ± 3.0*</td>
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<tr>
<td>MnTBAP</td>
<td>76.8 ± 2.0</td>
<td>71.1 ± 2.7</td>
<td>60.3 ± 6.6</td>
<td>81.1 ± 2.3</td>
<td>78.1 ± 2.4</td>
<td>80.5 ± 1.9</td>
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<tr>
<td>CG</td>
<td>75.7 ± 2.2</td>
<td>77.8 ± 2.8</td>
<td>61.3 ± 4.8</td>
<td>82.7 ± 3.4</td>
<td>79.0 ± 5.0</td>
<td>76.0 ± 3.13</td>
</tr>
<tr>
<td>t-NAMe</td>
<td>75.9 ± 3.9</td>
<td>76.5 ± 3.3</td>
<td>67.1 ± 5.3</td>
<td>86.4 ± 2.0*</td>
<td>85.0 ± 1.2*</td>
<td>86.3 ± 1.7*</td>
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</table>

Values are means ± SE; $n = 16$ hearts per experiment. Efficiency is measured as mmHg·beat$^{-1}$·g$^{-1}$·μl O2. *P < 0.05 vs. baseline values; †P < 0.05, each treatment vs. the control group; ‡P < 0.05, other treatments vs. the MnTBAP group.
Fig. 1. Time course of changes in systolic-diastolic left ventricular pressure (developed LVP) (A) and coronary flow (B) for each group. On warm reperfusion, developed LVP remained lower than baseline in all groups with the Mn(III) tetrakis(4-benzoic acid)porphyrin (MnTBAP) group showing the least recovery and the MnTBAP + catalase + glutathione (MCG) group the best recovery. *P < 0.05 values vs. baseline within each group; ‡P < 0.05, each treatment vs. the control (Con) group, ¶P < 0.05, other treatments vs. the MnTBAP group.
Fig. 2. Time course of changes in reduced NADH (A) autofluorescence and oxidized FAD (B) autofluorescence in each group. NADH increased slightly, but nonsignificantly, during hypothermic perfusion before ischemia in all groups, except for the MnTBAP and CG groups. NADH increased during the first hour of ischemia in each group; on warm reperfusion, NADH was lower than baseline in each group but the MCG group had the best preservation of NADH, whereas the MnTBAP group had the least preservation of NADH. FAD decreased in each group during ischemia; on warm reperfusion the MCG group displayed the least oxidized FAD state. *P < 0.05 values vs. baseline within each group; ‡P < 0.05, all treatments vs. Con group, ¶P < 0.05, other treatments vs. MnTBAP.
Fig. 3. Time course of changes in superoxide (O$_2^-$) (A) and mitochondrial Ca$^{2+}$ (m[Ca$^{2+}$]) (B) levels assessed with dihydroethidium (DHE) fluorescence intensity and indo 1-AM, respectively. Cold perfusion and cold ischemia increased O$_2^-$ in each group, but less so in the MCG group. On rewarming and reperfusion, O$_2^-$ was reduced in all groups but more so in the MCG group (A). During ischemia, m[Ca$^{2+}$] continued to increase in all groups, with the largest increase in the MnTBAP group. On rewarming and reperfusion, m[Ca$^{2+}$] decreased toward baseline values more rapidly in the MCG group (B). *P < 0.05 values vs. baseline within each group; ‡P < 0.05, treatment group vs. the Con group; ¶P < 0.05, other treatments vs. the MnTBAP group.
treatment did not significantly reduce m[Ca^{2+}] compared with the no treatment Con group.

Infarcted ventricular tissue as a percentage of total heart weight was greatest in the MnTBAP (60 ± 3%) group and least in the MCG (39 ± 2%) group (Fig. 4). There was no significant difference in infarct size between the Con, CG, and L-NAME-treated hearts.

**DISCUSSION**

This study shows that the ROS scavenger cocktail, MCG, perfused in isolated guinea pig hearts during a 27°C period before 2-h cold ischemia, protected against mechanical and metabolic dysfunction better than the SOD mimetic MnTBAP alone. Cardioprotective effects of MCG were characterized by better normalization of the mitochondrial redox state (NADH and FAD), decreased O$_2^*$ levels and m[Ca^{2+}] during and after ischemia, higher contractile and relaxant indices on reperfusion, and a reduction in infarcted tissue. Administration of L-NAME or CG during cold perfusion before 2-h cold ischemia moderately improved cardiac function but without significantly improving mitochondrial redox state or decreasing O$_2^*$ levels and m[Ca^{2+}]. These drugs had no significant effects on baseline functions, so the mechanisms of action are probably via ROS-mediated or scavenging effects. Our results suggest that the increased presence of downstream products of O$_2^*$, such as H$_2$O$_2$ or OH$^*$ during perfusion at 27°C, contributes to enhanced mitochondrial damage and poor cardiac function after 2 h 27°C ischemia.

**Hypothermia and cardiac protection.** Hypothermia is key to successful preservation as it decreases the rate at which intracellular enzymes degrade essential cellular components for organ viability during ischemia (5). Mild hypothermia reduces the cellular demand for ATP and so protects electron transport chain (ETC) function and preserves ATP synthesis. This mild uncoupling of oxidative phosphorylation during cold ischemia leads to attenuated mitochondrial dysfunction during warm reperfusion (4, 44). Preservation of antioxidant capacity and reduction of free radicals during I/R may account for the protection afforded by hypothermia (21, 23, 29).

It is well known that the colder the heart during ischemia, the longer ischemia can be tolerated with minimal tissue damage (16, 43). However, it is now known that hypothermia, per se, can have a deleterious effect on function with rewarming. This has been attributed in part to hypothermia-induced disturbances in cellular ion homeostasis and ROS generation and scavenging, which may cause I/R injury in transplant organs (52). Rauen et al. (36) showed that cultured rat liver endothelial cells incubated at 4°C showed marked lipid peroxidation, which was abated when the cells were preincubated with antioxidants and free radical scavenging enzymes (24).

Cold perfusion of isolated hearts, i.e., without concomitant ischemia, enhanced ROS levels in a temperature-dependent manner, and both an increased ROS production and reduced ROS scavenging are likely factors (14). In a related study (39), we also showed that cardiac perfusion at 17°C before 17°C ischemia not only increased ROS levels but also increased NADH and m[Ca^{2+}]; subsequent exposure to 30 min of ischemia at 17°C protected better than 30 min ischemia at 37°C. Although protection against tissue damage by hypothermia during ischemia is to be expected, the increases in ROS, NADH, and m[Ca^{2+}] induced by hypothermic perfusion before ischemia indicated that mitochondrial function might be compromised so that a fully protective effect by hypothermia against ischemia may not be forthcoming.

**Hypothermia, ROS, and free radical accumulation.** It is interesting that ROS levels rose as the mitochondria became more oxidized (lower NADH, higher FAD) during later ischemia. Enhanced ROS generation is often observed in isolated mitochondria when there is a high proton motive force (high NADH/NAD$^+$ redox potential) due to slowed stepwise electron transfer to O$_2$ at cytochrome oxidase (complex IV), which allows for electron leak at complexes I and III. During early ischemia, decreased P$_o$ slows complex IV activity and cytochrome c$_1$ becomes reduced. This would lead to electron leak from upstream sites, such as complex III (17). Although O$_2$ is required to form ROS and P$_o$ is likely very low during late ischemia, it appears there must be enough available O$_2$ to become reduced to O$_2^*$ rather than to H$_2$O. How this occurs when electron flux is slower is not clear, but it could be due to an even greater inhibition of complex IV activity so that a greater electron leak proportional to electron flow leads to a greater proportion of O$_2^*$ than H$_2$O produced.

**Hypothermia, ROS, and cardiac protection.** Excess free radicals are implicated in cellular injury and apoptosis, either as effectors or as by-products (46). Mitochondrial proteins and cell membrane proteins are particularly susceptible to oxidative stress and thus account for myocardial structural and functional alterations (9, 10, 12). Failure of electrons to progress through the ETC to cytochrome $c$ oxidase (complex IV) can lead to attack by O$_2^*$ to form O$_2^*$ and its downstream reactants. It is possible that hypothermic stress alone can create a metabolic imbalance by impairing endogenous free radical scavenging mechanisms, or by generating O$_2^*$ (52). Dismutation of O$_2^*$, spontaneously or catalyzed by SOD, produces H$_2$O$_2$, which can diffuse readily through membranes or be further reduced to OH or H$_2$O. Manganese-containing SOD (MnSOD2), located in the mitochondrial matrix, scavenges O$_2^*$ by converting it to H$_2$O$_2$, which in turn can be detoxified to H$_2$O by glutathione peroxidase in the presence of glutathione, or by intra- and extramitochondrial catalase (52). During increased mitochondrial O$_2^*$ generation, or when the antioxidant systems are unable to cope with the prooxidant redox state, H$_2$O$_2$ may accumulate and react with mitochondrial Fe$^{2+}$ to form the...
highly reactive OH\(^{-}\) radical (Fenton reaction) and lead to a condition of exacerbated oxidative stress (11, 12).

It is likely that simultaneous scavenging of O\(_2\)\(^{-}\) and its H\(_2\)O\(_2\) product to form H\(_2\)O could prevent OH\(^{-}\) formation and subsequent injury from oxidative stress. In this study, we used the highly permeable MnSOD mimetic, MnTBAP alone, or with CG to scavenge ROS during cold perfusion. Hearts treated with MCG were better protected than hearts treated with MnTBAP alone (see RESULTS). This indicates the possibility that giving MnTBAP alone during cold perfusion could result in an even greater formation of H\(_2\)O\(_2\) and OH\(^{-}\), which, upon subsequent cold I/R, could contribute to reduced protection by hypothermia. This idea is consistent with a report by Keith (25) that SOD plus catalase added to a cardiac preservation solution provided greater protection than either SOD or catalase alone.

We have shown recently in pilot studies (Aldakkak M, Stowe DF, Camara AKS, unpublished observations) that O\(_2\)\(^{-}\) generated by mendainone, a quinone that undergoes a one-electron reduction to form the semiquinone radical and subsequently the O\(_2\)\(^{-}\) radical, generates a higher signal with the probe DCFDA in the presence of MnTBAP than in its absence. In addition, using the DCFDA probe, we found that cold-induced H\(_2\)O\(_2\) production increased the fluorescence signal generated by MnTBAP and pegSOD but decreased the signal generated by MCG. These experiments support our contention that MnTBAP scavenges O\(_2\)\(^{-}\) to downstream products, possibly H\(_2\)O\(_2\) or OH\(^{-}\), which contributes to the compromise of function during cold storage. Consistent with this notion, Ku et al. (27, 28) reported that formation of OH from O\(_2\)\(^{-}\) and H\(_2\)O\(_2\) is likely responsible for injury due to cold storage, and addition of nicaraven (OH scavenger) to the preservation solution protected against the injury.

Other noncardiac studies have reported enhanced cold-induced ROS production and the role of ROS in tissue preservation injury (3, 33–36). For example, Bailey et al. (3) reported that cold-induced (28°C) constriction of cutaneous blood vessels is mediated by ROS production, which was abolished by Mn(III)tetrakis[(1-methyl-4-pyridyl)porphyrin] (MnTMPyP), a cell permeable SOD + catalase mimetic (49). Interestingly, in another preliminary study, we observed that exposure to MnTMPyP before 2 hr of cold ischemia protected the heart better than MnTBAP treated or Con hearts (data not shown). These results provide further evidence that dismutation of O\(_2\)\(^{-}\) with a combination of scavengers during cold perfusion before I/R is beneficial.

NO\(^{-}\) is also a O\(_2\)\(^{-}\) scavenger and can therefore modify generation of downstream radicals or generate the toxic product peroxynitrite (ONOO\(^{-}\)). Aside from its toxicity, ONOO\(^{-}\) is also known to form OH radicals after protonation in an iron-independent reaction (18). Our experiments with l-NAME show that NO\(^{-}\) is an important modulatory factor in hypothermic cardioprotection, insofar as it reacts in a 1:1 stoichiometry with O\(_2\)\(^{-}\) (14). We showed inhibition of NO\(^{-}\) production by l-NAME would prevent ONOO\(^{-}\) or OH\(^{-}\) formation, or both, and thus abate hypothermia-induced injury during cold I/R. Other studies showed that l-NAME, N\(^{\bullet}\)-monomethyl-l-arginine and oxyhemoglobin, all NO\(^{-}\) scavengers, provide protection against I/R injury (50). However, Fagbemi and Northover (20) showed that the presence of NO\(^{-}\) donors and not inhibitors in the cold buffer improved coronary flow during subsequent warm reperfusion. In any case, these studies demonstrate a significant role of NO\(^{-}\) in the pathogenesis of cold-induced I/R injury. However, in our study, the functional protection afforded by l-NAME treatment before ischemia was not associated with a preservation of mitochondrial function after ischemia. This suggests that unlike protection provided by MCG, l-NAME’s protection may be mediated in part by improved coronary flow (Fig. 1B) after ischemia.

Redox state and cardiac protection. Either NADH-linked or FAD-linked autofluorescence can be used as a measure of the metabolic state of mitochondria, but differences between these redox markers may reflect differences in electron flow through the ETC (1, 2). In a recent study, we found that NADH is elevated during 17°C perfusion before ischemia (39); this increase could result from increased production of NADH, or more likely from decreased oxidation of NADH. Either effect alone would increase the proton gradient across the inner mitochondrial membrane. In this and other studies (1, 2), we showed that NADH (reduced state) and FAD (oxidized state) did not change significantly from baseline during perfusion at 27°C. This suggests that mitochondrial respiration, although slowed at 27°C, retains a relative balance between electron flux and oxidative phosphorylation compared with 17°C when NADH is elevated (39). As in our previous studies (1, 2), ischemia at 27°C caused a reversible increase and decrease in NADH and FAD, respectively. The NADH and FAD values for the MCG group on reperfusion indicate a higher redox state and/or a greater viable mitochondrial mass and suggest a greater availability of reducing equivalents and electrons for oxidative phosphorylation. In contrast, in the MnTBAP group, the larger decrease in NADH and larger increase in FAD fluorescence on reperfusion could represent greater cell death on reperfusion (19) and/or increased volume of irreversibly oxidized and energy depleted mitochondria (1, 2). We also showed a significant correlation of rate of NADH decline after ischemia and infarct size in individual hearts (38). Thus preservation of a reduced mitochondrial redox state is a prerequisite for improved functional and metabolic recovery.

mCa\(^{2+}\) accumulation and I/R damage. Our laboratory (13, 48) as well as others (30) have reported that hypothermia increases cytosolic [Ca\(^{2+}\)]. We further showed that cold perfusion, per se, before 30-min cold ischemia mildly increased m[Ca\(^{2+}\)], which did not alter cold-induced protection, as demonstrated by reduced m[Ca\(^{2+}\)] during I/R compared with warm I/R (39). In the present study, cold perfusion increased m[Ca\(^{2+}\)] in all groups, but more so in the MnTBAP group; on reperfusion, the MnTBAP-treated hearts also demonstrated greater m[Ca\(^{2+}\)]. In contrast, the lower m[Ca\(^{2+}\)] on reperfusion in MCG hearts suggests a more effective mechanism of maintaining m[Ca\(^{2+}\)].

Under normal conditions, a small increase in m[Ca\(^{2+}\)] during increased workload is believed to stimulate the mitochondrial TCA cycle and furnish NADH via Ca\(^{2+}\)-dependent mitochondrial dehydrogenases. However, mCa\(^{2+}\) overload during reperfusion may lead to depolarization of mitochondrial membrane potential with uncoupling of oxidative phosphorylation and impaired ATP synthesis. Decreased ATP synthesis in turn leads to alteration of membrane ion pumps and disturbance in cellular ion homeostasis and function. It is suggested that mitochondrial release of Ca\(^{2+}\) following an overload depends on a dynamic equilibrium “set point” between the
mitochondrion and cytoplasm (32). This “set point” could be achieved in instances where readily available ATP and functioning Ca\(^{2+}\) pumps establish normal cytosolic [Ca\(^{2+}\)] levels. Therefore, excessive [Ca\(^{2+}\)] during I/R coupled with poor ATP synthesis could contribute to the sustained increase in [Ca\(^{2+}\)] in the MnTBAP hearts.

**Summary and limitations.** Our purpose was to examine whether the increase in ROS during mild hypothermic perfusion has beneficial or deleterious effects during subsequent ischemia and reperfusion and to determine which ROS are involved. To this end, we found that the cardioprotection obtained by mild hypothermia during ischemia can be enhanced by the proper selection of ROS scavengers administered only during the period of cold perfusion before ischemia. The demonstration that dissmutation of O\(_2^-\) alone is insufficient and, indeed, deleterious to recovery of function indicates indirectly that O\(_2^-\) is itself not toxic. Scavenging of H\(_2\)O\(_2\), in particular, and inhibition of ONOO\(^-\) formation are partially protective, but the best approach to protection in this model appears to couple the scavenging of intra-matrix O\(_2^-\) with that of extra-matrix H\(_2\)O\(_2\).

The conditions of this study may not fairly imitate the clinical use of cold preservation solutions protection during cardiac surgery. For example, we used a nonejecting heart model, we did not use a high K\(^+\) solution to arrest the heart, and the perfusate solution did not contain blood cells, which can scavenge ROS. The washout kinetics and the distribution of glutathione and catalase could be limited by the brief washout period. However, we know from other drug studies that a new steady state is attained after 2 min with washout of H\(_2\)O\(_2\)-soluble drugs and maximally reversed with MnTBAP within 5 min (14). Another potential limitation of this study is the use of DHE to monitor O\(_2^-\) production rather than DCFDA, a probe specific for H\(_2\)O\(_2\) or OH\(^-\) (47). Therefore, the use of DHE does not show potential changes in downstream ROS, which would support our contention that it is these ROS that are responsible for the cold I/R-induced injury. Our recent preliminary data, however, does support our view that scavenging O\(_2^-\) with MnTBAP increases H\(_2\)O\(_2\) release during cold perfusion.

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