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Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart

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Camara, Amadou K. S., Matthias L. Riess, Leo G. Kevin, Enis Novalija, and David F. Stowe. Hypothermia augments reactive oxygen species detected in the guinea pig isolated perfused heart. *Am J Physiol Heart Circ Physiol* 286: H1289–H1299, 2004. First published November 26, 2003; 10.1152/ajpheart.00811.2003.—Hypothermic perfusion of the heart decreases oxidative phosphorylation and increases NADH. Because O_2 and substrates remain available and respiration (electron transport system, ETS) may become impaired, we examined whether reactive oxygen species (ROS) exist in excess during hypothermic perfusion. A fiberoptic probe was placed on the left ventricular free wall of isolated guinea pig hearts to record intracellular ROS, principally superoxide ($O_2^-\cdot$), and an extracellular reactive nitrogen reactant, principally peroxynitrite ($ONOO^-$), a product of nitric oxide (NO·) + $O_2^-\cdot$. Hearts were loaded with dihydroethidium (DHE), which is oxidized by $O_2^-\cdot$ to ethidium, or were perfused with L-tyrosine, which is oxidized by $ONOO^-$ to dityrosine (diTyr). Shifts in fluorescence were measured online; diTyr fluorescence was also measured in the coronary effluent. To validate our methods and to examine the source and identity of ROS during cold perfusion, we examined the effects of a superoxide dismutase mimetic Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), and several agents that impair electron flux through the ETS: menadione, sodium azide (NaN_3), and 2,3-butanedione monoxime (BDM). Drugs were given before or during cold perfusion. ROS measured by DHE was inversely proportional to the temperature between 37°C and 3°C. We found that perfusion at 17°C increased DHE threefold versus perfusion at 37°C; this was reversed by MnTBAP, but not by L-NAME or BDM, and was markedly augmented by menadione and NaN_3. Perfusion at 17°C also increased myocardial and effluent diTyr ($ONOO^-$) by twofold. L-NAME, MnTBAP, or BDM perfused at 37°C before cooling or during 17°C perfusion abrogated, whereas menadione and NaN_3 again enhanced the cold-induced increase in ROS. Our results suggest that hypothermia moderately enhances $O_2^-\cdot$ generation by mitochondria, whereas $O_2^-\cdot$ dismutation is markedly slowed. Also, the increase in $O_2^-\cdot$ during hypothermia reacts with available NO· to produce $ONOO^-$, and drug-induced $O_2^-\cdot$ dismutation eliminates the hypothermia-induced increase in $O_2^-\cdot$.

mitochondria; complexes I, III, and IV; radical scavengers

HYPOTHERMIA is the most effective method to temporarily and reversibly reduce myocardial metabolism. It is widely used to protect the heart from anticipated ischemia and reperfusion injury during cardiopulmonary bypass. The mechanism underlying cardiac protection by hypothermia is believed to be a slowing of enzymatic activity, most importantly of those reactions that require ATP. The result is a temperature-dependent reduction of mitochondrial respiration and oxidative phosphorylation. During a subsequent period of ischemia, hypothermia appears to protect by attenuating mitochondrial energy-processing mechanisms leading to reduced ATP synthesis; on warm reperfusion mitochondrial respiration is less dysfunctional and ATP is more readily regenerated.

Although the heart can remain nonperfused longer at increasingly lower temperatures with a similar degree of mechanical or structural damage (8–10, 46, 48) as the temperature is lowered, optimal protection is compromised because of the deleterious effects of hypothermia per se. Hypothermic perfusion injury, i.e., without concomitant cardiac ischemia, is thought to be caused by altered cellular ion homeostasis resulting from slowed membrane ion pump (primary ATP dependence) and ion exchangers (secondary ATP dependence) and/or to slowed activity of enzymes responsible for mitochondrial respiration and contractile activity (36).

Cold perfusion, like warm perfusion, furnishes a continued supply of O_2 to accept electrons and also protons and substrates to furnish electrons and reducing equivalents (NADH). But low temperatures could reduce mitochondrial performance by limiting mitochondrial oxidative capacity. Mitochondrial enzymes and redox carrier molecules ferry electrons from substrate to O_2 via the electron transport system (ETS). Cold-induced reduction of mitochondrial enzyme activities may inhibit NADH oxidation to cause a more reduced redox state ($NADH/NAD^+$). By slowing down oxidative phosphorylation, cold perfusion may also lead to reduced mitochondrial ATP formation and, when coupled with available O_2 and more than adequate reducing equivalents, lead to impaired electron flow through the ETS. Accumulation of electrons upstream can cause univalent reduction of molecular oxygen to $O_2^-\cdot$ and other downstream ROS. In tandem with increased $O_2^-\cdot$ formation, $O_2^-\cdot$ scavenger enzyme activity is likely reduced by hypothermia so that $O_2^-\cdot$ production exceeds its dismutation; this would cause a net increase in detectable reactive oxygen species (ROS).

Cellular sources of ROS are cyclooxygenase, lipoxygenase, NAD(P)H oxidases, and hypoxanthine-xanthine oxidase pathways, but a major source is the respiratory oxidoreductases (complexes I and III) of mitochondria where electron leaks normally occurs. In a recent article (40), we show that mito-

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chondrial Ca^{2+} (mCa^{2+}) and NADH are increased during cold perfusion but that the increase in mCa^{2+} was attenuated during cold compared with warm ischemia while NADH remained elevated during cold ischemia. Thus it is possible that a cold-induced increase in ROS generation and the coincident rise in NADH is a cause or result of increased mCa^{2+} overload. A small physiological increase in mCa^{2+} during normothermia is thought to increase mitochondrial dehydrogenase activity to increase reducing equivalents that drive ATP synthesis (2). However, the cold-induced increase in mCa^{2+} (40), caused by an increase in cytosolic Ca^{2+} (8–10) secondary to slowed Na^+ (23, 26) and Ca^{2+} pump function (25), and coupled to reduced forward (inward) Na^+/Ca^{2+} exchange (23), may lead to excess generation of reducing equivalents. Moreover, endogenous scavengers of ROS are enzymes so their function is likely reduced as temperature falls.

Knowledge of the role of hypothermia in generating $O_2^-\cdot$, as well as its dismutation to H_2O_2, and how this affects levels of downstream reactants could lead to better therapeutic measures to protect the heart against ischemia-reperfusion injury. We proposed that myocardial ROS increases as an inverse function of temperature below 37°C and that this initial ROS is $O_2^-\cdot$. We also proposed that the mitochondrion is the principle source of $O_2^-\cdot$ generation during hypothermia and that nitric oxide (NO·) plays a permissive role in reacting with $O_2^-\cdot$ during hypothermia. In previous studies (20), we showed that ROS (principally $O_2^-\cdot$) can be monitored online in the intact heart and that a reactant (peroxynitrite, $ONOO^-$) can be measured in cardiac effluent (29) by using the method of Yasmin et al. (54). In this study, we again used the fluorescence probe dihydroethidium (DHE, Molecular Probes; Eugene, OR) to identify changes in intracellular $O_2^-\cdot$ levels, and for the first time, we used dityrosine (diTyr) fluorescence to assess changes in interstitial as well as effluent $ONOO^-$ levels from the reaction of the free radicals $O_2^-\cdot$ and NO·. We demonstrate in this study that hypothermia incrementally increases $O_2^-\cdot$ levels in the intact beating heart. Because the generation and scavenging of ROS are enzyme dependent, we proposed that reductions in temperature causes dysfunctional ETS that leads to enhanced generation of $O_2^-\cdot$ as well as to reduced dismutation of the generated $O_2^-\cdot$.

MATERIALS AND METHODS

Langendorff heart preparation. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Pub. No. 85-23, Revised 1996). Approval was obtained from the Medical College of Wisconsin animal studies committee. Our preparation has been described in detail previously (8, 10, 20, 29, 46, 48). Before being cooled, guinea pig hearts ($n = 88$) were perfused in the Langendorff mode at constant pressure (55 mmHg) and at 37°C with a modified Krebs-Ringer (KR) solution equilibrated with 95% O_2-5% CO_2 and containing (in mM) 138 Na^+, 4.5 K^+, 1.2 Mg^{2+}, 2.5 Ca^{2+}, 134 Cl^-, 14.5 HCO_3^-, 1.2 $H_2PO_4^-$, 11.5 glucose, 2 pyruvate, 16 mannitol, and 0.05 EDTA and included 5 U/l insulin. Perfusate and bath temperatures were maintained initially at 37°C using a servo-controlled water circulator; hearts were suspended in the bath containing the same solutions as that perfused.

Left ventricular pressure (LVP) was measured isovolumetrically by using a transducer connected to a saline-filled latex balloon placed in the left ventricle through an incision in the left atrium. Measured characteristics of LVP were diastolic and systolic LVP. Coronary flow (CF) was measured by an ultrasonic flowmeter (Transonic T106X; Ithaca, NY). Atrial and ventricular bipolar leads were used to measure spontaneous heart rate. Coronary inflow and coronary venous Na^+, K^+, Ca^{2+}, P_{O_2}, pH, and P_{CO_2} were measured offline with an intermittently self-calibrating analyzer (Radiometer ABL 505; Copenhagen, Denmark). Coronary sinus P_{O_2} tension ($P_{V_{O_2}}$) was also measured continuously online with a Clark electrode (model 203B, Instech; Plymouth Meeting, PA). Percent O_2 extraction was calculated as $100 \times (Pa_{O_2} - Pv_{O_2}/Pa_{O_2})$, where Pa_{O_2} is arterial P_{O_2}; myocardial O_2 consumption ($M\dot{V}_{O_2}$) was calculated as CF/g $\times$ ($Pa_{O_2} - Pv_{O_2}$) $\times$ O_2 solubility at 760 mmHg; O_2 solubility is 24 μl/ml H_2O at 37°C and 33 μl/ml H_2O at 17°C.

Intracellular detection of superoxide radical in intact hearts. Our fluorescence methods have been described previously for detection of ROS (20) cytosolic Ca^{2+} (48), intracellular Na^+ (52), mitochondrial Ca^{2+} (39), and NADH (38). ROS were measured near continuously in the beating heart before, during, and after cooling and during drug treatment. The fluorescent dye DHE was used to measure ROS formation in a transmural layer of myocardium excited by light directed onto the left ventricular wall by use of a bifurcated fiber-optic cable system. DHE enters cells and is oxidized by ROS, with a relative selectivity for $O_2^-\cdot$ (6, 51), to form ethidium, which intercalates with DNA and causes the nucleus to exhibit a red shift in fluorescence. DHE and ethidium are retained within cells with minimal leakage.

Light intensity, in arbitrary fluorescence units (afu), was measured in a light-blocking Faraday cage. A small piece of net-like nylon fabric was secured on one side of the heart to ensure optimal contact between the left ventricle and the fiberoptic probe tip without impeding contractility and relaxation. The fiberoptic cable was connected to a modified spectrophotofluorometer (SLM Aminco-Bowman II; Spectronic Instruments; Urbana, IL). Fluorescence emission (λ_{em}) at 590 nm (bandwidth 4 nm) was amplified by a photomultiplier tube (700 V applied) and recorded during excitation with light (150-W xenon arc lamp) filtered via a monochromator at fluorescence excitation (λ_{ex}) of 540 nm (bandwidth 4 nm). Signals were sampled every 12 s for 100 ms during the entire protocols for a total recording time of 95 s.

DHE was initially dissolved in 1 ml DMSO containing 16% (weight/volume) Pluronic I-127 (Sigma Chemical) to facilitate intracellular permeation; final DMSO concentration was 3 μM. Some hearts ($n = 40$) were loaded with 10 μM DHE in KR solution for 20 min; this was followed by washout of residual DHE with modified KR for 20 min. DHE loading increased diastolic LVP by 8% and CF by 10%; these effects were essentially reversed on washout of DHE. During DHE loading, fluorescence intensity increased gradually from 0.31 ± 0.02 afu before loading to 2.20 ± 0.10 afu after washout. Other hearts ($n = 6$) were perfused with the vehicle for DHE to measure background autofluorescence before, during, and after cold perfusion, with or without drug treatment, using the same protocols as for the DHE experiments. Autofluorescence (absence of DHE) did not change significantly between 37 and 3°C. The specificity of DHE for $O_2^-\cdot$ and the effects of possible artifacts, including movement and induced changes in LVP, pH (7–8), and flow, were examined previously and found not to influence the DHE signal (20). Inflow O_2 tensions between 220 and 650 mmHg also did not alter the DHE signal. Manganese (III) tetrakis(4-benzoic acid)porphyrin (MnTBAP) decreased autofluorescence up to −9% of the baseline, but this was much less than the decrease in fluorescence intensity obtained with DHE present. All subsequent recorded values of DHE fluorescence were corrected for any change in autofluorescence elicited by a given drug.

Extracellular detection of peroxynitrite in intact hearts. DiTyr fluorescence was used to assess $ONOO^-$ formation using the same fiberoptic probe in the same manner as for DHE. $ONOO^-$ is a product of $O_2^-\cdot$ and NO· that reacts with L-tyrosine in physiological buffer to form the fluorescent dimer diTyr (54). This technique assesses formation of diTyr in extracellular fluid as an indicator of $ONOO^-$ formed in or out of cells in a cone of left ventricular tissue underlying the fiber-optic probe. The chemical reaction proceeds with a rate constant (k) three times faster than the reaction between $O_2^{-}\cdot$ with SOD ($k = 2 \times 10^9\ M^{-1}\cdot s^{-1}$) (13) and is probably many times faster at lower temperatures as SOD activity is reduced. The sensitivity and linearity of this reaction in a crystalloid solution containing 0.3 mM L-tyrosine and authentic $ONOO^-$ (0.1–10 μM) or the equivalent volume of decomposed $ONOO^-$ was tested at 37°C (29) by a method reported previously (28). [DiTyr], measured by HPLC, is linearly related to its fluorescence intensity ($r^2 > 0.99$); the detection limit for diTyr is reported as 0.05 μM (54). The reaction of L-tyrosine with $ONOO^-$ to produce diTyr occurs immediately, and the product is stable at 25°C for several hours when pH is >7.3 (3).

After 30 min of stabilization and 10–15 min to take background fluorescence measurements, hearts were perfused with KR solution containing 0.3 mM L-tyrosine throughout the experiment. Myocardial fluorescence intensity was recorded near continuously (every 12 s for 100 ms) via the fiber-optic probe at the appropriate spectra λ_{ex} 320 nm (bandwidth 5 nm) and λ_{em} 410 nm (bandwidth 4 nm) amplified by a photomultiplier tube (820 V applied). Background autofluorescence recordings were also taken in hearts not perfused with L-tyrosine under the same conditions used to detect diTyr fluorescence ($n = 4$). No drug altered myocardial autofluorescence at diTyr wavelengths more than ±8%, and these values were subtracted from the signals obtained after L-tyrosine was perfused. Each drug was also tested for changes in autofluorescence in KR buffer alone; none had any appreciable effect.

Detection of peroxynitrite in effluent of intact hearts. In hearts perfused with L-tyrosine, 1-ml coronary effluent samples were collected at specified temperatures and kept at 3°C until measured for diTyr fluorescence within 1 h at 25°C. In several experiments ($n = 4$), hearts were ramp cooled (37, 27, 17, and 7°C) to 7°C. Effluent samples were collected at 37, 30, 27, 20, 17, and 7°C and on rewarming back to 37°C to assess diTyr fluorescence as a function of temperature. In control experiments, effluent was collected from hearts perfused without L-tyrosine and with or without drugs at 37°C, as well as during cooling and rewarming to 37°C, to measure for any drug or temperature-induced changes in autofluorescence. DiTyr was measured in cuvettes at 25°C at λ_{ex} 320 nm and λ_{em} 410 nm using spectrofluorometry (Perkin-Elmer model LS 50B, Beaconsfield, Buckinghamshire, UK). There was no change in baseline diTyr (0.73 ± 0.03 afu) between vehicle- and L-tyrosine-containing solutions not perfused, indicating no spontaneous diTyr formation in the absence of $ONOO^-$. With perfusion of L-tyrosine, the effluent diTyr signal increased to 42.3 ± 5.6 afu, indicating cardiac formation of $ONOO^-$ at 37°C. Effluent formation of $ONOO^-$ at 17°C was about twice that formed during 37°C perfusion. Because effluent samples were measured for diTyr fluorescence at 25°C, this verified that the apparent ROS formed was independent of a temperature-induced effect on the diTyr signal.

Drugs used to alter formation of ROS and reactants. Effects of drugs expected to alter fluorescence attributed to $O_2^{-}\cdot$ and $ONOO^-$ were compared at 37°C and 17°C. Selected drugs were the following: NO· synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (100 μM L-NAME); mitochondrial ETC complex inhibitor 2-methyl-1,4-naphthoquinone (10 μM menadione-vitamin K_3); nonspecific complex I inhibitor 2,3-butanedione monoxime (10 mM BDM); complex IV cytochrome oxidase inhibitor NaN_3 (1 mM) (all from Sigma; St. Louis, MO); and intracellular $O_2^{-}\cdot$ dismutase mimetic ($O_2^{-}\cdot \rightarrow H_2O_2$) 10 μM MnTBAP (AG Scientific; San Diego, CA). L-NAME and BDM were dissolved initially in KR solution; NaN_3 was initially dissolved in 0.9% NaCl. Menadione and MnTBAP were initially dissolved in DMSO before being added to the KR solution to attain the appropriate drug concentration. Final DMSO concentration for each drug was 2 μM; this concentration of DMSO alone had no apparent effect on ROS formation. In experiments involving more than one drug, each drug was perfused for 10 min with a 20-min washout period between drugs. No interaction of drugs was observed.

Experimental protocol. Each heart underwent an initial 30-min stabilization period and 10–15 min of baseline recording at 37°C before DHE was loaded and washed out or before continuous perfusion with L-tyrosine. Fluorescence signals were recorded near continuously in intact hearts and intermittently in effluent during all phases of the study.

There were six experimental protocols including their corresponding autofluorescence controls.

Protocol I: $n = 4$. Experiments were conducted to determine temperature-dependent changes in ROS signal intensity in DHE-loaded hearts. Perfusate temperature was gradually lowered from 37°C to 27, 17, or 7°C; these temperatures were held at steady state for 10 min, after which hearts were rewarmed to 37°C for 20 min between each test temperature. A more rapid change from 37°C to 7°C over 90 s was accomplished by precooling the perfusate to 17°C and overcooling the perfusate system to near 0°C. This rapid change in temperature produced an equivalent change in DHE fluorescence, as did the gradual change. With the use of the same protocol in L-tyrosine-loaded ($n = 4$) hearts, online and coronary effluent diTyr signals were assessed at 37, 27, 17, and 7°C and on rewarming to 37°C to assess the temperature effect on $ONOO^-$ release. In another group of DHE-loaded hearts ($n = 4$), perfusate temperature was changed continuously from 37°C to 3°C and back to 37°C.

Protocol II: $n = 8$. DHE and diTyr signals, indicative of $O_2^{-}\cdot$ and $ONOO^-$, were measured during *1*) normothermic perfusion (37°C), *2*) progressive cooling from 37°C to 17°C (20 min), and *3*) progressive rewarming from 17°C to 37°C (10 min). Perfusion at 17°C was maintained for 10 min before hearts were rewarmed to 37°C. Perfusion at 37°C was maintained for 20 min before the hearts were recooled to 17°C and after rewarming.

Protocol III: $n = 24$. The ROS-altering effects of L-NAME, MnTBAP, BDM, and menadione were examined during both perfusion at 37°C and continuous perfusion during cooling to 17°C. Drugs were randomly chosen and only two of the four drugs were perfused in a given heart. Fluorescence signals were recorded during warm and cold perfusion before the protocol was repeated in the presence of the two selected drugs. Hearts were perfused (37°C) for 10 min with L-NAME, MnTBAP, BDM, or menadione; this was followed by cooling with the drug to 17°C and then rewarming without the drug to 37°C.

Protocol IV: $n = 24$. This protocol examined the ROS-altering effects of L-NAME, MnTBAP, BDM, or menadione during perfusion only at 17°C. A given heart was perfused with two of four randomly selected drugs for 10 min at a constant 17°C after being initially cooled to 17°C and rewarmed in the absence of drugs. These two protocols were conducted to distinguish the ROS-altering effect of temperature on a drug response and of a drug on a temperature response.

Protocol V: $n = 4$. In DHE-loaded hearts, experiments using the complex IV inhibitor NaN_3 were conducted during warm and cold perfusion (37°C to 7°C) to further confirm the mitochondrial source of ROS during hypothermia. In additional hearts, 100 μM allopurinol ($n = 2$), 100 μM oxypurinol ($n = 2$), or 500 μM glutathione (GSH) alone ($n = 3$) and with 50 U/ml catalase ($n = 3$) were given before and during perfusion at 17°C to assess for an extra mitochondrial source of ROS and to confirm the identity of the ROS, respectively.

Protocol VI: $n = 16$. The above protocols were conducted in the absence of fluorescent probes to determine background autofluorescence measured in the heart and effluent for each drug at each temperature. Thus the wavelengths for the background fluorescence spectrum were the same used for a particular fluorescent probe.

Statistical analysis. All data are expressed as means ± SE. Among-group data were compared using analyses of variance at these time points: baseline (37°C), during and after cooling to 17°C, and during drug treatment with or without cold perfusion. Post hoc Student-Newman-Keuls tests were utilized where differences were found (Prisma version 3.0a, GraphPad Software; San Diego, CA). Groups compared were the following: perfusion at 17°C versus 37°C (no drugs) and perfusion at 37°C or at 17°C with drug versus control at same temperature (no drug). Differences among means were considered significant at $P < 0.05$ (two tailed).

RESULTS

Hypothermia caused an increase in each fluorescence signal for ROS/RNS ($O_2^{-}\cdot$ and $ONOO^{-}$) measured online in the myocardium with DHE and diTyr, respectively, and offline in the effluent with diTyr. Specifically, online DHE increased from 2.1 ± 0.1 afu at 37°C to 2.8 ± 0.1 afu at 17°C. Online diTyr increased from 0.73 ± 0.03 afu at 37°C to 0.96 ± 0.06 afu at 17°C, and effluent diTyr increased from 42.3 ± 5.6 afu at 37°C to 82.7 ± 6.6 afu at 17°C; all signals returned to basal levels (37°C) on rewarming. Effects of cooling and rewarming on enhancing or reducing formation of ROS and RNS were reproducible during repeated perfusions in the same heart (data not shown).

Figure 1*A* shows a representative tracing of graded temperature-dependent increases in myocardial DHE fluorescence. At each steady-state temperature the ROS signal remained relatively flat. Figure 1*B* shows that a continuous decline in temperature from 37°C to 3°C resulted in an inversely proportional increase in DHE; this was fully reversible on rewarming

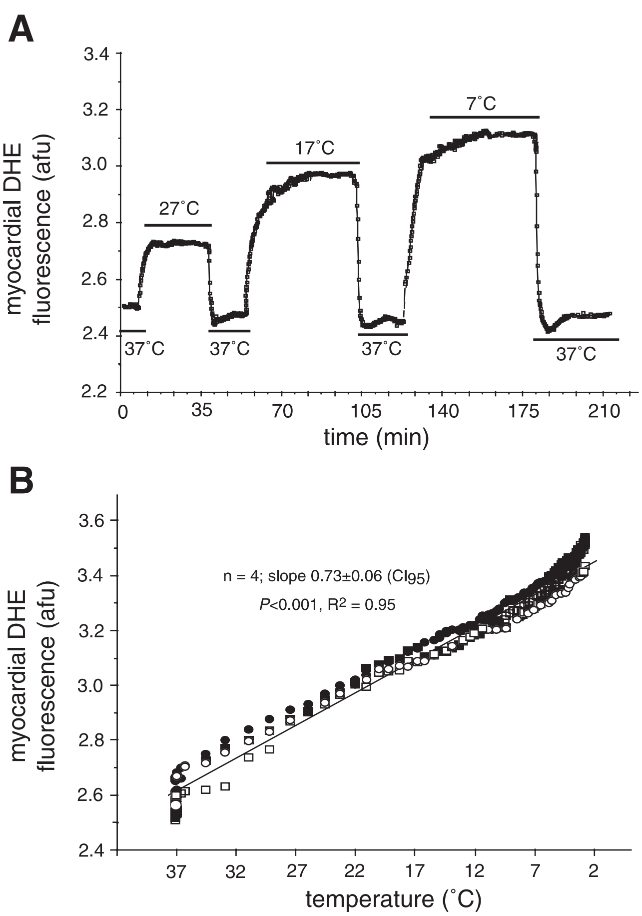

Fig. 1. Representative online dihydroethidium (DHE) fluorescence signals (superoxide, $O_2^{-}\cdot$) recorded during four steady-state temperature changes (*A*) and during a continuous change in temperature from 37 to 3°C and back to 37°C (*B*) in perfused hearts. DHE signal intensity is inversely proportional to temperature. afu, arbitrary fluorescence unit; CI_{95}, 95% confidence interval; R^2, regression coefficient. See text for details.

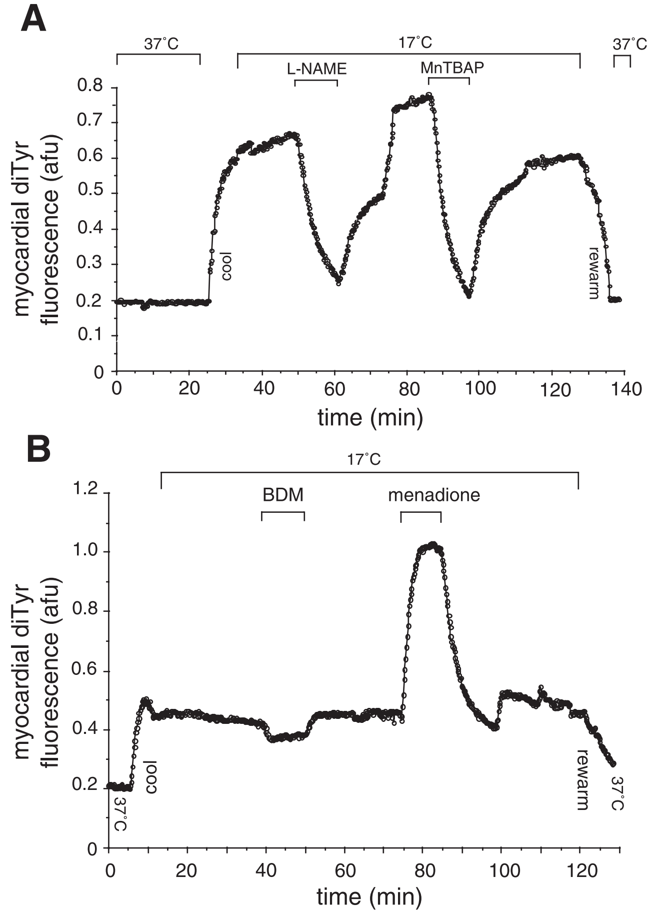

Fig. 2. Representative online dityrosine (diTyr) fluorescence signals ($ONOO^{-}$) recorded during 37°C perfusion and cooling to 17°C with or without drugs. N^G-nitro-L-arginine methyl ester (L-NAME) and Mn(III) tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP) blocked cold-induced diTyr signals; this effect was reversible with drug-free perfusion (*A*). 2,3-Butanedione monoxime (BDM) slightly attenuated the diTyr signal, whereas menadione markedly enhanced the signal (*B*). See text for details.

back to 37°C, and there was no hysteresis between the ascending and descending changes in temperature. Note that the change in temperature between 37°C and 7°C was not linear over time but faster at higher and slower at lower temperatures. Similar cold temperature-dependent changes in myocardial and coronary effluent $ONOO^{-}$ levels were observed in a different group of perfused hearts (data not displayed).

Figure 2 shows representative tracings of online $ONOO^{-}$ assessed by diTyr during cooling to 17°C alone and during perfusion with L-NAME (NOS inhibitor), MnTBAP (SOD mimetic), BDM, or menadione (mitochondrial ETS complex inhibitors). Incremental cooling caused a graded increase in $ONOO^{-}$ that attained a steady state at 17°C; both L-NAME and MnTBAP blocked the cold-induced increase in $ONOO^{-}$; these effects were reversible on washout of the drugs (Fig. 2*A*). BDM slightly attenuated $ONOO^{-}$, whereas menadione doubled the hypothermia-induced increase in $ONOO^{-}$ (Fig. 2*B*). DiTyr signals returned to baseline when rewarmed to 37°C.

Figure 3 summarizes graphically the results for online diTyr during warm and cold perfusion with drugs given either before (Fig. 3*A*) or during (Fig. 3*B*) perfusion at 17°C. Cold perfusion significantly increased $ONOO^{-}$, and, on rewarming, $ONOO^{-}$

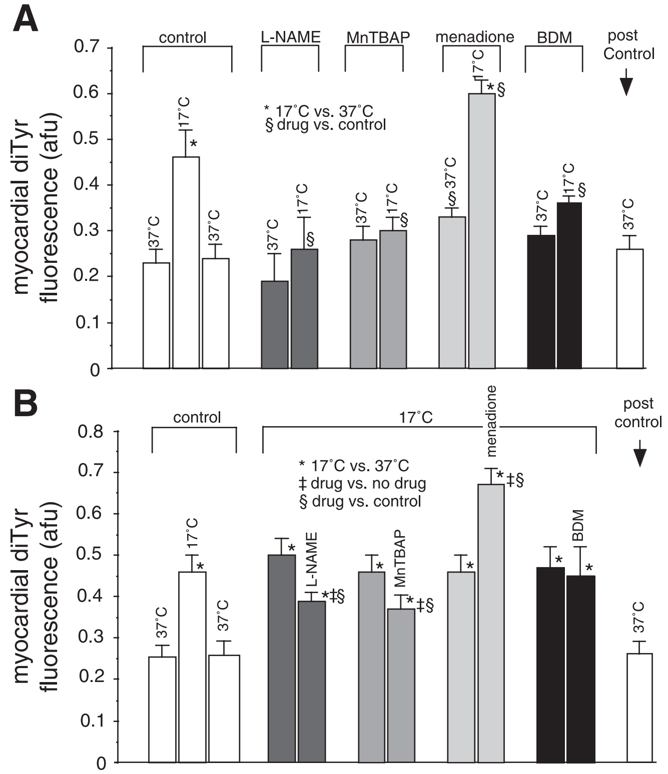

Fig. 3. Summary data for online diTyr fluorescence signals during warm and cold perfusion with drugs given either before (*A*) or during (*B*) 17°C perfusion. DiTyr fluorescence increased significantly during cold perfusion, and L-NAME and MnTBAP blunted the diTyr signal in both protocols (*A* and *B*). Menadione increased the diTyr signal during cold perfusion when given before or during cold perfusion; BDM attenuated the signal when perfused at 37°C before cooling (*A*) but did not change diTyr fluorescence during 17°C perfusion (*B*).

returned to the basal level. At 37°C, L-NAME and MnTBAP had no significant effect on basal $ONOO^-$ levels but abolished the cold-induced increase in $ONOO^-$. Menadione significantly increased $ONOO^-$ at 37°C and more so at 17°C. BDM did not significantly change $ONOO^-$ at 37°C but abolished the increase observed at 17°C. On rewarming to 37°C, diTyr signals returned to the basal level. Figure 3*B* shows that L-NAME and MnTBAP significantly attenuated $ONOO^-$ during 17°C perfusion, whereas menadione significantly increased cold-induced $ONOO^-$, and BDM had no effect on $ONOO^-$ during 17°C perfusion. On rewarming to 37°C, the diTyr signal returned to basal levels. These experiments show that cold-induced increases in $O_2^-\cdot$ cause corresponding increases in $ONOO^-$ and that blocking constitutive NO· synthesis or dismutating $O_2^-\cdot$ to H_2O_2 can block hypothermia-induced increases in $ONOO^-$.

To provide additional evidence that $O_2^-\cdot$ radicals exist in excess during cold perfusion, the above drug studies were repeated using DHE as a relatively selective probe for detecting $O_2^-\cdot$. Figure 4 shows representative online traces of DHE fluorescence during cooling to 17°C, at 17°C, and during rewarming to 37°C, in the absence and presence of BDM, menadione, MnTBAP, or L-NAME. Cooling to 17°C markedly increased the DHE signal for $O_2^-\cdot$; on rewarming the DHE signal returned to baseline. BDM perfused at 37°C or 17°C had no effect on warm or cold-induced $O_2^-\cdot$ (Fig. 4*A*), whereas MnTBAP not only decreased the $O_2^-\cdot$ signal at 37°C but also markedly depressed the increase in signal at 17°C. L-NAME perfused at 37°C or 17°C did not alter the warm-induced or the cold-induced $O_2^-\cdot$ signals, whereas menadione exacerbated the increase in $O_2^-\cdot$ at 17°C (Fig. 4*B*). The DHE ($O_2^-\cdot$) signal returned to baseline values (37°C, no drugs) in all these studies.

Figure 5 summarizes graphically the results for online $O_2^-\cdot$ detected during warm and cold perfusion with drugs given either before (Fig. 5*A*) or during (Fig. 5*B*) cold perfusion. $O_2^-\cdot$ levels during perfusion at 17°C were about threefold higher than those at 37°C; the DHE signal returned to baseline on rewarming. Neither BDM nor L-NAME perfused at 37°C or 17°C (Fig. 5, *A* and *B*) altered $O_2^-\cdot$ levels. MnTBAP tended to dismutate/scavenge the basal (37°C) levels of $O_2^-\cdot$ (Fig. 5*A*) and blocked the increased DHE signal for $O_2^-\cdot$ observed during cold perfusion (Fig. 5, *A* and *B*). Menadione enhanced the $O_2^-\cdot$ signal detected both at 37°C and at 17°C (Fig. 5*B*).

To further elucidate the role of mitochondria as the primary sources for increased ROS, specifically $O_2^-\cdot$, experiments were conducted in DHE-loaded hearts with NaN_3 (complex IV inhibitor); this drug did not alter DHE autofluorescence. Baseline DHE fluorescence (2.14 ± 0.08 afu at 37°C and 3.71 ± 0.18 afu at 3°C) increased from 2.14 ± 0.04 to 2.91 ± 0.05 afu

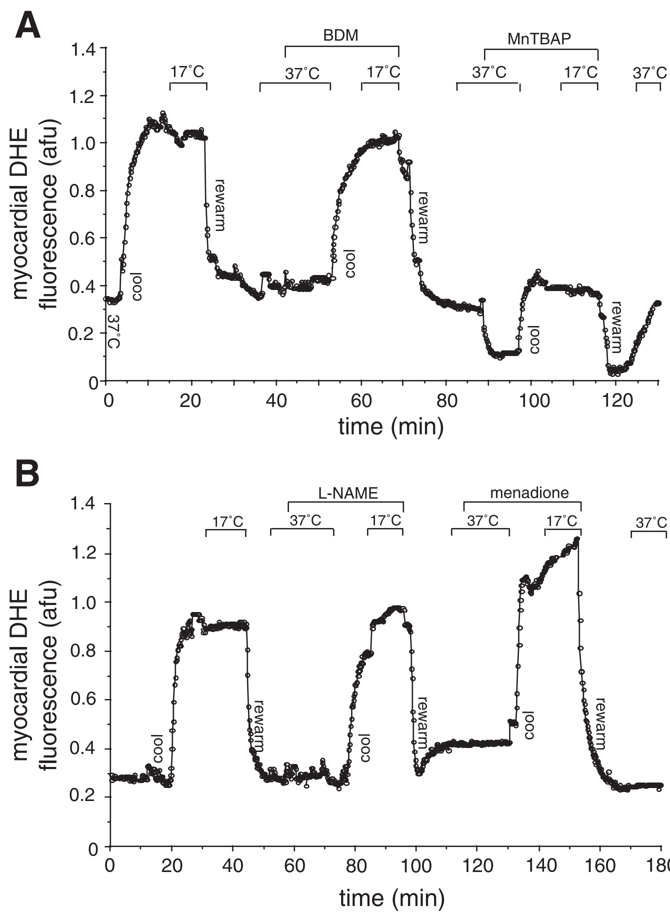

Fig. 4. Representative online DHE fluorescence ($O_2^-\cdot$) during 37°C perfusion and cooling to 17°C with or without drugs. BDM perfused before and during cold perfusion slightly reduced the DHE signal (*A*), whereas MnTBAP reduced the basal 37°C signal and almost completely eliminated the increased in the DHE fluorescence signal at 17°C (*A*). L-NAME did not alter the DHE signal; menadione increased DHE fluorescence during both warm and cold perfusion (*B*).

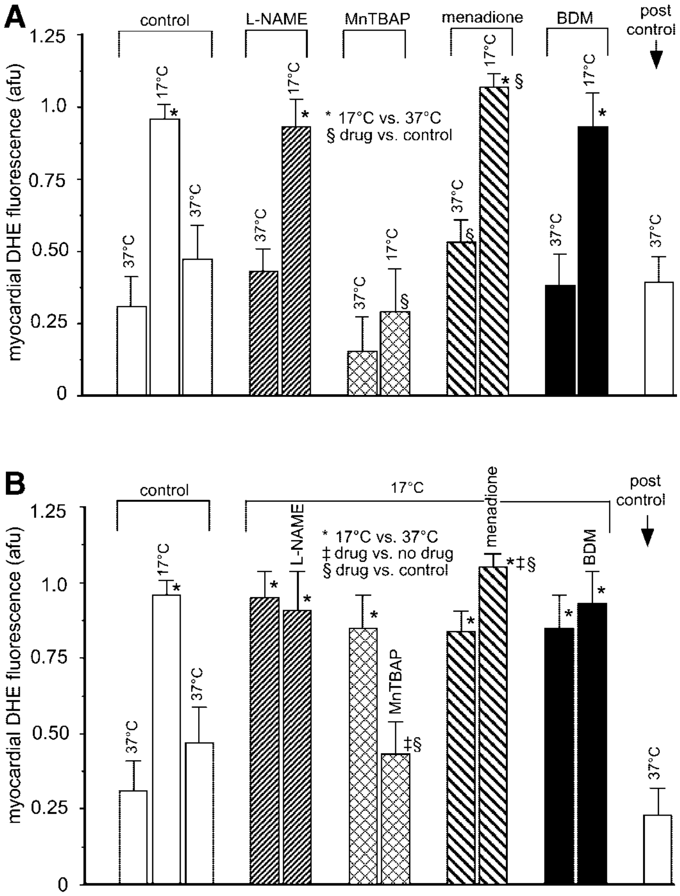

Fig. 5. Summary data for online DHE fluorescence ($O_2^{-}\cdot$) during warm and after cold perfusion with drugs given either before (*A*) or during (*B*) cold perfusion. L-NAME and BDM had no effect on DHE fluorescence when perfused before or during cold perfusion (*A* and *B*); MnTBAP abrogated the signal when perfused before or during cold perfusion (*A* and *B*). Menadione enhanced cold-induced DHE fluorescence.

after NaN_3 at 37°C and doubled to 6.1 ± 0.4 afu after NaN_3 at 3°C. This added increase in DHE signal by NaN_3 was reversed (3.47 ± 0.21 afu) by washout of NaN_3 during cold perfusion and was fully reversed on rewarming. In separate experiments, neither allopurinol nor oxypurinol altered the cold-induced increase in DHE when given before or during 17°C perfusion. Similarly, neither glutathione (GSH) alone nor GSH with catalase altered the cold-induced increase in DHE when given before or during 17°C perfusion with or without menadione (data not displayed).

To complement the online myocardial $ONOO^-$ and $O_2^{-}\cdot$ recordings during cooling and rewarming and to further verify enhanced ROS levels during hypothermic perfusion, samples of coronary effluent were collected periodically from hearts perfused with L-tyrosine at several temperatures. Effluent was measured offline spectrofluorometrically for changes in the diTyr signal at 25°C. Figures 6 and 7 show that $ONOO^-$ increased as the temperature fell to 17°C and returned to basal levels on rewarming to 37°C. Both L-NAME (Fig. 6*A*) and MnTBAP (Fig. 6*B*) reduced effluent $ONOO^-$ levels at 37°C compared with no drugs and completely blocked cold-induced increases in $ONOO^-$. BDM did not significantly alter $ONOO^-$ detected at 37°C but blunted the cold-induced increase in $ONOO^-$ (Fig. 7*A*). Menadione moderately increased $ONOO^-$ at 37°C and nearly doubled $ONOO^-$ detected at 17°C (Fig. 7*B*).

The effects of drugs and hypothermia on heart rate, systolic and diastolic LVP, LVP contractility ($dLVP/dt_{max}$), LVP relaxation ($dLVP/dt_{min}$), coronary flow, $\dot{M}V_{O_2}$, and percent O_2 extraction in hearts perfused at 37° and 17°C are displayed in Table 1. BDM, menadione, and L-NAME decreased heart rate, and this effect was reversed on washout. Compared with controls, BDM and menadione significantly depressed systolic LVP, $dLVP/dt_{max}$, and $dLVP/dt_{min}$ by 83%, 87%, and 91%, respectively, and by 25%, 28%, and 26%, respectively; BDM increased diastolic LVP from 1 to 6 mmHg. L-NAME and MnTBAP had no effect on systolic and diastolic LVP, $dLVP/dt_{max}$, and $dLVP/dt_{min}$. MnTBAP and L-NAME reduced CF by 24% and 18%, respectively; BDM and menadione did not alter CF. BDM, MnTBAP, and L-NAME, respectively, reduced $\dot{M}V_{O_2}$ by 33%, 19%, and 26%; menadione did not alter $\dot{M}V_{O_2}$, and BDM was the only drug that significantly reduced the percent O_2 extraction by 38%. Perfusion at 17°C without drugs depressed all functional values compared with perfusion at 37°C. NaN_3 perfusion (37°C) reversibly depressed developed LVP by 70% and increased CF by 15%; allopurinol, oxypurinol, GSH, and catalase had no effects on function at any temperature. Effluent pH was 7.27 ± 0.01 at 37°C and 7.42 ± 0.01 at 17°C.

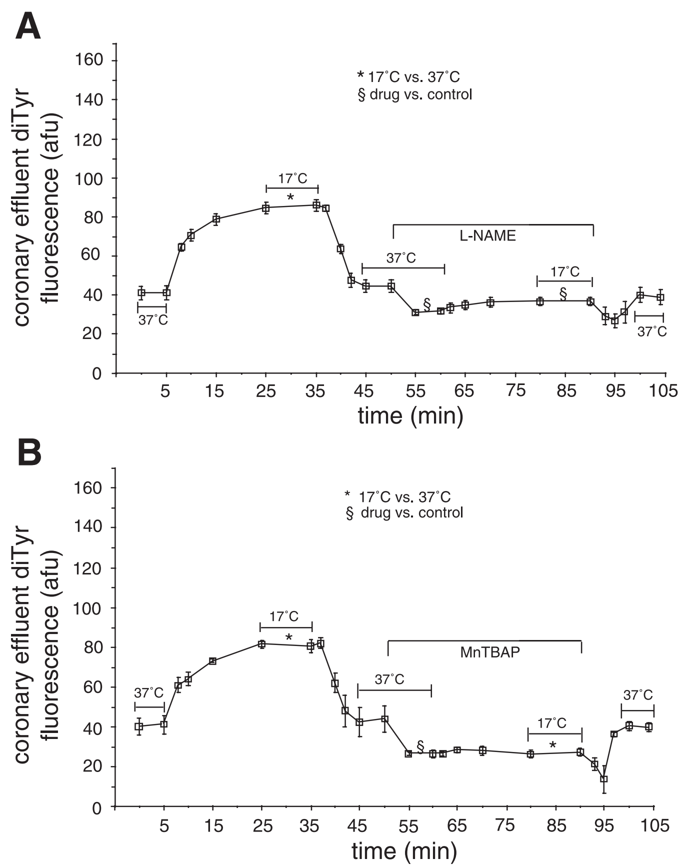

Fig. 6. Summary data for coronary effluent diTyr fluorescence ($ONOO^-$) during 37°C and 17°C perfusion and then at 17°C and 37°C in the presence of L-NAME (*A*) or MnTBAP (*B*). L-NAME slightly blunted basal (37°C) effluent diTyr and completely blocked the rise in effluent diTyr fluorescence during cold perfusion (*A*); MnTBAP also blunted effluent diTyr and completely blocked the rise in diTyr signal during cold perfusion (*B*).

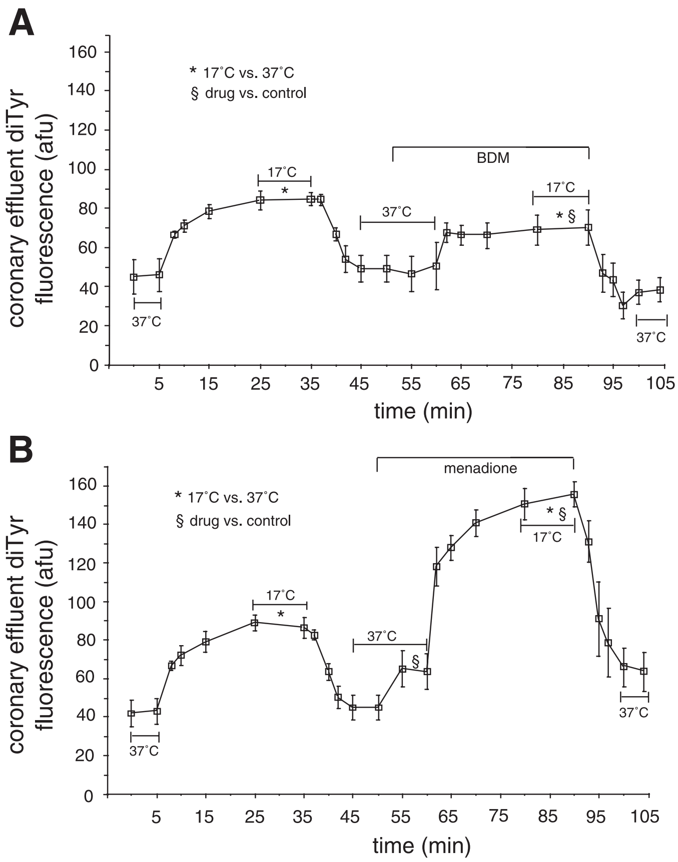

Fig. 7. Summary data for coronary effluent of diTyr fluorescence ($ONOO^-$) during 37°C and 17°C perfusion and then at 17°C and 37°C in the presence of BDM (*A*) or menadione (*B*). BDM did not alter baseline (37°C) effluent diTyr fluorescence but significantly attenuated effluent diTyr release during 17°C perfusion. Menadione caused a small but significant increase in effluent diTyr fluorescence during warm perfusion. Perfusion of menadione at 17°C resulted in an additional increase in effluent diTyr fluorescence that was reversed upon drug washout.

DISCUSSION

The role of mitochondria in cold-induced ROS/reactive nitrogen species (RNS) generation was examined by using pharmacological agents that directly or indirectly modulate mitochondrial ETS without causing deleterious and irreversible damage to the heart. We used two fluorescent probes (DHE and diTyr) and online myocardial and offline coronary effluent fluorescent techniques to demonstrate that ROS ($O_2^-\cdot$) and reactants ($ONOO^-$) detected in crystalloid-perfused hearts increase markedly during cold perfusion. Our overall findings in this model of cardiac hypothermia are the following. *1*) The magnitude of ROS/RNS detected increased inversely with a decrease in temperature. *2*) Use of pharmacological agents that either reversibly increase or decrease DHE and diTyr fluorescence signals confirmed that cold perfusion increases ROS levels and that this species was $O_2^-\cdot$; in turn $O_2^-\cdot$ reacts with NO· to form $ONOO^-$ released into the effluent. *3*) The NOS inhibitor L-NAME blocked the hypothermia-induced increase in myocardial $ONOO^-$ but not $O_2^-\cdot$. *4*) The SOD mimetic MnTBAP, a dismutator of $O_2^-\cdot$, blocked hypothermia-induced increases in both $O_2^-\cdot$ and $ONOO^-$. *5*) Menadione, an inhibitor of mitochondrial ETS at complexes I and III. and NaN_3, a cytochrome oxidase (complex IV) inhibitor, each caused an added increase in cold-induced $O_2^-\cdot$, whereas allopurinol and oxypurinol, inhibitors of xanthine oxidase, did not alter the cold-induced increase in $O_2^-\cdot$. *6*) BDM, a nonspecific inhibitor of mitochondrial ETS (19, 42), attenuated the cold-induced increase in $ONOO^-$ but had no effect on $O_2^-\cdot$. *7*) GSH, a substrate for glutathione peroxidase, given with catalase, did not significantly alter the cold-induced increase in $O_2^-\cdot$.

Thus our results strongly indicate that myocardial ROS increases in proportion to a cold-induced alteration of mitochondrial bioenergetics and suggest that generation of $O_2^-\cdot$ occurs due to increased electron leak. Moreover, we believe that reduced $O_2^-\cdot$ dismutation contributes more likely to the cold-induced rise in $O_2^-\cdot$ than does the enhanced $O_2^-\cdot$ generation. This is based on the finding that the relative increase in $O_2^-\cdot$ induced by cold alone could be increased by ETS inhibitors menadione and NaN_3 and chemically decreased by NO· and MnTBAP but neither by the temperature-dependent scavenger enzyme like catalase or glutathione, a substrate for glutathione peroxidase. In addition, there was no appreciable difference in response if these drugs were given before or during cold.

Increased ROS detection during cold perfusion in the isolated heart. Mild (27°C) to moderate (17°C) hypothermia decreases mechanical function, metabolic needs, and enzymatic function (47). Most enzyme activity decreases 50% for each 10°C fall in temperature (4); therefore, even at 17°C, $M\dot{V}O_2$ is maintained at ∼25% of that at 37°C (47). Hypothermia also increases intracellular Ca^{2+} in part by slowing Na^+-K^+-ATPase pump activity (23); this leads to Na^+ accumulation and activation of the reverse-mode Na^+/Ca^{2+} exchanger

Table 1. *Effects of BDM, menadione, MnTBAP, and L-NAME and cold perfusion on mechanical and metabolic function during warm (37°C) and cold (17°C) KR crystalloid perfusion*

Treatment	HR	sysLVP	diaLVP	$dLVP/dt_{max}$	$dLVP/dt_{min}$	CF	$M\dot{V}O_2$	%O_2 Extraction
Control (37°C)	240±4	95±3	1±0.4	3,106±183	−2,536±123	9.0±0.6	120±7	76±2
BDM (37°C)	198±4*	16±3*	6±1*	413±95*	−238±54*	8.6±0.6	80±9*	47±5*
Menadione (37°C)	216±5*	71±4*	2±0.4	2,240±212*	−1,868±181*	8.8±0.6	126±11	80±2
MnTBAP (37°C)	227±4	88±3	1±0.4	2,910±307	−2,473±236	6.8±0.6*	97±7*	81±3
L-NAME (37°C)	218±7*	88±3	1±0.4	2,730±294	−2,444±266	7.4±0.6*	89±7*	73±3
Control (17°C)	30±1*	76±6*	15±3*	373±36*	−288±55*	6.9±0.5*	44±2*	36±2*

Values are means ± SE. KR, Krebs-Ringer; BDM, 2,3-butanedione monoxime (10 mM); MnTBAP, manganese (III) tetrakis (4-benzoic acid)porphyrin chloride (10 μM); L-NAME, N^G-nitro-L-arginine methyl ester (100 μM); HR, heart rate (beats/min); sys- and diaLVP, systolic and diastolic left ventricular pressure, respectively (mmHg); $dLVP/dt_{max}$, maximum rate of contractility (mmHg/s); $dLVP/dt_{min}$, maximum rate of relaxation (mmHg/s); CF, coronary flow (ml·g^{-1}·min^{-1}); $M\dot{V}O_2$, myocardial O_2 consumption (μl·g^{-1}·min^{-1}). *$P < 0.05$, drug vs. control.

(1, 8, 46, 52). We have shown previously that cardiac hypothermia increases cytosolic [Ca^{2+}] (8–10, 47, 48). In support of a mitochondrial basis for hypothermia-induced oxidant stress leading to ROS generation, we have shown that cold perfusion also increases mCa^{2+} and NADH (40). Others have shown that hypothermia depolarizes mitochondrial membrane potential ($\Delta\Psi_m$) (44).

We used two fluorescent probes with different spectral characteristics and specificities to monitor online and offline changes in ROS signals in the heart subject to cold temperatures. Given that $ONOO^-$ is formed from NO· and O_2^-·, inhibition of either NO· or O_2^-· generation should attenuate the increase in $ONOO^-$ observed during cold perfusion. To reinforce our findings that both reactive species are present during cold perfusion, we used the NOS inhibitor L-NAME and the intracellular SOD mimetic MnTBAP (11) to demonstrate that the cold-induced increase in $ONOO^-$ fluorescence, measured either online or offline, is markedly attenuated by these agents. However, the O_2^-· signal indicated by DHE fluorescence was completely blocked by MnTBAP, whereas the O_2^-· signal was not blocked by L-NAME during either warm or cold perfusion. Moreover, menadione and NaN_3 enhanced the hypothermia-induced increase in O_2^-·; this suggests that it is the O_2^-· radical that is generated at mitochondrial sites along the ETS during hypothermia.

Despite our finding that hypothermia enhances ROS detection, it is well known that the hypothermic heart is much better protected against ischemia-reperfusion injury (8–10, 48). We did not examine in this study how hypothermic ischemia, per se, alters ROS production, but we reported recently that ROS formation is reduced during cold versus warm ischemia and reperfusion (40). Other studies have suggested that ROS play a role in cold-induced cellular injury. Rauen et al. (35–37) showed that cultured rat hepatocytes or endothelial cells incubated in 4°C buffer were injured under normoxic conditions and protected under hypoxic conditions; this injury was largely decreased when cells were incubated with one of several ROS scavengers. Cold aerobic perfusion (5–7°C) of the intact rat heart with Tyrode solution deteriorated electrical and mechanical function and increased lipid peroxidation; this was prevented when deferoxamine, a ROS scavenger, was added to the cold perfusate (27). A lazaroid scavenger was also found to decrease injury caused by lipid peroxidation in endothelial cells at 4°C (21). Our results in the intact, perfused heart demonstrate, unequivocally, that increased amounts of O_2^-· are detected during hypothermia and that this O_2^-· can be adequately dismutated and/or scavenged by exogenous means.

Because NO· is also a free radical, it can modify downstream radicals formed from a greater amount of O_2^-· available during cold perfusion. The NO· present during cold perfusion may originate from several sources. A well-known source is vascular endothelial cells, but studies also indicate the presence of inducible NOS and endothelial NOS in the cardiomyocytes (22, 43) and, in particular, in mitochondria (17, 32, 34). Mitochondrial NO· is proposed to modulate consumption of O_2 to H_2O by attenuating complex I and IV function and thereby enhancing ROS generation (32, 33). Regardless of the source of NO·, our experiments with L-NAME show that NO· either continues to be generated or remains in abundance during hypothermia to react in a 1:1 stoichiometry with O_2^-· to generate $ONOO^-$.

Possible sources and causes of enhanced O_2^-· during hypothermia in the isolated heart. ROS are continuously formed in tissue as byproducts of a variety of metabolic pathways including redox cycling in the ETS by complex I and III and also by NAD(P)H oxidases (45, 53) in vascular smooth muscle cells (18) and in cardiac myoctyes (14). One nonmitochondrial metabolic pathway known to cause O_2^-· generation is the hypoxanthine-xanthine oxidase pathway. Hypothermic perfusion is unlikely to enhance adenosine and hypoxanthine concentrations to drive this pathway and produce O_2^-·. Indeed, our experiments showed that neither allopurinol nor oxypurinol alone nor GSH with or without catalase attenuated the cold-induced increase in O_2^-·. Moreover, the menadione-induced increase in O_2^-· during cold perfusion was blocked by MnTBAP but not by allopurinol or oxypurinol or by GSH with or without catalase.

Why would hypothermia result in impaired mitochondrial bioenergetics? Any alteration in the balance between the generation and removal of ROS is considered an oxidative stress. The steady-state reduction of NAD^+ to NADH is normally a balance between delivery of reducing equivalents from substrates and the rate of dissipation of the mitochondrial membrane gradient by ATP synthase (complex V). This balance is likely altered during hypothermia due to O_2^-·, and therefore its reactants are generated in increased amounts. Indeed, it is well known that hypothermia slows mitochondrial oxidative phosphorylation (12), although it has not been well recognized that this could lead to impairment of electron transport through mitochondrial oxidases and thereby allow electron leak and reduction of molecular O_2 to O_2^-·. Interestingly, it was reported that the rate of mitochondrial ROS generation is inversely proportional to the rate of electron transport, increasing when ATP requirement declines or when components of the ETS are inhibited (7).

Modulation of O_2^-· generated by reduced scavenging activity during hypothermia. Our recent article (40) shows that hypothermia increases mCa^{2+} and NADH; this is associated with cold-induced increases in cytosolic Ca^{2+} in the intact heart (8–10, 48). It is likely that hypothermia not only increases O_2^-· generation but also decreases removal of O_2^-· because of the slowed activity of enzymes responsible for scavenging O_2^-·. Our study suggests that a large component of the increase in O_2^-· detected is due to reduced enzyme activity for the following reasons: If the mitochondrial matrix and cellular activities of SOD at 17°C are reduced to 25% of that at 37°C based on the Q_{10} principle, then MnTBAP, a nonenzyme, and NO· remain capable of scavenging most of the O_2^-· generated. Also, the added increase in O_2^-· generated by menadione and NaN_3 during hypothermia can be abolished by MnTBAP (unpublished observations). These online myocardial fluorescence techniques, as well as our offline effluent fluorescence techniques (29), provide unique methods to show mitochondrial dysfunction during cold and support our results that O_2^-· is increased by hypothermia.

Electron leakage from mitochondrial ETS is likely the major source of ROS generation in nonphagocytic cells. This has been shown traditionally by simple blockage of the electron transport at complex I or III by respiratory inhibitors such as rotenone or antimycin A, which enhance production of O_2^-· (24, 31). Because of their toxicity and irreversible damage, rotenone and antimycin A are not suitable for examining mitochondrial function in intact perfused hearts (preliminary studies). However, enhanced $O_2^-\cdot$ generation during hypothermia was clearly evidenced by the markedly reduced level of $O_2^-\cdot$ when MnTBAP was given before or during cold perfusion. MnTBAP is believed to penetrate into the mitochondrial matrix to dismutate $O_2^-\cdot$ because MnTBAP substituted well for SOD in a mitochondrial SOD knockout mouse model (16). MnTBAP is also effective in reducing oxidant-induced injury (11). We showed recently the efficacy of MnTBAP to dismutate/scavenge $O_2^-\cdot$ generated during ischemic preconditioning (IPC) pulses; this effect led to a greater increase in $O_2^-\cdot$ generation during later ischemia and reperfusion and reversal of the protection afforded by IPC (20).

BDM reversibly depresses muscle contractility in part by reducing myofibrillar sensitivity to Ca^{2+} and by inhibiting actomyosin ATPase and oxidative phosphorylation, as demonstrated in the isolated guinea pig heart (19). It is thought that BDM attenuates oxidative phosphorylation in part by slowing ETS at complex I (42); this effect was associated with increased NADH, particularly when perfusate Ca^{2+} was elevated. In preliminary experiments, however, we did not observe an additional change in NADH with 10 mM BDM during 17°C perfusion. Moreover, it was interesting that BDM attenuated $ONOO^-$ production but did not alter $O_2^-\cdot$ levels. Thus we speculate that BDM could reduce NOS activity directly or, by chelating Ca^{2+}, reduce Ca^{2+}-dependent NOS activity.

Menadione is a quinone that may undergo a one- or two-electron reduction (15). One-electron reduction of menadione catalyzed by NADH-ubiquinone oxidoreductase results in the formation of the semiquinone radical (14), which interacts with O_2 to form $O_2^-\cdot$ and H_2O_2 (15, 41). By accepting electrons from the NADH-ubiquinone complex, menadione may prevent the formation of ubisemiquinone, the reduced intermediate of the respiratory chain, and in this way increase mitochondrial ROS generation. Menadione was perfused to test whether the source of cold perfusion-induced $O_2^-\cdot$ generation was the mitochondrion. We found that perfusion of menadione at 37°C slightly increased the basal $O_2^-\cdot$ and $ONOO^-$ signals but markedly increased these signals during perfusion at 17°C. These findings corroborate the menadione-induced $O_2^-\cdot$ generation and also support our contention that cold perfusion may in part disrupt electron flow along the ETS and thus triggers ROS production. In addition to disrupting the ETS to make ROS, menadione (49) and cold perfusion (50) deplete the antioxidant GSH that may lead to ROS accumulation. However, in our study, GSH perfusion did not alter cold or menadione-induced increase in ROS signal detection (data not shown).

The mitochondrial source of $O_2^-\cdot$ was further supported by perfusion of the complex IV inhibitor NaN_3. NaN_3 binds to the Fe^{2+} of the heme prosthetic group and blocks the transfer of one electron to O_2. This blockade leads to sustained chemical reduction of the upstream components of the respiratory chain (5, 30), and the accumulated electrons univalently reduce O_2 to $O_2^-\cdot$. We showed that warm perfusion of NaN_3 caused an increase in $O_2^-\cdot$, a finding consistent with those reported by others (30). The additional increase in $O_2^-\cdot$ by NaN_3 during cold perfusion also suggests that hypothermia reduces ETS enzyme activity including at complex IV. Coupled with a cold-induced decrease in scavenging activity, this may lead to a further increase in the $O_2^-\cdot$ detected.

Possible limitations. It would be difficult to state with certainty the specific ROS involved in hypothermia using one probe, so we used two detection techniques. Both probes gave similar results. The detection of diTyr indeed confirmed the detection of $O_2^-\cdot$ by DHE during cold perfusion. Most investigators (6, 20) support the notion of the relative specificity of the DHE probe for $O_2^-\cdot$ detection; furthermore, we observe (not reported) in preliminary studies in isolated hearts that the DHE probe is insensitive to the administration of H_2O_2. A possible change in the binding affinity of the probes with a reduction in temperature could not be assessed. Thus only relative changes in DHE and diTyr signals were monitored and reported in this study. Nonetheless, experiments using the probes in cell-free cuvettes and in L-tyrosine- or DHE-loaded but exanimate, hearts showed that the signals did not change between 37°C and 3°C (data not shown). These findings suggest that the change in ROS signal during cold stress is a function of a specific reaction between the probes and living heart tissue and not due to the probes per se.

In summary, these studies show that ROS ($O_2^-\cdot$) and reactants ($ONOO^-$) are generated in crystalloid-perfused hearts during cold perfusion. The magnitude of ROS production is inversely proportional to temperature. MnTBAP blockade of the DHE signal confirms that $O_2^-\cdot$ is elevated moderately during cold perfusion, and attenuation of the diTyr signal by both MnTBAP and L-NAME confirms the increased availability of $O_2^-\cdot$ and its reaction with NO· to generate $ONOO^-$. The additional increase in cold-induced ROS generation by menadione and NaN_3 confirms that cold perfusion generates $O_2^-\cdot$ and that functional disruption of the mitochondrial ETS enzyme complexes coupled with reduced enzyme-scavenging efficiency during hypothermia are likely responsible for the temperature-dependent increases in $O_2^-\cdot$. From a clinical stand point it will be important to know whether it is possible to reduce ROS generation and to enhance $O_2^-\cdot$ scavenging during cold perfusion, therapies that may consequently lead to reduced ROS during subsequent cold ischemia and warm reperfusion to improve function and reduce cellular injury.

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