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Original Contributions

Hydrogen peroxide generation by mitochondria isolated from regionally ischemic and nonischemic dog myocardium

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Summary: We occluded the left anterior descending coronary artery of anesthetized, open-chest dogs, for 1 or 2 h. Some hearts were reperfused for 1 h after 1 h of ischemia. We isolated mitochondria from the central ischemic zone (CIZ) and a surrounding nonischemic zone (NIZ) of the left ventricle, and assayed H_2O_2 production using a horseradish peroxidase-dual wavelength spectrophotometric technique. Mitochondria, studied in the absence of exogenous respiratory chain inhibitors, generated H_2O_2 during State 4 respiration with succinate as the substrate. NIZ mitochondria in all groups produced ca. 1.5 nmols H_2O_2/min/mg protein (no significant differences between groups). The State 4 O_2 consumption rates of NIZ mitochondria from hearts subjected to 1 h ischemia plus reperfusion, or 2 h of ischemia (ca. 30 nmols/min/mg) were significantly higher than that of NIZ mitochondria of hearts subjected to only 1 h of ischemia (23 nmols/min/mg). Thus, the ratio between H_2O_2 produced and State 4 O_2 consumption fell from 6.5% to 5%. Mitochondria from all CIZ samples had State 4 O_2 consumption rates that were not different from corresponding NIZ values. However CIZ mitochondria of hearts subjected to 1 h ischemia without reperfusion produced less H_2O_2 (1.1 ± 0.1 nmols/min/mg), and had a slightly reduced H_2O_2/O_2 ratio (4.4 ± 0.7%), compared with their NIZ samples (1.5 ± 0.1 nmols/min/mg; 5.3%). Reperfusion after 1 h of ischemia abolished these regional differences. The CIZ mitochondria from hearts subjected to 2 h ischemia produced only 0.75 ± 0.22 nmols H_2O_2/min/mg (2.5% of State 4 O_2 consumption). These values were 50% of corresponding NIZ values, and were significantly less than for any other group or tissue region. If similar phenomena occur in conscious animals subjected to incomplete regional ischemia, especially of relatively brief duration or if accompanied by reduced intracellular defenses against oxidants such as H_2O_2, they suggest that mitochondria persist as H_2O_2 sources and so may contribute to the oxidant load and myocardial dysfunction.

Key words: heart; hydrogen peroxide; ischemia (regional); mitochondria; reperfusion

Introduction

Many studies implicate univalent reduction products of molecular oxygen – particularly superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) – as contributors to myocardial hypoxic or ischemic damage. Neutrophils are one source of oxidants in blood-perfused hearts (32), but administration of drugs or enzymes that affect oxygen radical generation or reactivity (17, 37, 38, 44) can reduce ischemic or hypoxic damage to hearts studied under asanguineous conditions. This suggests that the myocardium

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has endogenous sources of damaging oxygen metabolites. One potential endogenous source is xanthine oxidase (8, 34). However, this enzyme is absent, or at least is minimally active, in rabbit (10, 20, 21) and human (11, 19) hearts.

Mitochondria normally produce small amounts of H_2O_2 (4, 9, 16, 29, 48), largely via dismutation of $O_2^-\cdot$, and these organelles are ubiquitous. Although H_2O_2 is not a free radical, studies with isolated hearts (36–38) suggest that it contributes importantly to ischemic or hypoxic damage, whether directly or through its reaction with $O_2^-\cdot$ to form ·OH. Previously (45), we found that mitochondria isolated from rabbit hearts that were made globally and completely ischemic continued to generate H_2O_2, although at rates much lower than those obtained with mitochondria isolated from nonischemic controls. In this study we assessed the effects of 1 or 2 h of regional ischemia (induced by left anterior descending coronary artery ligation in dogs) on mitochondrial H_2O_2 generation. Regional ischemia is generally associated with a reduction of local blood flow and oxygen delivery that is not complete, yet is sufficient to cause contractile dysfunction. Based on this assumption, we assessed the hypothesis that regional ischemia would be accompanied by lesser reductions of mitochondrial H_2O_2 generation than those measured with complete, global ischemia. The methods used allowed simultaneous isolation and study of mitochondria from both ischemic and nonischemic zones of each heart. The data we obtained, especially with regional ischemia lasting no more than 1 h, support the hypothesis.

Materials and methods

Regional ischemia

Healthy mongrel dogs weighing approximately 25 kg were anesthetized with an intravenous injection of 30 mg/kg sodium pentobarbital, then intubated and ventilated with room air using a Harvard respirator. The chest was opened and the left anterior descending (LAD) coronary artery was occluded with a 2-0 silk ligature. Seven dogs were subjected to 1 h of LAD occlusion, at which time the hearts were excised and sampled as discussed below. Eight others were subjected to 1 h of ischemia followed by 1 h of reperfusion, caused by releasing the ligature. Seven more dogs were subjected to 2 h of LAD occlusion without reperfusion.

At the end of each experiment the hearts were excised quickly and samples of the central ischemic zone (CIZ; the area rendered cyanotic by occlusion) and a distant nonischemic zone (NIZ; perfused by the circumflex artery) were removed and placed in 4 °C bath of 0.9 % NaCl for further processing.

Mitochondrial isolation

Samples from the NIZ and CIZ were processed and analyzed simultaneously. All of the following preparative steps were performed at 4 °C. The tissue was quickly rinsed in fresh 0.9 % NaCl to remove blood, then placed in a beaker of homogenizing medium (0.18 M KCl, 10 mM Tris-EDTA, 0.5 % w/v bovine serum albumin; pH 7.4 at 4 °C). Fat, connective tissue, large blood vessels, and a few millimeters of epicardial and endocardial surfaces were removed with scissors, and the remaining tissue was weighed wet. The samples were minced finely with scissors, transferred to separate centrifuge tubes, and additional homogenizing medium (4 ml/g wet tissue weight) was added. The mince was homogenized with three 5-s bursts of a Tekmar Tissuemizer (Tekmar Instruments, Cincinnati, Ohio, USA). Mitochondria were isolated by differential centrifugation as described previously (45). Isolation did not involve limited protease digestion because our earlier work (45) showed that either protocol yielded mitochondria with qualitatively similar O_2 consumption and H_2O_2 generation values as affected by ischemia.

The final mitochondrial preparation was resuspended in a medium identical to that used for isolation except for omission of EDTA. Protein concentration in the suspension was assayed (Biuret) and the amount of protein contributed by the albumin in the medium, measured in mitochondria-free blanks, was subtracted. Additional EDTA-free medium was added to give a final mitochondrial protein concentration of 10 mg/ml. This preparation was placed on ice and assayed immediately.

Mitochondrial oxygen consumption

Oxygen consumption was measured polarographically (Gilson Oxygraph, Clark Electrode) in a medium containing final concentrations of 250 mM sucrose, 15 mM morpholinopropane sulfonic acid (pH 7.4), 5 mM K_2HPO_4 and 0.1 mM Tris-EDTA (30 °C). Mitochondrial protein concentration was 0.5 mg/ml in a total cuvette volume of 1.725 ml. Substrates used were Tris salts of glutamate, malate (3 mM each, studied alone or in combination), and succinate (10 mM). State 4 respiratory rates were measured for approximately 1.5 min, at which time Na_2ADP (310 µM final concentration; determined spectrophotometrically in stock solutions using the extinction coefficient) was added to induce State 3 respiration. When glutamate and/or malate were used as substrates, a second ADP addition was made after the mitochondria returned to stable State 4 respiration. ADP was added only once when succinate was the substrate.

Respiratory rates are expressed as natoms O or nmols O_2/min per mg of mitochondrial protein. Oxygen solubility in the medium was corrected for temperature and ambient barometric pressure. Respiratory control ratios (RCR) were calculated as the quotient of the State 3 respiratory rate and the subsequent State 4 rate. The ADP:O ratio was calculated from the amount of ADP added and the amount of oxygen consumed during the State $4 \rightarrow 3 \rightarrow 4$ transition.

Calibration and measurement of hydrogen peroxide generation

Glucose plus glucose oxidase, in the absence of mitochondria, was used to generate H_2O_2 for calibrating subsequent assays of mitochondrial H_2O_2 production (49). The medium described above was supplemented with 1 mM glucose, 13 units/ml horseradish peroxidase (HRP; Sigma Type VI), and 0.5–3.2 units/ml of glucose oxidase (Sigma Type X). Oxygen consumption during this calibration was measured polarographically with a vibrating platinum electrode (SLM-Aminco, Urbana, Illinois, USA). This electrode was used instead of the Clark electrode because it was small enough to fit into the spectrophotometer (SLM-Aminco DW-2c) cuvette to be used for measuring H_2O_2 production. The same media were used to quantify formation of an H_2O_2-HRP complex using dual-wavelength spectrophotometry (417–402 nm; extinction coefficient of the complex, 50 $mM^{-1}cm^{-1}$). Prior comparison (45) of oxygen consumption and absorbance changes using these methods and the glucose-glucose oxidase system indicated that the assay detected 69 ± 1 % of H_2O_2 formed. This value was used to correct measurements of mitochondrial H_2O_2 formation, assessed in the presence of 0.5 mg/ml mitochondrial protein and 13 U/ml HRP. Oxygen consumption was measured simultaneously. The HRP did not affect mitochondrial function. We did not assess the effect of exogenous respiratory chain inhibitors such as antimycin or rotenone.

Cytochrome $a+a_3$ assay

Cytochrome activity was determined using the method of Williams (51).

Statistics

Data from each assay condition from each heart sample were obtained, at the least, in duplicate. Values reported are arithmetic means ± one standard error of the mean (SEM). Data for mitochondria isolated from nonischemic and ischemic zones of the same heart were compared using paired *t*-tests. Between-group differences were analyzed first using analysis of variance (ANOVA). If ANOVA revealed a statistically significant difference ($p < 0.05$), Student's *t*-tests with a Bonferroni correction (to account for multiple comparisons) were used to compare data between groups.

Results

Mitochondrial protein yields and cytochrome activities

Table 1 summarizes the yields and cytochrome $a+a_3$ activities of mitochondria isolated from NIZ and CIZ left ventricular tissue in each of the three groups. The pooled mean yield for NIZ mitochondria was 5.1 ± 0.4 mg protein/g wet weight (no significant between-group differences, ANOVA). Mitochondrial yields from the CIZ samples were less than those

Table 1. Yields and cytochrome a + a_3 activities of mitochondria from regionally ischemic or ischemic reperfused canine hearts.

Group	Yield (mg protein/g ventricle wet weight)	Cytochrome a + a_3 (nmols/mg protein)
1-h ischemia, no reperfusion		
NIZ	4.17 ± 0.32	0.53 ± 0.05
CIZ	3.16 ± 0.43	0.59 ± 0.05
1-h ischemia plus 1-h reperfusion		
NIZ	5.94 ± 0.69	0.70 ± 0.04
CIZ	3.78 ± 0.43*	0.81 ± 0.05
2-h ischemia, no reperfusion		
NIZ	5.19 ± 0.91	0.66 ± 0.05
CIZ	3.53 ± 0.36	0.65 ± 0.09
Overall group means for NIZ samples	5.10 ± 0.38	0.62 ± 0.03

(NIZ = nonischemic zone of left ventricle; CIZ = central ischemic zone.)
* = Significant difference ($p < 0.05$) from value in NIZ samples in same group, based on paired *t*-tests.

Table 2. Oxidative phosphorylation parameters (glutamate plus malate as substrates) of mitochondria isolated from regionally ischemic or ischemic reperfused canine hearts.

Group	Oxygen consumption (nAtoms O/min/mg) State 3	State 4	Respiratory control ratio	ADP:O ratio
1-h ischemia, no reperfusion				
NIZ	231 ± 11	14 ± 2	19.5 ± 3.3	2.87 ± 0.05
CIZ	185 ± 15*	16 ± 3	13.8 ± 3.1*	2.81 ± 0.06
1-h ischemia plus 1-h reperfusion				
NIZ	225 ± 8	28 ± 2†	9.3 ± 1.3†	2.81 ± 0.06
CIZ	215 ± 30	27 ± 2†	8.4 ± 1.2†	2.71 ± 0.08
2-h ischemia, no reperfusion				
NIZ	248 ± 31	26 ± 5†	11.6 ± 2.0†	2.61 ± 0.07†
CIZ	151 ± 27*	24 ± 4†	8.5 ± 2.3*, †	2.39 ± 0.15†
Overall group means for NIZ samples	234 ± 11	23 ± 2#	13.3 ± 1.6#	2.77 ± 0.4#

(NIZ = nonischemic zone of left ventricle; CIZ = central ischemic zone.)
* Significantly different ($p < 0.05$) from corresponding value for NIZ samples in same group, based on paired *t*-tests.
† Significantly different ($p < 0.05$) from corresponding NIZ or CIZ value in 1-h ischemia no reperfusion group, based on nonpaired *t*-tests corrected for repeated measures.
# Significant difference ($p < 0.05$) was detected among values in NIZ samples, based on analysis of variance.

Table 3. Oxidative phosphorylation parameters (succinate as substrate) of mitochondria isolated from regionally ischemic or ischemic-reperfused canine hearts.

Group	Oxygen consumption (nAtoms O/min/mg) State 3	State 4	Respiratory control ratio	ADP:O ratio
1-h ischemia, no reperfusion				
NIZ	248 ± 18	40 ± 3	6.6 ± 0.7	1.66 ± 0.05
CIZ	201 ± 16*	45 ± 3	4.8 ± 0.6*	1.58 ± 0.02
1-h ischemia plus 1-h reperfusion				
NIZ	257 ± 16	56 ± 3†	4.7 ± 0.3†	1.60 ± 0.03
CIZ	225 ± 34	56 ± 4†	4.0 ± 0.4	1.48 ± 0.08
2-h ischemia, no reperfusion				
NIZ	255 ± 22	52 ± 4†	4.7 ± 0.3†	1.52 ± 0.04
CIZ	173 ± 29*	51 ± 6	3.3 ± 0.3*, †	1.33 ± 0.08*
Overall group means for NIZ samples				
	254 ± 10	49 ± 2#	5.3 ± 0.3#	1.59 ± 0.3

(NIZ = nonischemic zone of left ventricle; CIZ = central ischemic zone.)

* Significantly different ($p < 0.05$) from corresponding value for NIZ samples in same group, based on paired *t*-tests.

† Significantly different ($p < 0.05$) from corresponding NIZ or CIZ value in 1-h ischemia, no reperfusion group, based on nonpaired *t*-tests corrected for repeated measures.

Significant difference ($p < 0.05$) was detected among values in NIZ samples, based on analysis of variance.

from corresponding nonischemic samples, but the difference was statistically significant ($p < 0.05$, paired *t*-tests) only for hearts subjected to 1 h of ischemia plus reperfusion. The pooled mean value for mitochondrial cytochrome $a+a_3$ activity was 0.62 ± 0.03 nmols/mg protein, with no statistically significant differences between values for any group or between ischemic and nonischemic zones.

Mitochondrial oxygen consumption, respiratory control, and ADP:O ratios

Table 2 summarizes mitochondrial oxygen consumption rates, and the respiratory control and ADP:O ratios, obtained when glutamate plus malate were used as substrates. Table 3 shows comparable data for succinate.

We observed several trends in mitochondrial performance within and across tissue regions of hearts subjected to the various protocols. Mitochondria isolated from the NIZ of hearts subjected to only 1 h of ischemia were tightly coupled. With glutamate plus malate, for example, the mean RCR was 19.5 ± 3.3. In this group, and with either substrate(s) used, the State 3 oxygen consumption rates of CIZ mitochondria were significantly lower ($p < 0.05$, paired *t*-tests) than those of NIZ mitochondria. This accounted for significantly lower RCR values. Reperfusion after 1 h of ischemia reduced RC ratios further, compared with data obtained from hearts not reperfused after the same ischemia duration. This was because State 4 oxygen consumption rates increased to values that were significantly greater than those obtained with mitochondria in the 1 h ischemia-no reperfusion group. State 3 rates were not different between the same tissue regions of these two groups.

The increased State 4 rates measured with mitochondria of hearts reperfused after 1 h of ischemia persisted in hearts made ischemic for 2 h but not reperfused. In the latter group,

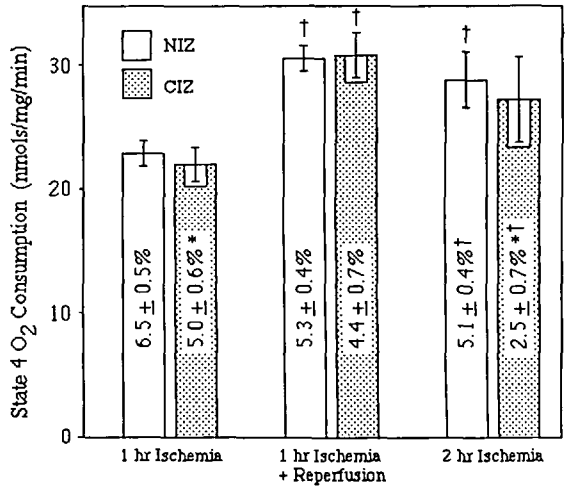

Fig. 1. Succinate-supported, State 4 oxygen consumption by mitochondria isolated from nonischemic zones (NIZ) and central ischemic zones (CIZ) of the three groups. Bar height indicates arithmetic mean ± 1 SEM. Units are nmols O_2 consumed/min per mg protein, rather than nAtoms O (cf. Table 3), to facilitate comparison with H_2O_2 production data in Fig. 2. Rates shown here are not precisely one-half the corresponding values in Table 3 because oxygen consumption during the assay of H_2O_2 production was measured by a slightly different method (see text). Numbers inside each bar indicate the ratio (mean ± 1 SEM) H_2O_2 produced/oxygen consumed, expressed as a percent. Note that within a group, mitochondrial oxygen consumption rates in the two tissue zones did not differ. Symbols: † significantly different ($p < 0.05$, *t*-tests adjusted for repeated measures) from value measured in same zone of hearts subjected to 1-h ischemia, no reperfusion. * Significantly different ($p < 0.05$, paired *t*-tests) from NIZ data in same group.

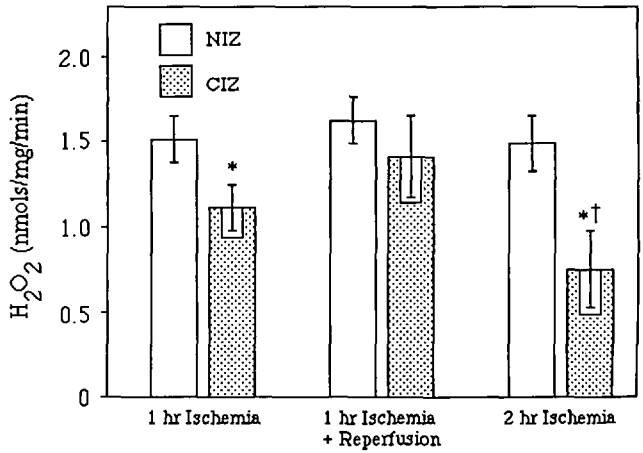

Fig. 2. Hydrogen peroxide production by mitochondria isolated from nonischemic zones (NIZ) and central ischemic zones (CIZ) of the three groups. Symbols: † significantly different ($p < 0.05$, *t*-test) from value measured in same zone of hearts subjected to 1-h ischemia, no reperfusion. * Significantly different ($p < 0.05$, paired *t*-tests) from NIZ data in same group.

however, CIZ mitochondrial State 3 oxygen consumption rates were significantly lower than those of the NIZ mitochondria. This, CIZ mitochondria had significantly lower RCR values. Significant changes of ADP:O ratios were detected only with mitochondria isolated from hearts made ischemic for 2 h.

Mitochondrial H_2O_2 production

Under the conditions we used, mitochondrial H_2O_2 generation was detected only in the presence of succinate and only under State 4 conditions (i.e., before the addition of ADP). Adding ADP caused rapid disappearance of the spectral complex, based on the absorbance change.

Figure 1 summarizes data for succinate-linked State 4 oxygen consumption rates, and its percentage relationship to H_2O_2 production. Figure 2 summarizes the rates of H_2O_2 production. Oxygen consumption data in Fig. 1 are expressed in nmols/O_2/min/mg, rather than units reported in the Tables, to facilitate comparison with units for H_2O_2 production shown in Fig. 2. When expressed in the same units, data for succinate-supported oxygen consumption measured with the platinum electrode during H_2O_2 assays were qualitatively and quantitatively similar to those obtained with the Clark electrode used for oxidative phosphorylation studies.

Although there were no significant within-group differences of State 4 oxygen consumption by NIZ and CIZ mitochondria, in hearts that were made ischemic but not reperfused there were regional differences of H_2O_2 production rates under these conditions. When hearts were subjected to 1 h of ischemia without reperfusion, succinate-linked State 4 oxygen consumption by NIZ mitochondria was 23.1 ± 1.0 nmols/min/mg. The amount of H_2O_2 produced concomitantly was 1.52 ± 0.14 nmols/min/mg, or approximately 6.5 % of the State 4 oxygen consumption rate. Despite identical oxygen consumption rates, however, CIZ mitochondria in this group produced only 1.11 ± 0.13 nmols H_2O_2/min/mg ($p < 0.05$ vs data for NIZ samples, paired *t*-tests). Thus, the ratio between H_2O_2 produced and O_2 consumed fell.

With 1 h of ischemia plus 1 h of reperfusion, State 4 oxygen consumption for both NIZ and CIZ mitochondria was approximately 30 nmols/min/mg. These values were significantly greater than those for mitochondria from corresponding regions of hearts subjected to 1 h of ischemia without reperfusion. With reperfusion following ischemia, however, the mean rate of H_2O_2 production by NIZ mitochondria (1.61 ± 0.14 nmols/min/mg) did not differ significantly from that of CIZ mitochondria (1.41 ± 0.24 nmols/min/mg). These H_2O_2 production rates were not significantly different from values measured in corresponding tissue regions of the 1-h ischemia-no reperfusion group (nonpaired *t*-tests). With reperfusion, the ratio of H_2O_2 produced to oxygen consumed was 5.3 ± 0.4 % for NIZ mitochondria, and 4.4 ± 0.7 % for CIZ samples (not significantly different from one another or from values for the 1-h ischemia-no reperfusion group).

Prolonging ischemia duration to 2 h did not change H_2O_2 production rates by NIZ mitochondria, although the H_2O_2 produced/O_2 consumed ratio fell, compared with data from the 1-h ischemia-no reperfusion group. However 2 h of ischemia did significantly reduce H_2O_2 production by CIZ mitochondria, to 0.75 ± 0.22 nmols/min/mg. This value was roughly half that of corresponding NIZ samples in the same group, and of other samples in other groups. Such reduced H_2O_2 production by CIZ mitochondria occurred despite maintenance of State 4 oxygen consumption at rates not different from those of NIZ mitochondria from the same hearts. Thus, only 2.5 % of oxygen consumed during State 4, succinate-supported respiration, was associated with production of detectable H_2O_2 ($p < 0.05$ vs NIZ value in same group and vs NIZ or CIZ values in other groups).

Discussion

One essential functional role of the mitochondrion is to generate ATP. Our data show that 1 or 2 h of ischemia, with or without reperfusion, alters oxidative phosphorylation parameters in both ischemic and nonischemic heart regions. Although ischemic conditions and mitochondrial isolation and assay methods differ from study to study, qualitatively similar changes, including those affecting ostensibly nonischemic regions, have been reported before (7, 28, 33, 46, 52).

Mitochondria also divert a small fraction of oxygen flux through the electron transport system to form oxygen metabolites that are potentially cytotoxic. With intact mitochondria the H_2O_2 that can be detected extramitochondrially arises (5, 31) from dismutation of $O_2^{-}\cdot$, largely by Mn-superoxide dismutase (SOD) within the mitochondrial matrix. The rate of extramitochondrial H_2O_2 release reflects stoichiometrically the rate of intramitochondrial $O_2^{-}\cdot$ production (9, 48). Although there is some species-dependency (4, 47) for the in vitro conditions required to demonstrate H_2O_2 production by intact mitochondria, the dependence on the presence of succinate and on the absence of ADP (i.e., State 4 conditions) has been reported before (29, 41, 45). Ischemia, depending on its severity, is associated with depletion of cellular ADP levels and reduction of the respiratory chain components. Such changes are mimicked by State 4 conditions in vitro, and could therefore be associated with increased mitochondrial generation of activated oxygen metabolites (3, 6, 30, 41, 47), particularly once the heart is reperfused and the oxygen supply is reestablished.

With the longest duration of regional ischemia we studied, 2 h, we found that H_2O_2 formation by NIZ mitochondria was reduced by half. This large and significant change suggests that with such prolonged or severe ischemic conditions, mitochondrial H_2O_2 formation might not be an important contributor to overall tissue damage and dysfunction. In both absolute and relative terms, however, the suppression of H_2O_2 formation by mitochondria isolated from ischemic regions of canine hearts subjected to 2 h of regional ischemia was less than that observed with rabbit hearts subjected to only 1 h of global, complete (zero flow) ischemia (45). When the effects of identical ischemic times, 1 h, are compared, regional ischemia appeared to have much less of an effect on mitochondrial H_2O_2 production than did global ischemia. These differences could be due to species-related factors, even though H_2O_2 production rates by mitochondria from nonischemic samples of rabbit (45) and canine hearts (present study) did not differ. Thus, another explanation is that with regional and incomplete ischemia, collateral blood flow renders portions of the affected tissue only partially ischemic. This could provide reduced but nonetheless sufficient amounts of oxygen to support production of intermediates such as H_2O_2, and might be associated with lesser disruption of pathways responsible for H_2O_2 production or degradation. We did not measure collateral blood flow or the region-at-risk in this study. Using a similar model of proximal left anterior descending coronary artery occlusion, however, we (43) found that after 1 h of ischemia mean transmural blood flow in the CIZ averaged 0.14 ± 0.09 ml/min/g. This was 12% of preischemic values, a significant reduction, but not total cessation, of ischemic zone flow. The region-at-risk ranged from 27.8% to 37.0% of the left ventricle. Other investigators (1, 12, 18, 39, 42) using similar protocols obtained comparable data.

Of more importance, perhaps, are our data with 1 h of ischemia. Such a time is consistent with clinically encountered episodes of coronary occlusion and subsequent intervention to restore flow and salvage tissue. Compared with data for ischemia alone, reperfusion significantly increased succinate-dependent State 4 oxygen consumption rates by comparable amounts in both the ischemic and nonischemic zones. However reperfusion did not change the concomitant rate of H_2O_2 production significantly. As noted in Table 3, the increased State 4 oxygen consumption rate was not accompanied by a change of the State 3 rate. Thus, there was also evidence of reduced respiratory control.

The mechanism for increases of State 4 oxygen consumption, yet decreases of H_2O_2 formation and of the H_2O_2/O_2 consumed, may involve ischemia-induced reductions of Mn-SOD levels (2, 13, 14) or activities. Such changes were particularly apparent in mitochondria isolated from the CIZ of hearts subjected to 2 h of ischemia. In that group, for example, we found no significant differences between CIZ and NIZ succinate-supported State 4 oxygen consumption rates. However, there was a reduced H_2O_2/O_2 ratio because of reduced H_2O_2 production by NIZ mitochondria. Our findings would be consistent with such an explanation. Related to this is the possibility that a greater fraction of mitochondrially generated $O_2^{-}\cdot$ escapes enzymatic dismutation, and so may be able exert adverse effects directly or via interaction with lesser yet sufficient amounts of H_2O_2 to form hydroxyl radical.

Data from other experimental approaches support biochemical evidence favoring the postulate that mitochondria can produce H_2O_2 following ischemia. Vandeplassche et al. (50) used an in situ electron microscopic-histochemical technique, applied to portions of canine hearts subjected to regional ischemia (left anterior descending coronary artery ligation), to assess mitochondrial H_2O_2 generation by a cyanide-independent, NADH-oxidase-dependent system (40) that functions independent of the overall mitochondrial coupling state. They found evidence of increased H_2O_2 generation in areas of myocardium that were ischemically damaged but still viable, compared with data obtained from areas that were nonischemic or irreversibly damaged owing to ischemia. Such damaged but potentially viable regions are likely to be those in which respiratory chain activity is depressed, and oxidation of NADH and reduction of molecular oxygen are inhibited. Thus, one pathway for H_2O_2 production may take over when conditions are unfavorable for another, yet the tissue is still viable.

Considered alone, the ability of mitochondria to generate H_2O_2 under the conditions we imposed does not prove that they or any source of H_2O_2 contribute to or account for ischemic cardiac dysfunction. However, a simultaneously diminished capacity to degrade H_2O_2 (or $O_2^{-}\cdot$), even if generated in "normal" amounts, may strengthen the potential biologic importance of this concept. Such changes have been documented. For example, ischemia reduces mitochondrial levels or activities of SOD (2, 13, 14, 45) and glutathione peroxidase (45). There are also more generalized (cellular or cytosolic) ischemia- or hypoxia-induced losses in the myocyte content or activity of glutathione peroxidase, catalase, SOD, and other compounds with antioxidative properties (14, 17, 22, 23, 27, 35, 45). Mitochondrial function is also vulnerable to the effects of cytotoxic oxygen metabolites (15, 24–26, 41). Therefore, mitochondria may not only be sources of damaging oxidants, but also targets of their effects, regardless of from where the oxidants arise.

If the current data apply to intact hearts of conscious animals subjected to regional but incomplete ischemia, especially of relatively brief duration, they would suggest that mitochondria continue as a source of H_2O_2, and in doing so they may contribute to the myocyte's oxidant load and the resulting dysfunction.

References

1. Ambrosio G, Weisfeldt ML, Jacobus WE, Flaherty JT (1987) Evidence for a reversible oxygen radical-mediated component of reperfusion injury: reduction by recombinant human superoxide dismutase administered at the time of reflow. Circulation 75:282–291
2. Arduini A, Mezzetti A, Porreca E, Lapenna D, DeJulia J, Marzio L, Polidoro G, Cuccurullo F (1988) Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in reperfused rat heart. Biochim Biophys Acta 970:113–121
3. Baker JE, Kalyanaraman B (1989) Ischemia-induced changes in myocardial paramagnetic metabolites: Implications for intracellular oxy-radical generation. FEBS Lett 244:311–314

4. Boveris A (1977) Mitochondrial production of superoxide radical and hydrogen peroxide. In: Reivich M, Coburn R, Lahiri S, Chance B (eds) Tissue Hypoxia and Ischemia. Plenum Press, New York, pp 67–82
5. Boveris A, Cadenas E (1975) Mitochondrial production of superoxide anion and its relationship to the antimycin-insensitive respiration. FEBS Lett 54:311–314
6. Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. Biochem J 134:707–716
7. Bush LR, Shlafer M, Haack DW, Lucchesi BR (1980) Time-dependent changes in canine cardiac mitochondrial function and ultrastructure resulting from coronary occlusion and reperfusion. Basic Res Cardiol 75:555–571
8. Chambers DE, Parks DA, Patterson G, Roy R, McCord JM, Yoshida S, Parmley LF, Downey JM (1985) Xanthine oxidase as a source of free radical damage in myocardial ischemia. J Mol Cell Cardiol 17:145–152
9. Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527–605
10. Downey JM, Miura T, Eddy LJ, Mellert T, Hearse DJ (1987) Xanthine oxidase is not a source of free radicals in the ischemic rabbit heart. J Mol Cell Cardiol 19:1053–1060
11. Eddy LJ, Stewart JR, Jones HP, Engerson TD, McCord JM, Downey M (1987) Free radical-producing enzyme, xanthine oxidase, is undetectable in human hearts. Am J Physiol 253(Heart Circ Physiol):H709–H711
12. Farber NS, Pieper GM, Gross GJ (1987) Regional differences in postischemic recovery in the stunned canine myocardium. Am Heart J 114:1086–1095
13. Ferrari R, Albertini A, Curello S, Ceconi C, DiLisa F, Raddino R, Visioli O (1986) Myocardial recovery during post-ischaemic reperfusion: Effects of nifedipine, calcium and magnesium. J Mol Cell Cardiol 18:487–498
14. Ferrari R, Ceconi C, Curello S, Guarnieri C, Caldarera CM, Albertini A, Visioli O (1985) Oxygen-mediated myocardial damage during ischemia and reperfusion: Role of the cellular defences against oxygen toxicity. J Mol Cell Cardiol 17:937–946
15. Flamigni F, Guarnieri C, Toni R, Caldarera CM (1982) Effect of oxygen radicals on heart mitochondrial function in alpha-tocopherol deficient rabbits. Internat J Vit Nutr Res 52:402–406
16. Forman HJ, Boveris A (1982) Superoxide radical and hydrogen peroxide in mitochondria. In: Free radicals in biology, Vol 5. Academic Press, London, pp 65–90
17. Gauduel Y, Duvelleroy MA (1984) Role of oxygen radicals in cardiac injury due to reoxygenation. J Mol Cell Cardiol 16:459–470
18. Gross GJ, Farber NE, Hardman HF, Warltier DC (1986) Beneficial actions of superoxide dismutase and catalase in stunned myocardium of dogs. Am J Physiol 250(Heart Circ Physiol 19):H372–H377
19. Grum CM, Gallagher KP, Kirsh MM, Shlafer M (1989) Absence of detectable xanthine oxidase in human myocardium. J Mol Cell Cardiol 21:263–267
20. Grum CM, Ketai LH, Myers CL, Shlafer M (1987) Purine efflux patterns after cardiac ischemia: Relevance to allopurinol cardioprotection. Am J Physiol 252(Heart Circ Physiol 21):H368–H373
21. Grum CM, Ragsdale RA, Ketai LH, Shlafer M (1986) Absence of xanthine oxidase or xanthine dehydrogenase in the rabbit myocardium. Biochem Biophys Res Commun 141:1104–1108
22. Guarnieri C, Flamigni F, Caldarera CM (1980) Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J Mol Cell Cardiol 12:797–808
23. Guarnieri C, Flamigni F, Rossoni-Caldarera C (1979) Glutathione peroxidase activity and release of glutathione from oxygen-deficient perfused rat heart. Biochem Biophys Res Commun 89:678–684
24. Guarnieri C, Muscari C, Ceconi C, Flamigni F, Caldarera CM (1983) Effect of superoxide generation on rat heart mitochondrial pyruvate utilization. J Mol Cell Cardiol 15:859–862
25. Harris EJ, Booth R, Cooper MB (1982) The effect of superoxide generation on the ability of mitochondria to take up and retain Ca^{++}. FEBS Lett 146:267–272
26. Hillered L, Ernster L (1983) Respiratory activity of isolated rat brain mitochondria following in vitro exposure to oxygen radicals. J Cereb Blood Flow Metabol 3:207–214

27. Julicher RHM, Tijburg LBM, Sterrenberg L, Bast A, Koomen JM, Noordhoek J (1984) Decreased defense against free radicals in heart during normal reperfusion after hypoxic, ischemic and calcium-free perfusion. Life Sci 35:1281–1288
28. Kane JJ, Murphy ML, Bissett JK, deSoyza N, Doherty JE, Straub KD (1975) Mitochondrial function, oxygen extraction, epicardial S-T segment changes and tritiated digoxin distribution after reperfusion of ischemic myocardium. Am J Cardiol 36:218–224
29. Loschen G, Azzi A (1976) On the formation of hydrogen peroxide and oxygen radicals in heart mitochondria. In: Harris P, Bing RJ, Fleckenstein A (eds) Recent advances in studies on cardiac structure and metabolism, Vol 7, Biochemistry and pharmacology of myocardial hypertrophy, hypoxia, and infarction. University Park Press, Baltimore, pp 3–12
30. Loschen G, Flohe L, Chance B (1971) Respiratory chain linked H_2O_2 production in pigeon heart mitochondria. FEBS Lett 18:261–264
31. Loschen G, Azzi A, Richter C, Flohe L (1974) Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett 42:68–72
32. Lucchesi BR, Mullane KM (1986) Leukocytes and ischemia-induced myocardial injury. Ann Rev Pharmacol Toxicol 26:201–224
33. Matsubara T, Itoh K, Nanki M, Nishimura K, Kambe T, Sugiyama S, Ozawa T, Sakamoto N (1983) Relationship between regional myocardial blood flow and mitochondrial function. Basic Res Cardiol 78:706–715
34. McCord JM (1985) Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 312:159–163
35. Meerson FZ, Kagan VE, Kozlov YP, Belkina LM, Arkhipenko YV (1982) The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. Basic Res Cardiol 77:465–485
36. Miki S, Ashraf M, Salka S, Sperelakis N (1988) Myocardial dysfunction and ultrastructural alterations mediated by oxygen metabolites. J Mol Cell Cardiol 20:1009–1024
37. Myers CL, Weiss SJ, Kirsh MM, Shlafer M (1985) Involvement of hydrogen peroxide and hydroxyl radical in the "oxygen paradox": reduction of enzyme leakage by catalase, allopurinol or deferoxamine, but not by superoxide dismutase. J Mol Cell Cardiol 17:675–684
38. Myers CL, Weiss SJ, Kirsh MM, Shepard BM, Shlafer M (1986) Effects of supplementing hypothermic cardioplegia solutions with catalase, superoxide dismutase, allopurinol, or deferoxamine on functional recovery of globally-ischemic and reperfused hearts. J Thorac Cardiovasc Surg 91:281–289
39. Myers ML, Bolli R, Lekich RF, Hartley CJ, Roberts R (1985) Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. Circulation 72:915–921
40. Nohl F (1987) A novel superoxide generator in heart mitochondria. FEBS Lett 214:269–273
41. Otani H, Tanaka H, Inoue T, Umemoto M, Omoto K, Tanaka K, Sato T, Osako T, Masuda A, Nonoyama A, Kagawa T (1984) In vitro study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury. Circ Res 55:168–175
42. Przyklenk K, Kloner RA (1986) Superoxide dismutase plus catalase improve contractile function in the canine model of the "stunned myocardium". Circ Res 58:148–156
43. Schott RJ, Nao BS, McClanahan TB, Simpson PJ, Todd RF, Stirling MC, Gallagher KP (1988) Anti-Mo1 monoclonal antibody does not alter myocardial stunning. Circulation 78:II–78 (abstract)
44. Shlafer M, Kane PF, Kirsh MM (1982) Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischemic, reperfused heart. J Thorac Cardiovasc Surg 83:830–839
45. Shlafer M, Myers CL, Adkins S (1987) Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia. J Mol Cell Cardiol 19:1195–1206
46. Sugiyama S, Ozawa T, Kato T, Suzuki S (1980) Recovery time course of ventricular vulnerability after coronary reperfusion in relation to mitochondrial function in ischemic myocardium. Am Heart J 100:829–837
47. Turrens JF, McCord JM (1990) Mitochondrial generation of reactive oxygen species. In: Zelenock

GB, D'Alecy LG, Fantone JC, Shlafer M, Stanley JC (eds) Clinical ischemic syndromes: mechanisms and consequences of tissue injury. CV Mosby, St. Louis, pp 203–212
48. Turrens JF, Alexandre A, Lehninger AL (1985) Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. Arch Biochem Biophys 237:408–414
49. Turrens JF, Freeman BA, Crapo JD (1982) Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. Arch Biochem Biophys 217:411–421
50. Vandeplassche G, Hermans C, Thoné F, Borgers M (1989) Mitochondrial hydrogen peroxide generation by NADH-oxidase activity following regional myocardial ischemia in the dog. J Mol Cell Cardiol 21:383–392
51. Williams JN, Jr (1964) A method for the simultaneous quantitative estimation of cytochromes a, b, c_1 and c in mitochondria. Arch Biochem Biophys 107:537–547
52. Wood JM, Hanley HG, Entman ML, Hartley CJ, Swain JA, Busch U, Chang C-H, Lewis RM, Morgan WJ, Schwartz A (1979) Biochemical and morphological correlates of acute experimental myocardial ischemia in the dog. IV. Energy mechanisms during very early ischemia. Circ Res 44:52–61

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