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MITOCHONDRIAL GENERATION OF OXYGEN RADICALS DURING REOXYGENATION OF ISCHEMIC TISSUES

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Ischemia and reperfusion causes severe mitochondrial damage, including swelling and deposits of hydroxyapatite crystals in the mitochondrial matrix. These crystals are indicative of a massive influx of Ca^{2+} into the mitochondrial matrix occurring during reoxygenation. We have observed that mitochondria isolated from rat hearts after 90 minutes of anoxia followed by reoxygenation, show a specific inhibition in the electron transport chain between NADH dehydrogenase and ubiquinone in addition to becoming uncoupled (unable to generate ATP). This inhibition is associated with an increased H_2O_2 formation at the NADH dehydrogenase level in the presence of NADH dependent substrates. Control rat mitochondria exposed for 15 minutes to high Ca^{2+} (200 nmol/mg protein) also become uncoupled and electron transport inhibited between NADH dehydrogenase and ubiquinone, a lesion similar to that observed in post-ischemic mitochondria. This Ca^{2+}-dependent effect is time dependent and may be partially prevented by albumin, suggesting that it may be due to phospholipase A_2 activation, releasing fatty acids, leading to both inhibition of electron transport and uncoupling. Addition of arachidonic or linoleic acids to control rat heart mitochondria, inhibits electron transport between Complex I and III. These results are consistent with the following hypothesis: during ischemia, the intracellular energy content drops severely, affecting the cytoplasic concentration of ions such as Na^+ and Ca^{2+}. Upon reoxygenation, the mitochondrion is the only organelle capable of eliminating the excess cytoplasmic Ca^{2+} through an electrogenic process requiring oxygen (the low ATP concentration makes other ATP-dependent Ca^{2+} transport systems non-operational). Ca^{2+}-overload of mitochondria activates phospholipase A_2, releasing free fatty acids, leading to uncoupling and inhibition of the interactions between Complex I and III of the respiratory chain. As a consequence, the NADH-dehydrogenase becomes highly reduced, and transfers electrons directly to oxygen generating O_2^-.

KEY WORDS: Calcium, hydrogen peroxide, ischemia, mitochondria, swelling.

INTRODUCTION

The production of partially reduced oxygen molecules such as O_2^- and H_2O_2 has been associated with a variety of toxic processes affecting living matter, including oxygen toxicity, carcinogenesis and reperfusion injury.[1]

The mammalian mitochondrion was first identified as a source of H_2O_2 in the early seventies.[2,3] Further studies indicated that the release of H_2O_2 was the consequence of O_2^- dismutation occurring in the mitochondrial matrix.[4] It was later pointed out that most of the O_2^- was being generated in the ubiquinone-cytochrome *b* area[3,5-7] and also by the NADH dehydrogenase (the first component of the respiratory chain[8]). Approximately 2% of the oxygen consumed when mitochondria are incubated with

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substrates in the absence of ADP undergoes partial reduction to H_2O_2.[2] Assuming that the resting myocardium consumes approximately 5 μmoles O_2/min/g tissue, the mitochondrial production of H_2O_2 could be as high as 0.1 mM/min.

In addition to these estimates, there is increasing evidence supporting the idea that mitochondria undergo substantial oxidative stress *in vivo*, even under normal circumstances. It has been indicated that the steady state damage to mitochondrial DNA is about 15-fold that observed in nuclear DNA, assessed as accumulation of hydroxydeoxoguanosine.[9] Moreover, the enzyme MnSOD (the mitochondrial form of SOD) is induced under a variety of oxidative insults even when other antioxidant enzyme activities do not increase[10-13] suggesting that this induction may have evolved to prevent an increased mitochondrial production of oxygen radicals.

The mitochondrial production of O_2^- is a non enzymatic process, therefore, it increases linearly with oxygen concentration.[14] These reactions will be more significant as the concentration of oxygen increases (during exposure to hyperoxia or hyperbaric oxygen), or when the respiratory chain becomes inhibited leading to an increased concentration of reducing equivalents. In this case, the relative oxygen concentration in the matrix also increases, due to decreased oxygen consumption.

Direct microscopic studies indicate that mitochondria are one of the first targets of reoxygenation injury. Mitochondrial lesions include swelling and the formation of dense hydroxyapatite crystals,[15] a consequence of the uptake of large amounts of cytoplasmic Ca^{2+} during reoxygenation. Calcium transport into the mitochondrial matrix is an electrogenic process that requires mitochondrial electron transport, dissipating the energy that otherwise should be used for ATP production. If Ca^{2+} accumulates in the cytoplasm either during ischemia or in the early stages of reperfusion, upon reoxygenation most of the Ca^{2+} will be taken up by the mitochondria since under low energy conditions the excess Ca^{2+} cannot be pumped into the sarcoplasmic reticulum. The decrease in ATP during ischemia should also lead to increased cytoplasmic Na^+, since the Na^+/K^+ ATPase will cease to operate. The increased cytoplasmic Na^+ may be exchanged with Ca^{2+} through the Na^+/Ca^{2+} antiport, also contributing to an increased cytoplasmic Ca^{2+} concentration. Finally, direct damage to the sarcoplasmic reticulum and other Ca^{2+} transporters (also described as occurring during ischemia[16]) will favor the accumulation of cytoplasmic Ca^{2+}. If the cytoplasmic concentration of Ca^{2+} is high enough, mitochondria will become swollen and irreversibly damaged. This process is favored by inorganic phosphate and ADP which also accumulate during ischemia as a consequence of ATP hydrolysis.

Several results suggest that oxygen radicals are involved in the reoxygenation damage occurring after ischemia, including the fact that reperfusion injury is oxygen-dependent. It was first postulated that during ischemia the enzyme xanthine dehydrogenase was reversibly or irreversibly modified into an oxidase form, capable of transferring electrons directly to oxygen instead of NAD^+,[17] thus forming O_2^- and H_2O_2. Yet, oxygen-dependent damage still occurs in organs such as human or rabbit hearts where no xanthine oxidase has been detected.[18,19] This paper indicates that reoxygenation of ischemic rat hearts leads to an inhibition of electron flow and respiratory control in heart mitochondria, as previously reported.[20,21] These results could be mimicked by exposing control mitochondria to high Ca^{2+} (i.e. 200 nmol/mg protein) or unsaturated fatty acids. In every case, mitochondrial H_2O_2 formation was increased. Thus, heart mitochondria should be considered among the sources of intracellular H_2O_2 during reoxygenation of ischemic tissues.

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METHODS

Isolation of heart mitochondria

Heart mitochondria were isolated from either rat or rabbit hearts by differential centrifugation. The hearts were first perfused free of blood prior to homogenization, minced and homogenized by passing the tissue through a small tissue grinder. The tissue fragments were completely disrupted using a potter Elvejehm homogenizer in a buffer containing 0.23 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, 0.2% bovine serum albumin and 5 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 400 × *g* to eliminate large pieces of tissue and the mitochondria were isolated from the supernatant by centrifugation at 7000 × *g*. With this procedure mitochondria from either rat or rabbit hearts had respiratory controls between 5 and 8.

Determination of oxygen consumption and H_2O_2 generation by mitochondria isolated from control or reoxygenated hearts

Oxygen consumption was determined using a Clark electrode in a buffer containing 0.23 M mannitol, 0.07 M sucrose, 5 mM potassium phosphate, 3 mM $MgCl_2$, 0.1 mM EDTA and 30 mM Tris-HCl (pH 7.4). Hydrogen peroxide production was determined using a turbine-driven dual wavelength spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania, PA) using either cytochrome *c* peroxidase (at 419–407 nm) or horseradish peroxidase (at 417–402 nm).[2] As it has been previously pointed out[2] H_2O_2 production determined as the rate of Compound I formation with horseradish peroxidase is underestimated by 60%.[22] We used both methods in order to confirm the generation of H_2O_2 by heart mitochondria.

Cytochrome *c* peroxidase was isolated from Bakers yeast based on the procedure first described by Yonetani[23] as modified by English *et al.*[24]

RESULTS AND DISCUSSION

Figure 1 shows a scheme of the respiratory chain, indicating the sites where O_2^- is known to be formed. According to Mitchell's hypothesis (Q-cycle) cytochrome *b* cannot be reduced unless there is an oxidant to receive an electron from fully reduced Coenzyme Q. Cytochrome *b* receives one electron from the semiquinone form of ubiquinone (coenzyme Q), not from the fully reduced form. Ubisemiquinone, however, cannot be formed unless oxygen is present to accept the electrons coming from Rieske's iron sulfur protein. Under normal circumstances O_2^- can be formed by direct electron transfer from two different components in the respiratory chain: the enzyme NADH dehydrogenase[8] and ubisemiquinone.[6,7] The stoichiometry among the components of the respiratory chain in mitochondria from any organ shows a large excess (about 10-fold) of ubiquinone relative to other respiratory components (i.e. cytochromes). Although the excess reduced ubiquinone accumulating during anoxia could autoxidize immediately upon reperfusion, its reduction is actually inhibited when there is no electron flow through the cytochrome c_1-cytochrome oxidase segment (i.e. in the presence of KCN, or in cytochrome *c*-depleted mitochondria[7]). Thus, we concluded that the mitchondrial damage observed after reoxygenation of ischemic tissues must be caused by the interaction between mitochondria and another chemical species present in the cytoplasm prior to reoxygenation.

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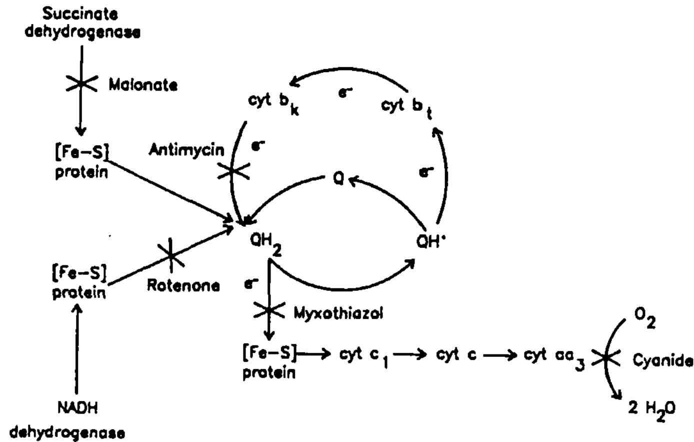

FIGURE 1 Scheme of the respiratory chain indicating the sites where inhibitors block electron transport.

Reoxygenation of ischemic hearts and Ca^{2+} overload have similar effects on mitochondrial respiration

Several years ago Scarpa and Lindsay[25] indicated that energy-dependent processes in isolated mitochondria could be preserved for longer times if nupercain was added to the mitochondrial preparations. Those authors attributed the protective effect of nupercain to the blockage of phospholipase A_2 activation by Ca^{2+}, which otherwise would have disrupted the mitochondrial inner membrane. In a more recent report Malis and Bonventre indicated that the combination of mitochondrial Ca^{2+} overload with externally generated oxygen radicals had a synergistic effect in inhibiting the respiratory chain between the NADH dehydrogenase and ubiquinone,[26,27] through a process that could involve the activation of phospholipase A_2. As an external source of oxygen radicals those authors added xanthine and xanthine oxidase, a widely used method for generating both O_2^- and H_2O_2.

Those two reports led us to propose that during ischemia, Ca^{2+} could accumulate in the cytoplasm, and be taken up by mitochondria during reoxygenation. Ca^{2+} overload could stimulate phospholipase A_2, thereby producing swelling, inhibiting the electron transport chain, stimulating the production of O_2^- at the NADH dehydrogenase level (Figure 1). The possible role of Ca^{2+} in mitochondrial disruption was tested using three different mitochondrial preparations: a) mitochondria from control rat hearts; b) mitochondria from post ischemic reoxygenated rat hearts; and c) control mitochondria exposed to large Ca^{2+} concentrations.

Mitochondria isolated from post-ischemic rat hearts had several lesions. First, the maximal rate of NADH-dependent oxygen consumption by intact rat heart mitochondria (using malate, glutamate and ADP as substrates) was significantly inhibited. Succinate-supported respiration was not affected, indicating that there was a blockage in the respiratory chain between the NADH-dehydrogenase and ubiquinone (Table I). Similar results could be obtained when control mitochondria were exposed to high Ca^{2+} (200 nmol/mg protein, Table I) suggesting that the inhibition observed in mitochondria isolated from post-ischemic tissues could be the consequence of Ca^{2+}

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TABLE I
Oxygen consumption by mitochondria isolated from control or reoxygenated ischemic rat hearts and by control heart mitochondria exposed for 20 min to 200 nmol Ca^{2+}/mg protein

	Mitochondrial Substrates					
	Malate + glutamate			Succinate		
Treatment	−ADP	+ADP	R.C.R.	−ADP	+ADP	R.C.R.
Control	29 ± 3[a]	151 ± 16	5.3 ± 0.7	47 ± 4	166 ± 7	3.6 ± 0.4
Post ischemic	23 ± 5	25 ± 5	1.1 ± 0.1	142 ± 20	160 ± 40	1.1 ± 0.1
Ca^{2+}-loaded	2.5 ± 0.5	2.7 ± 0.5	1.08 ± 0.04	102 ± 9	118 ± 7	1.2 ± 0.1

[a]The values correspond to rates of oxygen consumption in nmol/min/mg protein, as Mean ± SEM of 3 to 6 different experiments.

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overload. The respiratory control ratio in either preparation fell to 1.0 even using succinate as a substrate, indicating that these mitochondria were also completely uncoupled. Externally added NADH did not restore respiration, indicating that the inhibition was not due to NADH leakage out of the mitochondrial matrix.

It has been reported[26,27] that exogenous oxygen radicals (i.e. generated by xanthine oxidase) potentiated the inhibitory effect of high Ca^{2+}. Our results suggest that xanthine oxidase does not have to be present for the inhibition to occur, but rather Ca^{2+} at higher concentrations could lead to the same effects. When rat heart mitochondria were exposed to relatively high concentrations of Ca^{2+} (0–300 nmol/mg protein, from 0 to 0.5 mM) we found that the inhibition in both respiration and respiratory control was Ca^{2+} concentration-dependent (Figure 2). At a constant Ca^{2+} concentration the inhibition was also time-dependent, a result that was confirmed using rabbit heart mitochondria (Figure 3). Rabbit heart mitochondria, however, appeared more resistant to high Ca^{2+}. The respiratory control ratio of rabbit mito-

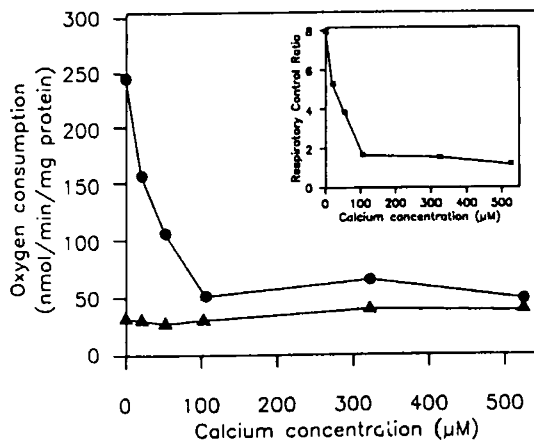

FIGURE 2 Ca^{2+} overload inhibits NADH-dependent oxygen consumption and respiratory control in intact rat heart mitochondria. Mitochondria were exposed to the indicated amounts of Ca^{2+} for 15 minutes. The mitochondria were then diluted to a final concentration of 0.2 mg/ml for monitoring oxygen consumption. Oxygen consumption was determined using 2 mM malate and 1 mM glutamate as substrates in the presence and in the absence of 50 μM ADP.

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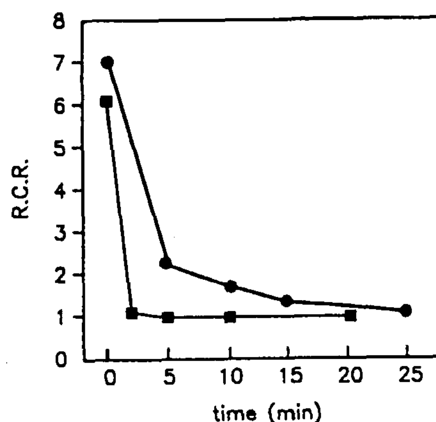

FIGURE 3 Effect of time on Ca^{2+}-dependent damage to the respiratory chain of rabbit (●) or rat (■) heart mitochondria. For these experiments mitochondria were incubated with 300 nmol Ca^{2+}/mg protein during several periods of time. Mitochondrial damage is produces a decrease in the Respiratory Control Ratio (Ratio between the rates of respiration in the presence and in the absence of ADP to phosphorylate). The experiments were carried out in the presence of 4 mM malate and 2 mM glutamate.

chondria was close to 2.0 after 5 minutes of incubation with Ca^{2+}, while rat mitochondria became completely uncoupled a minute or two after exposure to Ca^{2+} (Figure 3).

Several mitochondrial enzymes may be activated by Ca^{2+} (i.e. phospholipase A_2 and pyruvate dehydrogenase). An enzyme likely to be involved in Ca^{2+}-mediated damage to the mitochondrial membrane is phospholipase A_2, catalyzing the hydrolysis of membrane lipids. It has been reported that the inhibition of phospholipase A_2 increased the degree of coupling in isolated mitochondria.[25]

Possible role of fatty acids or fatty acid-derivatives as inhibitors of respiration

Our results indicate that Ca^{2+}-dependent uncoupling of the respiratory chain was (at least in part) caused by a non polar product since incubation of rat heart mitochondria with Ca^{2+} in the presence of bovine serum albumin partially prevented the decrease in the respiratory control ratio (Table II).

Peroxidized forms of arachidonic acid have been reported to uncouple membrane

TABLE II
NADH-dependent oxygen consumption, H_2O_2 production and R.C.R. by either control, post-ischemic, Ca^{2+}-treated or arachidonic acid-treated rat heart mitochondria (0.5 mg/ml). H_2O_2 formation was determined using cytochrome *c* peroxidase at 419–407 nm. The values represent Mean ± S.E.M.

Mitochondria	Oxygen consumption (nmol/min/mg protein)	H_2O_2 production (nmol/min/mg protein)	Respiratory Control Ratio
Control	29 ± 3	Non Detectable	6.0
Ischemic	23 ± 5	1.1 ± 0.3	1.0
Ca^{2+}-treated	2.5 ± 0.5	0.86[a]	1.0
Ca^{2+} + BSA (300 mg/ml)	12.4[a]	Non Detectable	3.0
Control + 15 µg/ml arachidonate	8.3[a]	2.2[a]	1.0

[a]These values correspond to the average of two separate experiments.

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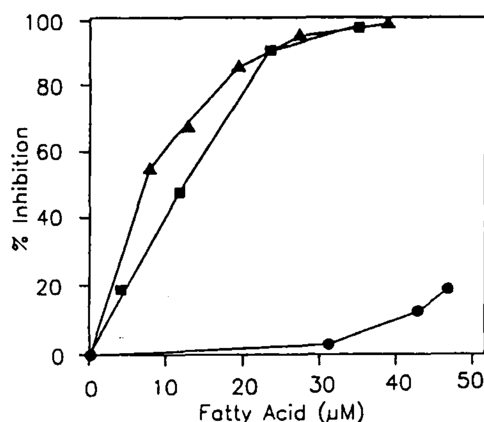

FIGURE 4 Inhibition of mitochondrial respiration by rat heart mitochondria incubated with malate and glutamate, in the presence of increasing concentrations of either arachidonic (■), linoleic (▲) or palmitic (●) acids. Respiration was determined as indicated in the legend for Figure 2.

bound ATPases,[28] suggesting that some of these products could be responsible for the mitochondrial lesions observed in either ischemic or Ca^{2+}-loaded mitochondria. Addition of either arachidonic or linoleic acids to control rat or rabbit mitochondria in the absence of added Ca^{2+} mimicked the lesions observed in mitochondria isolated from reoxygenated hearts. Unsaturated fatty acids inhibited NADH- but not succinate-dependent oxygen consumption (Figure 4). Palmitic acid did not inhibit electron transport to the same extent (Figure 4). Given the high concentrations used, it is possible that some of the oxidized products of unsaturated fatty acids may also have been responsible for mitochondrial inhibition. The inhibition cannot be attributed to a "detergent" effect of these fatty acids because the segment succinate dehydrogenase-cytochrom oxidase (involving three protein complexes) was still operating (not shown). These results suggest that Ca^{2+} accumulation may inhibit electron transport through a double mechanism including swelling and release of fatty acids through the activation of phospholipase A_2, which directly or upon oxidation, block electron flow. In addition to the inhibitory effect on oxygen consumption, fatty acids produced mitochondrial swelling (not shown) and stimulated H_2O_2 formation from isolated rat heart mitochondria (Table II).

These results indicate that both Ca^{2+} and fatty acids have similar effects on the respiratory chain, probably by causing swelling and inhibiting the collisional interaction between Complexes I and III of the respiratory chain.[29] Electron transport between the succinate dehydrogenase and Complex III is not affected, since succinate can still stimulate oxygen consumption. The interaction between the cytochrome bc_1 region and cytochrome oxidase is not affected since it depends upon cytochrome c movements in the aqueous phase of the mitochondrial inter-membrane space. As a consequence of the inhibition of electron flow in the mitochondrial respiratory chain, the enzyme NADH dehydrogenase becomes highly reduced. This enzyme is one of the primary mitochondrial sources of $O_2^{\cdot-}$, transferring electrons directly to oxygen when it is reduced.[8] Mitochondria isolated from control rat hearts did not produce any significant H_2O_2 when incubated with malate and glutamate (Table II). Both mitochondria isolated from reoxygenated ischemic hearts or control mitochondria pre-

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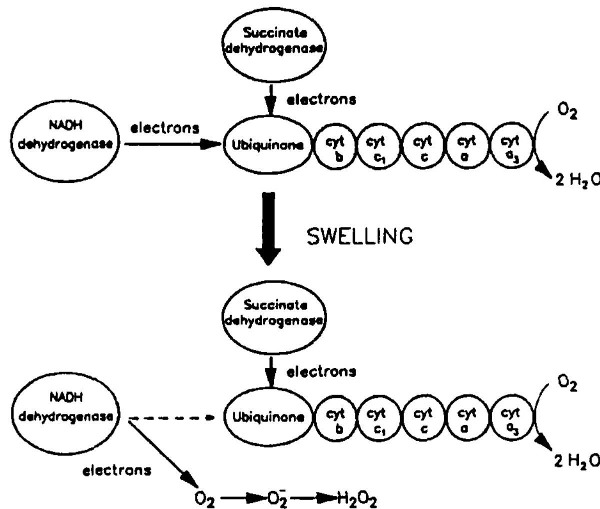

FIGURE 5 Scheme suggesting a possible mechanism for the inhibition of electron transport and stimulation of H_2O_2 production by mitochondria from post-ischemic hearts.

incubated with Ca^{2+} produced H_2O_2 due to the inhibition of electron transport (Table II). Addition of unsaturated fatty acids to control mitochondria mimic the effect of Ca^{2+} overload or ischemia and reperfusion as they too inhibit respiration. All these results point to a common mechanism (perhaps just swelling) by which electrons leak out at the NADH dehydrogenase level, stimulating H_2O_2 formation (Figure 5).

The results presented in this paper suggest that mitochondria from ischemic hearts are likely to generate increased H_2O_2 during reoxygenation. In addition, the inhibition and uncoupling of the respiratory chain will also contribute to the overall injury since the post-ischemic organ will have a very limited capacity to recover its normal steady state energy level.

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