Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 59-64

SUPEROXIDE RADICAL AS ELECTRON DONOR FOR OXIDATIVE PHOSPHORYLATION OF ADP

Kathleen Mailer[1]

Saint Mary's University, Halifax, N.S., Canada B3H 3C3

Received May 30, 1990

When isolated rat heart mitochondria are subject to xanthine/xanthine oxidase generated free radicals, nmol quantities of ADP are phosphorylated to ATP. This effect is proportional to xanthine oxidase concentration, and is relatively independent of ADP concentration. Exogenous superoxide dismutase partially suppresses the phosphorylation. Micromolar concentrations of iron salts completely eliminate the phosphorylation. Catalase has no effect. The likely electron source, then, is superoxide radicals. The reduced minus oxidised spectra of superoxide-bombarded mitochondria show that superoxide enters the electron transport chain by reducing cytochrome c and complex IV. Mitochondria retain their ability to phosphorylate ADP in more traditional ways under the experimental conditions described. Superoxide under physiological conditions in vivo may be a source of electrons for the oxidative phosphorylation of ADP.

Mitochondria from a variety of sources have been shown to produce 1-2% partially reduced oxygen species ($O_2^{\cdot-}$, H_2O_2, and $OH^{\bullet}$) from metabolized dioxygen (1). Because of the reactivity of these oxygen species, much work has concentrated on their pathology in living systems and on the use of exogenous free radical traps as therapy (2,3). Oxygen is not in unending suppy in vivo (compared with in vitro experiments). For this reason, and because radicals are ubiquitous in vivo, mitochondria may have a mechanism for reabsorbing partially reduced oxygen at a lower potential site. Partially reduced oxygen (particularly superoxide) may act as an electron donor for the oxidative phosphorylation of ADP. Lippman (4) has demonstrated that ATP can be formed from ADP and $O_2^{\cdot-}$ in a non-aqueous environment in the absence of any biological material. We suggest that ATP can also be formed in living systems from ADP and $O_2^{\cdot-}$ using the electron transport system of the mitochondrion.

The redox potential(s) of superoxide indicate that it can act as a reductant in bulk water (E_0' = -0.27 to -0.33 volts (5)); but it is a much more powerful reductant under non-aqueous

[1] Financial assistance from the Natural Sciences and Engineering Research Council of Canada and the New Brunswick Heart Foundation, technical assistance by Ms. Sheryl Crosby, and help with [^{32}P] measurement by Ms. Susan Powell are gratefully acknowledged.

Abbreviations: superoxide, $O_2^{\cdot-}$; hydroxyl radical, $OH^{\cdot}$; xanthine, X; xanthine oxidase, XO; manganese superoxide dismutase, MnSOD; copper-zinc superoxide dismutase, SOD; ethyleneglycol-bis-(b-aminoethyl ether) N,N'-tetraacetic acid, EGTA; tris(hydroxymethyl) amino methane, Tris; Coenzyme Q, CoQ.

0006-291X/90 $1.50

conditions (E_0' = -0.58 volts (6)). Under normal physiological conditions, the proximity of $O_2^{\cdot-}$ production to an electron acceptor in the oxidised state, a steady production of $O_2^{\cdot-}$, and the presence of a membrane environment would facilitate the net flow of electrons from superoxide to the electron transport chain. It is well known that cytochrome c in solution is readily reduced by superoxide. The experiments, then, were to determine 1) if $O_2^{\cdot-}$ can reduce cytochrome c in situ; 2) if reduction of cytochrome oxidase follows; 3) if net ATP production results. Heart tissue, a rich source of both mitochondria and of mitochondrially produced free radicals, was the test material.

METHODS AND MATERIALS

Mitochondria were obtained from male Sprague Dawley rats, 225-250 g. The animals were sacrificed using fluothane vapors and the hearts homogenized in 8 mL of solution A: 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA, which also contained 1 mg Nagase. Cell debris was pelleted at 1000xg and mitochondria recovered from the supernatant at 20,000xg. Mitochondria were rinsed once and suspended: 1 heart in 1.5 mL solution A. Control experiments using cervical dislocation rather than fluothane, and also eliminating Nagase, gave comparable mitochondria. Respiratory function was assayed in Solution B: 225 mM sucrose, 20 mM KCl, 17 mM K_2HPO_4, 7 mM $MgCl_2$, 15 mM Tris base, pH 7.2. Succinate, final concentration 22 mM, was the electron donor. Mitochondria typically had a respiratory control index of 3-4. Mitochondrial purity was also assayed by measuring cytochrome oxidase spectrophotometrically (7). Lysosomal contamination of the mitochondrial preparation (measured as acid phosphatase activity, Sigma Chemical Co. kit 104-AS) was minimal. There was no evidence for contamination by peroxisomes (urate oxidase activity (7)) or by microsomes (glucose-6-phosphatase activity (8)).

Experimental Conditions: Typically, a test mixture contained 0.5-1.0 mg mitochondrial protein, 200 μM X, 2 μM ADP, and 10 μM rotenone in 1 mL solution B at 20°C. Reaction was begun by the addition of 0.6 U XO. Aliquots of the test mixtures were added to an equal volume of 12% trichloroacetic acid or 5 M formic acid 0, 5, 10, and 15 min. after XO addition in order to stop the reaction. All experiments were done in the presence and also in the absence of 3 U catalase (from bovine liver, 2x crystallized; Sigma Chemical Co.).

ATP was quantitated enzymatically (Sigma Chemical Co. kit 366 UV, where NADH consumed equals ATP in solution); by spectrophotometry, and by measurement of [γ-^{32}P](ATP). For the latter two quantitations the test solution was acidified to 2.5 M formic acid and applied to 2 mL DEAE.formate in a pasteur pipet. After rinsing uncharged species, AMP and ADP from the gel with 3 M formic acid, ATP was eluted with 6 mL 5 M formic acid containing 0.8 M ammonium formate. ATP was quantitated at 260 nm(ε_{260} = 15.4 x 10^3 $Lmol^{-1}cm^{-1}$) or [γ-^{32}P](ATP) was measured in a Beckman LS7800 scintillation counter after the eluate was suspended in Aquasol-2.

DEAE.formate was prepared by successive washings of DEAE Sepharose CL-6B (Pharmacia) in 2.0 M formic acid (60-70°C)x4, 2.0 M sodium formatex2, 0.1 M sodium formatex2 and deionized waterx10.

Reduced minus oxidised spectra of mitochondria were generated by adding 0.2 U XO or 1 mM pyrogallol to the sample cuvet and 0.14 mM $K_3Fe(CN)_6$ to the reference. In addition both cuvets contained 2-4 mg mitochondrial protein, 200 μM X, 10 mM rotenone, and 2.4 mM NaCN in 1 mL solution B.

All reagents were analytical grade. Pyrogallol was purified by sublimation before use. XO (Sigma Grade III), from buttermilk, was substantially free of uricase.

RESULTS

Effect of Reaction Conditions on ATP Production: Up to 200 nmol ADP can be phosphorylated to ATP by rat heart mitochondria when the only external source of electrons to drive the reaction is the X/XO couple. Figure 1 indicates that this effect is proportional to XO, varied over a ten-fold range from 0.15-1.5 U. The non-zero concentrations of ATP synthesis at t=0 reflect ATP synthesis during the time taken to mix the samples and transfer aliquots to acid.

Figure 2 shows that the amount of ATP produced is relatively independent of mitochondrial protein from 0.3 - 1.3 mg. At protein concentrations $\geq$ 2 mg the amount of ATP, high at t=5 min., decreased over 15 min. This is not an effect unique to the X/XO system. When mitochondrial respiration is being measured with succinate as electron donor, respiratory control is sometimes lost when high concentrations of mitochondrial protein are used. Also, in experiments where mitochondria are omitted (not shown), added ATP is not affected by X/XO. Varying X from 35-450 mM or varying ADP concentration from 0.48-3.32 mM had no effect on the amount of ATP produced (data not shown).

Control experiments were performed in which X, XO, ADP, or mitochondria were omitted from the reaction mixture. The background level of ATP measured enzymatically was 20-60 nmol and this was subtracted to give the results in Figures 1 and 2. Rotenone was included in all reaction mixtures to prevent endogenous substrates fuelling oxidative phosphorylation. When rotenone was omitted approximately 5% more ATP was formed. The presence or absence of 3 U catalase (to remove H_2O_2) did not affect the results. However, ATP production was suppressed approximately 50% when 200 U SOD was added to the reaction mixtures. Adding 10-100 μM $FeCl_3$ to the reaction completely suppressed ATP produciton.

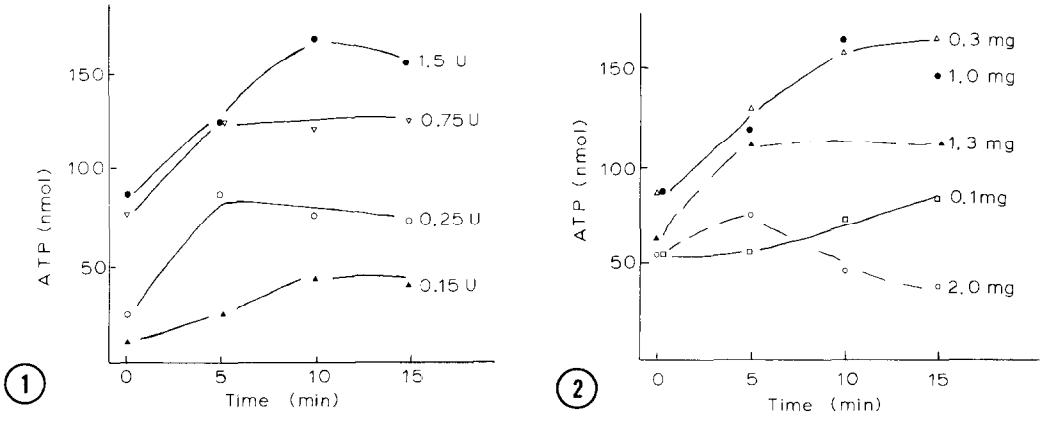

Figure 1. Effect of various XO concentrations on ATP production by heart mitochondria. ATP was measured enzymatically as described in the Methods section. The reaction mixture contained 1.3 mg mitochondrial protein, 240 μM X, 10 μM rotenone, and 2 mM ADP in 1 mL solution B. The reaction was begun by the addition of varying amounts of XO, as indicated in the Figure.

Figure 2. Effect of various mitochondrial concentrations on ATP production in the presence of X/XO. ATP was measured enzymatically. The reaction mixture contained 0.2-2.0 mg mitochondrial protein, 240 μMX, 10 μM rotenone, and 2 mM ADP in 1 mL solution B. The reaction was begun by the addition of 0.6 U XO.

TABLE 1

A. **Respiratory Control Index of Xanthine/Xanthine Oxidase Treated Mitochondria**

	t = 0	5	10	15 min.	Avg.
Mitochondria Suspended in:					
Solution B containing X/XO		1.85	2.33	2.21	2.13
Solution B		2.23	2.03	3.13	2.46
Solution A (Control)	2.88				

B. **[γ-^{32}P](ATP) Production by Mitochondria**

	Total [^{32}P] in eluate	[γ-^{32}P](ATP)
Succinate as Electron Donor	299,673 cpm	278,645 cpm
X/XO as Electron Donor	97,868	76,840
X/XO plus SOD and Catalase	17,349	0
X/XO plus SOD	9,394	0
Background [^{32}P] in Eluate	21,028	-
Total [^{32}P] in Reaction	10^6 cpm	-

Respiration of X/XO Bombarded Mitochondria: Mitochondria exposed to X/XO for 15 min. were centrifuged, rinsed, and resuspended in solution B. Respiratory control was then measured (Table 1). A 26% drop in respiratory control index was noted. A drop of 15% in respiratory control is noted when mitochondria are diluted in solution B without added XO.

ADP + [^{32}P] $\rightarrow$ [γ-^{32}P](ATP): To test that the measured ATP was produced in response to X/XO rather than preexistent, 1.5 μCi [^{32}P] was added to the reaction mix. Results in the Table show that eluted ATP contains [^{32}P] and that no radiolabelled ATP is apparent when SOD is in the reaction mixture. For comparison ATP production driven by succinate is also shown in the Table. This experiment is a crude measure of ATP production since 10^6 more Pi than ADP is present in the reaction. However, the results do show that X/XO can generate as much as 28% of the ATP produced by succinate.

Difference Spectroscopy: The spectrum of X/XO reduced mitochondria vs. $K_3Fe(CN)_6$ oxidised mitochondria is shown in Figure 3. Spectral peaks at 550 nm and 605 nm indicate reduction of cytochrome c and cytochrome oxidase, respectively. Also shown is the spectrum when succinate is the electron donor. Additional spectral peaks at 554 nm (seen only as a shoulder) and 563 nm are due to reduction of cytochromes c1 and b, respectively (9, 10). Succinate, entering the respiratory chain at CoQ, reduces mitochondrial complexes III and IV. Antimycin, which inhibits complex III, eliminates the succinate-driven spectra but has no effect on X/XO generated spectra.

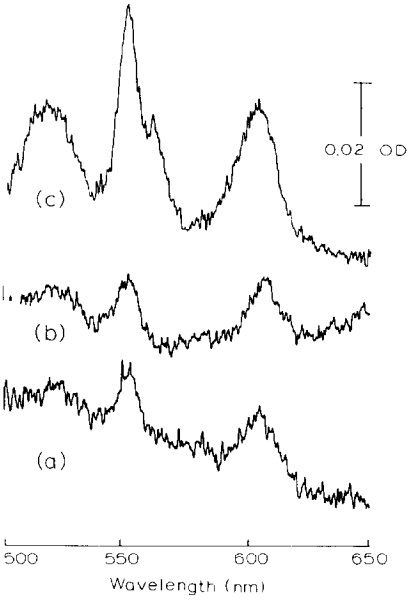

Figure 3. Spectra of reduced mitochondria vs. $K_3Fe(CN)_6$-oxidised mitochondria. Reaction conditions are noted in the Methods section. Spectra were recorded on a Pye Unicam SP8-400 UV/VIS Spectrophotometer
a) Reduction by 0.2 U XO; b) reduction by 1 mM pyrogallol; c) reduction by 22 mM succinate.

Therefore, X/XO reduces only the final portion of the electron transport chain and a maximum of one ATP per reduction event can be expected. Pyrogallol, which also generates $O_2^{\cdot}$, gave the same mitochondrial difference spectrum as X/XO (Figure 3).

DISCUSSION

The results outlined in this paper show that X/XO generated product(s) can reduce the heart mitochondrial electron transport chain (at cytochrome c) and generate ATP. The action of XO on X yields both $O_2^{\cdot}$ and H_2O_2; the hydroxyl radical may also be present as a reaction product of $O_2^{\cdot}$ and H_2O_2. H_2O_2 is eliminated as the electron donor since catalase does not affect the results. The hydroxyl radical is also eliminated since micromolar concentrations of iron salts (which catalyze $O_2^{\cdot} + H_2O_2 \rightarrow OH^{\bullet}$) prevent X/XO generated mitochondrial ATP. This strongly suggests $O_2^{\cdot}$ as the reductant.

A problem arises however with $O_2^{\cdot}$ as electron donor to mitochondria. Mitochondria contain endogenous MnSOD capable of dismutating $O_2^{\cdot}$. Also, exogenous SOD does not completely eliminate ATP production in this system (although it is noted that X/XO spectroscopic reduction peaks are delayed in the presence of SOD). The most likely explanation is the location of $O_2^{\cdot}$ production. Endogenous MnSOD is a matrix enzyme not available to dismutate externally generated $O_2^{\cdot}$. Cytochrome c, on the other hand, is a peripheral enzyme on the outside of the mitochondrial membrane and in a position to compete with added SOD for $O_2^{\cdot}$. The position of the $O_2^{\cdot}$ generator (xanthine in this experiment, mitochondrial components in vivo) (11) is critical.

Any $O_2^{\cdot}$ generated in or near the non-aqueous environment of the mitochondrial membrane is in a position to escape soluble SOD and reduce cytochrome c. Further, the redox potential of $O_2^{\cdot}$ in a lipid environment makes it a very good electron donor. Thus cytochrome c in vivo is serving two functions: first as a conveyor of electrons to cytochrome oxidase in the intact mitochondrion, and second as a membrane-protecting anti-oxidant, scavenging electrons for ATP production and regenerating molecular oxygen. That respiratory control is decreased at least partially in the presence of $O_2^{\cdot}$ is well known (ref. 12 and results in this paper). It would seem, then, that cytochrome c is not completely successful as an anti-oxidant. However, even partial success as a scavenger of electrons from superoxide, would be of great benefit to the heart in vivo.

REFERENCES

1. Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. **59,** 527-603.
2. Baker, J.E. and Kalyanaraman, B. (1989) FEBS Lett. **244,** 311-314.
3. Gupta, M. and Singal, P.K. (1989) Can. J. Physiol. Pharmacol. **67,** 1549-1559.
4. Lippman, R.D. (1982) J. Biochem. Biophys. Meth. **6,** 81-87.
5. Sawada, Y., Iyanagi, T. and Yamazaki, I. (1975) Biochemistry **14,** 3761-3764.
6. Peover, M.E. and White, B.S. (1966) Electrochim. Acta. **11,** 1061-1067.
7. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J.W., Fowler, S. and de Duve, C. (1968) J. Cell. Biol. **37,** 482-513.
8. Swanson, M.A. (1955) in Methods in Enzymol. **2,** 541-542.
9. Williams, J.N. (1964) Arch. Biochem. Biophys. **107,** 537-543.
10. Subba Reddy, K.V. and Hendler, R.W. (1983) J. Biol. Chem. **258,** 8568-8581.
11. Nohl, H. (1987) FEBS Lett. **214,** 269-273.
12. Nohl, H., Breuninger, V. and Hegner, D. (1978) Eur J. Biochem. **90,** 385-390.