Superoxide activates mitochondrial uncoupling protein 2
from the matrix side: studies using targeted antioxidants

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Running title: Superoxide activation of UCP2
Abbreviations: UCP, uncoupling protein; UCP-KO, uncoupling protein knockout mice; BAT, brown adipose tissue; TPMP, triphenylmethylphosphonium; Q, ubiquinone; mitoQ, 10-(6’-ubiquinonyl)decylntriphenylphosphonium; mitoQuinone and mitoQuinol, oxidized and reduced mitoQ; mitoVit E, 2-[2-(triphenylphosphonio)ethyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol bromide; decylQ, decylubiquinone; TROLOX, 6-hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; ROS, reactive oxygen species; X, xanthine; XO, xanthine oxidase;
Summary

Superoxide activates nucleotide-sensitive mitochondrial proton transport through the uncoupling proteins UCP1, UCP2 and UCP3 (Echtay, et al. (2002) Nature (London) 415, 1482-1486). Two possible mechanisms were proposed: direct activation of the UCP proton transport mechanism by superoxide or its products; or a cycle of hydroperoxyl radical entry coupled to UCP-catalyzed superoxide anion export. Here we provide evidence for the first mechanism and show that superoxide activates UCP2 in rat kidney mitochondria from the matrix side of the mitochondrial inner membrane. (i) Exogenous superoxide inhibited matrix aconitase, showing that external superoxide entered the matrix. (ii) Superoxide-induced uncoupling was abolished by low concentrations of the mitochondrially-targeted antioxidants mitoQ or mitoVit E, which are ubiquinone (Q) or tocopherol derivatives targeted to the matrix by covalent attachment to triphenylphosphonium cation. However, superoxide-induced uncoupling was not affected by similar concentrations of the non-targeted antioxidants Q0, Q1, decylQ, vitamin E or TROLOX or of the mitochondrially targeted but redox-inactive analogues decyltriphenylphosphonium or 4-chlorobutyltriphenylphosphonium. Thus matrix superoxide appears to be necessary for activation of UCP2 by exogenous superoxide. (iii) When the reduced to oxidized ratio of mitoQ accumulated by mitochondria was increased by inhibiting cytochrome oxidase it induced nucleotide-sensitive uncoupling that was not inhibited by external superoxide dismutase. Under these conditions quinols are known to produce superoxide and as MitoQ is localized within the mitochondrial matrix this suggests that production of superoxide in the matrix was sufficient to activate UCP2. Furthermore, the superoxide did not need to be exported or to cycle across the inner membrane to cause uncoupling. We conclude that superoxide (or its products) exerts
its uncoupling effect by activating the proton transport mechanism of uncoupling proteins at the matrix side of the mitochondrial inner membrane.
Introduction

Brown adipose tissue (BAT) is a major site of adaptive thermogenesis. The main component responsible for heat generation is uncoupling protein 1 (UCP1), a member of the mitochondrial anion carrier family (1,2). The primary function of UCP1 is to dissipate the proton motive force used by the ATP synthase during oxidative phosphorylation by catalyzing a proton leak across the mitochondrial inner membrane. Thus ATP synthesis is largely bypassed and the energy is released as heat. The proton transport activity of UCP1 is tightly controlled: it is activated by fatty acids and inhibited by purine nucleoside di- and triphosphates (1-4).

In 1997, new UCP1 homologs termed UCP2 and UCP3 were described in other mammalian tissues (5-8). These proteins show sequence homology to UCP1 (55% and 57% respectively) but are present at very low concentrations (9,10) compared to UCP1, which is abundant in BAT (1,2). UCP2 protein is expressed in several tissues, including spleen, lung, stomach and white adipose tissue (9). It is also expressed in kidney and pancreatic β-cells, as judged from GDP-sensitive superoxide-stimulated proton conductance measurements (11), although the evidence from Western blots in kidney is ambiguous because of poor antibody specificity (8,9,12). UCP3 is expressed primarily in skeletal muscle (and in BAT in rodents) (10,13,14).

The functions of UCP2 and UCP3 have been obscure but are slowly becoming clearer. On the basis of sequence similarity to UCP1, it was initially suggested that they catalyze the basal mitochondrial proton conductance. Work in which the proteins were overexpressed in yeast, mammalian cells and transgenic mice supported this hypothesis, but these results now appear to be artifacts of unphysiological expression for several reasons, particularly the lack of GDP-sensitive superoxide-stimulated proton conductance (10,15-24). In E. coli, several groups have achieved
abundant expression of these proteins in inclusion bodies (25-29). UCP2 and UCP3 solubilized from inclusion bodies and reconstituted in phospholipid vesicles show proton transport activities (26,28). However, different claims are made for the regulation of these proteins by coenzyme Q, fatty acids and nucleotides. The main problem with these studies is the renaturation of the solubilized inclusion bodies. Nevertheless, they have identified ubiquinone as an essential cofactor for uncoupling in vitro by UCP1 and its homologs UCP2 and UCP3 (27,28). The basal proton conductance of mitochondria from UCP2 or UCP3 knockout mice is the same as that of wild-type controls (12,23) and these knockout mice have unchanged thermoregulation, thermogenesis, energy expenditure and body weight (12-14,23,30), supporting the view that neither UCP2 nor UCP3 contributes significantly to the basal proton conductance of mitochondria, which is an important component of whole-body energy expenditure (31).

Several studies have suggested a role for UCP3 in the metabolism of fatty acids (32,33). However, UCP3 KO mice do not display any major defect in fatty acid or glucose oxidation (13,14). On the other hand, mice lacking UCP2 secrete more insulin, suggesting a role of UCP2 in the regulation of glucose-stimulated insulin secretion (34). Interestingly, mice lacking UCP2 or UCP3 show signs of oxidative stress, suggesting a role of these proteins in the regulation of oxygen free radical metabolism (13,35-37).

Recently, we showed that superoxide activates the inducible proton conductance of mitochondria through effects on UCP1, UCP2 and UCP3, and that this activation is very sensitive to inhibition by purine nucleoside di- and triphosphates (11). The superoxide-induced uncoupling correlates with the tissue expression of UCP2: it is found in mitochondria from kidney, spleen and pancreatic
β-cells but not in those from liver or heart. It is present in skeletal muscle mitochondria from wild-type mice (which contain UCP3) but absent in those from UCP3 KO mice. It is present in mitochondria from BAT (which contain UCP1), and in yeast mitochondria expressing mammalian UCP1, but is absent in mitochondria from wild-type yeast. Observations by others of decreased proton conductance in mitochondria from UCP3 knockout mice (13,14) and in thymocytes from UCP2 knockout mice (38) may reflect disruption of inducible proton conductance that is dependent on superoxide or other endogenous activators.

Two possible mechanisms were proposed for the activation of inducible proton conductance by superoxide (11): direct activation of the UCP proton transport mechanism by superoxide or its products; or a cycle of hydroperoxyl radical entry coupled to UCP-catalyzed superoxide anion export. In the present report we explore inhibition and activation of superoxide-induced uncoupling by mitochondrially targeted antioxidants (39-41) to investigate these mechanisms. Uncoupling by exogenous superoxide was inhibited by targeted antioxidants but not by non-targeted analogues showing that matrix ROS are necessary in the uncoupling mechanism. Uncoupling was activated by generating superoxide selectively in the matrix by using a targeted quinol in conjunction with cyanide, and this uncoupling was insensitive to exogenous superoxide dismutase, showing that matrix ROS are sufficient to activate the uncoupling mechanism without cycling through the external medium. These observations provide evidence for the first mechanism, and show that superoxide-stimulated uncoupling through UCPs occurs at the matrix side of the mitochondrial inner membrane.
Experimental Procedures

Chemicals: The mitochondrial-targeted compounds mitoQ, mitoVit E, 4-chlorobutyltriphenylphosphonium and decyltriphenylphosphonium were synthesized as described previously (40,41) (see Fig. 1 for structures). MitoQ is a mixture of two redox forms of quinone (ubiquinol and ubiquinone) attached to triphenylphosphonium. Q_o, Q_1, decylQ, vitamin E (α-tocopherol, type VI), triphenylmethylphosphonium chloride, xanthine oxidase (buttermilk) and isocitrate dehydrogenase were from Sigma. TROLOX was from Aldrich and xanthine was from Fluka.

Isolation of kidney mitochondria: Two female Wistar rats (4-8 weeks old) were killed by stunning followed by cervical dislocation. Kidneys were immediately removed and placed in ice-cold medium, containing 250 mM sucrose, 5 mM Tris-HCl (pH 7.4) and 2 mM EGTA (isolation medium). Mitochondria were isolated essentially as described previously (42), with all steps carried out at 4 °C. Tissue was minced with sharp scissors, rinsed and gently homogenized using a glass Dounce homogenizer (10-15 passes with medium fit plunger). The homogenate was centrifuged at 1047 x g for 3 min. The supernatant was centrifuged at 11621 x g for 10 min. Mitochondrial pellets were resuspended in isolation medium and centrifuged at 11621 x g for 10 min. This step was repeated. The pellet was resuspended in isolation medium and protein concentration was determined by the biuret method.

Proton leak measurements: To measure the kinetics of proton conductance, the respiration rate used to drive proton leak (in the presence of oligomycin to block ATP synthesis) was measured as a function of mitochondrial membrane potential, the driving force for the leak (43). Proton leak rates can be calculated from respiration rates by multiplying by the H+/O ratio of 6. Respiration rate and membrane potential
were measured simultaneously using electrodes sensitive to oxygen (Clark electrode - Rank Brothers Ltd., UK) and to the potential-dependent probe triphenylmethylphosphonium cation (TPMP⁺) (44). Kidney mitochondria (0.35 mg/mL) were incubated in standard assay medium (total volume 3.5 mL) containing 120 mM KCl, 5 mM KH₂PO₄, 3 mM Hepes and 1 mM EGTA, pH 7.2, at 37 °C with addition of 5 µM rotenone, 80 ng of nigericin/mL and 1 µg of oligomycin/mL. The electrode was calibrated with sequential 0.5 µM additions up to 2.5 µM TPMP⁺. When other phosphonium compounds (mitoQ, mitoVit E, 4-chlorobutyltriphenylphosphonium or decyltriphenylphosphonium) were tested, these compounds were used instead of TPMP to calibrate the TPMP electrode and to monitor the mitochondrial potential. The binding correction was assumed to be 0.4/(µL per mg protein) (44) for all phosphonium compounds: the more hydrophobic analogues will bind more strongly than this, so membrane potentials estimated with these probes in Figs 4b, 6, 7a and 8 will be systematically overestimated to different extents. However, the pattern of changes using any particular phosphonium probe will not be affected by the binding correction. Respiration was initiated with 4 mM succinate and the membrane potential was titrated by sequential addition of malonate up to about 1 mM. After each run, 0.2 µM FCCP was added to release the phosphonium probe for baseline correction. Where indicated, exogenous superoxide was generated using xanthine (50 µM) and xanthine oxidase (0.01U/3.5 mL assay medium). Xanthine was prepared at 0.35 mM in assay medium and 500 µL of stock was added to 3 mL of assay medium to give 50 µM xanthine in a total volume 3.5 mL in each run. Xanthine oxidase was prepared in assay medium at 0.2 U/100 µL and 5 µL (0.01 U) was added to the assay medium. Xanthine and xanthine oxidase were
added before the TPMP\textsuperscript{+} calibration and incubated with mitochondria for about 10-15 min before addition of succinate.

\textit{Aconitase:} Mitochondrial aconitase activity was measured by following the appearance of NADPH at 340 nm (45). Kidney mitochondria (0.35 mg/mL) were incubated for 20 min at 37 °C in 3.5 mL assay buffer, (120 mM KCl, 5 mM KH\textsubscript{2}PO\textsubscript{4}, 3 mM Hepes, 1 mM EGTA, 1 mM MgCl\textsubscript{2}, 0.2 mM NADP, 2 units isocitrate dehydrogenase and 5 mM citrate, pH 7.2), either with or without xanthine (50 \(\mu\)M) plus xanthine oxidase (0.01 U per 3.5 mL). After 20 min, superoxide dismutase (45 units) was added and the reaction was started by the addition of Triton-X 100 (0.12% v/v).
Results

Superoxide-induced uncoupling in kidney mitochondria: Since we use xanthine plus xanthine oxidase to generate superoxide and activate uncoupling by UCPs (11), there is a possible complication due to oxygen consumption by the xanthine oxidase (12). As shown in Fig. 2, the baseline rate of oxygen consumption of non-energized mitochondria in the absence (Fig. 2a) or presence of xanthine plus xanthine oxidase (Fig. 2b) was about 7 nmol O/min/mg protein; it was not inhibited by GDP (Fig 2c). After addition of succinate, oxygen consumption rates were much greater (Fig. 2a-c). The proton leak curves calculated from Fig. 2a-c are summarized in Fig 2d. The state 4 respiration of control mitochondria was about 100 µmol O/min/mg protein; in the presence of superoxide it increased to about 140 µmol O/min/mg protein. Even after correcting for the baseline rate, the state 4 respiration of superoxide-treated mitochondria was higher than controls and the proton leak curve was still deflected upwards, indicating increased mitochondrial uncoupling. The superoxide-induced uncoupling was inhibited by GDP (Fig 2c,d). Because of its insignificance to the superoxide-induced uncoupling, we show all results in this report (and in (11,23)) without subtracting the baseline rate. These results confirm that superoxide induces GDP-sensitive uncoupling in kidney mitochondria (11) and that the effect of xanthine plus xanthine oxidase is not simply one of increasing baseline oxygen consumption (12).

Exogenously generated superoxide reaches the mitochondrial matrix: We investigated whether exogenously generated superoxide reached the mitochondrial matrix by assaying the activity of aconitase, a matrix enzyme highly sensitive to superoxide (45). As shown in Fig 3, incubating kidney mitochondria with xanthine plus xanthine oxidase under our conditions significantly decreased the activity of
aconitase. Xanthine or xanthine oxidase alone had no effect. Addition of superoxide
dismutase, an enzyme that detoxifies superoxide to $\text{H}_2\text{O}_2$, together with xanthine plus
xanthine oxidase protected against damage to aconitase (data not shown). This
indicates that superoxide generated outside mitochondria using xanthine plus xanthine
oxidase reaches the matrix.

Prevention of superoxide uncoupling by mitoQ and mitoVit E: Exogenously generated
superoxide reaches the mitochondrial matrix, raising the question: is superoxide-
duced uncoupling mediated through interaction with the UCPs at the cytosolic or
matrix side of the membrane? To answer this question, we used a recently-introduced
approach for targeting antioxidants such as ubiquinone and tocopherol to the
mitochondrial matrix (39-41). These compounds are covalently linked to a lipophilic
triphenylphosphonium cation and termed mitoQ and mitoVit E. Such lipophilic
cations penetrate lipid bilayers and accumulate in mitochondria when a membrane
potential is present. Like TPMP$^+$, MitoQ (and mitoVit E, decyltriphenylphosphonium
and 4-chlorobutyltriphenylphosphonium), (see Fig. 1 for structures), are rapidly taken
up by energized mitochondria and immediately released by addition of uncoupler
FCCP (data not shown). State 4 respiration was slightly higher than controls in the
presence of mitoQ (Fig. 4b) or mitoVit E (Fig. 7a), suggesting that these compounds
improved substrate oxidation (and/or uncoupled slightly).

Fig. 4a shows the normal superoxide-induced, GDP-sensitive uncoupling that
is seen using kidney mitochondria (Fig. 2d, (11)). Fig 4b shows that the effect of
superoxide was completely abolished by the presence of 2.5 $\mu$M mitoQ.

To determine whether mitochondrial localization of mitoQ was required for
this protective effect, we compared mitoQ with the non-targeted antioxidant
analogues $Q_0$, $Q_1$ and decylQ. $Q_0$ and $Q_1$ have the quinone group with zero or one 5-
carbon isoprene side chains, while decylQ has a 10-carbon side chain and is mitoQ with the linking group but lacking the targeting triphenylphosphonium moiety (Fig. 1). These quinones will equilibrate into phospholipid membranes in the same way as mitoQ, but will not be accumulated into the mitochondrial matrix. None of these compounds, used at the same concentration at which mitoQ was fully inhibitory, blocked the uncoupling effect of superoxide (Fig. 5), showing that at these concentrations the antioxidant group had to be accumulated to prevent superoxide-induced uncoupling. We also tested non-antioxidant targeted compounds to eliminate any non-specific hydrophobic effects of mitoQ. The compounds used were decyltriphenylphosphonium, which is mitoQ lacking the quinone group, and 4-chlorobutyltriphenylphosphonium, which is mitoQ with the decylQ group replaced by a hydrophobic butyl chain and a chloro-endgroup (Fig. 1). As shown in Fig. 6, neither of these non-antioxidant targeted compounds abolished superoxide-induced uncoupling. These results demonstrate that mitoQ must be accumulated and redox-active to be effective at inhibiting superoxide-induced uncoupling. They show that the presence of ROS on the matrix side of the membrane is necessary during uncoupling by exogenously-generated superoxide, and imply that superoxide or its products must activate UCPs from the matrix side of the mitochondrial inner membrane.

We also tested another mitochondrial-targeted antioxidant, mitoVit E (α-tocopherol attached to triphenylphosphonium, Fig. 1). This compound, like mitoQ, abolished superoxide-induced uncoupling. As shown in Fig. 7a, exogenous superoxide had no effect on proton conductance in the presence of 2.5 µM mitoVit E. However, the non-targeted compounds vitamin E (α-tocopherol) or TROLOX (a more water-soluble analog of vitamin E) at the same concentration did not prevent
uncoupling by superoxide (Fig 7b,c), showing once again that at these low concentrations, accumulation of the antioxidant was required for inhibition of superoxide-activated uncoupling. This supports the idea that mitoQ abolishes superoxide-induced uncoupling because of its targeted antioxidant properties.

*Generation of superoxide within the mitochondrial matrix:* We have previously shown that addition of $Q_{10}$ to kidney mitochondria respiring on succinate increased proton conductance under conditions in which $Q_{10}$ was likely to be largely reduced to ubiquinol (when the mitochondria were titrated with cyanide, to decrease oxidation of the electron transport chain) but not when the ubiquinol/ubiquinone ratio was lower (when mitochondria were titrated with malonate, to decrease reduction of the electron transport chain). The effect was GDP-sensitive, and operated through the production of superoxide in the medium, since it was inhibited by exogenous superoxide dismutase (46). In Fig. 8 we show experiments to test whether reduced mitoQ could also affect mitochondrial proton conductance by acting as a source of superoxide, and whether this superoxide was produced in the medium or in the mitochondrial matrix.

The protective effect of mitoQ against uncoupling by superoxide (Fig. 4b) was seen using mitochondria respiring on succinate and titrated with malonate, where the ratio of mitoQuinol to mitoQuinone will decrease as the titration progressed. However, as shown in Fig. 8, when mitochondria were titrated with cyanide (an inhibitor of Complex IV), mitoQ caused an increase in proton conductance without the need for addition of xanthine plus xanthine oxidase. Under these conditions, mitoQ will have become more reduced (i.e. mitoQuinol) as the titration progressed. Uncoupling induced by mitoQuinol was fully inhibited by GDP, suggesting that it occurred through UCP2. However, unlike the situation with $Q_{10}$ (46), addition of superoxide dismutase had no effect on uncoupling induced by mitoQuinol. This
suggests that, just as with ubiquinol, when the ratio of reduced to oxidized mitoQ is increased by inhibiting the respiratory chain with cyanide, mitoQ generates superoxide. However, in contrast to ubiquinol, mitoQ is on the matrix side of the inner membrane and consequently this superoxide is not accessible to the exogenous added superoxide dismutase. This shows that matrix superoxide is sufficient to cause UCP2 activation, demonstrating that activation is not through cycling of superoxide across the membrane to the bulk external phase, followed by its protonation and reuptake.
Discussion

Recent discoveries imply a new function of the UCPs as part of the cellular defenses against oxidative stress (9,11,13,35-37,47,48). We recently showed that superoxide interacts with UCP1, UCP3 and very probably UCP2, leading to an increase in mitochondrial proton conductance (11). The superoxide-induced uncoupling requires fatty acids and is inhibited by purine nucleoside di- and triphosphates. This led us to propose (11) that the interaction of superoxide or its products with the UCPs may be a mechanism for decreasing mitochondrial ROS. It could do this by causing mild uncoupling, which will protect by oxidizing the electron transport chain and lowering the local oxygen concentration, both of which will decrease ROS production (49,50), or by transporting superoxide from the mitochondrial matrix for disposal in the intermembrane space. The mechanism of the observed superoxide-induced uncoupling in vitro would be either (i) an activation of the inducible proton transport mechanism of the UCPs, or (ii) a futile cycle of superoxide protonation in the medium to form hydroperoxyl radical, the diffusion of this radical to the matrix where it can deprotonate and regenerate superoxide, followed by UCP-catalyzed export of superoxide anion from the mitochondrial matrix back to the medium (11).

Many of the experiments in (11) and the present paper were performed using isolated mitochondria in the presence of high concentrations of exogenous superoxide, generated using xanthine plus xanthine oxidase. Several controls have been performed to check for artifacts. Xanthine or xanthine oxidase alone had no effect on the proton conductance of kidney mitochondria. The oxygen consumption by xanthine oxidase during generation of superoxide did not affect the measurements of uncoupling induced by superoxide (Fig. 2). The high sensitivity and specificity of
uncoupling to nucleotides and the lack of any uncoupling effect in liver or heart mitochondria under the same conditions as for kidney or skeletal muscle mitochondria (11) also argue against any non-specific effects of the superoxide generating system.

Since our studies were performed in the presence of exogenous superoxide, we first checked whether the superoxide anion reaches the mitochondrial matrix. The decrease in the activity of aconitase (a matrix enzyme whose activity is highly sensitive to superoxide) in the presence of xanthine plus xanthine oxidase (Fig. 3) clearly demonstrates that exogenously generated superoxide reaches the mitochondrial matrix. Superoxide anion is believed to diffuse through the mitochondrial membrane as hydroperoxyl radical (11,51). In the matrix, hydroperoxyl radical, superoxide anion (preferentially regenerated because of the more basic pH), or a breakdown product then inhibits aconitase by displacing one Fe atom from the catalytic [4Fe-4S]$_2^{2+}$ center (45).

We used mitochondrially-targeted antioxidants to destroy matrix superoxide and to probe the topology of the interaction of superoxide with UCPs. These targeted compounds are quinone (mitoQ) and tocopherol (mitoVit E) derivatives targeted to the mitochondrial matrix by covalent attachment to a lipophilic triphenylphosphonium cation. The uncoupling observed in kidney mitochondria in the presence of xanthine plus xanthine oxidase was completely inhibited by the addition of mitoQ or mitoVit E (Figs 4 and 7). This protective effect was due to their mitochondrial localization and antioxidant properties, since neither the non-targeted antioxidants $Q_{0}$, $Q_{1}$, decylQ, vitamin E or TROLOX (Figs 5 and 7) nor the non-antioxidant targeted compounds decyltriphenylphosphonium or 4-chlorobutyltriphenylphosphonium (Fig. 6), blocked the uncoupling effect of superoxide. Thus it appears that mitoQ and mitoVit E prevent uncoupling induced by externally-generated superoxide by destroying
superoxide in the mitochondrial matrix. It follows that the presence of superoxide in the matrix is necessary for uncoupling by externally generated superoxide.

The quinone moiety of mitoQ cycles between the oxidized and reduced form by exchanging electrons with the respiratory chain (40,41), hence mitoQ will have protective antioxidant effects through radical and ROS scavenging by both redox forms. However, when mitochondria were titrated with cyanide (an inhibitor of Complex IV), conditions under which the electron transport chain will become progressively reduced, mitoQ induced proton conductance (Fig. 8). During cyanide titrations, mitoQ is likely to accept electrons from the electron transport chain, thereby greatly increasing the ratio of mitoQuinol to mitoQuinone. Under these conditions quinones are known to generate superoxide. As mitoQ is present in the matrix, we propose that during the cyanide titration mitoQ generates superoxide in the matrix, and that this activates UCPs. Exogenous $Q_{10}$ also increases mitochondrial proton conductance when it is likely to be reduced (46). $Q_{10}$ induced uncoupling is sensitive to added superoxide dismutase, indicating that $Q_{10}$ causes its effect through the production of superoxide in the medium surrounding the mitochondria. The same uncoupling effect was observed with mitoQ but it was insensitive to added superoxide dismutase, indicating that superoxide was generated in the matrix. However, uncoupling induced by either reduced $Q_{10}$ or reduced mitoQ was completely inhibited by GDP, indicating that it occurred through interactions with UCP2 in both cases.

Our observations show that exogenous superoxide can enter mitochondria, and that superoxide (or its products) in the matrix is both necessary and sufficient to activate UCPs with no requirement for it to enter the space accessible to added superoxide dismutase. We conclude that superoxide or its products activates proton
transport by the UCPs specifically from the matrix side, and that the mechanism does not involve futile cycling of superoxide through the extramitochondrial space.
Acknowledgement

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References


Figure legends

Figure 1: **Targeted and non-targeted Q and vitamin E analogs used in this study.**

Figure 2: **Effects of superoxide and GDP on the proton leak kinetics of kidney mitochondria.** (a), (b) and (c), Recordings of simultaneous measurements of mitochondrial respiration and membrane potential using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylphosphonium cation (TPMP⁺). For details see "Experimental Procedures". The upper trace in each panel (the baseline) shows oxygen concentration in the presence of mitochondria and all indicated reagents except succinate; the other traces show oxygen and TPMP⁺ concentration in the presence of succinate added where indicated. (a), control; (b) with 50 µM xanthine (X) plus 0.01 U/3.5 mL xanthine oxidase (XO); (c) with X+XO plus 0.5 mM GDP. Kidney mitochondria (0.35 mg/mL) were incubated in the oxygen electrode chamber in a total volume of 3.5 mL assay medium with rotenone, nigericin and oligomycin at 37 °C (see Experimental Procedures). The TPMP⁺ and reference electrodes were inserted into the chamber and calibrated with five sequential 0.5 µM additions up to 2.5 µM TPMP⁺ (the first addition is not shown), then succinate was added to 4 mM. As shown in the recordings, the TPMP⁺ electrode indicated uptake of TPMP⁺ to equilibrium in response to the generation of a membrane potential following addition of succinate. Membrane potential was titrated by sequential additions of malonate up to about 1 mM (1, 1, 2, 4 µL of 0.5 M). At the end of each run, FCCP was added to dissipate completely the membrane potential and release all TPMP⁺ back to the medium, allowing correction for any small electrode drift; (d), Proton leak kinetics calculated from the recordings in (a)-(c) ignoring (open symbols)
or subtracting (filled symbols) the baseline. ([squares]) control; (◊) with X+XO; (O) with X+XO and 0.5 mM GDP.

Figure 3: **Response of matrix aconitase activity to exogenous superoxide.** Aconitase activity was measured spectrophotometrically in kidney mitochondria after incubation in the presence and absence of the superoxide generating system (X+XO) as described under "Experimental Procedures". Results are normalized means ± s.e.m. of 8 independent paired experiments. * P < 0.001 by paired student's *t*-test. (Aconitase specific activity was 336±44 mU/mg protein).

Figure 4: **Prevention of superoxide-induced uncoupling by mitoQ.** Superoxide was generated by incubating kidney mitochondria with X+XO. Membrane potential was monitored using a TPMP⁺ electrode calibrated by sequential additions of TPMP⁺ up to 2.5 µM (a) or mitoQ up to 2.5 µM (b) as described in "Experimental Procedures" and Fig. 1. Membrane potential was generated using 4 mM succinate and varied by adding malonate up to about 1 mM. ([squares]) control; (◊) with X+XO added before TPMP⁺ or mitoQ; (O) with X+XO and 0.5 mM GDP added before TPMP⁺ or mitoQ. Data are means ± s.e.m. of four (a) or three (b) independent experiments each performed in duplicate.

Figure 5: **Effects of Q₀, Q₁ and decylQ on superoxide-induced uncoupling.** Kidney mitochondria were incubated with succinate as substrate and titrated with malonate in the presence of 2.5 µM Q₀ (a), 2.5 µM Q₁ (b) or 2.5 µM decylQ (c). For details, see “Experimental Procedures”. ([squares]) control; (◊) with X+XO added
before TPMP⁺; (O) with X+XO and 0.5 mM GDP added before TPMP⁺. Data are means ± s.e.m. of three independent experiments each performed in duplicate.

Figure 6: Effects of 4-chlorobutyltriphenylphosphonium and decyltriphenylphosphonium on superoxide-induced uncoupling. Mitochondrial membrane potential was monitored by TPMP⁺ electrode calibrated with sequential addition of 4-chlorobutyltriphenylphosphonium (a) or decyltriphenylphosphonium (b) up to 2.5 µM. ([squares]) control; (◊) with X+XO added before 4-chlorobutyltriphenylphosphonium or decyltriphenylphosphonium; (O) with X+XO and 0.5 mM GDP added before 4-chlorobutyltriphenylphosphonium or decyltriphenylphosphonium. Data are means ± s.e.m. of three independent experiments each performed in duplicate.

Figure 7: Effects of mitoVit E, vitamin E and TROLOX on superoxide-induced uncoupling. Mitochondrial membrane potential was monitored by sequential addition up to 2.5 µM of mitoVit E (a), or of TPMP⁺ in the presence of 2.5 µM vitamin E (b) or 2.5 µM TROLOX (c) added before TPMP⁺. ([squares]) control; (◊) with X+XO; (O) with X+XO and 0.5 mM GDP. Data are means ± s.e.m. of three independent experiments each performed in duplicate.

Figure 8: Effect of mitoQ and a reduced respiratory chain on the proton leak kinetics of kidney mitochondria. Proton leak kinetics were measured as described in Fig. 3b except that malonate up to about 1 mM ([squares]) (mitoQ control) or cyanide up to about 100 µM (◊,◊,Δ) were added to change mitochondrial membrane potential. (◊) cyanide titration, (O) cyanide titration in the presence of superoxide
dismutase (12 U/mL); (Δ) cyanide titration in the presence of 0.5 mM GDP. Data are means ± s.e.m. of three independent experiments each performed in duplicate.
Figure 2
Figure 3

Aconitase activity (%)
Figure 4

(a) Respiration rate (nmol O/min/mg) vs. Membrane potential (mV)

(b) Respiration rate (nmol O/min/mg) vs. Membrane potential (mV)
Figure 5

(a) Membrane potential (mV) vs. Respiration rate (nmol O/min/mg)

(b) Membrane potential (mV) vs. Respiration rate (nmol O/min/mg)

(c) Membrane potential (mV) vs. Respiration rate (nmol O/min/mg)
Figure 6

(a) Respiration rate (nmol O/min/mg) vs. Membrane potential (mV)

(b) Another graph with similar data presentation.
Figure 7

Respiration rate (nmol O/min/mg) vs. Membrane potential (mV)
Figure 8
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Studies using targeted antioxidants
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