Selective Targeting of a Redox-active Ubiquinone to Mitochondria within Cells

ANTIOXIDANT AND ANTIAPOTOTIC PROPERTIES*

With the recognition of the central role of mitochondria in apoptosis, there is a need to develop specific tools to manipulate mitochondrial function within cells. Here we report on the development of a novel antioxidant that selectively blocks mitochondrial oxidative damage, enabling the roles of mitochondrial oxidative stress in different types of cell death to be inferred. This antioxidant, named mitoQ, is a ubiquinone derivative targeted to mitochondria by covalent attachment to a lipophilic triphenylphosphonium cation through an aliphatic carbon chain. Due to the large mitochondrial membrane potential, the cation was accumulated within mitochondria inside cells, where the ubiquinone moiety inserted into the lipid bilayer and was reduced by the respiratory chain. The ubiquinol derivative thus formed was an effective antioxidant that prevented lipid peroxidation and protected mitochondria from oxidative damage. After detoxifying a reactive oxygen species, the ubiquinol moiety was regenerated by the respiratory chain enabling its antioxidant activity to be recycled. In cell culture studies, the mitochondrially localized antioxidant protected mammalian cells from hydrogen peroxide-induced apoptosis but not from apoptosis induced by staurosporine or tumor necrosis factor-α. This was compared with untargeted ubiquinone analogs, which were ineffective in preventing apoptosis. These results suggest that mitochondrial oxidative stress may be a critical step in apoptosis induced by hydrogen peroxide but not for apoptosis induced by staurosporine or tumor necrosis factor-α. We have shown that selectively manipulating mitochondrial antioxidant status with targeted and recyclable antioxidants is a feasible approach to investigate the role of mitochondrial oxidative damage in apoptotic cell death. This approach will have further applications in investigating mitochondrial dysfunction in a range of experimental models.

The mitochondrial respiratory chain is a major source of superoxide and, therefore, mitochondria accumulate oxidative damage more rapidly than the rest of the cell, contributing to mitochondrial dysfunction and cell death in degenerative diseases and in aging (1–5). Mitochondria are also central to activating apoptosis and oxidative damage can lead to cell death, however, the significance of mitochondrial oxidative damage for cell death is unclear (6–8). One approach to this problem is to selectively target antioxidants to mitochondria (9–11). This should allow the relative importance of mitochondrial and cytoplasmic oxidative stress for cell death to be distinguished, and also enable the contribution of mitochondrial damage to aging, diabetes, and cancer to be investigated in cell and animal models.

Derivatives of ubiquinol are promising antioxidants to target to mitochondria (11, 12). In mammals ubiquinone comprises a 2,3-dimethoxy-5-methylbenzoquinone core with a hydrophobic 45- to 50-carbon chain at the 6 position (13, 14). Mitochondrial ubiquinone is a respiratory chain component buried within the lipid core of the inner membrane where it accepts two electrons from complexes I or II becoming reduced to ubiquinol, which then donates electrons to complex III (14). The ubiquinone pool in vivo is largely reduced and ubiquinol is an effective antioxidant, as well as being a mobile electron carrier (15–18). Ubiquinol acts as an antioxidant by donating a hydrogen atom from one of its hydroxyl groups to a lipid peroxyl radical, thereby decreasing lipid peroxidation within the mitochondrial inner membrane (18–20). The ubisemiquinone radicals thus formed disproportionate to ubiquinone and ubiquinol (21), or react with oxygen to form superoxide and ubiquinone thereby transferring the radical to the aqueous phase for detoxification by superoxide dismutase and peroxidases (17, 20). The respiratory chain then recycles ubiquinone back to ubiquinol to restore its antioxidant function. Vitamin E is another important antioxidant within the mitochondrial inner membrane, and the tocopheroxyl radical thus formed is regenerated to active vitamin E by reaction with ubiquinol or ubisemiquinone (15, 17, 20, 22, 23). Therefore, in vivo ubiquinol probably acts as an antioxidant by direct reaction with peroxyl radicals and by regenerating vitamin E (16, 17, 20).

The low solubility of ubiquinone in water makes it difficult to use in vitro, and animals must be fed ubiquinone-enriched diets for several weeks to increase levels in subsequently isolated mitochondria (11, 14). Therefore, to manipulate mitochondrial ubiquinone content in vitro we synthesized a ubiquinone analog selectively targeted to mitochondria by addition of a lipophilic triphenylphosphonium cation. Such lipophilic cations easily permeate lipid bilayers and accumulate in mitochondria within cells, driven by the large mitochondrial membrane po-
tential (9, 10, 24). Here we report on the antioxidant and antiapoptotic properties of this mitochondrially targeted ubiquinone derivative.

**MATERIALS AND METHODS**

**Chemical Syntheses**—To synthesize 11-bromoundecanoic peroxide (1) 11-bromoundecanoic acid (4.0 g, 15.1 mmol) and SOCl2 (1.6 ml, 21.5 mmol) were heated at 90 °C for 15 min (23). Excess SOCl2 was removed by distillation under reduced pressure (15 mm Hg, 90 °C) and the residue (IR, 1799 cm⁻¹) was dissolved in diethyl ether (20 ml) and cooled to 0 °C. Hydrogen peroxide (30%, 1.8 ml) was added, followed by dropwise addition of pyridine (1.4 ml) over 45 min, then diethyl ether (10 ml) was added and after 1 h at room temperature the product was diluted with diethyl ether (150 ml), washed with H2O (2 × 70 ml), 1 M HC1 (2 × 70 ml), H2O (70 ml), 0.5 M NaHCO3 (2 × 70 ml), and H2O (70 ml). After drying over MgSO4, the solvent was removed at room temperature under reduced pressure, giving crude 1 as a white solid (3.51 g), which was used without delay. IR (Nujol mull) 1810, 1100 cm⁻¹.

6-(10-Bromodecy)ubiquinone (2) was synthesized by stirring crude 1 (3.51 g, 12.5 mmol), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (1.31 g, 7.19 mmol, Aldrich), and acetic acid (60 ml) for 20 h at 100 °C. After cooling to room temperature, the mixture was diluted with diethyl ether (600 ml), washed with H2O (2 × 400 ml), 1 M HC1 (2 × 450 ml), 0.5 M NaHCO3 (2 × 450 ml), and dried over MgSO4. Removal of the solvent under reduced pressure gave a reddish solid (4.31 g). Column chromatography on silica gel, eluting with CH2Cl2, gave 2 as a red oil (809 mg, 28%) and unreacted 2,3-dimethoxy-5-methyl-1,4-benzoquinone (300 mg, 1.6 mmol, 13%).

To synthesize 10-(6-ubiquinonyl)decyltriphenylphosphonium bromide (4), triphenylphosphine (387 mg, 1.48 mmol), 3 (541 mg, 1.34 mmol), and ethanol (95%, 2.5 ml) were sealed under argon in a 15-ml Schlenk tube and stirred under argon for 10 min (Scheme 1). Excess NaBH4 was added to the mixture, followed by addition of diethyl ether (600 ml), washed with diethyl ether (150 ml), and dried over MgSO4. Removal of the solvent under reduced pressure gave a reddish solid (3.51 g). Column chromatography on silica gel, eluting with CH2Cl2, gave 2 as a red oil (809 mg, 28%) and unreacted triphenylphosphine and 2,3-dimethoxy-5-methyl-1,4-benzoquinone (300 mg, 1.6 mmol, 13%).

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To synthesize phosphonium bromide, triphenylphosphine (4.09 mg, 15.6 μmol), 3 (63.6 μmol; 15.6 μmol), and 250 μl of ethanol containing [3H]triphenylphosphine (74 μCi, Moravek Biochemicals, Brea, CA; 1 Ci/μmol) were sealed under argon in a Kimax tube and stirred in the dark for 55 h at 80 °C. After cooling the product was precipitated by addition of diethyl ether, and the orange solid was dissolved in CH2Cl2 and precipitated with diethyl ether. This was repeated four times to remove unreacted triphenylphosphine and 3. Two separate syntheses of [3H]-enriched 10-(6-ubiquinonyl)decyltriphenylphosphonium bromide were carried out giving products of 2.6 and 2.46 μCi/μmol, respectively, which gave the same results in experiments with isolated mitochondria, and their UV absorption spectra were as expected for a mixture of 4 and 5. TLC followed by scintillation counting of sectioned plates and comparison with the Rf values of the unlabeled compounds confirmed radioisotopic purity.

**Characterization of Products**—Stock solutions containing a mixture of 4 and 5 in ethanol were stored at -80 °C, and their concentrations were confirmed by 31P NMR. Fully oxidized solutions were generated by incubation in basic 95% ethanol on ice (13) or with beef heart mitochondrial membranes at room temperature. Both procedures gave an extin-
tion coefficient of 10,400 m\(^{-1}\) cm\(^{-1}\) at 275 nm for the quinone, with shoulders at 263 and 286 nm corresponding to the triphenylphosphonium moiety (26, 27) and a broad shoulder at 290 nm due to the quinone (13) (Fig. 1A). Reduction with NaBH\(_4\) gave the quinol, which had local maxima at 290 nm (\(\epsilon = 1800\) m\(^{-1}\) cm\(^{-1}\)) and at 285 nm (\(\epsilon = 3000\) m\(^{-1}\) cm\(^{-1}\)) (27). The absorbance at 275 nm in 0.1 M NaOH was 7000 m\(^{-1}\) cm\(^{-1}\). The quinone extinction coefficient (10,400 m\(^{-1}\) cm\(^{-1}\) at 275 nm) was slightly lower than that reported for other quinones (12,500 m\(^{-1}\) cm\(^{-1}\)) in aqueous buffer (28). This difference was not due to an intermolecular interaction between the phenol and the quinone, because the absorbances of 2 and the simple phosphonium methylnitrite (UHMP) were additive after either and the simple phosphonium methylnitrite (UHMP) were additive after either mixed together in either ethanol or aqueous buffer. To prepare mitoquinol an ethanolic solution was diluted in 0.5–1 ml of water and a few grains of NaBH\(_4\) were added. After incubation on ice in the dark for 5 min, excess NaBH\(_4\) was quenched with 5% HBr (0.2 ml) and the extract was then washed with 2 ml of NaCl, then the CH\(_2\)Cl\(_2\) was removed under nitrogen stream. Other samples were examined neat or as Nujol mulls over 100°C. Peroxynitrite was synthesized from acidified H\(_2\)O\(_2\) and NaNO\(_2\) in a simple flow reactor as described previously (35), concentrated by freeze fractionation and stock solutions in 1.5 M NaOH quantitated (\(\epsilon_{280} = 1.67 \times 10^4\) m\(^{-1}\) cm\(^{-1}\)) (36).

**Yeast Experiments**—The Saccharomyces cerevisiae strains used were D.\(\text{caq}^{+}\) (CEN.PK2-1C), \(\text{COQ3}^{+}\) (CEN.PK2-1C), \(\text{COQ2}^{+}\) (CEN.PK2-1C), and \(\text{COQ2}^{+}\) in CEN.PK2-1C (COQ2-1C). Yeasts were grown in Erlenmeyer flasks at 28°C under air with shaking at 200 rpm. For growth analysis, cultures in YPD (1% bacto yeast extract, 2% bactopeptone, 2% dextrose) were diluted into YPEG (1% bacto yeast extract, 2% bactopeptone, 3% ethanol, 3% glycerol) to an \(A_{595}\) of 0.1 and then grown in the dark while the \(A_{595}\) was monitored. For studies on yeast mitochondria, mitochondria were prepared from lactate-grown yeast of the CY4-\(\text{COQ3}^{+}\) and CY4 wild type strains (38). Briefly, lactate-grown yeast was isolated by centrifugation, the cell wall was removed by digestion with Zymolyase, spheroplasts were homogenized, and mitochondria were isolated by differential centrifugation. Mitochondria were stored at -80°C in 0.6 M sorbitol, 20 mM HEPES, pH 7.4, supplemented with 10 mg/ml fatty acid-free bovine serum albumin. For spectrophotometric assays, yeast mitochondria were washed in 0.6 M sorbitol, 20 mM HEPES and freeze-thawed in 50 mM potassium phosphate, pH 7.2.

**Mammalian Cell Culture**—Human osteosarcoma 143B cells were cultured at 37°C under humidified 95% air/5% CO\(_2\) in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 10% fetal calf serum. For toxicity studies, cells were grown to confluence in 24-well culture dishes and incubated for 24 h with DMEM/serum containing the compound. The supernatants were then harvested, and the amount of LDH released was assayed and compared with that present in untreated wells lysed with 0.1% Triton. For uptake studies cells were suspended in 0.5 ml of DMEM supplemented with 10 mM HEPES and 5 mM [\(^3\)H]mitoQ. After incubation, cells were pelleted by centrifugation, and the radioactivity in the pellet was quantitated by scintillation counting. For digitonin fractionation, cells were incubated with 0.1% digitonin then 500 ml of the cell suspension was mixed rapidly with 1.2 ml of ice-cold 250 mM sucrose, 20 mM MOPS pH 6.7, 3 mM EDTA, and 1 mg of digitonin, then 1 ml was layered onto 350 ml of oil (66% silicone oil/34% dioctyl phthalate) over 100 ml of 500 mM sucrose, 0.1% Triton and separated into mitochondrial and cytoplasmic fractions by centrifugation. The two fractions were assayed for citrate synthase and LDH activity or for content of radioactivity by scintillation counting.

**General Procedures**—Column chromatography was on Silica Gel type 60, 200–400 mesh, 40–63 \(\mu\)m (Merck). TLC was on Silica Gel G 60F\(_254\) (Merck) or on C-18 silica (Whatman). Nuclear magnetic resonance spectra were acquired on Varian 500 MHz or Varian 300 MHz instruments in CDCl\(_3\). Chemical shifts are in \(\delta\) units (ppm) downfield from tetramethylsilane for \(^1\)H NMR and \(^13\)C NMR and 85% phosphoric acid for \(^31\)P NMR. In some cases the total integral ratios in the \(^1\)H NMR were not precisely as expected; however, all other structural data are fully consistent with the proposed structures. Infrared absorption spectra were acquired using a PerkinElmer 1600 FTIR spectrometer. Phosphorus-31 NMR spectra were examined using a membrane permeation conform solution to NaCl discs followed by evaporation of the solvent in a nitrogen stream. Other samples were examined neat or as Nujol mulls between NaCl discs. Mass spectra were obtained from the Chemistry Department, University of Canterbury. Data are presented as \(m/z\) values for the parent molecular ion. Fluorescence measurements were made using a PerkinElmer MPP-3L fluorescence spectrophotometer.

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TABLE I
The octanol/PBS partition coefficients were determined at 37 °C and are means ± S.E.M. of three separate determinations

| Compound                | Octanol/PBS partition coefficient |
|-------------------------|----------------------------------|
| MitoQ                   | 160 ± 9                          |
| TPMP                    | 0.35 ± 0.02                      |
| Bromodecyl ubiquinone 2 | 310 ± 60                         |

FIG. 2. Reduction of mitoquinone and oxidation of mitoquinol by mitochondria. A, beef heart mitochondrial membranes (20 μg of protein/ml) were incubated with rotenone (8 μg/ml) and mitoQ (50 μM). A275 was monitored continuously and succinate (5 mM) and malonate (20 mM) were added where indicated. B, mitochondrial membranes were incubated as above with mitoquinol (50 μM), rotenone (8 μg/ml), and malonate (20 mM). Myxothiazol (10 μM) was added where indicated. When this experiment was repeated in the presence of ferricytochrome c (50 μM) and KCN (200 μM) the ferricytochrome c was reduced and this reduction was decreased by 60–70% by myxothiazol (data not shown). C and D, rat liver mitochondria (100 μg of protein/ml) were incubated in KCl, medium, and A275 was monitored. For the experiments shown in C, rotenone (8 μg/ml) and succinate (5 mM) were present and mitoQ (20 μM) was added where indicated. This experiment was repeated in the presence of malonate (20 mM) or FCCP (300 nM; not shown but identical to the experiment in the presence of malonate). For the experiments shown in D glutamate and malate (5 mM of each) were present and mitoQ (20 μM) was added where indicated. This experiment was repeated in the presence of rotenone (8 μg/ml) or FCCP (300 nM; not shown but identical to the experiment in the presence of rotenone).

FIG. 1. Absorption spectra of mitoquinol and mitoquinone. A, mitoQ (50 μM) was incubated with beef heart mitochondrial membranes (20 μg of protein/ml) for 1 h to oxidize it to mitoquinone. Reduction with NaBH4 (~250 μg) gave mitoquinol. B, 50 μM mitoQ was incubated with beef heart mitochondrial membranes, and the spectrum of mitoquinone was recorded (t = 0). Then antimycin (5 μM) and succinate (5 mM) were added, and further spectra were acquired at 5-min intervals (t = 5–25).

RESULTS AND DISCUSSION

Redox Activity of Mitoquinone and Mitoquinol—The mitochondrially targeted quinol, 10-(6′-ubiquinonyl)decyltriphenylphosphonium (4), and quinone, 10-(6′-ubiquinonyl)decyltriphenylphosphonium (5), were synthesized as shown in Scheme 1. Here they are called mitoquinol (reduced) and mitoquinone (oxidized), respectively, and mitoQ refers to a mixture of redox forms. As shown in Table I, mitoQ was intermediate in hydrophobicity between the simple phosphonium salt TPMP and the ubiquinone precursor bromodecyl ubiquinone (2). The distinctive absorption spectra of mitoquinone and mitoquinol are shown in Fig. 1A.

Electron Transfer between Mitochondria and mitoQ—To determine whether the respiratory chain could reduce mitoquinone, we incubated it with beef heart mitochondrial membranes and recorded its spectrum (Fig. 1B, t = 0). Addition of the respiratory substrate succinate reduced mitoquinone to mitoquinol (Fig. 1B). Mitoquinone-mitoquinol interconversion was then measured continuously by monitoring mitoquinone at 275 nm (Fig. 2). Mitoquinone was reduced by beef heart mitochondrial membranes and succinate, and this reduction was blocked by the complex II inhibitor malonate (Fig. 2A). Chemically reduced mitoquinol was also oxidized by membranes, and this oxidation was blocked by the complex III inhibitor myxothiazol (Fig. 2B). Mitoquinol and mitochondrial membranes reduced ferricytochrome c and myxothiazol inhibited this reduction by 60–70% (data not shown). Rat liver mitochondria respiring on succinate or glutamate/malate reduced mitoquinone, and this activity was blocked by the respiratory inhibitors malonate (Fig. 2C) or rotenone (Fig. 2D), respectively. Dissipation of the membrane potential with the uncoupler FCCP also eliminated the reduction of mitoquinone by preventing its uptake into mitochondria (Fig. 2, C and D).

Reduction of Mitoquinone by Respiratory Complexes—To distinguish between mitoQ reactions with respiratory complexes and the endogenous ubiquinone/ubiquinol pool, we extracted beef heart mitochondrial membranes with pentane to remove endogenous ubiquinone. These mitochondria still oxidized mitoquinol and, in the presence of succinate, reduced mitoquinone by a malonate-sensitive pathway (Fig. 3A). This strategy was extended to investigate yeast that entirely lacked endogenous ubiquinone due to inactivation of ubiquinone biosynthesis. These yeast did not grow on the nonfermentable carbon source YPEG until the short-chain ubiquinone analog Q2 was added, but addition of mitoQ did not lead to cell growth (Fig. 3B). However, when respiratory chain activity was measured in mitochondria isolated from these yeast, there was no electron flux from NADH or succinate to cytochrome c until Q2 was added (Fig. 3, C and D). MitoQ did stimulate myxothiazol-sensitive cytochrome c reduction, but less so than Q2 (Fig. 3, C and D). When ubiquinone reduction was analyzed directly, mitochondria lacking ubiquinone reduced both Q2 and mitoquinone, however, the rate for mitoquinone was slower than for Q2 (Fig. 3E). The rate of reduction of mitoquinone by mitochondria was reduced ferricytochrome c and myxothiazol inhibited this reduction by 60–70% (data not shown). Rat liver mitochondria respiring on succinate or glutamate/malate reduced mitoquinone, and this activity was blocked by the respiratory inhibitors malonate (Fig. 2C) or rotenone (Fig. 2D), respectively. Dissipation of the membrane potential with the uncoupler FCCP also eliminated the reduction of mitoquinone by preventing its uptake into mitochondria (Fig. 2, C and D).
This FCCP-sensitive accumulation of mitoQ was substantial upon addition of the uncoupler FCCP caused its immediate efflux (Fig. 4A). Tritiated mitoQ was taken up rapidly by energized mitochondria, and addition of FCCP up to 250 μM or daily additions of 5 μM mitoQ did not lead to cell growth (data not shown). Results show a typical experiment that was repeated several times and similar results were obtained when the CenAΔCoq3 yeast strain was used. C–E, mitochondria from the CY4 ΔCoq3 yeast strain were freeze-thawed and suspended at 50 μg of protein/ml and ferricytochrome c reduction measured at 550 nm. C, 125 μM NADH, 50 μM ferricytochrome c, and 2 mM KCN were present with or without myxothiazol (myx; 10 μM). 10 μM Q2 or 25 μM mitoQ were added as indicated. D, 10 mM succinate was present; myxothiazol (myx; 10 μM), 10 μM Q2, or 25 μM mitoQ were added as indicated; and ferricytochrome c reduction was measured at 550 nm. E, 10 μM Q2 or 25 μM mitoQ were added to freeze-thawed mitochondria respiring on 10 mM succinate, and ubiquinone reduction was monitored continuously at 275 nm. The inhibition of mitoQ reduction by malonate (20 mM) is also shown. F, the experiments shown in E were repeated using normal mitochondria isolated from the wild type CY4 strain.

similar in the presence or absence of endogenous ubiquinone (Fig. 3, E and F). We conclude that mitoQ can be reduced and oxidized by the mitochondrial respiratory chain and that this is primarily through the active sites of respiratory complexes rather than via the endogenous ubiquinone pool (14, 28).

Uptake of mitoQ by Isolated Mitochondria—Tritiated mitoQ was taken up rapidly by energized mitochondria, and addition of the uncoupler FCCP caused its immediate efflux (Fig. 4A). This FCCP-sensitive accumulation of mitoQ was substantial up to 20 μM mitoQ (Fig. 4B). The mitoQ accumulation ratios were measured relative to the simple lipophilic cation TPMP over a range of membrane potentials (Fig. 4C). At low membrane potential, the mitoQ accumulation ratio was greater than for TPMP, but at high potentials the maximum accumulation ratio for mitoQ (~500–600) was slightly less than that for TPMP (~1300), suggesting that the greater hydrophobicity of mitoQ decreases its uptake slightly relative to TPMP.

Location of mitoQ within Mitochondria—Alkyltriphenylphosphonium cations equilibrate between the bulk phase and a potential energy well on the membrane surface where they adsorb as a monolayer close to the carbonyl groups of the phospholipid fatty acids (41–43). This adsorption is described by the slope, b, in a plot of the surface density of adsorbed ions to their volume density in the bulk phase (42). We measured the non-specific adsorption of mitoQ and TPMP to de-energized mitochondrial preparations over a range of concentrations and determined the ratio of adsorbed to free cations from double-reciprocal plots (Fig. 4D). From these and the surface area of rat liver mitochondria (155 and 520 cm²/mg of protein for the outer and inner mitochondrial membranes, respectively) the ratio of the surface density of adsorbed ions to the volume density in the bulk phase was calculated.
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FIG. 5. Uptake of mitoQ by cells. A, 143B cells (5 × 10⁶) were incubated in 500 μl of DMEM/HEPES supplemented with 5 μM [3H]mitoQ (filled squares). These incubations were repeated in the presence of 4 μM FCCP (open triangle) or with a mixture of FCCP, valinomycin, ouabain, and oligomycin (open circles). Data are means ± range of two determinations, and this is a typical experiment that was repeated on three separate cell incubations. B, cells were incubated as above for 60 min with 5 μM mitoQ, and then intact cells were pelleted through oil (no additions) or the cells were treated with digitonin and the mitochondria pelleted through oil (plus digitonin). Citrate synthase (open bars) and lactate dehydrogenase (closed bars) activities were then measured in the pellet and supernatant fractions, and the proportion of the total activity that was found in the pelleted was calculated. Data are means ± S.D. of determinations on three separate cell preparations. C, cells were incubated as above with 5 μM [3H]mitoQ in the presence or absence of 4 μM FCCP, and then intact cells were pelleted through oil (cell) or the cell suspension was treated with digitonin and the mitochondria were pelleted through oil (mito). Data are means ± S.D. of experiments on four independent cell preparations.

FIG. 6. Effect of mitoQ on mitochondrial and cell function. A, the membrane potential of mitochondria respiring on succinate was measured in the presence of mitoQ. Data are expressed as a percentage of the membrane potential in the absence of mitoQ and are the means ± S.E. of measurements on three separate mitochondrial preparations. B and C, rates of coupled (closed bars), phosphorylating (open bars), and uncoupled (stippled bars) respiration were measured for mitochondria respiring on succinate (B) or glutamate and malate (C). Data are a percentage of the corresponding respiration rates in the absence of mitoQ and are the means ± S.E. of determinations on three separate mitochondrial preparations. In D mitoQ was incubated with 143B cells for 24 h, and LDH release into the culture medium was measured and expressed as a percentage of the total amount of LDH present in untreated wells. Data are means ± S.D. of three independent experiments.

Uptake of mitoQ by Mitochondria within Cells—Tritiated mitoQ incubated with a suspension of 143B osteosarcoma cells was taken up over 20–40 min, and this uptake was decreased by disrupting the mitochondrial membrane potential (Fig. 5A). To determine the location of mitoQ within cells, we disrupted the plasma membrane of cells with the detergent digitonin and pelleted the mitochondria by centrifugation through oil (Fig. 5, B and C). This separated cells into mitochondrially enriched and cytosolic fractions, as confirmed by the distribution of the mitochondrial and cytosolic marker enzymes citrate synthase and lactate dehydrogenase (Fig. 5B). About half the mitoQ within the cell was found in mitochondrial fraction, similar to the proportion of mitoQ uptake sensitive to FCCP (Fig. 5C). Therefore, substantial amounts of mitoQ are taken up by both isolated mitochondria and mitochondria within cells, driven by the mitochondrial membrane potential.

Low Toxicity of mitoQ to Mitochondria and Cells—The toxicity of mitoQ was investigated in mitochondria and cells. Up to 10 μM mitoQ had little effect on the membrane potential of isolated mitochondria but at 25 μM and above the potential decreased (Fig. 6A). For mitochondria respiring on either succinate or glutamate/malate, 10 μM mitoQ had little effect on uncoupled or phosphorylating respiration, but was inhibitory at 25–50 μM (Fig. 6, B and C). MitoQ stimulated coupled respiration by increasing the proton leak though the inner membrane; this effect was minimal for succinate at 10 μM but was noticeable at 10 μM for glutamate/malate (Fig. 6C). MitoQ toxicity to human 143B cells was determined from the release of lactate dehydrogenase into the culture medium over 24 h (Fig. 6D). MitoQ up to 10 μM did not affect cell viability, and
whether mitoquinone or mitoquinol was the effective antioxidant caused by oxidative stress (Fig. 7, A).

Membrane potential as indicated by a decrease in the membrane potential (Fig. 7 B). This oxidative damage also disrupted mitochondrial function.

To investigate the antioxidant efficacy of mitoQ, A, rat liver mitochondria were incubated with 10 mM succinate, 8 μg/ml rotenone, 50 μM ferrous sulfate, 100 μM ascorbic acid, and 1 mM hydrogen peroxide in the presence or absence of 5 μM mitoQ. Where indicated, 3 μM cis-parinaric acid was added and the fluorescence measured. Data are from a typical experiment repeated on three separate mitochondrial preparations. TPMP (5 μM) did not block cis-parinaric acid oxidation (data not shown). B, mitochondria were preincubated with 10 mM succinate and mitoQ for 5 min, and then a sample was taken for TBARS analysis (zero time). Ferrous sulfate (100 μM) and 300 μM ascorbic acid were then added, and 40 min later MDA formation was quantitated. Data are means ± range of duplicate determinations and are typical of experiments repeated on three separate mitochondrial preparations. C, mitochondria were incubated as described for B, isolated by centrifugation, and their membrane potential determined from the uptake of TPMP while respiring on glutamate/malate. Data are means ± range of a typical experiment. D, mitochondria were incubated as in B supplemented with 8 μg/ml rotenone. Succinate (5 mM) and mitoQ or TPMP (5 μM) were added to some incubations, whereas for the other incubations malonate (20 mM) was present and mitoQ was oxidized completely to mitoquinone before addition by incubation at basic pH. After preincubation for 5 min, ferrous sulfate (50 μM) was added, and 40 min later MDA formation was quantitated. Data are means ± range of duplicate determinations.

Concentrations of 25–50 μM were required for substantial cell death (Fig. 6 D). In summary, mitoQ concentrations up to 10 μM do not disrupt mitochondrial or cell function and, therefore, concentrations of 1–5 μM were used in subsequent experiments.

**Antioxidant Properties of mitoQ—** To investigate the antioxidant efficacy of mitoQ, we incubated mitochondria with cis-parinaric acid (Fig. 7 A). This fatty acid fluoresces within a lipid environment, and its conjugated double bond fluorophore is susceptible to oxidation. Consequently, the disappearance of fluorescence is a measure of lipid peroxidation (34). In the presence of mitochondria, cis-parinaric acid disappeared due to its insertion into lipid bilayers (Fig. 7 A). MitoQ prevented the oxidation of cis-parinaric acid by hydrogen peroxide and ferrous iron, demonstrating that mitoquinol is an antioxidant (Fig. 7 A). To quantify the antioxidant efficacy of mitoQ, mitochondria were incubated with ferrous iron, and the accumulation of MDA was measured as a marker of lipid peroxidation (Fig. 7 B). This oxidative damage also disrupted mitochondrial function as indicated by a decrease in the membrane potential (Fig. 7 C). Incubation with mitoQ prevented both the accumulation of MDA and the disruption to mitochondrial function caused by oxidative stress (Fig. 7, B and C). To determine whether mitoquinone or mitoquinol was the effective antioxidant, we oxidized mitoQ to mitoquinone and prevented its reduction by the respiratory chain by including malonate and rotenone in the incubation. Under these conditions, mitoquinone did not block lipid peroxidation (Fig. 7 D). In contrast, when mitoQ was reduced to mitoquinol by the respiratory chain, oxidative damage was prevented (Fig. 7 D). The simple lipophilic cation TPMP did not prevent lipid peroxidation (Fig. 7 D). Therefore, the antioxidant activity of mitoQ is due to its ubiquinol moiety.

Recycling of mitoQ by the Respiratory Chain—To determine whether mitoquinol was recycled by the respiratory chain after detoxifying a reactive oxygen species, we studied its interaction with peroxynitrite, a biologically significant oxidant formed from nitric oxide and superoxide (45–47). Because the half-life of peroxynitrite is only 1–2 s, mitoquinol regeneration from mitoquinone can be studied after all the added peroxynitrite has decomposed (45). Mitoquinol was rapidly oxidized to mitoquinone by peroxynitrite, however, mitoquinone was only detected when its reduction by the respiratory chain was prevented by malonate (Fig. 8 A, upper trace). Continuous monitoring of the mitoquinone concentration showed that peroxynitrite rapidly oxidized the mitoquinol to mitoquinone, which was then reduced to mitoquinol by the respiratory chain (Fig. 8 B). When malonate was present, this reduction by the respiratory chain was prevented (Fig. 8 B). We conclude that mitoQ is an effective antioxidant that can be recycled to its active form by the respiratory chain after detoxifying a reactive oxygen species.

**Prevention of Apoptosis by mitoQ—** A range of stimuli induce apoptosis by releasing cytochrome c from mitochondria into the cytoplasm, where it activates caspases. These include the oxidant hydrogen peroxide (8), the protein kinase C inhibitor staurosporine (40), and tumor necrosis factor-α (48). The mechanisms by which these stimuli cause cytochrome c release from mitochondria are unclear, but some or all may involve increased mitochondrial oxidative stress. Of particular interest is whether cytochrome c release induced by hydrogen peroxide is caused directly by mitochondrial oxidative damage or is a secondary consequence of cytoplasmic redox changes (8). A mito-
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Fig. 9. Prevention of apoptosis by mitoQ. A, Jurkat cells (5 × 10^6) in 5 ml of medium were preincubated for 30 min with no additions (open circles) or with 1 μM mitoQ (filled circles), then 150 μM hydrogen peroxide was added and cells were harvested at various times and their caspase activity was measured as the rate of DEVD-AMC cleavage. Without hydrogen peroxide there was no caspase activation either in the absence (open squares) or presence of 1 μM mitoQ (filled squares). Preincubation with 1 μM TPMP did not decrease caspase activation by hydrogen peroxide (filled triangle). The figure shows a typical experiment repeated on five separate cell preparations. B, Jurkat cells incubated with 1 μM mitoQ or Q, were treated with 150 μM hydrogen peroxide and 6 h later their caspase activity compared with cells treated with hydrogen peroxide alone. This figure shows typical experimental traces for caspase assays. C and D, Jurkat cells were treated with 150 μM hydrogen peroxide in the presence or absence of 1 μM mitoQ, cells were harvested at various times and annexin V binding analyzed by flow cytometry. C shows results from a typical experiment to measure the annexin V binding of cells harvested 6 h after treatment with 150 μM hydrogen peroxide in the presence or absence of 1 μM mitoQ. D shows measurements of the proportion of annexin V-positive cells at various times after addition of 150 μM hydrogen peroxide in the absence (open circles) or presence (filled circles) of 1 μM mitoQ. Cells incubated in the presence (filled squares) or absence (open squares) of 1 μM mitoQ without hydrogen peroxide treatment are also shown. Data are means ± range of duplicate determinations, and the experiment was repeated on two cell preparations with similar results.

The situation with hydrogen peroxide, mitoQ did not prevent apoptosis in Jurkat cells treated with staurosporine (40) or in WEHI 164 cells treated with tumor necrosis factor-α (49) (data not shown). We conclude that mitoQ blocks apoptosis induced by hydrogen peroxide. Because mitochondrial localization of the antioxidant is required to prevent apoptosis, mitochondrial oxidative stress may be a critical step in hydrogen peroxide-induced apoptosis but not for apoptosis following treatment with staurosporine or tumor necrosis factor-α.

Conclusion—To provide new approaches to investigate the role of mitochondrial oxidative damage in cell death, we synthesized a mitochondrially targeted antioxidant, mitoQ, comprising a ubiquinone attached to a triphenylphosphonium lipophilic cation. The ubiquinone moiety was found to cycle between its oxidized (mitoquinone) and reduced (mitoquinol) forms by exchanging electrons with the respiratory chain. Mitoquinol was an effective antioxidant protecting mitochondria from oxidative damage and was rapidly regenerated by the respiratory chain after detoxifying a reactive oxygen species. As anticipated, the triphenylphosphonium cation led to the rapid and reversible accumulation of mitoQ by isolated mitochondria and by mitochondria within cells. Therefore, mitoQ is a mitochondrial-specific antioxidant.

We then used mitoQ to help elucidate the role of mitochondrial oxidative damage in apoptotic cell death. As a first step we showed that mitoQ prevented apoptosis caused by hydrogen peroxide but not that caused by staurosporine or tumor necrosis factor-α. This suggests that mitochondrial oxidative damage plays an important role in hydrogen peroxide-induced apoptosis but is not required for apoptosis induced by staurosporine or tumor necrosis factor-α. Further work using these and other mitochondrially targeted compounds to dissect out the role of mitochondrial oxidative changes in hydrogen peroxide-induced apoptosis is ongoing. The findings reported here demonstrate that mitochondrially targeted antioxidants such as mitoQ can be used to investigate the role of mitochondrial oxidative stress in cell death. This strategy also has potential for unraveling the contribution of oxidative stress to other pathologies involving mitochondrial dysfunction.

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REFERENCES
1. Wallace, D. C. (1999) Science 283, 1482–1488
2. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922
3. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1995) Biochim. Biophys. Acta 1271, 165–170
4. Beckman, K. B., and Ames, B. N. (1998) Physiol. Rev. 78, 547–581
5. Michikawa, Y., Mazzucchelli, F., Bresolin, N., Starlato, G., and Attardi, G. (1999) Science 286, 774–779
6. Poylaak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) Nature 389, 306–305
7. Kroemer, G., Dallaporta, B., and Resche-Rigon, M. (1998) Ann. Rev. Physiol. 60, 619–642
8. Hampton, M. B., and Orenius, S. (1997) FEBS Lett. 414, 552–556
9. Murphy, M. P. (1997) Trends Biotechnol. 15, 326–330
10. Murphy, M. P., and Smith, B. A. J. (2000) Adv. Drug Delivery Rev. 41, 235–250
11. Matthews, R. T., Yang, L., Browne, S., Bail, M., and Beal, M. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8892–8897
12. Lass, A., Forster, M. J., and Sohal, R. S. (1999) Free Radic. Biol. Med. 26, 1557–1592
13. Crane, F. L., and Barr, R. (1971) Methods Enzymol. 18C, 137–165
14. Crane, F. L. (1977) Annu. Rev. Biochem. 46, 439–469
15. Lass, A., and Sohal, R. S. (1998) Arch. Biochem. Biophys. 352, 229–236
16. Kagan, V. E., Serbinova, E. A., Stoyanovskaya, D. A., Khvaja, S., and Packker, L. (1994) Methods Enzymol. 234, 343–354
17. Maguire, J. J., Wilson, D. S., and Packer, L. (1989) J. Biol. Chem. 264, 21462–21465
18. Ernst, L., Forshark, P., and Nordenbrand, K. (1992) Biofactors 3, 241–248
19. Takada, M., Ikenoya, S., Yuzuriha, T., and Katayama, K. (1994) Methods Enzymol. 105, 147–155
20. Ingold, K. U., Bowry, W. V., Stocker, R., and Walling, C. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 45–49
21. Land, E. J., and Swallow, A. J. (1970) J. Biol. Chem. 245, 1890–1894
22. Stoyanovsky, D. A., Osipov, A. N., Quinn, P. J., and Kagan, V. E. (1995) Arch. Biochem. Biophys. 323, 343–351
23. Mukai, K., Ikikuchi, S., and Urano, S. (1990) Biochim. Biophys. Acta 1035, 77–82
24. Liberman, E. A., Topali, V. P., Tsolfa, L. M., Jasaitis, A. A., and Skulachev, V. P. (1969) Nature 222, 1076–1078
25. Yu, C. A., and Yu, L. (1995) Methods Enzymol. 322, 60–68
26. Cabrini, L., Landi, L., Pasquali, P., and Lenaz, G. (1981) Arch. Biochem. Biophys. 208, 11–19
27. Burns, R. J., Smith, R. A. J., and Murphy, M. P. (1995) Arch. Biochem. Biophys. 322, 60–68
28. Chappell, J. B., and Hansford, R. G. (1972) in Subcellular Components: Preparation and Fractionation (Birnie, G. D., ed) pp. 77–91, Butterworths, London
30. Smith, A. L. (1967) Methods Enzymol. 10, 81–86
31. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) J. Biol. Chem. 177, 745–756
32. Scott, I. D., and Nicholls, D. G. (1980) Biochem. J. 186, 21–33
33. Brown, G. C., and Brand, M. D. (1985) Biochem. J. 225, 399–405
34. Tribble, D. L., van den Berg, J. J. M., Motchnik, P. A., Ames, B. N., Lewis, D. M., Chait, A., and Krauss, R. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1183–1187
35. Packer, M. A., and Murphy, M. P. (1994) FEBS Lett. 345, 237–240
36. Hughes, M. N., and Nicklin, H. G. (1968) J. Chem. Soc. A, 450–452
37. Grant, C. M., Maciver, F. H., and Dawes, I. W. (1997) FEBS Lett. 410, 219–222
38. Glick, B. S., and Puisis, L. (1995) Methods Enzymol. 260, 213–233
39. Burns, R. J., and Murphy, M. P. (1997) Arch. Biochem. Biophys. 339, 33–39
40. Scarlett, J. L., Sherd, P. W., Hughes, G., Ledgerwood, E. C., Ku, H. H., and Murphy, M. P. (2000) FEBS Lett. 475, 267–272
41. Ono, A., Miyachi, S., Demura, M., Asakura, T., and Kano, N. (1994) Biochemistry 33, 4312–4318
42. Flewelling, R. F., and Hubbell, W. L. (1986) Biochem. J. 49, 531–540
43. Flewelling, R. F., and Hubbell, W. L. (1986) Biochem. J. 49, 541–552
44. Schwierzmann, K., Cruz-Orive, L. M., Eggman, R., Sanger, A., and Weibel, E. R. (1986) J. Cell Biol. 102, 97–103
45. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624
46. Murphy, M. P., Packer, M. A., Scarlett, J. L., and Martin, S. W. (1998) Gen. Pharmacol. 31, 179–186
47. Schopfer, F., Riobo, N., Carreras, M. C., Alvarez, B., Radi, R., Boveris, A., Cadenas, E., and Poderoso, J. J. (2000) Biochem. J. 349, 35–42
48. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481–490
49. Faraco, P. R., Ledgerwood, E. C., Vandenabbeele, P., Prins, J. B., and Bradley, J. R. (1999) Biochem. Biophys. Res. Commun. 263, 385–392