Supplementation of Endothelial Cells with Mitochondria-targeted Antioxidants Inhibit Peroxide-induced Mitochondrial Iron Uptake, Oxidative Damage, and Apoptosis*

Received for publication, April 12, 2004, and in revised form, June 24, 2004
Published, JBC Papers in Press, June 25, 2004, DOI 10.1074/jbc.M404003200

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The mitochondria-targeted drugs mitoquinone (Mito-Q) and mitovitamin E (MitoVit-E) are a new class of antioxidants containing the triphenylphosphonium cation moiety that facilitates drug accumulation in mitochondria. In this study, Mito-Q (ubiquinone attached to a triphenylphosphonium cation) and MitoVit-E (vitamin E attached to a triphenylphosphonium cation) were used. The aim of this study was to test the hypothesis that mitochondria-targeted antioxidants inhibit peroxide-induced oxidative stress and apoptosis in bovine aortic endothelial cells (BAEC) through enhanced scavenging of mitochondrial reactive oxygen species, thereby blocking reactive oxygen species-induced transferrin receptor (TfR)-mediated iron uptake into mitochondria. Glucose/glucose oxidase-induced oxidative stress in BAECs was monitored by oxidation of dichlorodihydrofluorescein that was catalyzed by both intracellular H₂O₂ and transferrin iron transported into cells. Pretreatment of BAECs with Mito-Q (1 μM) and MitoVit-E (1 μM) but not untargeted antioxidants (e.g. vitamin E) significantly abrogated H₂O₂- and lipid peroxide-induced 2,7'-dichlorofluorescein fluorescence and protein oxidation. Mitochondria-targeted antioxidants inhibit cytochrome c release, caspase-3 activation, and DNA fragmentation. Mito-Q and MitoVit-E inhibited H₂O₂- and lipid peroxide-induced inactivation of complex I and aconitase, TfR overexpression, and mitochondrial uptake of ⁵⁵Fe, while restoring the mitochondrial membrane potential and proteasomal activity. We conclude that Mito-Q or MitoVit-E supplementation of endothelial cells mitigates peroxide-mediated oxidative stress and maintains proteasomal function, resulting in the overall inhibition of TfR-dependent iron uptake and apoptosis.

Enhanced mitochondrial oxidative damage is a prominent feature of most age-related human diseases including neurodegenerative disorders. Aberrant electron leakage from mitochondria in the respiratory chain in oxidant-stressed cells triggers the formation of reactive oxygen species (ROS), leading to enhanced oxidative damage in mitochondria (1). The increased oxidative damage in mitochondria induces the mitochondrial permeability transition resulting in apoptotic or necrotic cell death (2). Mitochondria play a major role in regulating apoptosis through enhanced release of cytochrome c that results in the activation of caspasases and subsequent cell death (3, 4). Several mitochondrial diseases, including amyotrophic lateral sclerosis, Parkinson’s, Alzheimer’s, and Huntington’s diseases, are characterized by dysfunctional or defective respiratory chain components (e.g. complexes I and II) that exacerbate ROS formation (2, 5–7). In addition, levels of mitochondrial iron increased under these pathological conditions (8–12).

The selective mitigation of oxidative damage in mitochondria is therefore an effective therapeutic strategy in such age-related human disorders. However, a major limitation of antioxidant therapy in the treatment of mitochondrial diseases has been the inability to enhance antioxidant levels in mitochondria (13). Recently, there was a breakthrough in mitochondrial targeting of antioxidants (14). Antioxidants were covalently coupled to a triphenylphosphonium cation, and these compounds were preferentially taken up by mitochondria (13–15). These agents initially accumulated in the cytoplasmic region of cells because of the negative plasma membrane potential (30–60 mV) (14–16). The lipophilic cations easily permeate through the lipid bilayers and subsequently accumulate several hundred-fold within mitochondria because of a large mitochondrial membrane potential (150–170 mV; negative inside).

Mito-Q, a derivative of ubiquinone, and MitoVit-E, a derivative of Vit-E, are two promising antioxidants (Fig. 1) that are specifically targeted to mitochondria (17, 18). Mitochondrial ubiquione is a respiratory chain component buried within the lipid core of the inner membrane where it accepts 2 electrons from complexes I or II forming the corresponding reduction product (i.e. ubiquinol) which then donates electrons to complex III (19). The ubiquinone pool in vivo exists largely in the reduced ubiquinol form acting as an antioxidant and a mobile electron carrier.

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Ubiquinol has been reported to function 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 (20–22). The ubisemiquinone radical formed during this process disproportionates into ubiquinone and ubiquinol (23, 24). The respiratory chain subsequently recycles ubiquinone back to ubiquinol, restoring its antioxidant function. Vitamin E (Vit-E) (or α-tocopherol) is another antioxidant within the mitochondrial inner membrane, and the tocopheroxyl radical formed from one-electron oxidation of Vit-E regenerates Vit-E by reacting with lipid peroxyl radical, thereby decreasing lipid peroxidation within mitochondria by undergoing redox cycling in the mitochondrial electron transport chain.

In this study, we tested the efficacy of two mitochondria-targeted antioxidants in preventing mitochondrial damage and apoptosis in bovine aortic endothelial cells (BAEC) treated with glucose/glucose oxidase and lipid hydroperoxide. Results suggest that mitochondria-targeted antioxidants are more effective inhibitors of mitochondrial damage and apoptosis than the corresponding “untargeted” counterparts (i.e. Vit-E).

EXPERIMENTAL PROCEDURES
Glucose oxidase was obtained from Sigma. 13-Hydroperoxyoctadecadienoic acid (13-HpODE) and 13-hydroxyoctadecadienoic acid were purchased from Cayman Chemical Co. 2’,7’-Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes. N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norleucinal (MG-132) was obtained from Biomol, and clasto-lactacystin-β-carbodermate (Lac) was purchased from Sigma. Mito-Q and MitoVit-E were synthesized as follows.

Mito-Q—Mitoquinone was synthesized according to the published method (15). Briefly, 11-bromoperoxycoumaric acid prepared from 11-bromoundecanoic acid was coupled with 2,3-dimethoxy-5-methyl-1,4-benzquinone to yield the 6-(10-bromodecyl) ubiquinone. The quinone was reduced to the quinol using sodium borohydride and heated with triphenylphosphine in dioxane for 4 days. The oily product separated from the reaction medium was purified and analyzed by mass spectrometry.

Endothelial Cell Culture—The cells were grown to confluence in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), insulin (10 μg/ml), transferrin (5 μg/ml), gluta mine (4 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Cells were used between passages 4 and 12, as described by Balla et al. (28). On the day of the treatment, the medium was replaced with DMEM containing 2% FBS. Mito-Q (1 μM) or MitoVit-E (1 μM) was added to the cells in the medium that contained 25 mM glucose as a substrate for glucose oxidase.

Cell Viability—Intracellular conversion of MTT to formazan was used as an indicator of cell viability (λmax = 562 nm). After treatment with Glu/GO in the presence and absence of antioxidants, the culture media were removed, and the cells were washed three times with control salt solution (CSS) (120 mM NaCl, 25 mM HEPES, pH 7.4, 25 mM KCl, 1.8 mM CaCl2, 4 mM MgCl2, and 15 mM glucose). Cells were incubated in CSS buffer containing 0.25 mg/ml MTT for 2 h at 37 °C. CSS buffer was removed, and cells were solubilized and mixed thoroughly in isopropyl alcohol, 0.08 N HCl (1:1) and the concentration of formazan measured optically as described previously (29).

Caspase Activity—After treatment with glucose/glucose oxidase (Glu/GO) and antioxidants, cells were washed twice with DPBS and lysed with cell lysis buffer (caspase-3 assay kit, Clontech). The caspase activities were measured as described previously (30). Cells were washed twice with DPBS following treatment with the aforementioned antioxidants and then lysed with 50 mM HEPES buffer, pH 7.4, containing 5 mM CHAPS and 5 mM dithiothreitol. After the cytosolic fraction was taken by centrifugation at 12,000 × g for 30 min, the caspase activities were measured in the supernatant using DEVD-pNA (acetyl-Asp-Glu-Val-Asp para-nitroanilide), acetyl-LEHD-pNA, and acetyl-IETD-pNA as substrates. The absorbance at 405 nm of the released pNA was monitored in a spectrophotometer and quantitated by using pNA as standard.

Fig. 1. Structures of mitochondria-targeted and untargeted antioxidants.

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**TUNEL Measurements**—The terminal deoxynucleotidyltransferase-mediated nick-end labeling (TUNEL) assay was used for microscopic detection of apoptosis (30). This assay was based on labeling of 3′-free hydroxyl ends of the fragmented DNA with fluorescein-dUTP catalyzed by terminal deoxynucleotidyltransferase. Procedures were followed according to the commercially available kit (ApoAlert) from Clontech. Apoptotic cells exhibit a strong nuclear green fluorescence that can be detected by using a standard fluorescein filter (520 nm). All cells stained with propidium iodide exhibit a strong red cytoplasmic fluorescence at 620 nm. The areas of apoptotic cells were detected by fluorescence microscopy equipped with rhodamine and fluorescein isothiocyanate filters.

**Fig. 2.** The effect of Mito-Q and mitovitamin E on glucose/glucose oxidase-induced caspase activation. A, the effect of Mito-Q (1 μM) on caspase-3 activity was determined in BAEC treated with Glu/GO (20 milliunits). Cells were pretreated with Mito-Q (1 μM) for 1 h and incubated with Glu/GO for 2, 4, 8, and 16 h. B, same as A except that MitoVit-E (1 μM) was used. C, treatment conditions are same as A except that caspase-9 activity was measured under these conditions. D, same as A, except that the effect of MitoVit-E (1 μM) on caspase-9 activity was measured in BAEC treated with Glu/GO (20 milliunits). E, the effect of Mito-Q and MitoVit-E on caspase-8 activation was measured in BAEC treated with Glu/GO (20 milliunits). Data shown are representative of three separate experiments. * represents p < 0.01 as compared with Glu/GO-treated group. F, BAECs were treated with Glu/GO (20 milliunits) for 8 h with or without Mito-Q (1 μM), and cells were stained for TUNEL-positive cells and examined by fluorescence microscopy as described under “Experimental Procedures.”
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Detection of Cytochrome c Release into Cytosol—The release of mitochondrial cytochrome c into the cytosol was measured according to methods described previously (31, 32). Briefly, BAECs were washed with DPBS and homogenized in PBS supplemented with 40 μg/ml saponin. Lysate was centrifuged at 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction to measure the released cytochrome c into the cytosol by Western blot analysis using a mouse anti-cytochrome c antibody (Pharmingen). Detection was by horseradish peroxidase-conjugated goat anti-mouse antibody using the ECL method.

Aconitase Activity—BAECs were washed three times with cold PBS and lysed with a lysing buffer containing 0.2% Triton X-100, 100 μM DTPA, and 5 mM citrate in PBS. The activity of aconitase in cell lysates was measured in 100 mM Tris-HCl, pH 8.0, containing 20 mM NADH and 20 μM aconitase inhibitor. An extinction coefficient for cis-aconitase of 3.6 mm−1 at 240 nm was used (33).

Complex I Activity—The complex I activity was measured as described previously (34). Briefly, cells were pelleted at 500 × g at 4 °C for 10 min and resuspended in 2.5 ml of TES buffer (0.25 M sucrose, 1 mM EGTA and 10 mM triethanolamine acetate, pH 7.0) after treatment. Cells were homogenized with a Dounce homogenizer with 15 strokes. Resulting material was transferred to a 2-ml tube and centrifuged at 1,500 × g. Postnuclear supernatant was centrifuged at 10,000 × g at 4 °C for 10 min to obtain a mitochondria-enriched pellet. The pellet was washed twice with 1 ml of homogenization buffer. The protein content of the pellet was assayed by the Bradford method. The mitochondrial pellet was subjected to “freeze-thawing” three times. Twenty microliters (0.3 mg of protein) of mitochondrial homogenate was mixed with 930 μl of 10 mM potassium phosphate buffer, pH 8.0, in a 1-ml cuvette containing 50 μl of 100 μM NADH. The rate of NADH oxidation was monitored at 340 nm for 2 min in a UV spectrophotometer. Then 5 μl of 10 mM ubiquinone-1 was added, and the stimulated rate of NADH oxidation was measured as complex I activity, using an extinction coefficient of 6.81 mm−1 cm−1 at 340 nm.

DCFH Staining—The determination of intracellular oxidant production is based on the oxidation of 2,7′-dichlorodihydrofluorescein (DCFH) to a fluorescent 2,7′-dichlorofluorescein (DCF). Following treatment of BAEC with Glu/GO and other targeted antioxidants, the medium was aspirated, and cells were washed twice with DPBS and incubated in 1 ml of medium without FBS. DCFH was added at a final concentration of 10 μM and incubated for 20 min. The cells were then washed once with DPBS and maintained in a 1-ml culture medium. The fluorescence was monitored after 30 min by using a Nikon fluorescence microscope equipped with a fluorescent isocyanate filter.

Flow Cytometry—Following different treatments, BAECs were washed in PBS three times and incubated with DCFH-DA or Mitosensor reagent for 15 min at 37 °C. The fluorescence was measured using a BD Biosciences FACScan flow cytometer with excitation and emission setting at 488 and 530 nm, respectively (35).

Glutathione Measurement—GSH levels were measured by high pressure liquid chromatography as the orthophthalaldehyde (OPA) adduct at pH 8.0 (36). Cells were washed twice with PBS, suspended in 250 μl of PBS, and lysed by sonication. After centrifugation at 10,000 × g for 2 min, 200 μl of the clear supernatant was derivatized by incubating with OPA for 30 min at room temperature. An aliquot of sample was injected into a column (Kromasil C-18) and eluted isocratically with a mobile phase consisting of 150 mM sodium acetate/methanol (91:5.5:5). The OPA-OPA adduct was monitored using a fluorescence detector operating at excitation and emission wavelengths at 250 and 410 nm, respectively. The levels of intracellular GSH were detected using a GSH solution as a standard.

Measurement of 55Fe Uptake in Endothelial Cells—BAECs were grown in DMEM containing 10% FBS until confluence. On the day of treatment the medium was replaced with DMEM containing 2% FBS, and the cells were allowed to adjust to the medium conditions. 0.2 μCi of 55Fe (as ferric chloride) was added to the medium, and its levels were measured after 4 h. Cells were washed with DPBS and lysed with PBS containing 0.1% Triton X-100, and the radioactivity was counted in a beta counter (36).

Measurement of 32P Glucose Uptake into Mitochondria of Endothelial Cells—BAECs were grown in DMEM containing 10% FBS until confluence. On the day of treatment the medium was replaced with DMEM containing 2% FBS, and the cells were allowed to adjust to the medium conditions. 0.2 μCi of 32P glucose (as ferric chloride) was added to the medium. Four hours later, the cells were washed twice with an isotope medium (320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris-HCl, pH 7.4). The cells were then scraped by using 1 ml of isolation medium and spun at 500 × g for 5 min at 4 °C. Cell pellets were resuspended in a 4-ml isolation medium and spun at 1500 × g. The supernatants were combined and spun at 1500 × g for 10 min at 4 °C. The supernatant was spun at 17,000 × g for 11 min at 4 °C. Final pellets were resuspended in a 200 μl of isolation medium. An aliquot was taken for protein estimation, and the remaining suspension was used for counting in a beta counter.

Detection of Transferrin Receptor Levels—BAECs were washed with ice-cold phosphate-buffered saline and resuspended in 100 μl of RIPA buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 100 mM NaCl, 100 mM sodium fluoride). To a 10-ml solution of the above, the following agents were added: 1 mM sodium vanadate, 10 μg/ml anti-apoptin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin inhibitors. Cells were homogenized by passing the suspension through a 25-gauge needle (15 strokes). The lysate was centrifuged for 15 min at 12,000 × g. Protein was determined by the Bradford method, and 20 μg were used for the Western blot analysis. Proteins were resolved on polyacrylamide gels and blotted onto nitrocellulose membranes. Membrane was washed twice with TBST (140 mM NaCl, 50 mM Tris-HCl, pH 7.2) containing 0.1% Tween 20 before blocking the non-specific binding with TBS containing 5% skim milk. Membrane was incubated with mouse anti-human transferrin receptor monoclonal antibody (1 μg/ml in TBS) (Zymed Laboratories Inc.) and 2% skim milk for 2 h at room temperature. Membrane was washed 5 times and detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5000) for 1 h at room temperature. The bands were detected by using the ECL method (Amersham Biosciences).

Detection of Mitochondrial Transmembrane Potential Changes—BAECs were treated in 6-well plates with Glu/GO with or without the targeted antioxidants. After treatment the cells were washed with serum-free media and incubated with the BD MitoSensor dye (Clontech) and incubated for 20 min according to the manufacturer’s instructions. This dye is sensitive to changes in mitochondrial transmembrane function.
potential changes. The fluorescence was monitored by using a fluorescein isothiocyanate and a rhodamine filter.

Proteasome Activity—26 S proteasome. Proteasomal activity was measured as reported earlier (39, 40). Briefly, BAECs were washed with buffer I (50 mM Tris, pH 7.4, 2 mM dithiothreitol, 5 mM MgCl₂, 2 mM ATP) and homogenized with buffer I containing 250 mM sucrose. Twenty micrograms of 10,000 g supernatant were diluted with buffer I to a final volume of 1 ml. The fluorogenic proteasome substrates N-succinyl-LLVY-AMC (chymotrypsin-like) and benzoxycarbonyl-Leu-Leu-Lys-amino-4-methylcoumarin (trypsin-like) were added in a final concentration of 100 and 80 μM, respectively. Proteolytic activity was measured by monitoring the release of the fluorescent group 7-amino-4-methylcoumarin (excitation 380 nm, emission 460 nm).

RESULTS

Measurements of Cell Viability—BAECs were incubated with Glu/GO for various times with or without Mito-Q (1 μM) and MitoVit-E (1 μM), and the cell viability was estimated by using the MTT assay. Glu/GO decreased the cell viability to 18% which was restored to 64 and 51%, respectively, in the presence of Mito-Q and MitoVit-E. Similar results with HpODE were observed, where treatment with Mito-Q and MitoVit-E restored the cell viability to 59 and 48%, respectively (data not shown).

Mitochondria-targeted Antioxidants Inhibit Apoptosis in BAEC Treated with Hydroperoxides—BAECs were incubated with Glu/GO (generator of H₂O₂) or 13-HpODE (lipoxigenase-catalyzed oxidative metabolite of linoleic acid) for different times, and caspase-3, -9, and -8 activities were measured (Figs. 2 and 3). The caspase-3 and -9 activities were markedly elevated after a 4-h treatment in Glu/GO-treated cells (Fig. 2, A–D). Unlike caspase-3 and caspase-9 activities, the caspase-8 activity increased only slightly in Glu/GO-treated cells (Fig. 2E). This is consistent with an intrinsic pathway involving mitochondria-mediated apoptosis. Exposure of BAEC to Glu/GO increased the percentage of TUNEL-positive cells (Fig. 2F). Pretreatment with mitochondria-targeted antioxidants, Mito-Q and MitoVit-E, inhibited the caspase-3 and caspase-9 proteolytic activation induced by Glu/GO. As shown in Fig. 2, A and C, Mito-Q-pretreated cells were markedly resistant to H₂O₂-induced caspase activation. Pretreatment with MitoVit-E also inhibited H₂O₂-mediated caspase activation, albeit...
to a lesser extent (Fig. 2, B and D), whereas treatment with ubiquinone and vitamin E elicited little or no inhibition of caspase-3 activation in H$_2$O$_2$-treated cells (Fig. 3, A and B). Pretreatment of cells with Mito-Q significantly decreased the fraction of TUNEL-positive cells induced by H$_2$O$_2$ generated from Glu/GO. These results indicate that mitochondria-targeted antioxidants are more potent in mitigating H$_2$O$_2$-induced apoptosis than their untargeted parent antioxidants.

Next, we investigated the effect of mitochondria-targeted antioxidants in BAEC treated with a lipid hydroperoxide, 13-HpODE. These results are shown in Fig. 4. Treatment with 13-hydroxyoctadecadienoic acid (two-electron reduction product of 13-HpODE) did not induce caspase activation in BAECs (Fig. 4A). As shown in Fig. 4, A and B, pretreatment of cells with Mito-Q or MitoVit-E caused a significant reduction in caspase-3 and -9 activities in 13-HpODE-treated cells. In contrast, pretreatment with “untargeted antioxidants” (e.g. vitamin E) did not elicit the same effects (Fig. 4).

**Effect of Mitochondria-targeted Antioxidants on Peroxide-induced Inactivation of Complex I and Aconitase**—As reported previously (31), treatment of BAEC with Glu/GO caused a significant depletion (less than 10% of control) in intracellular GSH levels, following an 8-h treatment (not shown). In the presence of Mito-Q (1 μM), GSH levels were restored to about 60% of control in cells treated with Glu/GO for 8 h (data not shown). Exposure of BAEC to Glu/GO and 13-HpODE caused a steady decrease in aconitase and complex I activities (Fig. 5). Pretreatment with Mito-Q prevented the inactivation of aconitase and complex I in the HpODE-exposed group rather than when exposed to Glu/GO under these conditions (Fig. 5). In contrast, treatment of cells with untargeted vitamin...
E or ubiquinone did not have any effect on Glu/GO-induced inactivation of aconitase or complex I activities, even at concentrations 10-fold higher than that of Mito-Q or MitoVit-E (Fig. 6).

Transferrin Receptor-mediated $^{55}$Fe Uptake in Glucose/Glucose Oxidase-treated BAEC—Previously, we reported an increase in TfR expression and $^{55}$Fe uptake in Glu/GO-treated BAEC (41), and we also reported that pretreatment of cells with anti-TfR antibody that specifically binds to the extracellular domain of TfR inhibited H$_2$O$_2$-induced iron signaling implying that the iron uptake is mediated by the transferrin receptor. In Glu/GO-treated cells, the TfR expression increased by 2.3-fold which was inhibited in Mito-Q-pretreated cells (Fig. 7A). Exposure of BAEC to Glu/GO or 13-HpODE caused an increase in intracellular and mitochondrial uptake of $^{55}$Fe. As shown in Fig. 7, C and D, Glu/GO treatment and HpODE induced an increase in $^{55}$Fe uptake by cells. The mitochondrial uptake of $^{55}$Fe also increased in Glu/GO-treated and HpODE-treated cells (Fig. 7, E and F). Mito-Q and MitoVit-E treatment inhibited the cellular and mitochondrial $^{55}$Fe uptake in BAEC (Fig. 7, C–F). In contrast, treatment with untargeted antioxidants (i.e. Vit-E) had little or no effect on HpODE-induced $^{55}$Fe uptake (Fig. 7, D and F). These findings indicate that mitochondria-targeted antioxidants are more potent in inhibiting peroxide-induced overexpression of TfR and iron uptake in BAEC.

Mito-Q and MitoVit-E Protect BAEC from Peroxide-mediated Oxidative Stress—The formation of a fluorescent DCF from oxidation of a nonfluorescent probe DCFH was used to detect intracellular oxidative stress and oxidant-induced TfR-dependent iron uptake (42–44). H$_2$O$_2$ itself does not react with DCF, but intracellular peroxidases or redox-active metal ions (e.g. iron) could catalyze the oxidation of DCF to DCF in the presence of H$_2$O$_2$ (42–44). Glu/GO treatment induced a time-dependent increase in DCF staining. DCF fluorescence showed an increase after a 4-h treatment with Glu/GO or 13-HpODE, which was inhibited by pre-incubation of cells with Mito-Q or MitoVit-E (Fig. 7B). The results indicate that Mito-Q and MitoVit-E protect BAEC from peroxide-induced oxidative stress.

**Fig. 6.** The effect of ubiquinone and vitamin E on aconitase activity and complex I activity in BAEC. Experimental protocols used are the same as described in Fig. 5, except that BAEC were pretreated with 1 μM vitamin E or ubiquinone.
GO. In the presence of Mito-Q or MitoVit-E, DCF green fluorescence was greatly inhibited (data not shown). FACScan analysis showed that Glu/GO caused a 10-fold increase in the fluorescence intensity as compared with the control. When cells were pretreated with Mito-Q and MitoVit-E, the fluorescence intensity decreased by 80 and 66%, respectively (Fig. 8). These results indicate that the intracellular oxidative stress caused by Glu/GO is abrogated by Mito-Q and MitoVit-E.

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**Fig. 7.** The effect of Mito-Q and mitovitamin E on peroxide-induced iron uptake in BAEC. A, BAEC were treated with Glu/GO in the presence and absence of Mito-Q, and TfR levels were measured by Western blotting at different time points. B, ^55^Fe uptake in cell lysates was measured in BAEC treated with Glu/GO (20 milliunits) with or without Mito-Q (1 μM) and MitoVit-E (1 μM) pretreatment for 2 and 4 h. C, ^55^Fe uptake was measured in BAEC treated with 13-HpODE (25 μM) in the presence and absence of Mito-Q and MitoVit-E (1 μM) pretreatment for 2 and 4 h. D, ^55^Fe uptake was measured in the mitochondrial fractions of BAEC. E, same as C, except that ^55^Fe levels in mitochondria isolated from BAEC were measured. Data are mean ± S.D. of three independent experiments. * represents p < 0.01 as compared with Glu/GO- or HpODE-treated groups.

*Mito-Q and MitoVit-E Preserve Mitochondrial Membrane Potential in Glucose/Glucose Oxidase-induced Apoptosis in BAEC*
and Prevent Cytochrome c Release into the Cytosol—Mitochondria play a central role in apoptotic cell death (45) and are critically involved in deciding whether a cell undergoes apoptosis. A plausible mechanism by which mitochondria trigger apoptosis is by the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm, where it activates the caspase cascade (46–51). Treatment of BAEC with Glu/GO leads to the release of cytochrome c from the inner mitochondrial membrane into the cytosol in BAEC (Fig. 9A). Mito-Q treatment prevented the cytochrome c release from the mitochondria in Glu/GO-treated BAEC, implying that the mitochondria are still intact in Mito-Q-treated cells.

Mitochondrial dysfunction is one of the earliest indicators of apoptosis (1, 2). The induction of mitochondrial permeability transition contributes to mitochondrial damage and dysfunction (52–54). A mitochondrial membrane sensor kit was used to assess whether the cells retain their mitochondrial membrane potential. MitoSensor is a cationic dye that fluoresces differently in apoptotic and non-apoptotic cells. The MitoSensor dye forms aggregates in mitochondria of healthy cells and exhibits a red fluorescence. In apoptotic cells, membrane potentials are altered, and the MitoSensor dye cannot accumulate in mitochondria and thus remain as monomers leading to a green fluorescence. The cells were mostly green after a 4-h treatment with Glu/GO, as compared with control. Pretreatment of cells with Mito-Q and MitoVit-E protected the cells from loss of mitochondrial membrane potential caused by oxidative stress induced by Glu/GO (data not shown). These results show that control cells exhibit red fluorescence ($R_2$ region), and when the cells undergo apoptosis they lose the membrane potential and their red fluorescence; consequently there is a shift to green fluorescence ($R_2$ region) (Fig. 9B). In Glu/GO-treated cells, the number of cells in the $R_2$ region increased to 97 from 6.7% in control experiments. In the presence of Mito-Q and Mito Vit-E, the number of cells in the $R_2$ region decreased to 13.2 and 15.7%, respectively. This trend was noticeable during a 4- and a 6-h treatment of BAEC with Glu/GO. Mito-Q and Mito Vit-E protected the cells from loss of membrane potential as evidenced by the large number of cells that retained the red fluorescence. These results are consistent with an earlier report (55) indicating that MitoVit-E was a less potent antioxidant than Mito-Q. These findings further confirm that mitochondria-targeted antioxidants protect endothelial cells from oxidant-induced mitochondrial membrane damage.

Mito-Q Restores the Proteasomal Activity in BAEC Treated with Glu/GO—Next, we determined the effect of mitochondria-targeted antioxidant, Mito-Q, on the proteasomal activity in BAEC treated with Glu/GO. Fig. 10, A and B, shows changes in the trypsin-like and chymotrypsin-like activities of the 26 S proteasome in BAEC treated with Glu/GO for different periods. There was a time-dependent decrease in the proteasomal activity (Fig. 10). Pretreatment of cells with Mito-Q and MitoVit-E considerably increased the proteasomal activity in Glu/GO-treated cells (Fig. 10). In contrast, pretreatment of cells with ubiquinone-10 and Vit-E, under these conditions, had little or no effect on the proteasomal inactivation induced by Glu/GO. In order to rule out other nonspecific reactions of Mito-Q and MitoVit-E, we investigated their effects in cells preincubated with Lac or MG-132. These proteasome-specific protease inhibitors covalently modify two $\beta$-subunits of the proteasome causing their inactivation (56). Under these conditions, both Mito-Q and MitoVit-E had only minimal effect on proteasomal inactivation (Fig. 11, A–D). These results clearly suggest that mitochondrial targeting of antioxidants markedly restore the proteasomal function in endothelial cells through mitigation of peroxide-induced oxidative stress.

**DISCUSSION**

In this study, we report that mitochondria-targeted antioxidants such as Mito-Q and MitoVit-E, at low concentrations, protect cells against peroxide-induced oxidative damage and apoptosis. These mitochondria-targeted antioxidants inhibit oxidative stress, restore proteasomal function, decrease mitochondrial uptake of Tfr iron, and ultimately help preserve the mitochondrial cell permeability. At low concentrations, the untargeted antioxidants (e.g., Vit-E and ubiquinone-10) did not elicit protection against oxidative damage/apoptosis. The antioxidant and antiapoptotic properties of Mito-Q and MitoVit-E were attributed to their ability to inhibit transferrin receptor-mediated iron uptake into mitochondria.

The mitochondrial membrane potential enables certain agents and drugs to be delivered selectively to mitochondrial compartments within cells (16, 57). The plasma membrane potential enables the uptake and accumulation of cationic compounds into the cell and mitochondria. At least 90–95% of the intracellular cation is localized into the mitochondria (58, 59). Consequently, selective mitochondrial delivery and sequestration were accomplished by covalently attaching a triphenylphosphonium cation to antioxidant drugs (Fig. 1). Triphenylphosphonium alone did not protect against oxidative damage/apoptosis. The antioxidant and antiapoptotic properties of Mito-Q and MitoVit-E were attributed to their ability to inhibit transferrin receptor-mediated iron uptake into mitochondria.

Antioxidants Inhibit Peroxide-induced Apoptosis

**FIG. 8.** The effect of Mito-Q and mitovitamin E on intracellular oxidative stress in BAECs treated with glucose/glucose oxidase. Cells were pretreated with Mito-Q or MitoVit-E (1 $\mu$M) for 1 h and incubated with Glu/GO (20 milliunits) for 4 h. Fluorescence intensity was measured by FACS analysis as described under “Experimental Procedures.” Filled area represents control cells in i–iii, and open area represents cells treated with Glu/GO (i), cells treated with Glu/GO and Mito-Q (ii), and cells treated with Glu/GO and MitoVit-E (iii) for 4 h.
protein activation. This leads to increased mRNA binding activity associated with the iron-responsive element (60, 61). Glucose/glucose oxidase treatment inhibits mitochondrial enzymes, aconitase and complex I, of the electron transport chain in BAEC (31). The partial inactivation of the mitochondrial iron-sulfur proteins (e.g., aconitase and other proteins present in the electron transport chain) is presumably sufficient to stimulate cellular iron signaling (31). A recent report (62) indicates that the 75-kDa Fe-S subunit of mitochondrial complex I in the respiratory chain is regulated by a novel increased iron-regulatory protein-iron-responsive element system.

Mitochondria play a key role in triggering apoptotic events (63–65) that are associated with transmembrane potential changes leading to the release of intermembrane proteins into the cytosol (e.g., cytochrome c) and to caspase activation (46–51). Mito-Q prevented both cytochrome c activation and caspase-3 and -9 activation in BAEC-treated with Glu/GO. However, the exact mechanism that causes ROS to stimulate cytochrome c release and apoptotic cell death is still unclear. Recently, oxidative modification of cardiolipin was thought to

**Fig. 9.** The effect of Mito-Q and mitovitamin E on changes in mitochondrial membrane potentials in BAEC treated with glucose/glucose oxidase. A, BAEC were pretreated with Mito-Q (1 μM) for 1 h and treated with Glu/GO (20 milliunits) for 2, 4, and 8 h. In control experiments, BAEC were treated with Glu/GO (20 milliunits). The cytochrome c levels were measured in the cytosolic extracts by Western blotting. Data shown are representative of three separate experiments. B, BAECs were treated under different conditions as indicated. Cells were washed in PBS three times and incubated with the Mito-sensor reagent for 15 min at 37 °C. The fluorescence was measured by using a BD Biosciences FACScan flow cytometer with excitation and emission settings of 488 and 530 nm, respectively. R1 represents the cells that have red fluorescence that still retain the mitochondrial membrane potential, and R2 represents the cells that have lost red fluorescence and attain green fluorescence due to the loss of membrane potential.
stimulate the release of cytochrome c (66). Cardiolipin is extremely susceptible to ROS-mediated peroxidation because of its high content of unsaturated fatty acid. Mitochondria-targeted antioxidants are clearly more potent because they can function as a potent chain-breaking antioxidant in the mitochondrial membrane. Recently cell-permeable peptide antioxidants targeted to the inner mitochondrial membrane have been found to potently inhibit intracellular ROS and cell death in neuronal cells (67).

Mitochondrial membrane integrity is essential for normal cell function. When cells are subjected to oxidative stress, mitochondria lose the membrane integrity, initiating a series of signaling events leading to apoptosis (68, 69). The present data show that Glu/GO induces severe oxidative stress resulting in mitochondrial membrane damage, as evidenced by the fluorescence data using the MitoSensor dye. Mito-Q and MitoVit-E pretreatment retained the membrane potential of these cells, thereby preventing cells from undergoing apoptosis.

The present data show that the 26 S proteasome system was inactivated by Glu/GO which was retained by pretreatment of cells with low concentrations of mitochondria-targeted antioxidants but not by untargeted parent antioxidants (Fig. 10). Activation of the proteasomes has been reported to play a critical regulatory role in apoptosis and cell survival by modulating Tfr-dependent iron uptake in oxidant-stressed endothelial cells (41). The observed decrease in Tfr levels in the presence of Mito-Q and MitoVit-E in endothelial cells treated with Glu/GO is likely to be due to its capacity to protect the proteasomal activity (Fig. 10). It is conceivable that mitochondria-targeted antioxidants mitigate peroxide-induced oxidative stress, TfR-dependent iron uptake, and endothelial apoptosis probably by restoring the inherent proteasomal activity. However, the targeted antioxidants were not capable of restoring the proteasomal activity when they were treated with proteasomal inhibitors. Recent work (70) also suggests that chronic low level proteasome inhibition leads to enhanced mitochondrial ROS formation and alters mitochondrial homeostasis.

Mitochondrial dysfunction is one of the most crucial factors

![Fig. 10. The effect of mitochondria-targeted and untargeted antioxidants on proteasomal activities in BAEC treated with glucose/glucose oxidase. BAEC were treated with Glu/GO (20 milliunits) in the presence of Mito-Q (1 μM), MitoVit-E (1 μM), Vit-E (1 μM), and ubiquinone-10 (1 μM) for various time periods. The chymotrypsin-like (A) and trypsin-like (B) activities of 26 S proteasome were measured in cell lysates as described under "Experimental Procedures." Data represent the mean ± S.D. of three separate experiments.](image-url)
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in regulating apoptosis in various neurological and age-related diseases such as Parkinson’s and Alzheimer’s and Friedreich’s ataxia (55). The present study provides new insights into the antiapoptotic mechanisms of mitochondria-targeted antioxidants in peroxide-treated endothelial cells. The use of such targeted antioxidants potentially enables one to “pinpoint” exactly whether mitochondria is the site of ROS generation and to unravel the role of ROS and iron in mitochondrial oxidative damage.

Acknowledgment—We thank Dr. Michael Murphy for the initial gift of Mito-Q.

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