Bioenergetic Effects of Mitochondrial Targeted Coenzyme Q Analogs in Endothelial Cells

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Running title: Bioenergetics and CoQ analogs

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Text pages: 14
Tables: 0
Figures: 11
Supplementary figures 2
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Words in:
  Abstract: 250
  Introduction: 596
  Discussion: 1627

Non-standardized abbreviations:
  BAE: bovine aortic endothelial
  CoQ: coenzyme Q
  DHE: dihydroethidium
  DHPA: 10-acetyl-3,7-dihydroxyphenoxazine
  DNP: dinitrophenol
  ECAR: extracellular acidification rate
  FCCP: carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone
  MitoQ: mitoquinol
  MnTMPyP: manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin
  mTPP: methyltriphenylphosphonium
  MTQAs: mitochondrial-targeted CoQ analogs
  NAC: N-acetylcysteine
  OCR: oxygen consumption rate
  OCR_{ATP}: oxygen consumption rate (OCR) directed at ATP turnover
  ROS: reactive oxygen species
  SkQ1: plastoquinonyl-decyl-triphenylphosphonium
  TPP: triphenylphosphonium

Recommended Section Assignment: Metabolism, Transport, and Pharmacogenomics
ABSTRACT

Mitochondrial-targeted analogs of coenzyme Q (CoQ) are under development to reduce oxidative damage induced by a variety of disease states. However, there is a need to understand the bioenergetic effects of these agents and whether or not these effects are related to redox properties, including their known prooxidant effects. We examined the bioenergetic effects of two mitochondrial-targeted CoQ analogs in their quinol forms, MitoQ (mitoquinol) and SkQ1 (plastoquinonyl-decyl-triphenylphosphonium), in bovine aortic endothelial (BAE) cells. We used an extracellular oxygen and proton flux analyzer to assess mitochondrial action at the intact cell level. Both agents, in dose-dependent fashion, reduced the oxygen consumption rate (OCR) directed at ATP turnover (OCR_{ATP}) (IC50 189 ± 13 nM for MitoQ and 181 ± 7 for SKQ1, difference non-significant) while not affecting or mildly increasing basal oxygen consumption. Both compounds increased extracellular acidification in the basal state consistent with enhanced glycolysis. Both compounds enhanced mitochondrial superoxide production assessed using mitochondrial-targeted dihydroethidium and both increased H_{2}O_{2} production from mitochondria of cells treated prior to isolation of the organelles. The MnSOD mimetic, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin, did not alter or actually enhanced the actions of the targeted CoQ analogs to reduce OCR_{ATP}. In contrast, N-acetylcysteine mitigated this effect of MitoQ and SkQ1. In summary, our data demonstrate important bioenergetic effects of targeted CoQ analogs. Moreover, these effects are mediated, at least in part, through superoxide production but dependent on conversion to H_{2}O_{2}. These bioenergetic and redox actions need to be considered as these compounds are developed for therapeutic purposes.
INTRODUCTION

Oxidative damage contributes to a variety of disease states; many of these primarily of vascular etiology. Concern over this problem has led to attempts at antioxidant therapy; in particular, directed at mitochondria, since these organelles likely represent the predominant cellular source of reactive oxygen species (ROS) (Boveris et al., 1972; Chance et al., 1979; Loschen et al., 1971; Raha and Robinson, 2000). Efforts are underway to develop effective antioxidant compounds targeted to mitochondria (Murphy, 2001, 2004). One approach involves the synthesis of compounds linking redox forms of coenzyme Q analogs to alkylated triphenylphosphonium (TPP+) compounds. The resultant antioxidant compounds in the form of lipophilic cations are avidly taken up into the relatively negative mitochondrial matrix (Kelso et al., 2001; Tauskela, 2007). Although much attention has been directed at such targeted redox therapy, an important issue that has received less attention is the metabolic consequence of this approach.

In the work reported here, we focus on the bioenergetic actions of two mitochondrial targeted antioxidants compounds, MitoQ and SKQ1. Both have been characterized in vitro and in vivo for potential therapeutic effects (Skulachev et al., 2009; Smith et al., 2008). MitoQ (mitoquinone, mitoquinol, or a mixture of these two redox cycling compounds) consists of the quinone/quinol moiety of CoQ and a shortened (10 carbon or two 5-carbon prenyl units) side chain linked to triphenylphosphonium. SKQ1 arose from efforts to modify the MitoQ structure to try and improve the balance between antioxidant to prooxidant effects as well as to facilitate increased delivery. A potentially important modification, converting methoxy groups on the quinone moiety of MitoQ to methyl groups, led to SKQ1 (redox forms of plastoquinonyl-decyl-triphenylphosphonium) which demonstrated greater permeability across synthetic lipid bilayers than MitoQ (Skulachev et al., 2009).

MitoQ and SkQ1, here termed mitochondrial-targeted CoQ analogs (MTQAs) are effective antioxidants by virtue of inhibiting lipid peroxidation during redox cycling of the quinol/quinone forms of the compounds (James et al., 2004). The major protective reaction transfers a hydrogen atom (H) from the quinol to a lipid radical (LO2•) forming LOOH and generating the semiquinone form of the MTQA
The respiratory chain then regenerates the MTQA through redox cycling similar to what occurs for native Coenzyme Q. An example of the effectiveness of this process is the prevention of cardiolipin peroxidation in heart mitochondria exposed to iron and ascorbate (Skulachev et al., 2010). On the other hand redox cycling generates electron leaks to oxygen leading to prooxidant properties as demonstrated in past studies by our laboratory and others (Doughan and Dikalov, 2007; James et al., 2004; O'Malley et al., 2006).

Although promising as therapeutic agents there are certain concerns. In particular, there is a need for better understanding of the bioenergetic effects of targeted CoQ analogs and whether these are direct and/or consequent to their redox properties. There is evidence that MTQAs induce mild respiratory uncoupling through reduction of mitochondrial membrane potential, possibly a protonophoric effect that may involve fatty acid transport (Skulachev et al., 2009; Skulachev et al., 2010). However, it is not clear to what extent this translates to bioenergetic action at the intact cell level.

Here, we used recently available intact cell respirometry technology to examine the dose-dependent effects of MitoQ and SKQ1 on mitochondrial function, extracellular acidification rate (ECAR), and reactive oxygen production in BAE cells. We show that both compounds have potent bioenergetic effects at the mitochondrial level and that these are, at least in part, due to their redox properties. We also provide evidence that MTQAs may, under conditions of cell stress, compromise mitochondrial substrate delivery.

METHODS

Reagents and supplies

MitoQ was synthesized from commercially available 11-bromoundecanoic acid and 2,3-dimethoxy-5-methyl-1,4-benzoquinone as described (Asin-Cayuela et al., 2004; Kelso et al., 2001). SkQ1 was synthesized from commercially available 2,3-dimethyl-1,4-hydroquinone using a three step procedure previously described by Antonenko and co-workers (Antonenko et al., 2008). Structural integrity and purity were documented using an Agilent LC/MS apparatus. Both SkQ1 and mitoquinol
eluted as single peaks on HPLC. Moreover, $^1$H and $^{13}$C NMR demonstrated the single component nature of these samples. Figure 1 depicts the structures of MitoQ and SKQ1.

Other reagents, kits, and supplies were as specified or purchased from standard sources.

**Cell culture**

BAE cells were grown in medium M199 (Invitrogen) supplemented with minimal essential medium amino acids (Invitrogen), minimal essential medium vitamins (Sigma), 1mM sodium pyruvate (Invitrogen), and 20% fetal bovine serum (HyClone, Logan, UT) as described (Moser et al., 1992). Cells were grown to near confluence in 150-cm$^2$ flasks and used between passages 5 and 10.

**Respirometry**

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using an intact cell respirometer designed for adherent cells (Seahorse, Inc). BAE cells were grown in 24 well plates designed for respirometer analyses. OCR and ECAR were determined in assay medium consisting of medium M199 lacking sodium bicarbonate and pyruvate (Invitrogen) over time periods up to 95 minutes with assessments at 8-10 minute intervals. Prior to analysis, cells within individual wells were exposed for 18 h or for 30 min to 0.3% vehicle (ethanol, in well volume of 600 µl) or to differing concentrations of MitoQ or SkQ1 (added in equivalent volume to vehicle alone) as described in the figure legends and/or text. In some experiments (see figure legends), cells were treated with manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) or N-acetyl cysteine (NAC) or vehicle for these compounds (water). During respirometry, wells were sequentially injected at the times indicated in the figures with: oligomycin (2 µM) to block ATP synthase to assess respiration required for ATP turnover (OCR$_{ATP}$); carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone (FCCP, 2 µM), a proton ionophore, to induce chemical uncoupling and induce maximal respiration; or Antimycin-A (0.5 µM) plus rotenone (2 µM) to completely inhibit electron transport and measure non-mitochondrial respiration. The FCCP concentration used in these studies was determined by titration of with differing amounts of the uncoupler using the least amount required for maximal uncoupling in cells unexposed to MTQAs.
OCR (pmoles/min/µg DNA) and ECAR (mpH/min/µg DNA) were determined as the average numbers recorded during time periods defined as intervals between the above sequential injections. Basal OCR was determined as respiration prior to injection of any compounds minus non-mitochondrial OCR. OCR_{ATP} was determined as basal OCR minus OCR after oligomycin injection. OCR accountable by the proton leak was calculated as OCR in the presence of oligomycin minus non-mitochondrial OCR. Maximal uncoupled respiration was calculated as OCR after FCCP minus non-mitochondrial OCR. All values for OCR and ECAR were normalized to DNA content of the individual wells. ECAR was quantified simply as the recorded acidification rate during the respiratory conditions delineated above. ECAR is expressed as milli pH (mpH) unit change per min per µg DNA where mpH = 1/1000^{th} pH unit.

IC_{50} and EC_{50} determinations

Half maximal inhibitory (IC_{50}) or stimulatory (EC_{50}) concentrations were determined by third order polynomial curve fitting (r^2 > 0.94 for all curve fits). Maximal inhibition of OCR_{ATP} and maximal leak respiration was considered as the theoretical maximum value. For both metrics, this is equal to the difference between basal OCR and non-mitochondrial respiration. Maximal FCCP respiration was considered as FCCP respiration in the presence of vehicle.

Quantification of DNA

After respirometry, well contents were extracted in 0.4% SDS and diluted to 0.01%. DNA content of each well was determined using a Sigma DNA Quantitation Kit (DNA-QF) using calf thymus DNA standards prepared in 0.01% SDS.

ROS detection

Superoxide was determined by fluorescent microscopy using the mitochondrial targeted (TPP+ conjugated) dihydroethidium (DHE) probe, MitoSOX (Invitrogen). Cells were loaded for 15 min with 5µM concentration of MitoSOX in 0.25mL medium 199 lacking bicarbonate and containing 5.55 mM D-glucose and 2mM HEPES. Dyes were washed out x 2 with 0.3 mL assay medium. Dye-loaded cells were then incubated at 37°C for 2.5 hours and imaged using an Olympus IX71 microscope and
tetramethylrhodamine isothiocyanate filter at 1 second exposure. A heat-controlled cell chamber is mounted in place on the microscope stage to maintain the cells at 37°C for the duration of photography.

Excitation and emission were set at 555 nm and 604 nm with bandwidths of 25 nm and 45 nm, respectively. To document specificity MitoSOX fluorescence was determined with and without the addition of MnTMPyP (25 µM). Quantitative data were obtained using the image analysis software application, ImageJ, from the NIH.

_H₂O₂ production by isolated BAE mitochondria:_

BAE cells were treated with MitoQ, SKQ1, or vehicle for 18h before mitochondria were prepared. The organelles were isolated as we previously described (O'Malley et al., 2006). After isolation, mitochondria remained unexposed to any further MTQA compound. Mitochondria isolated in this way were of good quality based on assay of cytochrome C oxidase (Sigma, St. Louis) revealing 5-10% outer membrane damage, well within an acceptable range compared to mitochondrial preparations from several sources (Wojtczak et al., 1972). There was also no difference in membrane damage between mitochondria from cells treated for 18h with MitoQ or SKQ1 compared to vehicle-treated cells. H₂O₂ production was measured as we previously described (O'Malley et al., 2006) using the probe, 10-acetyl-3,7-dihydroxyphenoxazine (DHPA or Amplex Red, Invitrogen), recognized as an optimal probe for isolated mitochondria (Brand, 2010). As we and others have previously shown, ROS detected in this way derives largely from superoxide converted to H₂O₂ by matrix MnSOD and released externally (O'Malley et al., 2006). Specificity was evident since fluorescence could be abolished by catalase. In addition, we determined fluorescence under no mitochondrial substrate conditions and subtracted to determine substrate induced fluorescence. Samples were prepared in 96-well plates containing 0.06 ml per well of respiratory buffer. Fluorescence was measured as we previously described (O'Malley et al., 2006) once every 60 seconds and carried out for 30 cycles. For quantification, a H₂O₂ standard curve ranging from 0-5 µM was prepared and included on each plate.

Statistics
Data were analyzed by one-way ANOVA or unpaired, two-tailed t-test as described in the figure legends and text. Analyses were performed using GraphPad Prism Software (San Diego, CA). Significant differences were determined as \( p < 0.05 \). Repetition numbers are given in the figure legends. For Seahorse experiments, where indicated, repetitions refer to the number of repeated experiments each involving the average of 2-3 wells per 24-well experimental plate (experimental run). In other cases, the numbers refer to the total number of wells. In these cases, each condition studied was included on each of the multiple plates utilized, avoiding bias by experimental run, all of which were carried out under identical conditions using the same instrument.

RESULTS

*MitoQ and SkQ1 alter mitochondrial respiration in intact BAE cells:*

MitoQ and SkQ1 both showed dose-dependent effects on mitochondrial function (figure 2). As expected based on past studies we carried out in isolated mitochondria (Fink et al., 2009), untargeted Coenzyme Q, at the same concentrations, had no effect (supplemental figure 1). Data were normalized to DNA, although essentially the same results were obtained if oxygen consumption were normalized per well. Figure 2A illustrates a representative experiment depicting changes in OCR under different respiratory conditions defined by the sequential injections of oligomycin, FCCP, and rotenone plus antimycin A. As shown, MitoQ and SkQ1 altered respiratory parameters in similar and dose dependent fashion. There was a trend towards enhanced basal respiration but significant only for the highest doses of MitoQ and SKQ1 (figure 2B). However, MitoQ and SkQ1 induced potent dose-dependent reductions in OCR\(_{ATP}\) with corresponding increases in the proton leak (figures 2C and D). MitoQ and SkQ1 decreased respiration in the presence of the chemical uncoupler FCCP (figure 2E). There was no difference in non-mitochondrial respiration (figure 2F). IC\(_{50}\) and EC\(_{50}\) values (figure 2C-E) did not significantly differ between MitoQ and SKQ1.

If the above effects of MitoQ and SKQ1 were due only to a protonophoric action, they would likely be observable much sooner than the 18h time period used for the studies of figure 2. Therefore, we
carried out analogous experiments, but adding MitoQ and SKQ1 either 18 h before or 30 minutes before the experimental runs. As shown (figures 3A-D), after 30 min these agents, at concentrations of 200 nM, had no significant effects on basal OCR, OCR\textsubscript{ATP}, or OCR related to the proton leak; although, FCCP uncoupled OCR was mildly reduced. This is in marked contrast to the effects of these agents when added 18 h before the experimental runs. At 300 nM concentrations (figure 3, panels E to G), acute (30 min) MitoQ and SKQ1 did alter OCR\textsubscript{ATP} and FCCP respiration, but were less effective than when added 18 h before study.

*MitoQ and SkQ1 enhance mitochondrial superoxide production in intact BAE cells:*

Overnight exposure of BAE cells to either MitoQ or SkQ1 enhanced superoxide production as demonstrated by fluorescence of the mitochondrial targeted dihydroethidium probe, MitoSOX (figure 4). The fluorescent signal localized near the cell nucleus and cytoplasm consistent with the distribution of mitochondria. The signal was abolished by addition of the cell permeable SOD mimetic, MnTMPyP (25 µM), documenting specificity for the superoxide radical.

*MitoQ and SkQ1 enhance mitochondrial H\textsubscript{2}O\textsubscript{2} production:*

ROS production was increased in mitochondria isolated from cells exposed for 18 h to MitoQ or SKQ1 (figure 5). Importantly, this increase was evident even though the mitochondria were not exposed to either agent after isolation or during *in vitro* incubation to assess H\textsubscript{2}O\textsubscript{2} generation. Production rates were normalized to the mean value for vehicle treated cells. Absolute rates of H\textsubscript{2}O\textsubscript{2} production by mitochondria of vehicle treated cells were 15.8 ± 1.3 pmol/min/mg mitochondria under state 4 conditions and 11.1 ± 0.9 under simulated state 3 conditions (see legend to figure 5). In these experiments, one of the MTQA concentrations (500 nM) was higher than used in other experiments. This was done in anticipation of some loss from mitochondria after isolation.

*Effect of an SOD mimic on the bioenergetic action of MTQAs:*

MnTMPyP did not significantly alter OCR in vehicle-treated cells under any of the respiratory conditions studied (figure 6). The SOD mimic also had no effect on basal OCR in the presence of
MitoQ or SkQ1 (figure 6A). Although MnTMPyP did not affect OCR<sub>ATP</sub> in the presence of vehicle, MnTMPyP reduced OCR<sub>ATP</sub> in the presence of MitoQ and non-significantly (p = 0.06) in the presence of SkQ1 (figure 6B). Corresponding effects were observed on the proton leak (figure 6C). MnTMPyP did not alter the effects of the MTQA compounds on FCCP or non-mitochondrial OCR (figures 6D and 6E).

**Effect of N-acetylcysteine on the bioenergetic action of MTQAs:**

N-acetylcysteine (NAC) did not alter basal OCR either in the presence or absence of MitoQ or SkQ1 (figure 7A). However, NAC mitigated the effect of the MTQA compounds (200 µM) on OCR<sub>ATP</sub> and on OCR due to the proton leak (figures 7B and 7C). NAC also altered non-mitochondrial respiration for vehicle as well as MTQA-treated cells. Note that non-mitochondrial OCR does not impact the effect of the MTQA compounds on OCR<sub>ATP</sub> or on the calculated proton leak. This is because non-mitochondrial OCR does not enter into the oligomycin sensitive calculation and is subtracted out of the proton leak calculation (see “methods”).

Interestingly, NAC appeared to completely abolish any action of FCCP; possibly a chemical interaction between these compounds. This phenomenon did not involve MTQAs since it occurred even in cells with no exposure to MTQAs (figure 7E). Therefore, we assessed NAC effects on MTQA modulated bioenergetics; this time using a different chemical uncoupler, 30 µM dinitrophenol (DNP), rather than FCCP (figure 8). These studies used 300 µM concentrations of MitoQ and SKQ1 rather than the 200 µM concentrations used in figure 7. NAC significantly mitigated the effects of 300 µM SKQ1 on OCR<sub>ATP</sub>, OCR attributed to the proton leak, and DNP respiration (figures 8C-8E). NAC had similar, but non-significant, effects to mitigate the action of 300 µM MitoQ on these parameters.

**Effect of MTQAs on FCCP respiration:**

Of note is that MTQAs blunted or completely prevented the expected rise in OCR upon addition of FCCP after oligomycin (figures 2A and 2E). This could, in part, be due to a non-specific interaction between the MTQAs and FCCP since the MTQAs had less effect on maximal DNP uncoupled respiration (figure 8). However, such interaction cannot explain the full effect of the MTQAs on FCCP respiration.
This is because, when the MTQAs were added 30 minutes before the respirometer runs (figure 3), there was much less reduction in FCCP respiration. Possibly oligomycin could deplete cellular ATP enough to impair the subsequent effect of FCCP to increase OCR (Brand and Nicholls, 2011). To determine whether this were the case, we examined the effect of the MTQAs on these parameters in the absence of prior oligomycin. However, MitoQ and SkQ1 (200 nM) had essentially the same effects independent of prior oligomycin (figure 9A).

The effect of MTQAs to reduce or prevent the expected rise in OCR upon addition of FCCP could be due to limitation of substrate supply through glycolysis. FCCP per se may limit substrate supply through ionophoric action on endosomes and alterations in cytoplasmic calcium (Brand and Nicholls, 2011). We reasoned that MTQAs might further limit substrate supply, thereby, reducing OCR in cells already stressed by FCCP. Therefore, we examined the effect of MitoQ and SkQ1 on OCR, in the presence of added pyruvate. Pyruvate significantly mitigated the effect of the MTQAs to reduce uncoupled (FCCP) respiration (figures 9B and 9C).

Pyruvate did not mitigate the effect of the MTQAs to reduce OCR_{ATP} (figure 9D).

mTTP\+ does not alter BAE bioenergetics:

Possibly, the cationic component of the MTQAs might contribute to the bioenergetic effects. However, overnight (18h) treatment with the cation moiety of the MTQAs, methyltriphenylphosphonium (mTPP\+), had no effect on the bioenergetic profile in BAE cells (figure 10). A repeat experiment showed the same result (not shown).

Effects of MitoQ and SkQ1 on extracellular acidification:

Both MTQAs enhanced ECAR under basal conditions and, at higher doses, after oligomycin administration (supplemental figure 2). As shown in figures 11A and 11B, MitoQ and SKQ1 increased basal OCR and ECAR with a greater increase in ECAR at higher concentrations, consistent with progressive glycolysis (Ferrick et al., 2008; Nicholls et al., 2010). Administering either compound for
18h, compared to 30 min, decreased the ratio of OCR to ECAR (figure 11C) consistent with a greater effect on glycolysis at 18h.

DISCUSSION

MTQAs have well documented antioxidant properties by preventing lipid peroxidation (James et al., 2004). However, we and others have described prooxidant effects due to redox cycling with superoxide production by the semiquinone form of the compounds (Doughan and Dikalov, 2007; James et al., 2004; O'Malley et al., 2006). Moreover, since these agents are CoQ analogs and targeted to mitochondria, metabolic effects should be anticipated. However, these have not been well described. Here, we examined the bioenergetic properties of two MTQA compounds in intact vascular endothelial cells. This cell type is of obvious importance to the problem of vascular disease, a state for which MTQAs are hypothesized to benefit. In fact, endothelial dysfunction is a well-known independent predictor of atherosclerotic events in humans (Yeboah et al., 2009).

Here we used recently available respirometer technology to assess the bioenergetic action of MTQAs. We show that higher MitoQ and SkQ1 mildly increase basal OCR. This is consistent with our past observation of increased oxygen use in BAE cells perfused on glass beads and exposed to a comparatively high concentration of MitoQ at 1000 nM (Fink et al., 2009). Of particular note is that, in spite of only mild change in basal OCR and no change in non-mitochondrial OCR, both MitoQ and SkQ1, in dose-dependent fashion, markedly reduced OCR_{ATP} with corresponding increases in OCR resulting from the proton leak. These data indicate respiratory uncoupling, reaching a marked extent at MTQA concentrations over 150 nM, and imply that any attempt to use MTQAs for therapeutic purposes must remain cognizant of bioenergetic action and employ dosing low enough to avoid critical limitation to cellular ATP generation. Of note is that most cellular studies demonstrating benefits of MTQAs involved concentrations in the upper range or above what we examined herein; for example, reduced telomere shortening in fibroblasts exposed to oxidative stress (Saretzki et al., 2003), reduced glucose-induced
oxidative damage in BAE cells (Dhanasekaran et al., 2004), or reduced endogenous DNA damage in human peripheral mononuclear cells (Marthandan et al., 2011).

The uncoupling effect of MTQAs could be due to a protonophoric effect. If so, we would expect rapid action upon addition to cells as these cations are highly permeable based on their positive charge and lipophilic side chains (Murphy and Smith, 2007). However, we observed much less effect when the MTQAs were added 30 minutes before the extracellular flux experiments (figure 3). Thus, it is unlikely that protonophoric action explains all the metabolic effect of these compounds.

The uncoupling effect of MTQAs might be due, in part, to their known prooxidant action (Doughan and Dikalov, 2007; James et al., 2004; O'Malley et al., 2006). Our current data support this demonstrating increased mitochondrial superoxide production in intact BAE cells (figure 4) and increased H₂O₂ production from mitochondria isolated after 18h antecedent treatment with MTQAs (figure 5). Since the isolated mitochondria were not further exposed to exogenous MTQAs, the increase in H₂O₂ production resulted from the antecedent intact cell treatment.

We then asked whether the uncoupling effects might improve by administering antioxidant agents along with MitoQ or SkQ1. However, in spite of the action of MnTMPyP to reduce superoxide, this agent did not mitigate the effect of MitoQ or SkQ1 on OCR_ATP and the proton leak. In contrast, MnTMPyP actually enhanced the effect of MitoQ on these parameters with trends in the same direction for SkQ1 (figure 6). This could occur if the action of MTQAs on OCR_ATP were, at least in part, due to generation of H₂O₂, since the SOD mimetic converts superoxide to H₂O₂.

The effects of NAC (figures 7 and 8) support a role for H₂O₂ in mediating the bioenergetic effects of MitoQ and SkQ1. NAC generates glutathione (Sen, 1998) which protects against H₂O₂ induced oxidation of thiol groups (Mallis et al., 2002). NAC also acts as a direct scavenger of hypochlorous and hydroxyl radicals and reacts slowly with H₂O₂ (but not with superoxide) (Aruoma et al., 1989). As shown (figures 7 and 8), NAC, opposite to MnTMPyP, partially reversed the effects of the MTQAs on OCR_ATP and the proton leak.
Interestingly, NAC completely abolished all action of FCCP even in the absence of an MTQA (figure 7E). This could represent chemical interaction between NAC and FCCP compounds abolishing the ionophoric effect of FCCP. In fact, there is precedent for such direct reactivity with impaired ionophoric action of the chemical uncoupler (Sulo, 1985). Of further note, NAC has been reported to reduce FCCP mediated GSH depletion in malignant Calu-6 cells (Han et al., 2009) and in As4.1 juxtaglomerular cells (Han and Park, 2011). Perhaps these are interactive chemical rather than physiologic effects.

The effects of MTQAs on uncoupling could be due to the TPP$^+$ cation moiety. However, this is not the case since the cation itself did not alter the bioenergetic profile (figure 10).

Possibly reduced ADP or phosphorous availability (for example, through oxidative impairment of transport proteins) might impair ATP production and explain the decrease in OCR$_{ATP}$. However, this does not seem likely since this would impair basal OCR as well (OCR$_{ATP}$ is a component of basal OCR).

Part of the effect of MTQAs on FCCP respiration could result from limitation of substrate availability. FCCP itself can diminish substrate supply through non-mitochondrial cytoplasmic effects (Brand and Nicholls, 2011). Possibly, MTQAs might exacerbate this leading to reduction in OCR in cells already stressed with FCCP. This is supported by the effect of pyruvate to mitigate the effect of MTQAs on FCCP respiration (figure 9C). We acknowledge that this interpretation might be confounded by possible antioxidant effects of pyruvate that may have improved FCCP respiration. However, adding pyruvate did not prevent the effect of MTQAs to reduce OCR$_{ATP}$ (figure 9D). Moreover, this lack of effect of pyruvate to mitigate MTQA effects on OCR$_{ATP}$, as well as the relatively small effect of MTQAs on uncoupled respiration due to DNP (figures 8B and 8E), add further support to the concept that MTQAs limit substrate supply, at least under the stress condition imposed by FCCP. Although, there may be little relevance to the effects of MTQA on substrate supply under usual physiologic conditions, it is possible that this effect may become important under certain cytoplasmic stress conditions.

As shown in supplementary figure 2 and figure 11, MTQAs enhanced ECAR under basal respiratory conditions suggesting that these compounds enhance glycolysis. This is consistent with a prior
report from our laboratory indicating that MitoQ induces a dose dependent (50 to 1000 nM) enhancement of glucose oxidation while reducing fatty acid oxidation (Fink et al., 2009). This is also consistent with an adaptive response to impaired capacity for ATP formation since glucose oxidation provides more ATP per unit oxygen consumed than fatty acids.

We can only speculate as to comparative effects between MitoQ and SkQ1. We observed no significant differences in IC₅₀ for OCRₐₜₚ or FCCP uncoupled respiration and no significant differences in EC₅₀ for the proton leak. However, SKQ1 appeared to have less prooxidant effect both for superoxide (figure 4) and H₂O₂ production (figure 5) and the effects of SKQ1 may have been mitigated more by NAC. It has been reported that SKQ1, compared to MitoQ, manifests less prooxidant action in aqueous medium and greater antioxidant effect against lipid peroxidation (Skulachev et al., 2009). Thus, our current observations are compatible.

Although our studies imply pause as to the therapeutic use of MTQAs, these compounds may be of metabolic benefit at low enough doses. It is plausible that low doses could induce subtle uncoupling in vivo that could, over time, result in cumulative energy dissipation and weight loss. Further, our past studies showed that MTQAs enhance glucose oxidation while reducing fatty acid oxidation (Fink et al., 2009). This effect might be advantageous to cells faced with an ischemic environment wherein glucose oxidation provides more ATP per unit oxygen consumed. In fact, a switch to glucose oxidation is a well-known adaptive cardiac response to limited blood supply (Boudina and Abel, 2007). Finally, the prevention of lipid peroxidation remains a potential benefit of MTQA treatment (Kelso et al., 2002; Skulachev et al., 2009) and there is suggestion that cytotoxic effects of MitoQ may provide a form of anticancer therapy (Rao et al., 2010).

There are limitations to our study. Targeting of DHE to mitochondria in the form of MitoSOX depends on mitochondrial membrane potential for localization. However, we observed an increase in fluorescence, not a decrease, which would occur if MTQAs reduce potential. We were unable to determine EC₅₀ values for the effect of the MTQA compounds on OCR or ECAR (figure 2 and
supplementary figure 2) since we do not know the maximal enhancement beyond 300 µM concentrations. However this does not seem important given the near maximal effect of the 300 µM concentrations on uncoupling and the proton leak; that is, effects that would likely render toxicity beyond tolerable limits. Another limitation is that we did not assess the effects if MTQAs on mitochondrial ultrastructure. Although we cannot rule this out, we doubt any significant impairment in mitochondrial structure since basal OCR was not adversely impacted and we did not see any greater cytochrome C loss from mitochondria of control versus MTQA-treated cells (see “methods”). Moreover, MitoQ actually protects mitochondria from structural changes due to ischemic reperfusion (Adlam et al., 2005) and SKQ1 protects against age-related mitochondrial damage in Drosophila flight wings (Anisimov et al., 2008) consistent with a general effect of MTQAs to protect against oxidative damage to lipid membranes (James et al., 2004; Skulachev et al., 2010). A further limitation is that we only administered the reduced forms of MitoQ and SKQ1. We would expect very similar effects due to the regenerative redox cycling of these compounds (James et al., 2004; Skulachev et al., 2010). Moreover, in the past, we treated isolated BAE mitochondria with both mitoquinone and mitoquinol and observed similar effects on ROS production (O’Malley et al., 2006).

In summary, new findings are as follows: 1) MTQAs act in dose-dependent fashion to reduce OCR\textsubscript{ATP} while increasing proton leak and mildly increasing (higher doses) basal respiration. Higher doses lead to marked uncoupling and might not be tolerated \textit{in vivo} depending on tissue concentrations achieved. 2) The bioenergetics effects of MTQAs on OCR\textsubscript{ATP} and the proton leak are, in part, consequent to their prooxidant action. The prooxidant effect likely occurs through generation of H\textsubscript{2}O\textsubscript{2} or downstream effects of this radical to induce oxidative modification of molecules such as proteins or compounds containing thiol groups. 3) MTQAs enhance the basal rate of acidification suggesting enhanced glycolysis. 4) MTQAs may limit substrate supply in cells subject to cytoplasmic stress.

We conclude that these effects of MTQAs and their dose dependency need to be considered as these or related compounds are developed for therapeutic purposes.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Fink, Yorek, Kerns, and Sivitz

Conducted experiments: Fink, Herlein, Fenner, Sivitz

Contribute new reagents: Fenner, Kerns

Performed data analysis: Fink, Herlein, Sivitz

Wrote or contributed to the writing of the manuscript: Fink, Yorek, Kerns, Sivitz
REFERENCES


*Circulation* **120**: 502-509.
FOOTNOTES

This work was supported by Veterans Affairs Medical Research Funds; the National Institute of Health [5R01HL073166], and by the Iowa Affiliate Fraternal Order of the Eagles. We also acknowledge predoctoral fellowship support for AMF, in part from the American Chemical Society Division of Medicinal Chemistry sponsored by Bristol-Myers Squibb and in part from a National Institute of Health Training Grant in Pharmacological Sciences [T32GM067795].
FIGURE LEGENDS

Figure 1. Reduced (quinol) forms of SKQ1 [10-(6'-plastoquinolyl)decyltriphenylphosphonium] and MitoQ [mitoquinol or 10-(6'-ubiquinolyl)decyltriphenylphosphonium].

Figure 2. Effect of vehicle (Veh), MitoQ (MQ) and SkQ1 (SK) in nanomolar concentrations (numbers following abbreviations) on oxygen consumption measured under different respiratory conditions as described under methods. MQ or SK were added 18h before respirometry. Panels: A) Representative respirometer experiment indicating the dose dependent effect of SKQ1 on OCR measured before and after sequential injections of the indicated compounds; oligomycin (2 µM), FCCP (2 µM), or antimycin A (Ant A, 0.5 µM) plus rotenone (Rot, 2 µM). MitoQ had similar effects (not shown for clarity). Each data point represents the mean of 2-3 determinations (individual wells) during basal, oligomycin, FCCP, and non-mitochondrial OCR. Data in subsequent panels represent this and five repetitions of this experiment (n = 6 for each data point, each data point representing the mean obtained in 2-3 wells per condition). Panels B-F) Basal OCR, OCR_{ATP}, OCR attributed to the proton leak, OCR after FCCP, and non-mitochondrial OCR as affected by SKQ1 or MitoQ in the concentrations shown. Numbers above bars indicate IC\textsubscript{50} ± SEM for OCR\textsubscript{ATP} and OCR after FCCP or EC\textsubscript{50} for the proton leak. Data represent mean ± SEM, * p < 0.05, ** p < 0.01 compared to vehicle by one-way ANOVA with repeated measures and Dunnett post tests. Note y-axis scale differences. IC\textsubscript{50} and EC\textsubscript{50} values did not differ significantly between SKQ1 and MitoQ.

Figure 3. Effect of MitoQ and SKQ1 added 30 min or 18 h prior to extracellular flux analyzer runs. A) Bioenergetic profile in cells exposed to Vehicle (Veh), 200 nM MitoQ (MQ), or 200 nM SKQ1 (SK) for the time periods indicated. Each data point represents mean ± SEM of values determined in 6 individual wells. B-D) Quantitative analysis of data in panel A demonstrating the effects of 200 nM MQ or 200 nM
SK on basal OCR, OCR_{ATP}, and OCR during FCCP uncoupled respiration. E-G) Quantitative effects of 300 nM MQ or 300 nM SK on the same parameters. Each data point represents mean ± SEM of values determined in 4-6 individual wells. * p < 0.05 or ** p < 0.01 compared to vehicle, † p < 0.01 compared to corresponding compound at 30 min by one-way ANOVA and Tukey post tests.

Figure 4. Superoxide release detected as MitoSOX fluorescence in BAE cells (magnification x 400) treated with MTQAs (300 nM) or vehicle. Panels: A-E) Cells treated as indicated with MitoQ, SKQ1, Vehicle (Veh), MitoQ plus the superoxide dismutase mimetic (MnTMPyP), or SKQ1 + MnTMPyP. F) Quantitative results. Each data point represents the average light density per unit area over 10 representative cells in each of 4 experiments comparing cells exposed to each indicated condition. MQ = MitoQ, SK = SKQ1, adjacent numbers = concentration (nM), M = MnTMPyP. Data represent mean ± SEM normalized to the average density of vehicle treated cells, * p < 0.05, ** p < 0.01 compared to vehicle by one-way ANOVA and Dunnett post tests.

Figure 5. H_2O_2 production by mitochondria (0.1 mg/ml) isolated from BAE cells after 18 h treatment as indicated on the x-axis. MQ = MitoQ, SK = SKQ1. H_2O_2 production is expressed relative to the mean value for vehicle treated cells (see text). A) Production rates were determined under state 4 conditions (no added ADP) in mitochondria respiring on 5 mM succinate + 5mM glutamate + 1mM malate. n = 10-12 determinations for each condition. B) Production rates determined as in panel A, but under simulated state 3 conditions created by adding 20 µM ADP. Hexokinase (5 U/ml) and 2-deoxyglucose (5 mM) were added to recycle ATP (formed by oxidative phosphorylation) back to ADP maintaining ADP availability through the incubation period. n = 10-12 determinations for each condition. * p < 0.01 compared to vehicle by one-way ANOVA and Dunnett post tests.
Figure 6. Effect of the SOD mimetic, MnTMPyP (25 µM; - absent, + present), on OCR in BAE cells treated for 18h with vehicle (VEH), 200 nM MitoQ (MQ 200), or 200 nM SKQ1 (SK 200). MnTMPyP was added at the time of addition of the MTQA or vehicle. Panels A-E depict OCR during respirometry assessed as described in figure 2 under the conditions indicated in the headings. Each bar represents mean values ± SEM, n = 6 wells per data point. * p < 0.05, ** p < 0.01 compared to corresponding condition in the absence of MnTMPyP by two-tailed, unpaired t-test.

Figure 7. Effect of 10 mM NAC (- absent, + present), on OCR in BAE cells treated for 18h with vehicle (VEH), 200 nM MitoQ (MQ 200), or 200 nM SKQ1 (SK 200). NAC was added at the time of addition of the MTQA or vehicle. Panels A-D) OCR during respirometry assessed as described in figure 2 under the conditions indicated in the headings. Bars represent mean values ± SEM, n = 8-10 wells per data point, * p < 0.05, ** p < 0.01 compared to corresponding condition in the absence of NAC by two-tailed, unpaired t-test. E) Effect of NAC on FCCP respiration. Cells were treated with NAC alone for 18 h or vehicle (water, for NAC) in the absence of exposure to either MitoQ or SkQ1 (n=3 wells per data point). Data points (mean ± SEM) are expressed as percent of baseline OCR determined immediately before addition of oligomycin.

Figure 8. Effect of 10 mM NAC (- absent, + present), on OCR in BAE cells treated for 18h with vehicle (VEH), 300 nM MitoQ (MQ 300), or 300 nM SKQ1 (SK 300). NAC was added at the time of addition of the MTQA or vehicle. A) Bioenergetic profiles of cells treated with SKQ1, vehicle, or SKQ1 plus NAC. B) Bioenergetic profiles of cells treated with MitoQ, vehicle, or MitoQ plus NAC. C-E) Quantitative data showing the effect of NAC on MTQA treated cells during respiration under the conditions indicated in the headings. Bars represent mean values ± SEM, n = 4-5 wells per data point, * p < 0.05, ** p < 0.01 compared to corresponding condition in the absence of NAC by two-tailed, unpaired t-test.
Figure 9. Modulation of respiration by MitoQ (MQ) and SkQ1 (SK) and by pyruvate (all treatments administered for 18h). Concentrations (nM) are indicated in numbers following the designations MQ or SK. A) Effects of Mito Q (200 nM) and SkQ1 (200 nM) on OCR as affected by FCCP (2 µM) added in the absence of prior oligomycin. B) Effects of Mito Q (200 nM) and SkQ1 (200 nM) on OCR in the presence of pyruvate (5 mM) added to the respirometer (Seahorse) medium. Each data point in panels A-B represents mean ± SEM of values determined in 6-7 individual wells. C) Quantitative effect of pyruvate on maximal uncoupled (FCCP) respiration in the presence of SKQ1 or MitoQ. Data are expressed as percent of FCCP respiration without added MTQA (only vehicle added) and represent mean ± SEM of values determined in 6-7 wells. * p < 0.05, ** P < 0.005, *** P < 0.001 compared to absence of pyruvate by unpaired, two-tailed t-test. D) Lack of effect of pyruvate to mitigate OCR\textsubscript{ATP} in the presence of SKQ1 or MitoQ, n= 6-7.

Figure 10. Bioenergetic profiles of cells treated for 18h with the cation moiety of the MTQAs, methyltriphenylphosphonium (MeTPP). Data represent mean ± SEM, n=3 for each data point. This experiment was repeated with the same result.

Figure 11. Basal OCR compared to ECAR in BAE cells treated with MitoQ or SKQ1. A) Cells were exposed to MitoQ for 18h at the concentrations (nM) indicated by the arrows. B) Corresponding data for SKQ1. C) Ratio of OCR to ECAR in cells exposed to 200 nM MitoQ or 200 nM SKQ1 for 30 min or 18 h. * p < 0.01, ** p < .001 for differences between 30 min and 18 h by two-way ANOVA (time x MTQA x interaction). Data in all panels represent mean ± SEM, n = 6 for each data point. Data in this figure derive from that included in figure 2, supplementary figure 2, and figure 3.
Figure 1
Figure 2
Figure 3

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Figure 4

A MitoQ  B SKQ1  C Vehicle

D SKQ1 + MnTMPyP  E MitoQ + MnTMPyP

F

Relative density

Vehicle  MitoQ  SKQ1  SKQ1 + MnTMPyP  MitoQ + MnTMPyP

**  **  *
Figure 5

(A) State 4

(B) ADP 20 μM
Figure 6
Figure 7
Figure 8
Figure 9
Figure 11