A Mitochondrial-Targeted Coenzyme Q Analog Prevents Weight Gain and Ameliorates Hepatic Dysfunction in High-Fat-Fed Mice

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ABSTRACT

We hypothesized that the mitochondrial-targeted antioxidant, mitoquinone (mitoQ), known to have mitochondrial uncoupling properties, might prevent the development of obesity and mitigate liver dysfunction by increasing energy expenditure, as opposed to reducing energy intake. We administered mitoQ or vehicle (ethanol) to obesity-prone C57BL/6 mice fed high-fat (HF) or normal-fat (NF) diets. MitoQ (500 μ M) or vehicle (ethanol) was added to the drinking water for 28 weeks. MitoQ significantly reduced total body mass and fat mass in the HF-fed mice but had no effect on these parameters in NF mice. Food intake was reduced by mitoQ in the HF-fed but not in the NF-fed mice. Average daily water intake was reduced by mitoQ in both the NF- and HF-fed mice. Hypothalamic expression of neuropeptide Y, agouti-related peptide, and the long

form of the leptin receptor were reduced in the HF but not in the NF mice. Hepatic total fat and triglyceride content did not differ between the mitoQ-treated and control HF-fed mice. However, mitoQ markedly reduced hepatic lipid hydroperoxides and reduced circulating alanine aminotransferase, a marker of liver function. MitoQ did not alter whole-body oxygen consumption or liver mitochondrial oxygen utilization, membrane potential, ATP production, or production of reactive oxygen species. In summary, mitoQ added to drinking water mitigated the development of obesity. Contrary to our hypothesis, the mechanism involved decreased energy intake likely mediated at the hypothalamic level. MitoQ also ameliorated HF-induced liver dysfunction by virtue of its antioxidant properties without altering liver fat or mitochondrial bioenergetics.

Introduction

Several mitochondrial-targeted organic compounds and peptides have been developed largely for antioxidant purposes (Kagan et al., 2009; Lyamzaev et al., 2011; Smith et al., 2011; Teixeira et al., 2012). Given their targeted localization, however, it is not surprising that changes in metabolic properties, including mitochondrial uncoupling, have also been documented (Fink et al., 2009; Skulachev, 2009; Skulachev et al., 2009; Fink et al., 2012b). One such compound, termed mitoquinone (mitoQ), is a derivative of endogenous coenzyme Q (CoQ) consisting of the quinone moiety, a 10-saturated-carbon side chain (as opposed to the 50-carbon unsaturated chain side chain of CoQ), and a cationic moiety, triphenylphosphonium, added to the end of the side chain (Fig. 1A). MitoQ has been extensively studied and well

documented to act as an antioxidant by virtue of its capacity to block lipid peroxidation (Kelso et al., 2002); however, we recently found that mitoQ, when administered to bovine aortic endothelial cells, has potent bioenergetic activity as an uncoupler of mitochondrial respiration (Fink et al., 2012a). How mitoQ or other mitochondrial-targeted CoQ analogs uncouple has not been resolved. One proposed mechanism posits that it occurs through interacting with membrane fatty acids to encourage a flip-flop process wherein the protonated carboxyl group transfers H^+ (Severin et al., 2010).

Since respiratory uncoupling should enhance energy expenditure, as mitochondrial charge is dissipated as heat rather than directed to energy storage, we hypothesized that mitoQ might prevent weight gain in mice susceptible to obesity. We further hypothesized that this would occur through increased energy expenditure, as opposed to reduced energy intake. Hence, we treated obesity-prone, normal-fat (NF) or high-fat (HF) fed C57BL/6 mice for up to 197 days with mitoQ or vehicle added to the drinking water. We describe the effects of mitoQ on body composition, bioenergetics, energy intake, and regulation of appetite. We report beneficial effects on body weight and fat mass, albeit by a mechanism other than expected.

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ABBREVIATIONS: 2DOG, 2-deoxyglucose; 2DOGP, 2DOG phosphate; AgRP, agouti-related peptide; ALT, alanine aminotransferase; ANOVA, analysis of variance; BSA, bovine serum albumin; CART, cocaine- and amphetamine-regulated transcript; CNS, central nervous system; CoQ, coenzyme Q; HF, high fat; LepRb, leptin receptor, long form; mitoQ, mitoquinone; NF, normal fat; NPY, neuropeptide Y; POMC, proopiomelanocortin; ROS, reactive oxygen species; VCO₂, carbon dioxide production; VO₂, oxygen consumption; $\Delta\Psi$, mitochondrial inner membrane potential.

Since HF-fed mice accumulate liver fat and manifest liver dysfunction, we also tested the hypothesis that mitoQ might mitigate these complications either by reducing lipid content and/or by reducing lipid peroxidation.

Materials and Methods

Reagents and Supplies. MitoQ was synthesized from the commercially available advanced intermediate idebenone, as previously described (Rao et al., 2010). Structural integrity and purity were documented using an Agilent liquid chromatography-mass spectrometry apparatus (Agilent Technologies, Santa Clara, CA), where mitoQ eluted as a single peak on high-performance liquid chromatography. Moreover, $^1\text{H-NMR}$ was the same as previously reported and further demonstrated the single component nature of the sample. Other reagents, kits, and supplies were as specified or purchased from standard sources.

Animal Procedures. Male C57BL/6 mice (age 4 weeks) were obtained from The Jackson Laboratories (Bar Harbor, ME). Mice were fed a normal rodent diet (13% kcal fat, diet 7001; Teklad, Harlan Laboratories, Madison, WI) until initiation of the dietary protocol at age 6 weeks and maintained according to National Institutes of Health guidelines. The protocol was approved by our institutional animal care committee.

The experimental protocol and procedures are depicted in Fig. 1B. Treatment was begun when the mice were 6 weeks of age with either mitoQ (500 μM) or vehicle (ethanol) added to the drinking water. The concentration of ethanol in the water of all mice was 0.1%. On day 3, mice in both groups were either continued on the normal-fat (NF) diet or begun on a high-fat (HF) diet (lard, 60% kcal fat; Research Diets, New Brunswick, NJ). Hence, the treatment groups consisted of NF-fed, vehicle-treated ($n = 9$); NF-fed, mitoQ-treated ($n = 9$); HF-fed, vehicle-treated ($n = 8$); and HF-fed, mitoQ-treated ($n = 8$). One HF-fed, vehicle-treated mouse developed a skin rash typical of idiopathic ulcerative dermatitis at the beginning of the final third of the protocol period and had to be euthanized. Data for that mouse were not included ($n = 7$ for the HF-fed, vehicle-treated group). Several procedures were carried out as indicated in Fig. 1. Within each group, two or three mice were housed per cage until day 159, after which mice were placed in individual cages. So, for the first 158 days, food and water intake reflected the average for

the number of mice per cage, after which the data reflected individual animals. Mice were weighed every 6 or 7 days, and food and water intakes were determined by the difference between the added and remaining supply. Mice were euthanized using isoflurane between day 188 and day 197 for collection of blood by cardiac puncture and isolation of liver and hypothalamic tissue.

Hypothalamic Gene Expression. Whole hypothalami were dissected free and immersed in liquid nitrogen for 5 minutes before storage at -80°C . Total RNA was isolated using RNeasy Plus Mini Kit from Qiagen N.V. (Venlo, The Netherlands); 5 μg of total RNA in a final volume of 100 μl was used to synthesize first-strand cDNAs with the Super-Script preamplification system. Then 10 μl of cDNA and 0.4 mmol/l of primers were added in a final volume of 25 μl polymerase chain reaction (PCR) mixture (iQ SYBR Green supermix; Bio-Rad Laboratories, Inc., Hercules, CA), and amplified in an iQ5 Multicolor Real Time PCR Detection System (Bio-Rad). The PCR conditions for all genes were as follow: denaturation for 5 minutes at 95°C , then 40 cycles for 30 seconds at 95°C and 30 seconds at 60°C . S18 ribosomal RNA expression was used as internal control to normalize mRNA expression of these genes. The primer set for each gene is S18, ACTGCCATTAAGGGCGTGG (sense), CCATCCTTCACATCCTTCTG (anti-sense); agouti-related peptide (AgRP), CAGAAGCTTTGGCGAGGT (sense), AGGACTCGTGCAGCCTTACAC (anti-sense); cocaine- and amphetamine-regulated transcript (CART), ATGGAGAGCTCCCGCCTG (sense), CAGCTCCTTCTCGTGGGAC (anti-sense); Neuropeptide Y (NPY), TCAGACCTCTTAATGAAGGAAAGCA (sense), GAGAACAAGTTTCATTTCCCATCA (anti-sense); proopiomelanocortin (POMC), CTGCTTCAGACCTCCATAGATGTG (sense), CAGCGAGAGGTCGAGTTTGC (anti-sense); the long form of the leptin receptor (LepRb), TGTITTTGGGACGATGTTCCA (sense), GCTTGGTAAAAAGATGCTCAAATG (anti-sense); and β -actin (CATCCTCTTCTCCCTGGAGA (sense), TTCCATACCAAGAAGGAAGG (anti-sense).

Leptin Concentrations. Plasma leptin was determined using a mouse leptin ELISA Kit (Sigma-Aldrich, St. Louis, MO). Plasma from the NF groups required no dilution. Plasma samples from the HF groups were diluted 1:8 to avoid exceeding the standard curve.

Body Composition. Total body, fat, lean, and fluid mass were determined by NMR spectroscopy using a Bruker mini-spec LF 90II instrument (Bruker Corporation, Billerica, MA). To analyze body composition, mice were placed into a restraint tube and inserted into

Fig. 1. Structure of mitoQ and treatment protocol. (A) Structure. (B) Protocol indicating times of treatments and procedures. Mice were treated with mitoQ or vehicle. Mice in each treatment group were fed NF or high-fat HF diets. Mice were sacrificed from day 188 to day 197 in staggered fashion so that the average number of days treated were 193 ± 1.5 for mice in each of the four groups.

the rodent-sized NMR apparatus, adjusting the volume of the chamber based on the size of the animal.

Stool Calorimetry. Stools were sampled by pooling two separate 19-hour collections of feces performed over a 3-day period. For each collection, mice were transferred to cages without bedding but lined with a single ply of Whatman 3MM chromatography paper (Fisher Scientific) covering the bottom of the cage. Feces were separated from the paper, dried in open air for 48 hours, and weighed. Digestive efficiency and total daily caloric absorption were determined by bomb calorimetry using a semi-microbomb calorimeter (Parr Instrument Co, Moline, IL). Desiccated food and fecal samples were analyzed for total caloric density. Total daily caloric absorption was calculated for individual mice by subtracting the total calories lost to the stool per day from the total number of calories ingested.

Indirect Calorimetry (Whole-Animal Gas Exchange). This was accomplished using a PhysioScan Metabolic System (Omnitech Electronics) to assess gas exchange in small animals. Mice were placed within the chamber for 20 minutes. Gas exchange was determined over the last 5 minutes at which oxygen consumption (VO_2) and carbon dioxide production (VCO_2) had reached a steady plateau in the resting animal.

Isolation of Mitochondria. Liver mitochondria were prepared by differential centrifugation as we described previously (O'Malley et al., 2006; Fink et al., 2009). In addition, mitochondria were purified on a self-generating Percoll (Sigma-Aldrich) gradient using a Beckman XL-80 ultracentrifuge (Beckman Coulter, Inc., Brea, CA) and SW60 swinging bucket rotor. Mitochondria were suspended in isolation medium (0.25 M sucrose, 5 mM HEPES pH 7.2, 0.1 mM EDTA, 0.1% defatted bovine serum albumin, BSA), layered over a solution of 3 parts Percoll/7 parts isolation medium, and centrifuged at 4°C for 30 minutes at 90,000g. The purified mitochondrial band near the bottom of the tube was transferred to 1.5-ml centrifuge tubes containing isolation medium lacking BSA, spun at 4°C in a microfuge at 8500g for 5 minutes at 4°C, and the pellet was washed a second time. The protein content of the final suspension was determined using the method of Bradford.

Generation of the 2-Deoxyglucose ATP Energy Clamp. We used a novel method that we recently described (Yu et al., 2013) to carry out bioenergetic studies of isolated mitochondria under conditions of clamped ADP and membrane potential ($\Delta\Psi$). Studies were carried out in the presence of excess 2-deoxyglucose (2DOG) and hexokinase and varying amounts of added ADP or ATP. ATP generated from ADP under these conditions drives the conversion of 2DOG to 2DOG phosphate (2DOGP) while regenerating ADP. The reaction occurs rapidly and irreversibly, thereby effectively clamping ADP concentrations and, consequently, also clamping $\Delta\Psi$, dependent on the amount of exogenous ADP or ATP added. This technique enabled bioenergetic studies to be carried out over respiratory states ranging from state 4 (no added ADP) to state 3 (ADP added in different amounts). For this method to be effective, the membrane potential should decrease in stepwise fashion to plateau levels with each incremental addition of ADP (or ATP), which is the case, as we have shown in the past for muscle (Yu et al., 2013), liver (Yu et al., 2014), and heart (Yu et al., 2014) mitochondria.

Respiration and Membrane Potential. Respiration and $\Delta\Psi$ were determined using an Oxygraph-2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria) fitted with a potential sensitive tetraphenylphosphonium electrode. Mitochondria (0.25 mg/ml) were fueled by combined substrates consisting of 5 mM succinate + 5 mM glutamate + 1 mM malate and incubated at 37°C in 2 ml of ionic respiratory buffer (99 mM KCl, 6 mM KOH, 10 mM NaCl, 5 mM Na_2HPO_4 , 2 mM MgCl_2 , 10 mM HEPES pH 7.2, 1 mM EGTA, 0.2% defatted BSA) with 5 U/ml hexokinase (Worthington Biochemical Corporation, Lakewood Township, NJ) and 5 mM 2-deoxyglucose. ADP was added sequentially to final concentrations of 2.5, 10, and 20 μM , with plateaus in respiration and potential achieved after each addition. A tetraphenylphosphonium standard curve was performed in each run by adding tetraphenylphosphonium chloride at concentrations of 4, 8, and 12 μM before the addition of mitochondria to the chamber.

Use of the 2DOG ATP Energy Clamp to Quantify ATP Production in Isolated Mitochondria and Simultaneous Assessment of H_2O_2 Production. Mitochondria (0.5 mg/ml) were added to individual wells of black polystyrene 96-well round-bottom plates in a total volume of 60 μl and incubated at 37°C in respiratory buffer plus 5 U/ml hexokinase (Worthington Biochemical) and 5 mM 2DOG in the presence of selected (0, 2.5, 10, and 40 μM) concentrations of ADP and fueled by the combined substrates, 5 mM succinate + 5 mM glutamate + 1 mM malate. After incubation for 20 minutes, the contents of the microplate wells were removed to tubes on ice containing 1 μl of 120 μM oligomycin to inhibit ATP synthase. Tubes were then centrifuged for 4 minutes at 14,000g to pellet the mitochondria. Supernatants were transferred to new tubes and stored at -20°C for quantification of 2DOGP by NMR spectroscopy. To prepare the NMR sample, 40 μl of assay supernatant was added to a 5 mm (o.d.) standard NMR tube (Norell, Inc., Landisville, NJ) along with 50 μl of deuterium oxide and 390 μl of a buffer consisting of 120 mM KCl, 5 mM KH_2PO_4 and 2 mM MgCl_2 , pH 7.2.

ATP production rates were calculated based on the percent conversion of 2DOG to 2DOGP, the initial 2DOG concentration, incubation volume, and incubation time. To assess simultaneously the H_2O_2 production, mitochondrial incubations were carried out in the presence of 10-acetyl-3,7-dihydroxyphenoxazine, described as follows.

NMR Spectroscopy. NMR spectra were collected at 37°C on a Bruker Avance II 500 MHz NMR spectrometer. Mitochondrial samples were studied by acquiring two-dimensional $^1\text{H}/^{13}\text{C}$ -HSQC NMR spectra using ^{13}C -labeled 2DOG at C6-position ($[\text{6-}^{13}\text{C}]\text{2DOG}$) as we recently described (Yu et al., 2013). The amount of 2DOG and 2DOGP present in the NMR samples were quantitatively measured using the peak intensities of the assigned resonances of these compounds. NMR spectra were processed with the NMRPipe package (Delaglio et al., 1995) and analyzed using NMRView software (One Moon Scientific, Westfield, NJ) (Johnson and Blevins, 1994).

Mitochondrial H_2O_2 Production. H_2O_2 production was assessed simultaneously with ATP production using the fluorescent probe 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red; Invitrogen, Carlsbad, CA), a highly sensitive and stable substrate for horseradish peroxidase and a well established probe for isolated mitochondria (Rhee et al., 2010). Fluorescence was measured and quantification carried out as we previously described (O'Malley et al., 2006). The addition of catalase,

Fig. 2. Effect of mitoQ (MQ) treatment on body mass in NF- and HF-fed mice. Mouse weights were measured at intervals of 6 or 7 days. Study end varied from 188 to 197 days, depending on the exact day of sacrifice for individual mice.

500 U/ml, reduced fluorescence to below the detectable limit, indicating specificity for H_2O_2 . The addition of substrates to respiratory buffer without mitochondria did not affect fluorescence. Amplex Red does not interfere with ATP production or with NMR detection of 2DOGP (Yu et al., 2013).

Hepatic Lipid Extraction and Liver Hydroperoxide Determination. Lipid hydroperoxides contained in total hepatic extracts from whole liver tissue were quantified using a commercially available lipid hydroperoxide assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. To extract lipids, portions of liver tissue were removed immediately after euthanasia, weighed (average weight, 0.16 g), and immersed in liquid nitrogen for 5 minutes before storage at -80°C . Frozen tissue samples were then placed in glass tubes containing 0.25 ml of water, homogenized for 30 seconds using an Omni TH hand-held tissue homogenizer, extracted using chloroform/methanol, and assayed for lipid hydroperoxides.

To determine hepatic total lipid content, 0.5 ml of lipid extracts were placed in microfuge tubes and evaporated overnight for complete solvent removal. Lipid weight was determined as the difference in tube weight before addition of extract and after solvent evaporation.

Liver Histology. Liver tissue was washed and immersed in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), frozen at -80°C , and sectioned with a cryostat at $10\text{-}\mu\text{m}$ thickness. Frozen slides were then fixed in cold Z-Fix (Anatech, Ltd., Battle Creek, MI), rinsed, stained with hematoxylin and eosin, and visualized using an Olympus IX71 inverted microscope.

Liver Triglycerides. Triglycerides in portions of whole liver were determined using a commercially available microplate based colorimetric assay kit (Cayman Chemical). Liver tissue was extracted by homogenizing in cold Dulbecco's phosphate-buffered saline (Invitrogen) at a tissue:

liquid ratio of 20% (w/v). Homogenates were centrifuged at 4°C for 15 minutes at $10,000g$. The lipid cake and supernatant were transferred to a tube and thoroughly mixed prior to loading of the assay plate.

Serum Chemistry. Serum alanine aminotransferase (ALT), sodium, urea nitrogen, and creatinine determinations were performed by our institution's (Iowa City VA Medical Center) clinical chemistry laboratory by automated methods. ALT was done by coupling the ALT-dependent reaction between L-arginine and 2-oxoglutarate to NADH oxidation by pyruvate and lactate dehydrogenase. Sodium was determined using an ion-selective electrode. Urea nitrogen was determined by urease catalyzed NH_4^+ formation and coupling to NADH reduction by 2-oxoglutarate and glutamate dehydrogenase. Creatinine was determined by the Jaffe method.

Statistics. Data were analyzed by two-factor analysis of variance (ANOVA) (drug $\times$ diet) or two-tailed, unpaired t test as indicated in the figures and legends. All comparisons between mitoQ- and vehicle-treated mice significant by two-factor ANOVA were also significant by individual t test ($n = 7\text{--}9$ per group except for the gene expression data wherein studies were performed on six mice per group or in the number indicated for serum chemistries where we did not have enough serum from all mice).

Results

Body Weight and Composition. As expected, body weight increased markedly in the HF-fed compared with the NF-fed mice. However, HF-fed mice treated with mitoQ gained less weight than did the vehicle-treated HF-fed mice (Figs. 2 and 3A).

Fig. 3. Effect of mitoQ treatment on total body mass determined by scale weight and on components of body mass determined by NMR spectroscopy after treatment times indicated on the x-axis. (A) Total body mass. (B) Fat mass. (C) Lean mass. (D) Fluid mass. * $P < 0.05$; ** $P < 0.01$; † $P < 0.001$ compared with vehicle-treated, HF-fed mice by two-way ANOVA (drug treatment $\times$ diet). Interaction was significant at both 61 days ($P = 0.027$) and 167 days ($P = 0.001$) for total body mass. Interaction was also significant at 61 days ($P < 0.001$) and 167 days ($P = 0.022$) for fat mass, at 61 days ($P < 0.001$) and 167 days ($P = 0.008$) for fluid mass, but it was not significant for lean mass.

MitoQ did not affect weight gain in the NF-fed mice. Body composition assessed by NMR spectroscopy revealed that mitoQ treatment of the HF-fed mice decreased fat mass, lean mass, and fluid mass relative to the vehicle-treated HF fed mice, again with no differences for the NF-fed mice (Fig. 3, B–D). There was significant interaction for total body mass, fat mass, and fluid mass, indicating that the effect of mitoQ depended on dietary group. Consistent with the changes in body fat mass, leptin concentrations were reduced in the mitoQ-treated, HF-fed mice compared with vehicle-treated (1047 ± 40 pg/ml versus 1223 ± 91 , $P < 0.05$ by two-factor ANOVA, drug $\times$ diet) and reduced, but not significantly, in the mitoQ-treated versus vehicle-treated NF fed mice (26.2 ± 7.1 pg/ml versus 39.7 ± 6.7).

Dietary Intake and Absorption. Average daily food intake was decreased in the HF-, mitoQ-treated mice compared with the vehicle-treated mice, whereas no difference was found in food intake in the NF mice (Fig. 4, A and B). Bomb calorimetry studies of stool composition revealed that the percent of calories absorbed did not differ as a result of mitoQ treatment in either the HF- or NF-fed mice (Fig. 4C). Average daily water intake was reduced in the mitoQ-treated mice for both the NF and HF groups (Fig. 4, D and E).

Appetite Neuropeptide Expression. Hypothalamic mRNA expression of NPY, AgRP, and LepRb were all reduced in the HF mice treated with mitoQ compared with HF-fed, vehicle-treated mice with no significant changes in CART or POMC mRNA (Fig. 5). Also, no change was seen in β -actin mRNA, included as a control or “housekeeping” transcript. None of these transcripts were altered by mitoQ treatment of the NF mice (Fig. 5). We choose to analyze this data using

t tests rather than two-way ANOVA as a result of the smaller number of data points and because our hypotheses involved the effect of mitoQ rather than that of diet.

Liver Mitochondrial and Whole-Body Energetics. Respiration, potential, and ATP production were assessed at different levels of clamped ADP availability, resulting in respiratory states ranging from states 4 to 3, depending on the amount of ADP added (Fig. 6, A–C). As expected, greater ADP availability resulted in greater respiration and ATP production and lower membrane potential as mitochondrial charge is used by ATP synthase. Based on area under the curve analyses, mitoQ did not significantly affect respiration, potential, or ATP production in either NF- or HF-fed mice (Fig. 6, D–F). Respiration and ATP production were greater in the HF-fed mice compared with NF-fed mice by two-factor ANOVA (drug $\times$ diet, $P < 0.001$ for overall diet effect). Inspection of Fig. 6C shows that some ATP (manifest as 2DOGP) appeared to be produced even in the absence of added ADP as a result of endogenous ADP and/or ATP present in the isolated mitochondria. To assess this, mitochondrial incubations were performed without added ADP but in the presence or absence of oligomycin. Generation of 2DOGP was observed only in the absence of oligomycin (data not shown), indicating endogenous ADP in the isolated liver mitochondria. Any endogenous ATP would have been used to generate 2DOGP both in the presence or absence of oligomycin.

Resting whole-body VO_2 , VCO_2 , and respiratory quotient (VCO_2/VO_2) were not significantly different in the HF-fed, mitoQ-treated mice compared with vehicle-treated, HF-fed mice (Fig. 6, G–I). As expected, given the known effects of

Fig. 4. Food and water intake by mitoQ- and vehicle (Veh)-treated mice fed NF or HF. (A) Food intake was measured at intervals of 6 or 7 days. Two or three mice within each group were maintained in one cage, as described in *Materials and Methods*, from day 1 to day 158, after which all mice were maintained in individual cages from days 159 to study end. (B) Mean daily food intake from day 159 to study end. (C) Percent of calories absorbed over intervals starting at day 39 and again at day 151. (D) Water intake measured at intervals of 6 or 7 days assessed along with food intake as in (A). (E) Mean daily water intake from day 159 to study end. * $P < 0.001$ compared with vehicle-treated, HF-fed mice by two-way ANOVA (drug treatment $\times$ diet). Interaction was significant for food intake but not for water intake, indicating that the mitoQ effect on food intake but not on water intake was dependent on diet.

Fig. 5. Effect of mitoQ treatment on hypothalamic mRNA expression by mice fed NF or HF. (A) AgRP; (B) NPY; (C) CART; (D) POMC; (E) LepRb; (F) β -actin. *P* values indicate significance compared with vehicle treated mice on the same diet by two-tailed, unpaired *t* test. *n* = 6 mice per group.

obesity with increased adipose mass, HF feeding markedly reduced VO_2 , VCO_2 , and VCO_2/VO_2 relative to NF feeding (Fig. 6, G–I).

Hepatic Fat and Triglyceride Content. As evident by direct imaging and quantitative biochemical measures, liver total fat and triglycerides were much greater in the HF-fed compared with the NF-fed mice (Fig. 7). However, we observed no change in tissue triglyceride or total fat content in either feeding group as a result of mitoQ treatment.

Hepatic Mitochondrial Reactive Oxygen Species Production, Lipid Peroxidation, and Liver Function. Neither mitoQ nor diet altered the production of reactive oxygen species (ROS) as measured by H_2O_2 generated by isolated liver mitochondria (Fig. 8, A and B). On the other hand, mitoQ treatment of the HF-fed mice resulted in a marked reduction in liver lipid hydroperoxides, whether expressed per gram of tissue (Fig. 8D) or normalized to total fat content (Fig. 8E). Lipid hydroperoxides were below the limit of assay sensitivity in extracts of liver from the NF-fed mice, so we could not determine the effect of mitoQ treatment of these mice. Liver function was assessed by measuring the activity of circulating ALT in the serum. HF-fed mice showed very high ALT activity compared with NF-fed mice (Fig. 8C), indicating liver dysfunction of HF-fed mice. MitoQ treatment did not affect the ALT activity of the NF-fed mice but significantly lowered the ALT activity of the HF-fed mice (Fig. 8C).

Sodium and Renal Function. MitoQ did not alter circulating urea nitrogen or sodium concentrations in either HF- or NF-fed mice (data represent mean $\pm$ S.E.). Sodium concentrations (mEq/l) in NF vehicle, NF mitoQ, HF vehicle,

and HF mitoQ-treated mice were 152 ± 1 (*n* = 4), 151 ± 1 (*n* = 4), 152 ± 1 (*n* = 7), and 151 ± 1 (*n* = 7), respectively. Corresponding values for urea nitrogen (mg/dl) were 29.3 ± 1.5 , 32.0 ± 1.5 , 27.4 ± 0.7 and 26.7 ± 0.7 (*n* = 7–9 for all groups). Creatinine levels in mice are normally lower than in humans (Wirth-Dziedziolowska, 2009), for whom the assay was designed. Although measureable and not different among the mouse groups, all creatinine values were below the lower limit of reliable assay sensitivity (*n* = 7 for all groups), whereas we would have expected higher values in the presence of renal dysfunction.

Discussion

As we hypothesized, mitoQ-treated mice fed HF gained less weight than vehicle-treated HF mice. However, our results were not as we hypothesized with regard to energy dissipation as the major mechanism. Rather, mitoQ-treated, HF-fed mice compared with vehicle-treated consumed fewer calories (Fig. 4) and manifested reduced hypothalamic mRNA encoding the orexigenic peptides NPY and AgRP (Fig. 5). Hence, our data suggest that the preventative effect of mitoQ on weight gain was mediated, at least in part, by the central nervous system (CNS). Interestingly, we observed no differences in body weight as a result of mitoQ treatment of the NF-fed mice. In accord with the lack of difference in body mass, there was no difference in caloric intake or in the expression of appetite-related genes in these NF-fed mice.

MitoQ also decreased LepRb mRNA with no change or non-significant decreases in the anorexigenic CART and POMC

Fig. 6. Bioenergetic effects of mitoQ in NF- and HF-fed mice. Respiration, inner membrane potential, and ATP production by liver mitochondria isolated at study end from mitoQ- or vehicle-treated mice fed NF or HF. (A–C) Respiration, mitochondrial inner membrane potential, and ATP production as a function of clamped ADP concentration. (D–F) Area under the curves depicted in (A–C), respectively. (G–I) Indirect calorimetry performed shortly before sacrifice. VO_2 , VCO_2 , and respiratory quotient (RQ) were all reduced as a result of HF feeding, but no significant differences were noted as a result of mitoQ treatment. * $P < 0.01$ compared with NF mice treated accordingly with vehicle or mitoQ by two-factor (drug $\times$ diet). Diet induced significant overall differences in mitochondrial respiration ($P < 0.05$), ATP production ($P < 0.001$), and all gas-exchange parameters ($P < 0.001$) with no significant interaction, whereas mitoQ had no significant effect on these parameters.

transcripts; changes that may be compensatory for decreased food intake. Although our intent was to focus on the effect of mitoQ rather than the effect of HF versus NF feeding, there appeared to be upregulation of LepRb and CART in the HF mice compared with NF mice. This is not surprising for CART, likely an anorexigenic compensatory response. However, for LepRb, this remains enigmatic. Past studies are controversial with respect to this issue. Dependent on rodent strain and time of treatment, HF feeding increased (Lin et al., 2000; Gamber et al., 2012), decreased (Lin et al., 2000; Madiehe

et al., 2000; Mitchell et al., 2009), or did not change (Madiehe et al., 2000; Peiser et al., 2000; Sahu et al., 2002) the hypothalamic expression of LepRb.

MitoQ treatment of HF-fed mice ameliorated lipid hydroperoxides and improved liver function as indicated by circulating ALT (Fig. 8, C–E). MitoQ did not alter liver fat or triglyceride content (Fig. 7) or liver mitochondrial bioenergetics (Fig. 6), suggesting that the mechanism by which mitoQ improved liver function involved the antioxidant action of the compound rather than perturbed energy intake or output. Although lipid

Fig. 7. Hepatic lipid content determined on liver tissue isolated at study end from mitoQ- or vehicle-treated mice fed NF or HF diets. (A–D) Representative H&E-stained histologic images (magnification, 100×) showing liver isolated from NF-fed, vehicle-treated (A); NF-fed, mitoQ-treated (B); HF-fed, vehicle-treated (C), and HF-fed, mitoQ-treated (D) mice. (E) Liver triglyceride content. (F) Total lipid content. No significant differences were observed for mitoQ- versus vehicle-treated NF- or HF-fed mice. $P < 0.001$ for diet effect by two-way ANOVA for both triglycerides and total lipids.

peroxidation was markedly reduced, mitoQ treatment did not alter ROS production by isolated liver mitochondria (Fig. 8, A and B). This finding was as expected and entirely consistent with knowledge that mitochondrial-targeted CoQ analogs do not scavenge ROS. Rather, they reduce lipid oxidative damage through the action of the semiquinone form of these compounds to block the chain reaction by which lipid peroxides are propagated (Kelso et al., 2002). Our observation of decreased lipid peroxides without significant liver mitochondrial uncoupling is also of interest because it supports the view that mitoQ directly impairs lipid peroxidation rather than acting indirectly by reducing membrane potential. Our results are consistent with observations by Mercer et al. (2012) that mitoQ reduced oxidative DNA damage in liver of $ATM^{+/-}/ApoE^{-/-}$ mice.

We were surprised that although our mitoQ treated, HF-fed mice gained less weight than did control HF mice, no difference was seen in hepatic triglyceride or total fat content. However, while this manuscript was in revision, a report appeared describing the effect of mitoQ on features of the metabolic syndrome in HF-fed rats (Feillet-Coudray et al., 2014). Consistent with our observations, these rats gained less weight than controls but also exhibited no change in liver fat content. These rats also showed improvement in markers of oxidative damage. This report also describes a mitoQ-induced reduction in food intake but did not assess liver function, whole-body energetics, or markers of CNS appetite regulation.

Both HF- and NF-fed mice drank less water when given mitoQ compared with vehicle. Conceivably, this could have resulted from a CNS affect regulating thirst; however, our studies were not designed to assess this issue. Alternatively, the decrease in water intake could have resulted from taste aversion. In any case, the question arises as to whether this could have caused the decrease in food intake and body mass in the HF-fed mice administered mitoQ. However, we do not think this was the case. First, the mitoQ-treated, HF-fed mice gained less fat mass than vehicle-treated HF mice with no disproportionate decrease in fluid mass and did not appear dehydrated, as further evidenced by no change in the serum sodium or urea nitrogen. Second, the mitoQ-induced decrease in body mass was only observed in the HF mice, not in the NF group (both groups drank less water). Third, the percent of food absorbed was similar for the mitoQ-treated mice compared with vehicle-treated mice so gastrointestinal toxicity was not likely. Finally, the mitoQ-treated mice did not appear ill, as we observed no decrease in mobility or abnormalities in hair or skin.

Consistent with the decrease in body-fat mass, circulating leptin concentrations at sacrifice were reduced in the mitoQ-treated, HF-fed mice compared with vehicle-treated mice, as was the expression of hypothalamic mRNA encoding *LepRb*. Again, mitoQ had no such effects in the NF-fed mice.

Although highly speculative, a means by which mitoQ, which does enter the CNS (Rodriguez-Cuenca et al., 2010),

Fig. 8. Hepatic oxidative stress and liver function in mitoQ- or vehicle (Veh)-treated mice fed NF or HF. (A) ROS production as a function of clamped ADP concentration. (B) Area under the curves depicted in (A). (C) Serum ALT. (D) Lipid hydroperoxides per gram of wet tissue. (E) Lipid hydroperoxides per milligram of hepatic lipid. Lipid hydroperoxides were determined on extracts of liver tissue isolated at study end. Extracts were the same as those used to assess total lipid content shown in Fig. 7B. ^{*} $P = 0.01$; ^{**} $P < 0.01$ by two-tailed, unpaired t test; [†] $P < 0.001$ compared with vehicle-treated HF mice by two-way ANOVA.

could induce satiety is plausible. Beyond its antioxidant action on lipid peroxidation, mitoQ has a prooxidant effect to increase mitochondrial superoxide production from redox cycling of the quinone moiety, as we and others have found in the mitochondria of endothelial cells (O'Malley et al., 2006; Doughan and Dikalov, 2007), HepG2 cells (Plecita-Hlavata et al., 2009), or heart (Skulachev et al., 2009). Since hypothalamic ROS production reportedly encourages satiety (Benani et al., 2007; Jaillard et al., 2009), it is conceivable that mitoQ-induced hypothalamic superoxide could act to restrain food intake.

Our whole-body bioenergetic data showed no increase in oxygen consumption by the mitoQ-treated mice compared with vehicle-treated mice (Fig. 6G). MitoQ also did not change liver mitochondrial respiration, potential, or ATP production (Fig. 6, A–F). Uncoupling of liver mitochondria would manifest as an increase in respiration with reduced potential, neither of which was observed. Of course, it remains possible that mitoQ at the dose administered did not induce sufficient uncoupling or that this effect could not be detected because of inadequate residual mitoQ after isolation of the mitochondria for in vitro incubation.

Rodriguez-Cuenca et al. (2010) administered MitoQ to normally fed wild-type mice for 20–28 weeks by adding the compound to the drinking water at 500 μM. These authors noted no significant effects on body weight, lean or fat mass, or markers of oxidative damage. Obese or HF-fed mice were not studied. Our current findings agree in that we observed no differences in any of the

phenotypic or biochemical parameters studied in our wild-type, NF-fed mice as a result of mitoQ treatment. In another study, Mercer et al. (2012) administered mitoQ via drinking water at the same dosage to mice that were genetically predisposed to atherosclerosis (ATM^{+/-}/ApoE^{-/-} mice). On a diet containing 19.5% casein milk fat and 0.05% cholesterol, the mitoQ-treated animals gained less weight, had less hypercholesterolemia, and hypertriglyceridemia, and showed improvements in hyperglycemia and hepatic steatosis.

Although not relevant to our main objective of assessing the effects of mitoQ, we were surprised that HF feeding, compared with NF feeding, increased hepatic mitochondrial respiration and ATP production (Fig. 6, A, C, D, and F). This finding differs from what we observed in a past study, wherein we actually found that HF feeding reduced ATP production (Yu et al., 2014). Although both studies used C57BL/6 mice and the diets were similar, their methods differed. In the current study, the mitochondria were prepared not only by differential centrifugation but also were subject to purification by centrifugation through a Percoll gradient, a step that was not carried out in our past study. So it is possible that our past data could have reflected some protein contamination, possibly more prominent in the mitochondria isolated from the HF-fed mice. Also, in the current study, mice were started on the diets at age 6 weeks and fed the diets for 28 weeks, compared with onset at 12 weeks and feeding duration of 18 weeks. Finally, all mice in the

current study received 0.1% ethanol (added to the water as vehicle or within the added mitoQ preparation).

Our study has certain limitations. We added mitoQ to the drinking water, but this may not be optimal for drug delivery. In the cited studies (Rodriguez-Cuenca et al., 2010; Mercer et al., 2012), the authors administered mitoQ as a β -cyclodextrin complex of the methane sulfonate salt and reported only an initial decrease in water intake during the early days of treatment. Another limitation is that although we show that mitoQ prevented weight gain, we did not actually mimic treatment of existing obesity (i.e., by administration to already obese mice). Further, the numbers of mice may not have provided sufficient power to detect significant changes in the markers we assessed for energy dissipation, and the dose of mitoQ might not have been high enough to induce uncoupling. However, this does not diminish the observation that weight gain was prevented and hypothalamic orexigenic gene expression markedly reduced. Another limitation is that, for practical workload reasons, we did not assess mitochondrial function in tissues or cells beyond liver. Finally, we assessed hypothalamic mRNA expression but did not address content in specific hypothalamic nuclei. However, the gene most affected by HF feeding, AgRP, is specifically expressed in the arcuate nucleus; CART is largely expressed therein; and for NPY at least, we assessed this in hypothalamic tissue as opposed to its otherwise widespread localization.

In spite of these limitations, our study supports the concept that mitochondrial-targeted coenzyme Q analogs might be used for prevention or therapy of obesity and/or mitigation of hepatic steatosis. Modifications in the structure that enhance potency or that favorably affect the therapeutic window might prove beneficial in the treatment of human obesity and related clinical issues.

In summary, we show that administration of mitoQ decreased weight gain and adipose tissue accumulation when administered to HF-fed, but not NF-fed, obesity-prone C57BL/6 mice. The mechanism, at least in part, involved suppression of food intake associated with reduced expression of hypothalamic orexigenic genes. Although we cannot absolutely rule out a role for mitochondrial uncoupling, we did not observe significant changes in markers of energy dissipation. MitoQ also mitigated HF-induced liver dysfunction, likely by reducing oxidative damage.

Authorship Contributions

Participated in research design: Fink, Yu, Grobe, Rahmouni, Kerns, Sivitz.

Conducted experiments: Fink, Herlein, Guo, Weidemann, Yu, Sivitz.

Contributed new reagents or analytic tools: Kulkarni, Kerns.

Performed data analysis: Fink, Guo, Weidemann, Yu, Grobe, Rahmouni, Sivitz.

Wrote or contributed to the writing of the manuscript: Fink, Yu, Grobe, Rahmouni, Kerns, Sivitz.

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