Resolution of Mitochondrial Oxidative Stress Rescues Coronary Collateral Growth in Zucker Obese Fatty Rats

Yuh Fen Pung, Petra Rocic, Michael P. Murphy, Robin A.J. Smith, Jennifer Hafemeister, Vahagn Ohanyan, Giacinta Guarini, Liya Yin, William M. Chilian

Objective—We have previously found abrogated ischemia-induced coronary collateral growth in Zucker obese fatty (ZOF) rats compared with Zucker lean (ZLN) rats. Because ZOF rats have structural abnormalities in their mitochondria suggesting dysfunction and also show increased production of $O_2^-$, we hypothesized that mitochondrial dysfunction caused by oxidative stress impairs coronary collateral growth in ZOF.

Methods and Results—Increased levels of reactive oxygen species were observed in aortic endothelium and smooth muscle cells in ZOF rats compared with ZLN rats. Reactive oxygen species levels were decreased by the mitochondria-targeted antioxidants MitoQuinone (MQ) and MitoTempol (MT) as assessed by MitoSox Red and dihydroethidine staining. Lipid peroxides (a marker of oxidized lipids) were increased in ZOF by $\approx$47% compared with ZLN rats. The elevation in oxidative stress was accompanied by increased antioxidant enzymes, except glutathione peroxidase-1, and by increased uncoupling protein-2 in ZOF versus ZLN rats. In addition, elevated respiration rates were also observed in the obese compared with lean rats. Administration of MQ significantly normalized the metabolic profiles and reduced lipid peroxides in ZOF rats to the same level observed in lean rats. The protective effect of MQ also suppressed the induction of uncoupling protein-2 in the obese rats. Resolution of mitochondrial oxidative stress by MQ or MT restored coronary collateral growth to the same magnitude observed in ZLN rats in response to repetitive ischemia.

Conclusion—We conclude that mitochondrial oxidative stress and dysfunction play a key role in disrupting coronary collateral growth in obesity and the metabolic syndrome, and elimination of the mitochondrial oxidative stress with MQ or MT rescues collateral growth. (Arterioscler Thromb Vasc Biol. 2012;32:325-334.)

Key Words: collateral circulation ■ coronary circulation ■ obesity ■ oxidized lipids ■ reactive oxygen species

In prosperous societies, there is a rapid increase in the incidence of metabolic syndrome (MS), a condition characterized by abdominal obesity, hyperglycemia, insulin resistance, and hyperinsulinemia. People with MS are particularly at increased risk for ischemic heart disease and the deleterious consequences of myocardial infarction. Importantly, a well-developed coronary collateral circulation reduces infarct size and the incidence of sudden death in patients experiencing ischemic heart disease.¹

The myocardium, which has high energy requirements, relies on mitochondrial aerobic metabolism to maintain a high ATP/ADP ratio. A byproduct of mitochondrial bioenergetic activity is the generation of reactive oxygen species (ROS), including the superoxide anion ($O_2^-$), the hydroxyl radical (HO·), and hydrogen peroxide ($H_2O_2$).² Mitochondrial $O_2^-$ is normally neutralized by superoxide dismutase 2 (SOD-2, also known as MnSOD or mitochondrial SOD), which is located within the mitochondrial matrix, and also by superoxide dismutase 1 (SOD-1, also known as Cu/ZnSOD or cytosolic SOD-1), which is found in the cytosol and also in the intermembrane space between the inner and outer mitochondrial membranes.³ Both SOD-1 and SOD-2 dismutate $O_2^-$ to $H_2O_2$ and $O_2$. $H_2O_2$ is then converted to $O_2$ and $H_2O$ by the antioxidant enzymes glutathione peroxidase-1 (GPx-1), peroxiredoxins, and catalase.⁴ However, if there is an imbalance between the mitochondrial prooxidant generation and antioxidant defenses, mitochondrial oxidative stress may ensue.

Because of the presence of large numbers of mitochondria and the relatively high oxygen tension, the myocardium is prone to oxidative damage from ROS produced by the mitochondrial electron transport chain. Chronic increases in mitochondrial ROS can lead to mitochondrial DNA fragmentation, functional decline, progression of myocardial remodeling, and heart failure.⁵ Moreover, mice null for SOD-2 show damage to cardiac myocytes 1 week.
after birth and die of cardiomyopathy within 3 weeks of birth.

In view of the above reports and results showing that diabetes and MS are associated with mitochondrial abnormalities,7,8 we hypothesized that mitochondrial oxidative stress leads to mitochondrial dysfunction and compromises coronary collateral growth (CCG) in ZOF rats (a rat model of human MS). In this study, excessive lipid peroxidation was observed in ZOF rats as compared with their lean littermates, indicating oxidative stress. We further demonstrated induction of antioxidant enzymes (except GPx-1) and uncoupling proteins (UCPs), as well as enhanced electron transfer activities in ZOF rats. Treatment of ZOF rats with MitoQuinone (MQ), a mitochondria–targeted antioxidant, normalized lipid peroxides, UCP-2, and metabolic rate of the obese rats to levels comparable to the lean control animals. In addition, attenuation of mitochondrial and cytosolic ROS production was observed in aortic endothelium and smooth muscle cells in ZOF rats. The protective effect of MQ treatment partially restored CCG to levels comparable to those of control ZLN rats. Another mitochondria-targeted free radical scavenger, MitoTempol (MT), also restored collateral growth. Our data provide evidence linking mitochondrial oxidative stress and dysfunction to impaired CCG in ZOF rats and indicate that mitochondria-targeted antioxidants may be a useful therapeutic strategy to decrease mitochondrial oxidative stress, improve bioenergetics, and restore collateral growth in the heart in this model of MS.

**Materials and Methods**

**Animal Model**
Male Zucker lean (Fa/Fa or Fa/fa) (ZLN) and obese (fa/fa) (ZOF) rats, of 6 to 8 months old, were obtained from the Charles River Laboratory (Wilmington, MA) and housed under pathogen-free conditions. Rats that were homozygous for the fa gene (fa/fa) were obese and had MS, whereas those that were heterozygous (Fa/Fa) were lean and metabolically normal.

**MQ and MT Administration**
ZOF rats were housed individually and were placed on a normal control for the obese

**Rat Heart Mitochondrial and Cytosolic Fraction Isolation**
For some molecular studies, crude mitochondria were prepared using mitochondrial extraction buffer (Mitosciences Inc, Eugene, OR). For bioenergetics studies, mitochondria were isolated according to the protocol by Miyamoto et al., with slight modifications. Briefly, left ventricular tissues were minced, rinsed, and homogenized in MSHE buffer (210 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, 1 mmol/L EGTA), and 0.1% fatty acid–free bovine serum albumin (pH7.2). The homogenate was centrifuged at 27 000g for 10 minutes. The supernatant was collected as cytosolic fraction. The pellet was supplemented with nargarse protease (1 mg/g wet weight tissue) for 4 minutes on ice and rehomogenized. The homogenate was centrifuged at 400,000 g for 10 minutes to remove nuclei and debris. The supernatant was poured through cheesecloth. The pellet obtained was subjected to the same homogenization and centrifugation processes, and the supernatant was poured through cheesecloth.

**Cytosolic Lipid Peroxidation Assay**
The thiobarbituric acid–reactive substance assay was determined according to Buege and Aust,11 Ohkawa et al,12 and the manufacturer’s protocol (Zoetemtrix Corp., Buffalo, NY), with modifications. In brief, 60 µL of either malondialdehyde standards or 500 μg of homogenate was added to 540 µL of thiobarbituric acid. The solution was heated at 95°C for 1 hour, chilled on ice, and centrifuged at 3000 rpm for 15 minutes. The supernatant was collected, and absorbance was read at 532 nm in 200-µL duplicates.

**Figure 1.** Protein carbonyls and lipid peroxides contents. A, Left: Immunoblot showing significantly higher total carbonyls content (total immunoreactive carbonyls) in Zucker obese fatty rats (ZOF) compared with Zucker lean rats (ZLN). Right: Carboxyl content was increase by ~70% in ZOF vs ZLN rats (*P<0.05). DNP indicates 2,4-dinitrophenylhydrazone. B, Lipid peroxidation assay as an index of oxidative stress from cytosolic fraction from left ventricles. Higher lipid peroxidation content was observed in ZOF rats (~47 vs ~32 nmol/mL per mg of total protein of thiobarbituric acid–reactive substances [TBA]) in ZOF vs ZLN; (*P<0.05; n=4 pairs). MitoQuinone (MQ) treatment significantly attenuated this effect in ZOF rats, indicating reduced oxidative stress in ZOF rats (*P<0.05; n=4 pairs).
Mitochondrial Protein Carbonyl Group Detection

Excessive ROS production or defective antioxidant defense leads to oxidation of protein, lipids, and DNA. Carbonyl groups (aldehydes and ketones) are produced on protein side chains, especially on Pro, Arg, Lys, and Thr, when they are oxidized. To detect carbonylated proteins, carbonyl groups in protein side chains were derivatized with 2,4-dinitrophenylhydrazone to form the 2,4-dinitrophenylhydrazone derivative (Oxyblot Protein Oxidation Detection Kit, Millipore, Billerica, MA). The lysates were then denatured with 12% SDS, incubated with 2,4-dinitrophenylhydrazine, and neutralized solution at 37°C incubator without CO2 overnight. Before the start of experiment, the injection ports on the sensor cartridge were loaded with the appropriate mitochondrial substrates or inhibitors at 10 × concentration. The sensor cartridge was placed into the XF24 analyzer for automated calibration. During the calibration, isolated mitochondria were then seeded in XF24 V7 cell culture microplate (except for background correction well). Following centrifugation of the plate at 2000g for 20 minutes at 4°C, MSHE with initial experiment conditions was gently added into the wells containing mitochondria, and the plate was placed at 37°C incubator without CO2 for 5 minutes. The plate was then transferred to the XF24 analyzer, and the experiment was initiated.

For electron flow assay, mitochondria (2.5 μg/well) were supplemented with electron flow initial medium containing 10 mM/L pyruvate, 2 mM/L malate and 4 mM/L L-carbamoyl cyanide p-trifluoromethoxyphenylhydrazine. Injections (10 × concentration of either mitochondrial substrates or inhibitors) were made as follows: 20 μmol/L rotenone, 100 μmol/L succinate, 20 μmol/L antimycin A, and 100 μmol/L ascorbate+1 mM/L tetramethyl-p-phenylenediamine. Typical mix, measure, and mix cycles for the electron flow assay were 30 seconds, 3 minutes, and 1 minute, respectively.

All data were analyzed using the XF software and displayed as “point-to-point” oxygen consumption rates (pmol O2/minute per
well), or absolute oxygen tension (mm Hg O₂). Data were presented as the average of 3 to 5 wells/condition. For most measurements, either the highest or lowest point-to-point rates were taken. For oligomycin rate, an average of the last 3 point-to-point rates were taken because the effect of oligomycin requires at least 1 minute to reach maximal effect and an average of the last 30 seconds of the measure provides the most consistent result.15

Rat Model of CCG
ZLN and ZOF rats, ~6 to 8 months of age, were used for chronic (10 days) implantation of a pneumatic occluder over the left anterior descending coronary artery as described by Toyota et al16 to produce repetitive ischemia (RI). The RI protocol for rat consisted of 8 40-second occlusions, 1 every 20 minutes over 2 hours and 20 minutes followed by a period of rest for 5 hours and 40 minutes. This 8-hour cycle was repeated 3 times per day over a period of 10 days.

MitoSox Red and Dihydroethidine Detection of Mitochondrial and Cytosolic ROS on Vascular Endothelial and Smooth Muscle Cells Ex Vivo
Vascular mitochondrial and cytosolic ROS productions were determined using MitoSox Red and dihydroethidine (DHE). MitoSox Red, a live cell permeant and derivative of hydroethidine, is rapidly and selectively targeted to mitochondria with positively charged triphenylphosphonium. Once in the mitochondria, the reagent is intercalated into the mitochondrial DNA and oxidized by O₂⁻, and it exhibits red fluorescence (emission ~580 nm). Freshly isolated aortic rings were incubated with MitoSox Red or DHE (Molecular Probes, Carlsbad, CA) in Krebs/HEPES buffer.17,18 Fluorescent images were captured using an Olympus (Melville, NY) IX71 fluorescent microscope at ×20 magnification and were further analyzed with National Institutes of Health imaging software.

Collateral Blood Flow Measurement
Flow to the collateral-dependent zone was measured by neutron-activated microspheres (2.5×10⁶ microspheres/mL) labeled with either Gold or Samarium (Biopal Inc, Worcester, MA).19 Microspheres were dispersed by agitation for 1 minute and injected into the left ventricle in 200 μL during left anterior descending artery occlusion using a 25-gauge needle over 20 seconds. Gold microspheres were administered during surgery, and Samarium microspheres were given at the time of harvesting. The normal zone (NZ) and collateral-dependent zone (CZ) were divided, and the total weight of each was measured. Radioactivity was measured following neutron bombardment, and collateral blood flow was measured as a ratio of the cpm/g of the 2 regions, ie, CZ/NZ flow ratio. Blood flow was also quantified by comparing the tissue counts per gram to the counts in an arterial blood sample obtained at 0.4 mL/minute. In brief flow to the tissue was calculated as follows: Flow (mL/minute per g)=counts/g tissue divided by (counts/withdrawal rate of blood sample).

Statistical Analysis
Data were normalized to the respective controls values and were expressed as mean±SEM. Statistical analysis of data were performed by i-way ANOVA followed by post hoc Bonferroni test, as appropriate. P<0.05 was considered statistically significant.

Results
Lipid Peroxidation and Protein Carbonyls
Mitochondrial proteins were derivatized with 2,4-dinitrophenylhydrazine and then monitored with 2,4-dinitrophenylhydrazonoce antibody for protein carbonyl content. As shown in Figure 1A (left and right panels), ZOF showed greater levels of mitochondrial protein carbonyl moieties (~70%, P<0.05) compared with the lean controls, indicating that mitochondrial oxidative stress was higher in ZOF as compared with ZLN rats. Left ventricular oxidized lipid content is shown in Figure 1B. Basal thiorbarbituric acid–reactive substance level in ZOF rats was significantly elevated compared with ZLN (32.3±1.2 versus 52.3±6.0 nmol/mL per mg of protein). MQ treatment significantly reduced peroxidation of lipids in ZOF rats to the level similar to that of the lean rats (27.3±0.9 nmol/mL per mg of protein; *P<0.05, n=4 pairs).
MQ Improved Cytosolic and Mitochondrial Antioxidant Defense in ZOF Rats

Expression of cytosolic and mitochondrial antioxidant proteins from the left ventricles of Zucker rats were evaluated using immunoblots against SOD-1, SOD-2, GPx-1, catalase, Prd-3, and Prd-6. Superoxide dismutase-1, which is located in the cytosol and between the inner and outer-membranes of mitochondria, was significantly increased in ZOF (≈80%; *P < 0.05; n=4–6 pairs) and ZOF+MQ (≈110%; *P < 0.05; n=4–6 pairs) rats as compared with the lean rats (Figure 2A). Expression level of SOD-2, which is located within the matrix, was also significantly higher in the ZOF (≈59%; *P < 0.05; n=4–6 pairs) and ZOF+MQ (≈33%; *P < 0.05; n=4–6 pairs) rats as compared with the lean rats (Figure 2B). Interestingly, MQ did not improve GPx-1 expression, which was found to be low in the obese rats, suggesting a reduced catabolism of H2O2 by GPxs (≈50% reduction in ZOF and ZOF+MQ versus ZLN; *P < 0.05; n=4–6 pairs) (Figure 2C). In contrast, enhanced expression of catalase, Prd-3 and Prd-6, was found in ZOF (≈20%, n=4–6 pairs) and ZOF+MQ (≈40%, *P < 0.05; n=4–6 pairs) rats compared with the lean rats (Figure 2D–2F, respectively).

MQ Improved Mitochondrial Function and Bioenergetics Profiles of ZOF Rats

Figure 3 illustrates the respiration profiles of mitochondria isolated from Zucker rats. In the presence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone as an uncoupler, mitochondria from the obese rats exhibited higher respiration rates with successive exposure to the following substrates or inhibitors: pyruvate+malate (≈39%; complex I–dependent respiration; *P < 0.05; n=6 pairs; 3–4 wells/condition) (Figure 3A), rotenone+succinate (≈30%, complex II–dependent respiration; n=6 pairs; 3–4 wells/condition) (Figure 3B), and antimycin+tetramethyl-p-phenylenediamine+ascorbate (≈28%; n=6 pairs; 3–4 wells/condition) (Figure 3C), as compared with the lean. MQ treatment of the obese rats in vivo normalized the respiration rates of the subsequently isolated mitochondria to the level similar to those found in the lean rats.

MQ Reduced the Expression of UCPs in ZOF Rats

To elucidate the mechanisms responsible for enhanced respiration rates in ZOF rat mitochondria and its reduction after MQ treatment, immunoblots on UCPs were performed (Figure 4). UCP-2 expression was significantly higher in ZOF rats (≈37%; ZOF versus ZLN; *P < 0.05; n=4 pairs). UCP-2 and UCP-3 protein expressions in ZOF+MQ group were significantly lower as compared with the ZOF controls rats (≈31% for UCP-2, *P < 0.05; and 21% for UCP-3, n=4 pairs).

MQ and MT Decreased Mitochondrial and Cytosolic ROS Production in the Aorta

Steady-state mitochondrial and cytosolic O$_2^-$ production in the aortic wall was significantly higher (>2-fold) in ZOF rats than in ZLN rats (Figure 5A and 5B for Mitosox Red ZLN versus ZOF, *P < 0.05, n=3 pairs; Figure 5E and F for DHE ZLN versus ZOF, *P < 0.05, n=3 pairs). Conversely, treatment with MQ and MT (50 μmol/L and 3 mmol/L, respectively) supplemented in their drinking water for 10 to 14 days significantly reduced mitochondrial and cytosolic ROS throughout the wall in ZOF rats to the similar level observed in the lean rats (Figure 5C and 5D compared with 5A for Mitosox Red; Figure 5G and 5H compared with 5E for DHE). These findings suggest that mitochondria are one of the major sources of ROS in the obese rats.

**Figure 4.** Expression of uncoupling proteins. Mitochondrial uncoupling proteins were immunoblotted with UCP (UCP)-2 (A), and UCP-3 (B) antibodies, and bar graphs summarize immunocaptured signals. Uncoupling protein-2 expression was significantly higher in Zucker obese fatty (ZOF) rats (≈37%; ZOF versus Zucker lean [ZLN] rats; *P < 0.05; n=4 pairs) (A), and MitoQuinone (MQ) treatment of the obese rats lowered expression of UCP-2 (≈31% ZOF vs ZOF+MQ, *P < 0.05; n=4 pairs) (A) and UCP-3 (≈21% ZOF vs ZOF+MQ; n=4 pairs) (B). Reduction in uncoupling proteins after MQ treatment appears to be in line with the respiration profiles, indicating that improvement in bioenergetics profile after MQ treatment appear to be regulated through reduction of oxidative stress and uncoupling proteins.
MQ and MT Treatment Induced CCG in ZOF Rats

CCG expressed in empirical units of flow (mL/minute per g tissue) or as the ratio of perfusion between the CZ and the NZ was shown in Figure 6. In ZLN rats, collateral flows increased from 0.23±0.02 to 0.84±0.02 mL/minute per g (before and after RI), but in sham-operated ZLN rats, collateral flows did not change (0.23±0.03–0.24±0.02 mL/minute per g) from the beginning to the end of the protocol. In ZOF rats, either sham (0.09±0.01–0.12±0.01 mL/minute per g) or control ZOF exposed to RI (0.08±0.01–0.15±0.05 mL/minute per g), collateral flows did not change. In ZOF rats treated with MQ or with MT, collateral flow increased from 0.08±0.03 to 0.72±0.01 mL/minute per g and from 0.08±0.01 to 0.64±0.03 mL/minute per g, respectively (Figure 6A). The CZ/NZ ratios also mimicked these changes, with significant increases in the ratio occurring in ZLN rats exposed to RI (≈0.81) and in ZOF rats subjected to RI with MQ (≈0.72) or MT (≈0.64). In the sham groups or in ZOF rats subjected to RI, the CZ/NZ ratio did not significantly change (Figure 6B). Surprisingly, VEGF expression was significantly lower in the obese rats as compared with the lean rats, and MQ treatment did not salvage this deficiency (Figure 6C, *P<0.05, n=4 pairs).

Discussion

Over the years, NADPH oxidase, xanthine oxidase, and endothelial nitric oxide synthase uncoupling have been the 3 major sources of cytosolic ROS that have been studied extensively in cardiovascular research.5,20 In line with the findings from other research groups,21,22 our laboratory had previously demonstrated blunted endothelial function and cytosolic oxidative stress in ZOF rats,23,24 a rat model that presents many characteristics of MS, including obesity, insulin resistance, hyperlipidemia, hyperinsulinemia, hypertriglyceridemia, and hyperphagia.25 We further reported compromised CCG of ZOF rats in response to episodic ischemia.26 When VEGF was transfected into smooth muscle cells and introduced into coronary circulation, we did not observe significant improvement in CCG in ZOF rats. However, correction of oxidative stress with extracellular superoxide dismutase coupled with the same VEGF smooth muscle–based gene delivery system restored coronary collateral development partially in the obese rats. These results indicated that amelioration of oxidative stress may help restore redox-dependent signaling in CCG of ZOF rats.26 To further decipher the role of redox-dependent signaling in mediating coronary collateral development, we hypothesized a role for mitochondrial dysfunction and oxidative stress in our rat model of MS. This hypothesis was in part based on literature showing mitochondrial dysfunction in the hearts of OVE26 mouse model of type I diabetes.27 Our hypothesis was also supported by unpublished observation from our own laboratory and others that ZOF rats were known to develop structural abnormalities of the mitochondria,28,29 suggesting a
possible role for oxidative stress. Thus, we focused our studies on the mitochondria.

Our major observations from this study were that in ZOF rats compared with lean controls, (1) lipid peroxides were higher in the myocardium, (2) antioxidant enzymes and UCPs (UCP-2 in particular) were induced in the myocardium, (3) maximal respiration rates were elevated in the myocardium, and (4) both mitochondrial and cytosolic ROS were increased throughout the vascular wall, suggesting an increase in ROS in both the endothelium and in smooth muscle. Administration of the mitochondrial-targeted antioxidant MQ ameliorated lipid peroxidation in the myocardium and ROS generation in the vascular cells. MQ also normalized the metabolic profile after treatment with MQ. During uncoupling with carbonyl cyanide p-trifluoromethoxyphenylhydrazone, mitochondria from obese rats had a significantly higher increase in oxygen consumption rate compared with those from lean rats. Chronic treatment with MQ prevented this increase. Moreover, MQ induced a protective effect in mitochondria from ZOF rats by normalizing the maximal respiration rates to the levels observed in the lean rats.

**MS and the Prediabetic Myocardium**

MS is a constellation of metabolic abnormalities that markedly increases the risk of developing ischemic heart disease, atherosclerosis, cardiomyopathy, and type II diabetes. Development of MS is tightly associated with impaired mitochondrial function and increased mitochondrial oxidative stress. Mitochondrial \( \text{O}_2^\bullet^- \) production occurs primarily at discrete points in the electron transport chain at CI and CIII and in components of the tricarboxylic acid cycle, including \( \alpha \)-ketoglutarate dehydrogenase. Mitochondrial-derived \( \text{H}_2\text{O}_2 \)
diffuses to the cytoplasm and ultimately leads to oxidation of cytoplasmic proteins. Chronic exposures of ROS result in oxidative damage to DNA, proteins, and lipids and inactivation of tricarboxylic acid cycle aconitase and the electron transport chain at Complex I, II, and III [19,20]. In this study, we observed enhanced lipid peroxidation in ZOF rats as compared with the lean. One possible explanation could be the excessive leak of electrons, which is passing down the respiratory chain before the reduction of oxygen to water at cytochrome c oxidase. These released electrons form superoxide with molecular oxygen. Superoxide and lipid peroxidation products, such as hydroxynonenal, activate uncoupling protein-2 (UCP-2). Activation of UCPs, UCP-2 in particular, has been proposed to act as a negative feedback loop to induce mild uncoupling that subsequently lowers ROS production [10]. This suggestion is further supported by the observation of decreased lipid peroxidation, as well as attenuated levels of UCP-2 in the obese on MQ treatment as compared with ZOF rats. However, whether a mitochondrial electron or proton leak is the cause or effect of impaired mitochondrial function in MS or vice versa remains elusive.

**MQ as a Potential Therapy for Diseases Related to Mitochondrial Oxidative Stress and Dysfunction**

MQ and MT are promising antioxidants that have been successfully targeted to mitochondria [21,22]. Of these 2 compounds, MQ has been used more widely to treat a variety of conditions associated with oxidative stress [21]. It contains a covalently attached lipophilic triphenylphosphonium cation that, because of the large negative potential (~150–180 mV) across the inner mitochondrial membrane, leads to MQ being concentrated several hundredfold within mitochondria. Once adsorbed to the matrix face of the mitochondrial inner membrane, MQ accepts electrons from Complex II to form the quinol reduction product, which is an effective antioxidant. It should be noted that the ubiquinone/ubiquinol pool exists mainly in the reduced ubiquinol form in vivo, and ubiquinol has been reported to function as an antioxidant by donating a hydrogen atom to a lipid peroxyl radical, thereby decreasing lipid peroxidation within the mitochondrial inner membrane [23,24]. The ubiquinol radical formed in this process rapidly disproportionates and electron transport chain subsequently recycles ubiquinone back to ubiquinol, restoring the antioxidant function.

The effects of long-term ad libitum oral administration of 500 μmol/L MQ on the behavior, metabolism, gene expression, and accumulation of oxidative damage markers of young C57BL/6 mice has been investigated and shown to have no effect on redox cycling or cause uncoupling in vivo [25]. In addition, MQ has been used in a wide range of animal studies, where it has shown beneficial effects in ameliorating cardiac ischemia/reperfusion injury [26,27], damage to endothelial cells in vivo associated with chronic exposures to nitroglycerin [28], increase in blood pressure in a spontaneously hypertensive rat model in which the increase in blood pressure is thought to arise from elevated mitochondrial oxidative damage in endothelial cells [29], sepsis [30,31], and against heart damage associated with the anticancer compound Adriamycin [32].

As mentioned previously, our former work using smooth muscle cells to deliver extracellular superoxide dismutase and VEGF had modest effects on increasing coronary collateral flows in ZOF rats. However, a striking result of the present study was the nearly complete restoration of coronary collateral flow in the ZOF rats by mitochondrial-directed antioxidants—MQ and MT. Thus, it seems that mitochondrial oxidative stress plays a key role in abrogating CCG in response to ischemia. Our connection between mitochondrial oxidative stress and the abrogation of collateral growth is somewhat tenuous, but we believe that altered mitochondrial bioenergetics and decreases in high energy phosphates play a critical role. Therefore, it may be possible that mitochondriatargeted antioxidants may be worth exploring as therapies in patients with this disorder. In this regard, it is useful to note that MQ has undergone 2 human clinical trials, 1 in Parkinson disease and another in protecting against liver inflammation [33,34]. These trials showed that MQ could be given safely to patients for up to a year and that it was effective in decreasing liver damage.

[Figure 7. Proposed mechanism for increased coronary collateral development in the obese and the prediabetic myocardium. In the insulin-resistant animals, augmented fatty acid oxidation would increase the delivery of reducing equivalents to the electron transport chain, leading to excessive superoxide production. The enhanced production of superoxide and lipid peroxides induce expression of uncoupling-protein-2. Increased levels of uncoupling-protein-2 lead to enhanced oxygen consumption, without an increment or even a reduction in the ATP synthesis. Reduced ATP synthesis and levels potentially contribute to compromised arteriogenesis/angiogenesis in response to repeated ischemic insults insofar as reduced energy levels would prevent a cell from undergoing a phenotypic switch which requires energy. Scavenging of mitochondrial-derived free radicals (MitoQuinone [MQ] and MitoTempol [MT] treatment) would normalize the electron transfer activities facilitated by ubiquinone (Q) in the obese rats to the level in lean rats, leading to reduced lipid peroxides. Reduction in lipid peroxides, which is the known agonist for uncoupling proteins, appears to improve bioenergetics profile in the obese myocardium. Improvement in bioenergetics is essential in mediating the phenotypic switch of vascular cells during arteriogenesis/angiogenesis. UCP indicates uncoupling protein.]

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**Figure 7.** Proposed mechanism for increased coronary collateral development in the obese and the prediabetic myocardium. In the insulin-resistant animals, augmented fatty acid oxidation would increase the delivery of reducing equivalents to the electron transport chain, leading to excessive superoxide production. The enhanced production of superoxide and lipid peroxides induce expression of uncoupling-protein-2. Increased levels of uncoupling-protein-2 lead to enhanced oxygen consumption, without an increment or even a reduction in the ATP synthesis. Reduced ATP synthesis and levels potentially contribute to compromised arteriogenesis/angiogenesis in response to repeated ischemic insults insofar as reduced energy levels would prevent a cell from undergoing a phenotypic switch which requires energy. Scavenging of mitochondrial-derived free radicals (MitoQuinone [MQ] and MitoTempol [MT] treatment) would normalize the electron transfer activities facilitated by ubiquinone (Q) in the obese rats to the level in lean rats, leading to reduced lipid peroxides. Reduction in lipid peroxides, which is the known agonist for uncoupling proteins, appears to improve bioenergetics profile in the obese myocardium. Improvement in bioenergetics is essential in mediating the phenotypic switch of vascular cells during arteriogenesis/angiogenesis. UCP indicates uncoupling protein.
Conclusion

There is increasing evidence to support the concept that mitochondria play a critical role in ischemic heart disease and various cardiovascular diseases. However, the question of whether abnormalities leading to mitochondrial oxidative stress and dysfunction are the cause or response to various cardiovascular diseases is far from resolved. A better understanding of the redox-dependent mitochondrial signal transduction pathways, availability of pharmacological agents that can alter the production and scavenging of mitochondrial ROS, and various animal models with impaired mitochondrial function would aid in resolving the question. In the context of our study, we conclude that mitochondrial dysfunction, induced by oxidative stress due to accelerated electron or proton leak, abrogates the process of CCG in a rat model of obesity and insulin resistance and that resolution of the oxidative burden rescues this impaired phenotype as illustrated in Figure 7.

Sources of Funding

This work was supported by National Institutes of Health Grants HL32788, HL83366, and RC1HL100828 (to W.M.C.) and by American Heart Association Postdoctoral Fellowship 09POST2290021 (to Y.F.P.).

Disclosures

None.

References


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Arterioscler Thromb Vasc Biol. 2012;32:325-334; originally published online December 8, 2011;
doi: 10.1161/ATVBAHA.111.241802
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure I

Percent body weight change of ZOF rats relative to day-0 with 50 μM MQ supplemented in drinking water over 10 to 14 days. Lean, ZOF and ZOF+MQ rats exhibited steady weight gain of ~4.5 ± 0.4 %, ~5.3 ± 0.9 % and ~5.2 ± 1.6 %, respectively. Note that MQ treatment to ZOF did not significantly alter the weight gain profile.
Figure II

Titration of mitochondria amount, ADP and FCCP concentrations. To determine the optimal amount of mitochondria for respiration studies, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0 and 50.0 µg mitochondria/well were tested, respectively. As shown in Supplementary Figure 2A, 0.5 and 1.0 µg mitochondria per well showed no appreciable deficit in oxygen tension in the well (~150 to 160 mmHg O₂) over the course of the experiment, suggesting that OCR does not exceed the ability of the analyzer to replenish oxygen tension after completion of measurement for each cycle (n=2 lean rats; 5 to 6 wells/condition/rat). However, 2.5 µg mitochondria/well (state 2 OCR: ~400 pMoles/min) was chosen for all the following experiments due to greater experimental resolution. Next, various concentrations of ADP and FCCP were used to ensure sustainable state 3 and maximal respiration rates. In this study, 5 mM ADP and 4 µM FCCP were used in the following experiments (n=2 lean rats; 5 to 6 wells/condition/rat; Supplementary Figure 2B and 2C).