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Uncoupling Protein 2 Impacts Endothelial Phenotype via p53-Mediated Control of Mitochondrial Dynamics

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Abstract

Rationale—Mitochondria, although required for cellular ATP production, are also known to have other important functions that may include modulating cellular responses to environmental stimuli. However, the mechanisms whereby mitochondria impact cellular phenotype are not yet clear.

Objective—To determine how mitochondria impact endothelial cell function.

Methods and Results—We report here that stimuli for endothelial cell proliferation evoke strong upregulation of mitochondrial uncoupling protein 2 (UCP2). Analysis in silico indicated increased UCP2 expression is common in highly proliferative cell types, including cancer cells. Upregulation of UCP2 was critical for controlling mitochondrial membrane potential (Δψ) and superoxide production. In the absence of UCP2, endothelial growth stimulation provoked mitochondrial network fragmentation and premature senescence via a mechanism involving superoxide-mediated p53 activation. Mitochondrial network fragmentation was both necessary and sufficient for the impact of UCP2 on endothelial cell phenotype.

Conclusions—These data identify a novel mechanism whereby mitochondria preserve normal network integrity and impact cell phenotype via dynamic regulation of UCP2.

Keywords

Endothelium; mitochondria; superoxide; uncoupling proteins; angiogenesis; endothelial function; ischemia

Introduction

The cellular requirement for ATP as a high-energy intermediate to fuel for many critical cellular reactions is universal. The production of ATP depends upon an extracellular source of carbohydrates or lipids that are initially metabolized in the cytosol to products that are
then transported into mitochondria for oxidative phosphorylation to produce a proton gradient (i.e., membrane potential, $\Delta \psi$) that powers ATP generation. Known mitochondrial functions extend beyond ATP production to include other cellular processes such as apoptosis, heme synthesis, calcium homeostasis, inflammation, and development. Thus, mitochondria have the capacity to impact cellular phenotype via energy-dependent and energy-independent mechanisms.

One means by which mitochondria can impact cellular phenotype is through participation in signaling paradigms and there is ample evidence from multiple organisms that mitochondria signal to the nucleus. Respiratory deficiency induced by either pharmacologic or genetic means is known to affect gene expression in the nucleus. Mitochondrial-derived heme coordinates nuclear regulation of genes encoding heme-dependent proteins such as cytochrome $c$, catalase, and cytochrome oxidase. The mitochondrial release of proteins such as apoptosis-inducing factor (AIF) and SMAC/DIABLO into the cytosol also regulates genes important for apoptosis. Thus, mitochondrial events are readily communicated to the nucleus to affect gene regulation.

The cellular responses to environmental stimuli also involve mitochondria. Proteolytic processing of cell surface receptors such as ErbB4 can release intracellular domains that trigger mitochondrial release of pro-apoptotic proteins. Energy deprivation responses, such as AMP kinase activation, induce mitochondrial gene upregulation that is critical for cellular stress adaptation. Uptregulation of endothelial VEGF involves perinuclear mitochondrial clustering and the local production of mitochondrial $\cdot O_2^-$. The latter has also been linked to the control of MAP kinase phosphatases and the coordination of NF-kB-dependent inflammatory responses. Finally, mitochondrial electron transport is known to impact the activation state of cell-surface growth factor receptors. Thus, the mitochondrion is emerging as an important organelle for the coordination of cellular environmental responses.

Although mitochondria clearly impact how cells respond to the environment, the mechanisms involved in this process are not well understood. Many mitochondrial actions are linked to the electron transport chain and the resultant membrane gradient, $\Delta \psi$. Control of $\Delta \psi$ involves the relative availabilities of substrates for electron transport (NADH and FADH$_2$), respiration (O$_2$), and ATP synthesis (ADP), as well as any proton leak. The latter is largely regulated via uncoupling proteins that belong to the mitochondrial carrier superfamily. Herein we report that endothelial cell proliferation and angiogenesis involve upregulation of mitochondrial uncoupling protein 2 (UCP2) in order to reduce $\Delta \psi$ and limit mitochondrial $\cdot O_2^-$ that otherwise promotes p53-dependent mitochondrial fragmentation resulting in premature senescence. These findings suggest a new function for UCP2 that has broad implications for processes that involve the vascular endothelium.

**Methods**

**Endothelial cell culture and transfection**

Bovine Aortic Endothelial Cells (BAECs) were purchased from Genlantis (San Diego, CA) and were cultured in EBM supplemented with EGM-MV Bullet Kit from Lonza (Walkersville, MD). Before each experiment, BAECs were made quiescent by 24-hour incubation in low-serum medium (EBM supplemented with 0.1% or 0.4% FBS). Human aortic endothelial cells (HAECs) were from Lonza and were cultured as described. Murine Lung Endothelial Cells (MLECs) from mice of both sexes were isolated as described, and cultured on gelatin- or collagen-coated plates and grown in MLEC medium containing 20% FBS, 38% DMEM, 38% Ham’s F-12 with 100 μg/ml endothelial mitogen (ECGS; Biomedical Technologies; Stoughton, MA), 4 mM L-glutamine, 100 μg/ml heparin.
and penicillin/streptomycin. Endothelial purity was confirmed by staining with Dil-Ac-LDL (Biomedical Technologies; Stoughton, MA) and anti-CD31 antibody (BD Biosciences; San Jose, CA).

**Quantitative Real-Time PCR**

Total RNA was extracted from cells and tissues with the RNeasy Mini Kit (Qiagen) or TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse transcribed with oligo(dT) primers for cDNA synthesis. Real-time PCR was performed in the iQ5 real-time PCR detection system (Bio-Rad Laboratories) and the products were detected using either SYBR Green dyes (Bio-Rad Laboratories) or TaqMan probes of the TaqMan Gene Expression Assays for specific genes (Applied Biosystems; Foster City, CA).

**Transfections**

For gene overexpression, BAECs or MLECs were incubated with adenoviruses at 50 - 100 MOI as described.14,20 For gene silencing, BAECs were transfected with 1.3 μg of siRNA oligonucleotides (Thermo Scientific Dharmacon; Lafayette, CO) against human UCP2 (Cat. Nos. D-005114-01, -02, -03, and -04), human SOD2 (D-009784-03, -04, -19, and -20); or negative control (D-001210-02, -03, -04, and -05) as described.14 Likewise, MLECs were transfected with mouse mitofusin 1 (Mfn1) (J-065399-09, -10, -11, and -12); mouse mitofusin 2 (Mfn2) (J-046303-05, -06, -07, and -08); mouse p21 (J-058636-05, -06, -07, and -08); mouse p53 (J-040642-09, -10, -11, and -12); or the non-targeting pool (D-001810-10-05).

**Cell proliferation and migration assays**

DNA synthesis was directly measured via the 3H-thymidine incorporation assay. Cell proliferation was determined with CyQUANT GR fluorescent dye (Invitrogen Molecular Probes; Eugene, OR) to determine the relative cell number with a fluorescence microplate reader (Gemini XPS, Molecular Devices). Cell migration was assayed with the *in vitro* scratch assay in which a monolayer of quiescent cells was uniformly scratched and the rate of cell migration to close the void was evaluated 20 hours after wounding using ImageJ.

**Mitochondrial membrane potential and superoxide measurements**

Mitochondrial membrane potential (Δψ) was determined using two complementary fluorescent methods. Cells were incubated with 1 μM JC-1 for 30 min and Δψ estimated as the fluorescence ratio of JC-1 aggregates (red; excitation 550nm, emission 600nm) to monomers (green; excitation 485nm, emission 535nm) formed as a function of inner mitochondrial membrane potential.21 For measurement of mitochondrial superoxide (•O2–), cells were loaded with 5 μM MitoSOX Red (Invitrogen) for 10 min and the fluorescence (excitation 390nm; emission 510nm) determined. Live cells were labeled with 0.5 μM MitoSOX for 20 min and the fluorescence images at both 405- and 514-nm excitation were captured using an Eclipse TE2000-S fluorescence microscope (Nikon; Melville, NY) with a CCD camera utilizing a SPOT Insight 2MP Firewire Color Mosaic (Diagnostic Instruments; Sterling Heights, MI).

**Antibodies and immunoblotting**

Antibodies against cytochrome c oxidase subunit IV (COX IV), p21cip/waf, p16ink16a, phospho-p53 (mouse Ser18), phospho-c-Jun (Ser63), phospho p38 MAP kinase (Thr180/ Tyr182) and -rabbit or mouse IgG were purchased from Cell Signaling Technology. Antibodies against cytochrome c were from BD Biosciences. We obtained antibodies against UCP1, UCP2, and UCP3 from Fitzgerald Industries International (Acton, MA) and specific UCP2 antibodies (N-19 and A-19) and native p53 antibody from Santa Cruz.
Biotechnology (Santa Cruz, CA). We obtained SOD2 antibody from Millipore (Temecula, CA) and actin antibody from Sigma-Aldrich (St. Louis, MO). Protein extracts in DTT-containing SDS sample buffer were separated in 10% or 12% SDS-polyacrylamide gels and transferred to Hybond ECL nitrocellulose membranes (GE Healthcare; Piscataway, NJ). Immunoblotting was then carried out and quantified as described. In cases where loading controls produced paired bands, both were used for quantification.

**Oxygen consumption and lactate**

Using a Clark-type oxygen electrode (Hansatech), respiration in whole cells was quantified as previously described. Briefly, 1 × 10⁶ cells were resuspended in 1 mL of respiration medium (DPBS, 2 mM glucose, 1 mM pyruvate, 2% fatty acid-free bovine serum albumin). To obtain proton leak, oligomycin was added to a final concentration of 2 μM. To measure maximal respiration, FCCP was added to a final concentration of 2.4 μM. Non-mitochondrial respiration, obtained by adding myxothiazol to a final concentration of 4 μM, was subtracted from total respiration measurements. For lactate, accumulation was determined by a lactate fluorometric assay kit (MBL International, Woburn, MA) according to the manufacturer's instructions and the concentration of secreted lactate was normalized to the cell number in each sample.

**Capillary sprouting assay in aortas**

Thoracic aorta was placed in EBM-2 media as described, periaortic tissue was carefully removed, and then the aorta was cleaned and sliced into 1 mm-long rings. Rings were then embedded in liquid collagen gel in 48-well plates (BD Biosciences), incubated at 37°C for 1h to polymerize the collagen, and the solid gel covered with MLEC medium diluted 1:2 with DMEM. Each aortic ring was examined daily and capillary sprouts counted along the sample perimeter under 100 and 200 × magnification. Vascular sprouts were distinguished from fibroblasts via morphology as described and CD31 staining. To infect aortic rings with adenoviral vectors, each ring was embedded in liquid collagen gel containing respective vectors (Ad-UCP2, Ad-SOD2, etc.) at 1.0 × 10⁸ pfu before polymerized. Transfection was validated in separate experiments with Ad-GFP transfection and after 4d by fixation of collagen gel-embedded tissue with 4% formaldehyde in PBS for 10 min at 4°C. Fixed sections underwent immunofluorescence staining with anti-GFP and anti-CD31 antibody and counterstained with DAPI before fluorescence imaging.

**Animals and hindlimb ischemia model**

Heterozygous UCP2-null animals on the C57 background were obtained from Dr. Bradford Lowell, Ph.D. (Harvard Medical School) and bred to homogeneity with age-matched controls. SOD2 heterozygous mice were obtained from Jackson Laboratories and bred to obtain heterozygous and control animals. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of University of Massachusetts Medical School. Male mice at 8 to 12 weeks of age were anesthetized with intraperitoneal injection of combination of 100 mg/kg ketamine hydrochloride and 5 mg/kg xylazine (Webster Veterinary, Devens, MA) prior to surgery. Unilateral hindlimb ischemia in the left leg was introduced in the mice as described. In selected experiments, 100 μl (2.0 × 10⁸ pfu) of adenoviral vectors encoding UCP2, or LacZ was injected into five different sites of the ischemic thigh muscles such as adductor longus, adductor magnus, and adductor brevis muscles. Hindlimb tissue perfusion was assessed with Moor LDI2-IR laser Doppler imaging system (Moor Instruments, Devon, UK). Blood flow images were obtained under conditions of constant body temperature (36±1.0°C) and average hindlimb blood flow was expressed as the ratio of ischemic to non-ischemic foot flow to account for minor variations in imaging conditions.
Cell cycle analysis

Endothelial cells at 50% confluence were synchronized in 0.4% serum overnight. Cells were then cultured in complete medium and at 24 and 48h harvested, stained with propidium iodide and subjected to FACS analysis as described.

Cellular senescence assay

MLECs were seeded onto 0.1% gelatin-coated 12-well plates and maintained for 14 days under normal conditions. Senescence was assayed as senescence-associated β-galactosidase (SA-βgal) activity using the senescence β-galactosidase staining kit (Cell Signaling Technology). Both bright-field and phase-contrast pictures were viewed through 4× or 10× objective on a microscope on the Eclipse TE2000-S microscope as above and images processed with SPOT Advanced Version 4.6 imaging software (Diagnostic Instruments, Inc.). SA-βgal-positive cells were quantified with the ImageJ software.

Mitochondrial length measurements

Mitochondrial length was measured as an index of mitochondrial fragmentation. Live endothelial mitochondria were labeled with 100 nM MitoTracker Green FM for 30 min and the fluorescence images were captured using a 100× oil immersion objective to acquire high resolution images of mitochondria as described. To determine mitochondrial length, ImageJ was applied in a blinded fashion to the measurement for each frame of a selected region of interest (ROI) as described for determining DNA contour lengths.

Statistical analysis

All data are expressed as means ± SE and the numbers of independent experiments are indicated. Statistical comparisons were conducted between two groups by use of Student’s t-test or Mann-Whitney U test as appropriate. Multiple groups were compared with either one-way Kruskal-Wallis or ANOVA with a post-hoc Tukey-Kramer multiple comparisons test as indicated in legends. A probability value < 0.05 was considered significant. All statistics were done using StatView 5.0 (SAS Institute; Cary, NC) or GraphPad Prism 5 (GraphPad Software; La Jolla, CA).

Results

Dynamic modulation of UCP2 and Δψ with endothelial proliferation

Quiescent BAEC monolayers stimulated to proliferate with FBS exhibited a reduction in Δψ (Fig. 1A), whereas increasing cell confluence was associated with an increase in Δψ (Figs 1B and C). Since mitochondrial UCPs are implicated in Δψ regulation, we probed endothelial UCP expression and observed mRNA and protein only for UCP2 and 3 (Fig. 1D). We then observed that UCP2 is upregulated with endothelial proliferation (Fig. 1A) and downregulated with increasing confluence, with no dynamic regulation of UCP3 (Figs. 1B and E). We could recapitulate the proliferation-induced changes in Δψ by molecular manipulation of UCP2 (Fig. 1F) and UCP2-null cells had increased Δψ (Fig. 1G), Thus, our data indicate that the proliferation-induced changes in Δψ can be explained, at least in part, by dynamic regulation of UCP2.

UCP2 and endothelial metabolism

Since we found that UCP2 is dynamically regulated in the endothelium, we probed its implications for basic mitochondrial functions. We found that UCP2-null cells had a trend for lower basal respiration rate (*P<0.07) than wild-type cells and similar rates of basal proton leak. UCP2-null cells also had similar maximal respiration (2.08 ± 0.30) than wild-type cells (2.61 ± 0.58, P=0.23 by two-tailed t-test; Fig. 2A). Proliferating UCP2-null cells
also produced less lactate than wild-type cells (Fig. 2B), consistent with a lower rate of glycolysis. Suppression of UCP2 was associated with reduced ATP levels (Fig. 2C) and endothelial ATP levels appeared greatest in endothelium with the highest proliferation rate (Fig. 2D). Collectively, these data indicate that UCP2-null cells have more prominent perturbations in glycolysis than respiration, and that endothelial ATP levels vary as a function of proliferation.

**UCP2 regulates endothelial phenotype via changes in Δψ**

To gain insight into the potential function of UCP isoforms, we examined UCP mRNA in silico as a function of cell type. We found that UCP1 exhibited relatively homogeneous mRNA expression across multiple cell types as did UCP3, with the exception of a 3-fold higher expression in skeletal muscle (Online Figure I). In contrast, UCP2 mRNA expression varied over 50-fold as a function of cell type with the highest transcript levels in rapidly dividing cells, including those harboring erythroid (CD71), endothelial progenitor (CD34) and endothelial angiogenic (CD105) markers (Online Figure I). Based upon this latter association, we tested known stimuli for angiogenesis such as VEGF and AMP kinase and found UCP2 upregulation (Fig. 3A). Serum- and VEGF-mediated UCP2 upregulation was associated with c-Jun N-terminal kinase (JNK) activation and JNK inhibition attenuated both UCP2 upregulation and VEGF-mediated endothelial cell proliferation (Online Figure II). We also manipulated endothelial UCP2 levels and found that endothelial cell proliferation (Fig. 3B) and migration (Fig. 3C) were directly related to UCP2. Consistent with these observations, UCP2-null endothelium exhibited impaired proliferation (Fig. 3D) and migration (Fig. 3E), without any compensatory upregulation of UCP3 (Online Figure IIIA).

Since endothelial proliferation and migration are features of angiogenesis, we examined the impact of UCP2 in a capillary sprout formation assay, an in vitro model of early angiogenesis. Wild-type aortic segments exhibited a higher rate and total extent of capillary sprout formation than did UCP2-null aortic segments (Fig. 3F), and reconstitution of UCP2 into UCP2-null endothelium rescued the defect in capillary sprout formation (Fig. 3G). Similarly, capillary sprout formation from UCP2-null adipose tissue was impaired relative to wild-type, and UCP2 reintroduction also rescued this defect (Figures IIIB and IIIC). To determine the physiologic relevance of our in vitro observations, we examined hindlimb ischemia-induced angiogenesis and observed a lower rate of blood flow recovery in UCP2-null mice compared to wild type controls (Fig. 3H) that was largely rescued by adenoviral-mediated restoration of UCP2 expression (Online Figure IIIID).

**UCP2 modulates endothelial proliferation via Δψ-dependent changes in mitochondrial •O_2^-**

Because Δψ modulates mitochondrial •O_2^- and UCP2 can impact Δψ, we investigated mitochondrial •O_2^- as a function of cell proliferation. Both BAECs (Fig. 4A) and murine endothelial cells (Fig. 4B) exhibited an inverse relation between mitochondrial •O_2^- and cell proliferation rate and this response was accentuated in UCP2-null endothelium (Fig. 4B). Reconstitution of UCP2 into UCP2-null endothelium (Fig. 4C) normalized mitochondrial •O_2^- and forced overexpression of UCP2 in wild type cells attenuated mitochondrial •O_2^- (Fig. 4C). Treatment of either wild type or UCP2-null endothelium with mitochondrial SOD (SOD2) also reduced the mitochondrial •O_2^- flux (Fig. 4C). Finally, attenuation of mitochondrial •O_2^- in UCP2-null cells with either UCP2 or SOD2 (Fig. 4C) enhanced endothelial cell proliferation (Fig. 4D). These data indicate UCP2 modulates endothelial cell proliferation via mitochondrial •O_2^-.
If mitochondrial •O$_2^-$ explains the UCP2-null phenotype, then independent mitochondrial •O$_2^-$ manipulation should produce qualitatively similar effects. To this end, we found that SOD2$^+/-$ endothelium exhibited increased mitochondrial •O$_2^-$ (Fig. 4E) and impaired proliferation (Fig. 4F) that were both corrected by either UCP2 or SOD2 transfection (Figs. 4G and H). Forced overexpression of SOD2 in BAECs enhanced migration in the scratch assay (Fig. 4I), whereas SOD2 suppression inhibited migration and increased mitochondrial •O$_2^-$ (Fig. 4I). Capillary sprouting in aortic segments from SOD2$^+/-$ animals was impaired compared to wild-type animals (Fig. 4J). In the hindlimb ischemia model, we found less blood flow recovery in SOD2$^+/-$ mice than in wild-type mice that was rescued via adenoviral overexpression of UCP2 (Fig. 4K) in a manner that suppressed mitochondrial •O$_2^-$ (Fig. 4G) and improved proliferation (Fig. 4H) in the endothelium. Thus, excess mitochondrial •O$_2^-$ produces impaired endothelial cell proliferation, migration, and angiogenesis.

**UCP2 does not limit endothelial antioxidants or impact basal NO• bioactivity**

Since endothelial antioxidants and •O$_2^-$ can impact nitric oxide (NO•) bioactivity, we investigated both as a function of UCP2. Acute suppression of UCP2 had no material impact on BAEC enzymatic antioxidant levels (Online Figure IVA). Also, stimulated NO• bioactivity with acetylcholine was no different between wild-type and UCP2-null vessels (Online Figure IVB) and basal NO• bioactivity was actually greater in UCP2-null vessels than wild-type vessels (Figures. IVC and IVD). Thus, the impact of mitochondrial •O$_2^-$ on endothelial phenotype is not dependent upon reduced antioxidant levels or basal NO• bioactivity.

**Limiting ΔΨ is sufficient to explain the impact of UCP2**

To confirm that ΔΨ changes can account for the impact of UCP2 effects on endothelial phenotype, we force expressed UCP1, a protein not expressed in endothelium, to decrease endothelial ΔΨ. We found that forced expression of UCP1 decreased ΔΨ (Fig. 5A), increased proliferation (Fig. 5B), increased migration (Fig. 5C), and limited mitochondrial •O$_2^-$ (Fig. 5D). Thus, independently decreasing ΔΨ with UCP1 produced qualitatively similar changes in endothelial cell phenotype as ΔΨ manipulation with UCP2. These data suggest that the impact of UCP2 on endothelial phenotype is due, in part, to its effect on mitochondrial ΔΨ.

**UCP2 regulates cell cycle progression via mitochondrial •O$_2^-$**

Because NO• bioactivity was not impaired, we examined other mechanisms of impaired endothelial function. Proliferating UCP2-null endothelium exhibited more cells in the G1-phase cells than wild-type endothelium (Fig. 6A), suggesting impaired G1-S cell cycle transition. This part of the cell cycle is controlled, in part, by cyclin-dependent kinases and we found that UCP2-null cells exhibited higher expression of the cyclin-dependent kinase inhibitors such as p16$^{ink4a}$ and p21$^{cip/waf}$, than did wild-type cells (Fig. 6B and C). Since cell cycle inhibition can lead to senescence, we examined the senescence indicator, β-galactosidase and found a 4.5-fold increase in senescence in UCP2-null endothelium compared to wild-type cells (Figs. 6D and E). Transfection of UCP2-null cells with either UCP2 or SOD2 (Fig. 6E) attenuated senescence. We observed a similar senescence increase in SOD2$^+/-$ cells (Fig. 6E) and transfection of either UCP2 or SOD2 also inhibited the development of senescence (Fig. 6E). Since p53 is both sensitive to ROS and can control p16$^{ink4a}$ and p21$^{cip/waf}$ expression, we investigated the role of p53 in the UCP2-null phenotype. We found that p53 suppression by siRNA rescued the proliferation defect of UCP2-null endothelium (Fig. 6F). Moreover, we observed that proliferating and hypoxic UCP2-null endothelium exhibited p53 phosphorylation at serine 18 (Fig. 6G), a residue involved in redox-sensitive p53 activation. Thus, UCP2-null endothelium exhibits a dysfunctional phenotype manifest as premature senescence via excess mitochondrial •O$_2^-$ in a p53-dependent manner.
UCP2 regulates endothelial phenotype via superoxide-dependent control of mitochondrial fragmentation

Since mitochondria undergo dynamic changes in fusion and fission during the G1-S cell cycle transition, we examined endothelial mitochondrial morphology as a function of UCP2. Compared to wild-type endothelium, proliferating UCP2-null cells exhibited superoxide-dependent mitochondrial fragmentation that was rescued by transfection with either UCP2 or SOD2 (Figs. 7A and B). Similarly, SOD2 heterozygous cells exhibited greater mitochondrial fragmentation than wild-type cells that was largely rescued by transfection with SOD2 or UCP2 (Figs 7A and B). This fragmentation was linked to p53, as p53 RNAi attenuated fragmentation (Fig. 7C), whereas p21cip/waf forced overexpression recapitulated mitochondrial fragmentation (Fig. 7D). To gain insight into the mechanism of mitochondrial fragmentation, we examined gene expression important for normal mitochondrial morphology (Fig. 7E) and found that genes required for fusion of mitochondria, mitofusin 1 and 2 (Mfn1, Mfn2), and optic atrophy 1 (Opa1) were significantly lower than genes required for mitochondrial fission such as fission 1 (Fis1) and dynamin-related protein 1 (Drp1) – a pattern associated with mitochondrial fragmentation.

To determine if mitochondrial fragmentation was sufficient to cause the UCP2-null phenotype, we suppressed mitofusin 1 and 2 expression and found that induction of mitochondrial fragmentation significantly reduced endothelial cell proliferation (Fig. 7F). Taken together, these results indicate that endothelial UCP2 is necessary for maintaining mitochondrial morphology, and that disrupting the normal balance between mitochondrial fusion and fission has implications for endothelial function.

Discussion

The principal finding of this work is that endogenous UCP2 modulates endothelial mitochondrial network morphology that dictates, in part, endothelial cell function. Through UCP2, endothelial ΔΨ decreases with cell proliferation and, as endothelial growth slows, reduced UCP2 levels produce an increase in ΔΨ. We found these changes in ΔΨ moved in parallel with mitochondrial •O2- production that, if left uncontrolled, resulted in p53-dependent mitochondrial network fragmentation that limited endothelial functions important for angiogenesis, including proliferation, migration, and blood flow recovery from tissue ischemia. The central role of ΔΨ was supported by observations that ΔΨ manipulation with UCP1 could phenocopy the effect of UCP2. Moreover, the key role of mitochondrial •O2- was consistent with observations that SOD2+/− endothelium, with excess mitochondrial •O2-, demonstrated mitochondrial network fragmentation and impaired endothelial cell function. Collectively, these data indicate that endogenous UCP2 is an important modulator of endothelial cell function, in part, via its impact on mitochondrial network integrity.

The precise biologic functions of uncoupling proteins remain a matter of debate. UCP1, the predominant uncoupling protein in brown adipose tissue, mediates ΔΨ proton leak that is critical for adaptive thermogenesis. In contrast, UCP2 and UCP3 are not necessary for adaptive thermogenesis or normal energy metabolism. These latter two uncoupling proteins modulate ΔΨ to a lesser extent than UCP1, even in the presence of requisite coactivators such as fatty acids and •O2- derived alkenals. Multiple models of UCP2 or UCP3 overexpression suggest a protective role against oxidative stress and endothelial dysfunction from diet-induced obesity. Considering that endothelial cell proliferation can be associated with excess •O2- and oxidative stress, one could argue that our data identify UCP2 as a stress-responsive protein. However, UCP overexpression is prone to artifact from improper membrane insertion, casting doubt on the ultimate role of UCPs in limiting •O2- mediated toxicity. Our data strongly support a role of UCP2 to limit mitochondrial •O2- and deserve particular attention, as we focused...
upon endogenous UCP2 regulation. Moreover, we used loss-of-function models with rescue by UCP2 complementation that are less prone to artifact. Consistent with the idea that UCP2 limits mitochondrial •O$_2^-$, we found that independent manipulation of mitochondrial •O$_2^-$ (via SOD2) copied the UCP2-null phenotype, increasing confidence that endogenous UCP2 importantly regulates mitochondrial ROS.

One might expect that mitochondrial •O$_2^-$ would limit NO• bioactivity in our system to explain the UCP2-null phenotype. Rather, we found intact NO• bioactivity in unstressed UCP2-null endothelium, but the cells exhibited premature senescence and upregulation of p21cip/waf and p16ink4a. These two gene products have been linked, in part, to activation of the tumor suppressor, p53. Our observations that correction of excess mitochondrial •O$_2^-$ prevented senescence is consistent with data that ROS can upregulate p53 to induce both cell cycle arrest and genes that tend to lower cellular ROS levels. In this context, it is germane to note that RNAi-mediated suppression of prohibitin, an inner mitochondrial protein, also upregulates mitochondrial •O$_2^-$, producing premature senescence and impaired endothelial proliferation. Studies in other cell types indicate prohibitins are required for proliferation, prompting speculation that suppressing mitochondrial •O$_2^-$ (perhaps to prevent p53 activation) may be a general requirement of cellular proliferation, perhaps via prevention of premature senescence. In this regard, we need to interpret our data with caution as we have not determined whether senescence alone could explain all of our experimental findings.

The notion that UCP2 suppression of mitochondrial •O$_2^-$ may be important for cell proliferation is supported by our observation that UCP2 mRNA is most prominent in very proliferative cells and tissues such as bone marrow, lymphomas, leukemias, erythroid precursors, T-cells, and CD105 positive endothelial cells (Online Figure I). Moreover, UCP2 gene silencing prevented epidermal cell tumor induction in a p53-dependent manner. Thus, data from diverse cell types link UCP2 upregulation (and mitochondrial •O$_2^-$ suppression) to rapid cell proliferation, suggesting that UCP2 impacts some fundamental component of the proliferative response. Since UCP2 is a target gene for peroxisome proliferator gamma coactivator-1 (PGC-1), the principal determinant of mitochondrial biogenesis, UCP2 may be needed to expand mitochondrial mass during cell proliferation, perhaps by limiting mitochondrial ROS-mediated damage. Alternatively, UCP2 is known to promote oxygen-insensitive glycolysis (The Warburg Effect) and glycolysis is required for the high proliferation rate of many cancer cells. Thus, UCP2 may be required for glycolysis-dependent growth, a finding consistent with our observations of reduced lactate production in UCP2-null endothelium (Fig. 2).

We observed excess mitochondrial fragmentation with UCP2-null mice that was linked to mitochondrial •O$_2^-$, as it was recapitulated in the SOD2+/- mice and attenuated with maneuvers that reduce mitochondrial •O$_2^-$. These findings suggest a novel relation between mitochondrial redox state and the balance between mitochondrial fusion and fission. This relation is consistent with our observations that mitochondrial fragmentation in UCP2-null cells involved a p53- and p21cip/waf-dependent mechanism, and literature that p53 can be activated by ROS via p53 phosphorylation on serine 20 (mouse Ser18). This latter event is known to involve the ataxia-telangiectasia mutated (ATM) kinase. Thus, one potential explanation for our data would be ATM kinase activation by mitochondrial •O$_2^-$. This contention is supported by observations that ATM and its p53 target (Ser18/20) have important implications for metabolism.

The findings presented here suggest a role for UCP2 in cell proliferation. With the increased metabolic flux that occurs as a result of growth stimulation, it is not surprising that mitochondrial Δψ and •O$_2^-$ would increase. The fact that forced expression of UCP1 limited
Δψ and recapitulated the effect of UCP2 suggests that UCP2 upregulation is an important mechanism for limiting Δψ and the resultant mitochondrial •O$_2^-$ flux that, if left unchecked, would activate pathways to limit cell proliferation. With regards to the endothelium, this paradigm has important therapeutic implications. For example, our findings suggest that targeting UCP2 may prove to be an important means of limiting tumor angiogenesis. Conversely, promotion of mitochondrial uncoupling could enhance the angiogenic response to ischemia and this strategy could prove fruitful in the setting of occlusive vascular diseases. Thus, our data highlight the importance of mitochondrial uncoupling and its dynamic regulation in the endothelial cell proliferative response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

| Δψ | Mitochondrial membrane potential |
| AIF | Apoptosis inducing factor |
| AMP kinase | Adenosine monophosphate activated protein kinase |
| ATM | Ataxia telangectasia-mutated |
| BAEC | Bovine aortic endothelial cell |
| COX | Cytochrome c oxidase |
| DMEM | Dulbecco's modified Eagle medium |
| DRP1 | Dynamin-related protein-1 |
| ErbB4 | Epidermal growth factor receptor tyrosine kinase B4 |
| FCCP | Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone |
| Fis1 | Fission-1 |

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<th>Abbreviation</th>
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<tr>
<td>HAEC</td>
<td>Human aortic endothelial cell</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>UCP2</td>
<td>Uncoupling protein 2</td>
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<tr>
<td>MLEC</td>
<td>Mouse lung endothelial cell</td>
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<tr>
<td>SMAC/DIABLO</td>
<td>Second mitochondrial activator of caspase/DIABLO gene product</td>
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<tr>
<td>JNK</td>
<td>C-Jun, N-terminal Kinase</td>
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<td>Mitofusin-1</td>
<td>Mfn1</td>
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<tr>
<td>Mitofusin-2</td>
<td>Mfn2</td>
</tr>
<tr>
<td>Opa1</td>
<td>Optic atrophy-1</td>
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<tr>
<td>P38 MAPK</td>
<td>P38 Mitogen-activated kinase</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SA-βgal</td>
<td>Senescence-associated beta glactosidase</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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Novelty and Significance

What Is Known?

- Mitochondria are known to participate in a number of cellular functions beyond that of energy production.
- The main driving force for many mitochondrial functions is the electrochemical gradient (Δψ) produced by the mitochondrial electron transport chain.
- The regulation of Δψ involves both the electron transport chain and uncoupling proteins that facilitate protein leak across the inner mitochondrial membrane.

What New Information Does This Article Contribute?

- Mitochondrial Δψ is dynamically regulated in endothelial cells by uncoupling protein-2 (UCP2) in response to external stimuli such signals to promote proliferation.
- The decrease in endothelial cell Δψ by UCP2 upregulation during proliferation limits mitochondrial superoxide generation.
- In the absence of UCP2, excess mitochondrial superoxide leads to p53-dependent mitochondrial fragmentation that is necessary and sufficient to impair endothelial cell proliferation and angiogenesis.

Mitochondria have long been known to be critical for cellular ATP production. More recently, mitochondrial have been implicated in other cellular processes, but their role in the endothelium is largely unknown. Thus, we sought to probe the role of mitochondria in endothelial proliferative responses. We found that endothelial cell proliferation was associated with a reduced Δψ due to upregulation of UCP2, a protein known to regulate Δψ. In the absence of UCP2, proliferating endothelial cells had higher Δψ and excess mitochondrial superoxide that prevented their proliferation, migration, and participation in angiogenesis. Similarly, endothelium lacking mitochondrial SOD had excess mitochondrial superoxide and mimicked the UCP2-null phenotype. This excess mitochondrial superoxide lead to p53-dependent mitochondrial fragmentation and endothelial cell senescence. Correction of excess mitochondrial superoxide corrected both the mitochondrial fragmentation and endothelial dysfunction. These data indicate that endothelial UCP2 is important to maintain both normal mitochondrial dynamics and endothelial function.
**Figure 1. Regulation of endothelial UCP2 and Δψ with proliferation**

BAECs were (A) stimulated with FBS or (B) cultured to specific confluence before assessment of Δψ by JC-1 fluorescence and expression of UCP2, UCP3, cytochrome c oxidase IV (COX IV), or actin by immunoblot. 5 μM Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a control for the lower limit of Δψ. Data represent n = 3; *P < 0.05 for trend from one-way ANOVA. (C) Mitochondrial Δψ by tetramethylrhodamine ethyl ester (TMRE) corrected for mitochondrial mass. N=3; *P < 0.05 for trend from one-way ANOVA (D) Expression of UCP2 and UCP3 mRNA and protein in BAECs. (E) Densitometric analysis of UCP2 protein by immunoblot as a function of confluence. N=5; *P < 0.05 vs. 50% by ANOVA with Tukey-Kramer post-hoc test. (F) BAECs (CTL) were transfected with UCP2 (Ad-UCP2 at the indicated MOI), β-galactosidase (Ad-LacZ; 100 MOI), or the indicated siRNA followed by assessment for Δψ or protein levels of transfected or endogenous UCP2, cytochrome c oxidase IV (COX IV), or actin by immunoblotting. N = 3. *P < 0.05 vs. LacZ adenovirus or siCTL. (G) Mitochondrial Δψ by TMRE as in (C). N=3; *P < 0.05 by unpaired t-test.
Figure 2. Metabolic signature of UCP2-null endothelium
(A) Oxygen consumption was determined in wild-type and UCP2-null MLECs in the presence or absence of oligomycin (oligo) or carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) using a Clark electrode as described in “Methods.” (B) Media from wild-type or UCP2-null MLECs in 0.1% serum was analyzed for lactate content and expressed as a function of cell count. N=3; *P<0.01 vs. WT by unpaired t-test. (C) BAEC ATP levels as a function of UCP2 status as described in “Methods.” N=3; *P<0.01 vs. siCTL by unpaired t-test. (D) BAEC ATP levels as a function of confluence or oligomycin treatment. N=4; *P<0.01 for trend by one-way ANOVA.
Figure 3. UCP2 modulates endothelial cell proliferation and migration
(A) Wild-type MLECs were treated with AICAR (0.5 mM) or VEGF (25 ng/mL) as indicated for 24h followed by immunoblots for the indicated proteins. Actin, cytochrome c (Cyt c) and cytochrome c oxidase subunit IV (COX IV) were loading controls for cytosol and mitochondria. (B) BAEC proliferation by [3H]-thymidine incorporation in response to VEGF or UCP2 manipulation as indicated. Data are normalized to the VEGF vehicle control. N=4-5; *P < 0.05 vs. respective controls by Mann-Whitney U test. (C) BAECs
underwent UCP2 manipulation as indicated and migration assessed 20h after scratch N=3-5; *P < 0.01 vs. respective control by Mann-Whitney U test. (D) Proliferation of wild-type (WT) and UCP2-/- MLECs by cell count. N=4; *P < 0.05 vs. WT by two-way repeated measures ANOVA. (E) Migration of WT and UCP2-/- MLECs after scratch wounding. N=5; *P < 0.01 vs. WT by Mann-Whitney U test. (F) Aortic segments from the indicated genotypes implanted in collagen gel after 7d (bar = 500 um) with capillary sprout counts as a function of time. N=6; *P < 0.001 vs. WT by two-way repeated-measures ANOVA. (G) Capillary sprouting in UCP2-/- aortic segments in collagen gel containing no additions (CTL) or the indicated adenoviral vector. N=6/group; *P < 0.01 vs. vehicle or Ad-LacZ by two-way repeated measures ANOVA. (H) Blood flow recovery in wild-type (WT) and UCP2-/- mice with unilateral femoral artery excision expressed as a fraction ratio of the ischemic (I) vs. non-ischemic (N) limbs with representative images of laser-Doppler tissue perfusion in hindlimbs. N=4; *P = 0.02 vs. WT by two-way repeated measures ANOVA.
Figure 4. UCP2 dictates endothelial phenotype via mitochondrial $\cdot O_2^-$
(A) BAECs stained with mitochondrial-targeted hydroethidine at the indicated level of confluence to assess mitochondrial $\cdot O_2^-$ (bar = 50 μm). (B) Mitochondrial $\cdot O_2^-$ in MLECs from the indicated genotype as a function of confluence. N=4; *P < 0.01 for WT vs. UCP2-/- by two-way factorial ANOVA. (C) MLEC mitochondrial $\cdot O_2^-$ in the indicated genotypes transfected with UCP2 (Ad-UCP2), SOD2 (Ad-SOD2), or LacZ. N=3; *P < 0.05 vs. UCP2-/- Ad-LacZ; †P < 0.05 vs. wild-type Ad-LacZ. (D) Proliferation of UCP2-null MLECs transfected as in (C) with data normalized to CTL. N=3; *P < 0.05 vs. Ad-LacZ by Kruskal-Wallis ANOVA. (E) Mitochondrial $\cdot O_2^-$ in wild-type vs. SOD2+/- endothelium.
N=4 – 8; *P<0.05 vs. WT by Mann-Whitney U test. (F) Proliferation of wild-type and SOD2 +/- MLEC as a function of time. N=4; *P < 0.05 for trend by two-way repeated measures ANOVA. Mitochondrial •O_2^- (G) and proliferation (H) in SOD2 +/- MLEC as a function of transfection with control (LacZ), UCP2, or SOD2 adenovirus. N=3; *P < 0.05 vs. LacZ by Kruskal-Wallis ANOVA. (I) Left, Migration of BAECs with manipulated SOD2 levels via the indicated adenovirus or siRNA, N=6; *P = 0.01 vs. respective controls by Mann-Whitney U test. Right, BAEC Mitochondrial •O_2^- as a function of treatment with CTL or SOD2 siRNA. N=3; *P < 0.01 vs. control siRNA by Student’s t-test. (J) Capillary sprouting in aortic segments from the indicated genotypes. N=6; *P < 0.001 vs. WT by two-way repeated measures ANOVA. (K) Blood flow recovery in WT and SOD2 +/- mice after unilateral hindlimb ischemia with our without hindlimb transfection with UCP2 or LacZ adenovirus. N=5/group; †P < 0.01 vs. WT and *P < 0.01 vs. Ad-LacZ by two-way repeated measures ANOVA.
Figure 5. Independent manipulation of $\Delta \psi$ impacts endothelial phenotype

BAECs were transfected with irrelevant (Ad-LacZ) or UCP1 adenovirus (ad-UCP1) and examined for (A) $\Delta \psi$, (B) proliferation, (C) migration, and (D) mitochondrial $\text{•O}_2^-$ as described in “Methods.” N=4 – 6; *$P<0.05$ vs. Ad-LacZ by unpaired t-test.
Figure 6. UCP2 and endothelial senescence

(A) Cell cycle analysis in WT and UCP2-null MLECs at the indicated time. (B) Expression of WT and UCP2-null mRNA for p16^{ink4a} and p21^{cip/waf}. N=3; *P<0.05 vs. WT by Mann-Whitney U-test. (C) Expression of WT and UCP2-null endothelial p16^{ink4a} and p21^{cip/waf} by immunoblot. (D) WT and UCP2-null senescence-associated β-galactosidase (SA-βgal) via x-gal staining. Bar = 750 μm. (E) Endothelial SA-βgal staining as a function of genotype and transfection with either UCP2 or SOD2. N = 3-6; *P < 0.05 vs. WT + Ad-GFP, †P < 0.05 vs. UCP2-/- + Ad-GFP, and ‡P < 0.05 vs. SOD2+/+ + Ad-GFP by Kruskal-Wallis ANOVA and post hoc comparison. (F) Cell proliferation in WT and UCP2-null MLECs treated with none, control, or p53 siRNA. N=4; *P < 0.05 vs. WT with control siRNA; **P < 0.01 vs. WT with no treatment; †P < 0.01 vs. WT with control siRNA; and ‡P < 0.01 vs. UCP2-/- with control siRNA by one-way ANOVA with Tukey-Kramer test. (G) MLECs of the indicated genotype under normoxic or hypoxic (1% O₂) conditions for 16h were lysed and probed for the indicated proteins by immunoblot.
Figure 7. Mitochondrial morphology and endothelial phenotype
(A) Representative images of MLEC mitochondrial morphology by Mitotracker green staining as a function of the indicated genotype and/or transfection with the indicated adenovirus (Ad). (B) Composite data indicating average mitochondrial length as an index of fragmentation in MLECs from the indicated genotype and/or transfected with the indicated adenovirus. N=13 – 16; *P < 0.01 vs. respective wild-type or LacZ control by Kruskal-Wallis ANOVA. (C) Mitochondrial length as an index of fragmentation in the indicated genotypes with and without suppression of p53. N=12 – 16; *P < 0.05 vs. respective control.
(D) MLEC mitochondrial morphology and average length with forced expression of p21cip/waf (bar = 5 μm; N=9 – 13; *P < 0.05 vs. LacZ by Kruskal-Wallis ANOVA. (E) MLEC expression of mitochondrial genes as a function of the indicated genotype. N=3 – 4; *P < 0.05, **P < 0.01 vs. WT by Mann-Whitney U-test. (F) WT MLEC proliferation with suppression of mitofusin 2 (Mfn2) or both mitofusin 1 (Mfn1) and Mfn2. N=10 each; *P < 0.05 vs. CTL siRNA by two-way repeated measures ANOVA.