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

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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. (Circ Res. 2013;113:891-901.)

Key Words: angiogenesis ■ endothelium ■ endothelial function ■ ischemia ■ mitochondria ■ superoxides ■ mitochondrial uncoupling proteins

The cellular requirement for ATP as a high-energy intermediate to fuel many critical cellular reactions is universal. The production of ATP depends on 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 (ie, membrane potential, Δψ) 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.

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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 and the second mitochondrial activator of caspase/DIABLO gene product, 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 epidermal growth factor receptor tyrosine kinase B4, can release intracellular domains that trigger mitochondrial release of proapoptotic proteins. Energy deprivation responses, such as AMP kinase activation, induce mitochondrial gene upregulation that is critical for cellular stress adaptation. Upregulation of endothelial vascular endothelial growth factor involves perinuclear mitochondrial clustering and the local production of mitochondrial •O₂-. The latter has also been linked to the control of mitogen-activated protein kinase phosphatases and the coordination of nuclear factor κB-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 (nicotinamide adenine dinucleotide and flavin adenine dinucleotide-2), respiration (O$_2$), and ATP synthesis (ADP).1 As well as any proton leak.18 The latter is largely regulated via uncoupling proteins that belong to the mitochondrial carrier superfamily.19 Herein, we report that endothelial cell proliferation and angiogenesis involve upregulation of mitochondrial uncoupling protein 2 (UCP2) to reduce $\Delta \psi$ and limit mitochondrial •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 endothelial basal medium 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 (endothelial basal medium supplemented with 0.1% or 0.4% fetal bovine serum). Human aortic endothelial cells were from Lonza and were cultured as described.14 Murine lung endothelial cells (MLECs) from mice of both sexes were isolated as described,19 and cultured on gelatin- or collagen-coated plates and grown in MLEC medium containing 20% fetal bovine serum, 38% Dulbecco’s modified eagle medium, 38% Ham’s F-12 with 100-μg/mL endothelial cell growth supplement (ECGS, Biomedical Technologies, Stoughton, MA), 4-mmol/L L-glutamine, 100-μL-glutamine, 100-μg/mL heparin, and penicillin/streptomycin.

**Quantitative Real-Time Polymerase Chain Reaction**

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 polymerase chain reaction was performed in the iQ5 real-time polymerase chain reaction 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 to 100 multiplicity of infection as described.18,20 For gene silencing, BAECs were transfected with 1.3 μg of small interfering RNA oligonucleotides (Thermo Scientific Dharmacon, Lafayette, CO) against human UCP2 (Cat. Nos. D-005114-01, -02, -03, and -04); human superoxide dismutase 2 (SOD2; D-009784-03, -04, -19, and -20); or negative control (D-002100-02, -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 nontargeting 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 ($\Delta \psi$) was determined using 2 complementary fluorescent methods. Cells were incubated with 1-µmol/L 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol-carboyanine iodide (JC-1) for 30 minutes and $\Delta \psi$ estimated as the fluorescence ratio of JC-1 aggregates (red, excitation 550 nm, emission 585 nm) to monomers (green, excitation 485 nm, emission 535 nm) formed as a function of inner mitochondrial membrane potential.21 For measurement of mitochondrial superoxide ($\cdot$O$_2^-$), cells were loaded with 5-µmol/L MitoSOX Red (Invitrogen) for 10 minutes and the fluorescence (excitation 590 nm; emission 510 nm) was determined. Live cells were labeled with L-0.5-µmol/L MitoSOX for 20 minutes and the fluorescence images at both 405- and 514-nm excitation were captured using an Eclipse TE2000U 2-fluorescence microscope (Nikon, Melville, NY) with a CCD camera using a SPOT Insight 2MP Firewire Color Mosaic (Diagnostic Instruments, Sterling Heights, MI).

**Antibodies and Immunoblotting**

Antibodies against cytochrome c oxidase subunit IV, p21 (upstate, p16601,6), phospho-p53 (mouse Ser18), phospho-c-Jun (Ser63), phospho p38 mitogen-activated protein kinase (Thr180/Tyr182), and -rabo- 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 performed and quantified as described.22 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.23,24 Briefly, 1×10^6 cells were resuspended in 1 mL of respiration medium (Dulbecco’s phosphate-buffered saline, 2 mmol/L glucose, 1 mmol/L pyruvate, 2% fatty acid-free bovine serum albumin). To obtain proton leak, oligomycin was added to a final concentration of 2 µmol/L. To measure maximal respiration, carbonyl cyanide 4-(trifluoromethoxy)
phenylhydrazine was added to a final concentration of 2.4 μmol/L. Nonmitochondrial respiration, obtained by adding myxothiazol to a final concentration of 4 μmol/L, 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 endothelial basal medium-2 as described,28 periaortia 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 1 hour to polymerize the collagen, and the solid gel covered with MLEC medium diluted 1:2 with Dulbecco’s modified eagle medium. 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 described26 and CD31 staining. To infect aortic rings with adenoviral vectors, each ring was embedded in liquid collagen gel containing respective vectors (adenovirus–UCP2, adenovirus–SOD2, etc) at 1.0×108 pfu before polymerized. Transfection was validated in the left leg was introduced in the mice as described.28 In selected ex-

Animals and Hindlimb Ischemia Model
Heterozygous UCP2-null animals26 on the C57 background were obtained from Dr Bradford Lowell (Harvard Medical School) and bred to homogeneity with age-matched controls. SOD2 heterozygous mice were anesthetized with intraperitoneal injection of combination of 100 mg/kg ketamine hydrochloride and 5 mg/kg xylazine (Webster Veterinary, Devens, MA) before surgery. Unilateral hindlimb ischemia was conducted between 2 groups by use of Student t test or Mann–Whitney U test as appropriate. Multiple groups were compared with either 1-way Kruskal–Wallis or ANOVA with a post hoc Tukey–Kramer multiple comparisons test as indicated in legends. A P value <0.05 was considered significant. All statistics were done using StatView version 5.0 (SAS Institute, Cary, NC) or GraphPad Prism version 5 (GraphPad Software, La Jolla, CA).

Results
Dynamic Modulation of UCP2 and Δψ With Endothelial Proliferation
Quiescent BAEC monolayers stimulated to proliferate with fetal bovine serum exhibited a reduction in Δψ (Figure 1A), whereas increasing cell confluence was associated with an increase in Δψ (Figure 1B and 1C). Because mitochondrial UCPs are implicated in Δψ regulation,26 we probed endothelial UCP expression and observed mRNA and protein only for UCP2 and UCP3 (Figure 1D). We then observed that UCP2 is upregulated with endothelial proliferation (Figure 1A) and downregulated with increasing confluence, with no dynamic regulation of UCP3 (Figure 1B and 1E). We could recapitulate the proliferation-induced changes in Δψ by molecular manipulation of UCP2 (Figure 1F) and UCP2-null cells had increased Δψ (Figure 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
Because 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 2-tailed t test; Figure 2A). Proliferating UCP2-null cells also produced lesser lactate than wild-type cells (Figure 2B), consistent with a lower rate of glycolysis,32 Suppression of UCP2 was associated with reduced ATP levels (Figure 2C) and endothelial ATP levels seemed greatest in endothelium with the highest proliferation rate (Figure 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
In contrast, UCP2 mRNA expression varied more than 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 on this latter association, we tested known stimuli for angiogenesis such as vascular endothelial growth factor and AMP kinase and found UCP2 upregulation (Figure 3A).

Serum- and vascular endothelial growth factor–mediated UCP2 upregulation was associated with c-Jun N-terminal kinase (JNK) phosphorylation (Figures 1C and 2A). Additionally, UCP2 mRNA and protein levels were lower in UCP2-null bovine aortic endothelial cells (BAECs) as compared to wild-type BAECs under similar conditions (Figures 1A and 2A). This was associated with increased mitochondrial membrane potential (Δψ; Figure 1C) and reduced oxygen consumption (Figure 2A).

Figure 1. Regulation of endothelial uncoupling protein 2 (UCP2) and Δψ with proliferation. Bovine aortic endothelial cells (BAECs) were (A) stimulated with fetal bovine serum (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 immunoblotting. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) of 5-μmol/L was used as a control for the lower limit of Δψ. Data represent n=3; *P<0.05 for trend from 1-way ANOVA. C, Mitochondrial Δψ by tetramethylrhodamine ethyl ester (TMRE) corrected for mitochondrial mass. n=3; *P<0.05 for trend from 1-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 (control [CTL]) were transfected with UCP2 (Ad-UCP2 at the indicated multiplicity of infection [MOI]), β-galactosidase (Ad-LacZ; 100 MOI), or the indicated small interfering RNA (siRNA) followed by assessment for Δψ or protein levels of transfected or endogenous UCP2, COX IV, or actin by immunoblotting. n=3; *P<0.05 vs Ad-LacZ or siRNA control (siCTL). G, Mitochondrial Δψ by TMRE as in C. n=3; *P<0.05 by unpaired t test. Ad indicates adenovirus; GADPH indicates glyceraldehyde 3-phosphate dehydrogenase; siUCP2, small interfering UCP2; and WT, wild-type.

Figure 2. Metabolic signature of uncoupling protein 2 (UCP2)-null endothelium. A, Oxygen consumption was determined in wild-type (WT) and UCP2-null murine lung endothelial cells (MLECs) in the presence or absence of oligomycin (oligo) or carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) using a Clark electrode as described in Methods section. 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, Bovine aortic endothelial cell (BAEC) ATP levels as a function of UCP2 status as described in Methods section. n=3; *P<0.01 vs siRNA control (siCTL) by unpaired t test. D, BAEC ATP levels as a function of confluence or oligomycin (oligo) treatment. n=4; *P<0.01 for trend by 1-way ANOVA. siUCP2 indicates small interfering UCP2.
kinase activation and inhibition attenuated both UCP2 up-regulation and vascular endothelial growth factor–mediated endothelial cell proliferation (Online Figure II). We also manipulated endothelial UCP2 levels and found that endothelial cell proliferation (Figure 3B) and migration (Figure 3C) were directly related to UCP2. Consistent with these observations, UCP2-null endothelium exhibited impaired proliferation (Figure 3A) and migration (Figure 3D), without any compensatory upregulation of UCP3 (Online Figure IIIA).

Because 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 this response was accentuated in UCP2-null endothelium (Figure 3E). BAECs (Figure 3F) and murine endothelial cells (Figure 3G) showed that UCP2-null endothelium rescued the defect in capillary sprout formation (Figure 3H). Similarly, capillary sprout formation from UCP2-null adipose tissue was impaired relative to wild-type, and UCP2 reintroduction also rescued this defect (Online Figure IIIB and IIIC). To determine the physiological 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 with wild type controls (Figure 3H) that was largely rescued by adenoviral-mediated restoration of UCP2 expression (Online Figure IID).

**UCP2 Modulates Endothelial Proliferation Via Δψ-Dependent Changes in Mitochondrial •O₂⁻**

Because Δψ modulates mitochondrial •O₂⁻ and UCP2 can impact Δψ,¹⁸ we investigated mitochondrial •O₂⁻ as a function of cell proliferation. Both BAECs (Figure 4A) and murine endothelial cells (Figure 4B) exhibited an inverse relation between mitochondrial •O₂⁻ and cell proliferation rate and this response was accentuated in UCP2-null endothelium (Figure 4B). Reconstitution of UCP2 into UCP2-null endothelium (Figure 4C) normalized mitochondrial •O₂⁻ and forced overexpression of UCP2 in wild-type cells attenuated mitochondrial •O₂⁻ (Figure 4C). Treatment of either wild-type
or UCP2-null endothelium with mitochondrial SOD (SOD2) also reduced the mitochondrial $\cdot O_2^-$ flux (Figure 4C). Finally, attenuation of mitochondrial $\cdot O_2^-$ in UCP2-null cells with either UCP2 or SOD2 (Figure 4C) enhanced endothelial cell proliferation (Figure 4D). These data indicate UCP2 modulates endothelial cell proliferation via mitochondrial $\cdot O_2^-$. If mitochondrial $\cdot O_2^-$ explains the UCP2-null phenotype, then independent mitochondrial $\cdot O_2^-$ manipulation should produce qualitatively similar effects. To this end, we found that SOD2-/- endothelium exhibited increased mitochondrial $\cdot O_2^-$ (Figure 4E) and impaired proliferation (Figure 4F) that were both corrected by either UCP2 or SOD2 transfection (Figure 4G and 4H). Forced overexpression of SOD2 in BAECs enhanced migration in the scratch assay (Figure 4I), whereas SOD2 suppression inhibited migration and increased mitochondrial $\cdot O_2^-$ (Figure 4I). Capillary sprouting in aortic segments from SOD2-/- animals was impaired compared with wild-type animals (Figure 4J). In the hindlimb ischemia model, we found less blood flow recovery in SOD2-/- mice after unilateral hindlimb ischemia with or without hindlimb transfection with UCP2 or LacZ adenovirus. Ad indicates adenovirus; and SOD2, superoxide dismutase 2.
UCP2 Regulates Cell Cycle Progression via Mitochondrial \( \Delta \psi \)

Because endothelial antioxidants and \( \cdot \mathrm{O}_2^- \) can impact nitric oxide (NO•) bioactivity,\(^{34}\) we investigated both as a function of UCP2. Acute suppression of UCP2 had no material impact on BAEC enzymatic antioxidant levels (Online Figure IVA). In addition, stimulated NO• bioactivity with acetylcholine was not 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 (Online Figure IVC and IVD). Thus, the impact of mitochondrial \( \cdot \mathrm{O}_2^- \) on endothelial phenotype is not dependent on reduced antioxidant levels or basal NO• bioactivity.

Limiting \( \Delta \psi \) Is Sufficient to Explain the Impact of UCP2

To confirm that \( \Delta \psi \) 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 \( \Delta \psi \). We found that forced expression of UCP1 decreased \( \Delta \psi \) (Figure 5A), increased proliferation (Figure 5B), increased migration (Figure 5C), and limited mitochondrial \( \cdot \mathrm{O}_2^- \) (Figure 5D). Thus, independently decreasing \( \Delta \psi \) with UCP1 produced qualitatively similar changes in endothelial cell phenotype as \( \Delta \psi \) manipulation with UCP2. These data suggest that the impact of UCP2 on endothelial phenotype is because, in part, of its effect on mitochondrial \( \Delta \psi \).

UCP2 Regulates Cell Cycle Progression via Mitochondrial \( \cdot \mathrm{O}_2^- \)

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 (Figure 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\(^{ink}}\) and p21\(^{cip/waf} \), than did wild-type cells (Figure 6B and 6C). Because cell cycle inhibition can lead to senescence, we examined the senescence indicator, \( \beta \)-galactosidase, and found a 4.5-fold increase in senescence in UCP2-null endothelium compared with wild-type cells (Figure 6D and 6E). Transfection of UCP2-null cells with either UCP2 or SOD2 (Figure 6E) attenuated senescence. We observed a similar senescence increase in SOD2\(^{+/−} \) cells (Figure 6E) and transfection of either UCP2 or SOD2 also inhibited the development of senescence (Figure 6E). Because \( \mathrm{p}53 \) is both sensitive to reactive oxygen species (ROS)\(^{29} \) and can control p16\(^{ink} \) and p21\(^{cip/waf} \) expression, we investigated the role of p53 in the UCP2-null phenotype. We found that \( \mathrm{p}53 \) suppression by small interfering RNA rescued the proliferation defect of UCP2-null endothelium (Figure 6F). Moreover, we observed that proliferating and hypoxic UCP2-null endothelium exhibited \( \mathrm{p}53 \) phosphorylation at serine 18 (Figure 6G), a residue involved in redox-sensitive \( \mathrm{p}53 \) activation.\(^{29} \) Thus, UCP2-null endothelium exhibits a dysfunctional phenotype manifest as premature senescence via excess mitochondrial \( \cdot \mathrm{O}_2^- \) in a \( \mathrm{p}53 \)-dependent manner.

UCP2 Regulates Endothelial Phenotype via Superoxide-Dependent Control of Mitochondrial Fragmentation

Because mitochondria undergo dynamic changes in fusion and fission during the G1-S cell cycle transition,\(^{35} \) we examined endothelial mitochondrial morphology as a function of UCP2. Compared with wild-type endothelium, proliferating UCP2-null cells exhibited superoxide-dependent mitochondrial fragmentation that was rescued by transfection with either UCP2 or SOD2 (Figure 7A and 7B). Similarly, SOD2 heterozygous cells exhibited greater mitochondrial fragmentation than wild-type cells that was largely rescued by transfection with SOD2 or UCP2 (Figure 7A and 7B). This fragmentation was linked to \( \mathrm{p}53 \), because \( \mathrm{p}53 \) RNA interference attenuated fragmentation (Figure 7C), whereas \( p21^{cip/waf} \) forced overexpression recapitulated mitochondrial fragmentation (Figure 7D). To gain insight into the mechanism of mitochondrial fragmentation, we examined gene expression important for normal mitochondrial morphology (Figure 7E) and found that genes required for fusion of mitochondria, such as 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.\(^{36} \) To determine whether 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 (Figure 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 •O$_2^-$ 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 •O$_2^-$ was consistent with observations that SOD2-/- endothelium, with excess mitochondrial •O$_2^-$, 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 biological 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.31 In contrast, UCP238 and UCP339 can mediate proton leak, but are not necessary for adaptive thermogenesis or normal energy metabolism.18,40 These latter 2 uncoupling proteins modulate Δψ to a lesser extent than UCP1,18 even in the presence of requisite coactivators, such as fatty acids and •O$_2^-$-derived alkenals.41,42 Multiple models of UCP2 or UCP3 overexpression suggest a protective role against oxidative stress38,43,44 and endothelial dysfunction from diet-induced obesity.45 Considering that endothelial cell proliferation can be associated with excess •O$_2^-$ and oxidative stress,46 one could argue that our data identify UCP2 as a stress-responsive protein. However, UCP overexpression is prone to artifact from improper membrane insertion,40 casting doubt on the ultimate role of UCPs in limiting •O$_2^-$-mediated toxicity. Our data strongly support a function of genotype and transfection with either UCP2 or SOD2.

Our data strongly support a role of UCP2 to limit mitochondrial •O$_2^-$, and 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.34 Rather, we found intact NO• bioactivity in unstressed UCP2-null endothelium, but the cells exhibited premature senescence and upregulation of p21<sub>WAF1/CIP1</sub> and p16<sub>INK4A</sub>. These 2 gene products have been linked, in part, to activation of the tumor suppressor, p53.47 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 arrest38 and genes that tend to lower cellular ROS levels.49,50 In this context, it is germane to...
note that RNA interference–mediated suppression of prohibitin, an inner mitochondrial protein, also upregulates mitochondrial \( \cdot \text{O}_2^\cdot \), producing premature senescence and impaired endothelial proliferation. Studies in other cell types indicate prohibitins are required for proliferation, prompting speculation that suppressing mitochondrial \( \cdot \text{O}_2^\cdot \) (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 \( \cdot \text{O}_2^\cdot \) 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+ 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 \( \cdot \text{O}_2^\cdot \) suppression) to rapid cell proliferation, suggesting that UCP2 impacts some fundamental component of the proliferative response. Because UCP2 is a target gene for peroxisome proliferator \( \gamma \) coactivator-1\( \alpha \), 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 (Figure 2).

We observed excess mitochondrial fragmentation with UCP2-null mice that was linked to mitochondrial \( \cdot \text{O}_2^\cdot \), because it was recapitulated in the SOD2\( ^{1-} \) mice and attenuated with maneuvers that reduce mitochondrial \( \cdot \text{O}_2^\cdot \). 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 p21<sup>cip1/waf1</sup>- dependent mechanism, and literature that p53 can be activated by ROS via p53 phosphorylation on serine 20 (mouse Ser18).<sup>29</sup>

This latter event is known to involve the ataxia-telangiectasia–mutated kinase. Thus, one potential explanation for our data would be ataxia-telangiectasia–mutated kinase activation by mitochondrial •O<sub>2</sub><sup>-</sup>. This contention is supported by observations that ataxia-telangiectasia–mutated and its p53 target (Ser18/20) have important implications for metabolism.<sup>56,57</sup>

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<sub>2</sub><sup>-</sup> 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<sub>2</sub><sup>-</sup> 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 helpful 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.

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Disclosures

None.

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30. Song W, Bossy B, Martin OJ, Hicks A, Lubitz S, Knott AB, Bossy-Wetzel E. Assessing mitochondrial morphology and dynamics using...
In the absence of UCP2, excess mitochondrial superoxide leads to endothelial cell senescence. Correction of excess mitochondrial superoxide limits endothelial cell senescence. These data indicate that endothelial UCP2 is important to maintain both normal mitochondrial dynamics and endothelial function.

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 ΔΨm because of upregulation of UCP2, a protein known to regulate ΔΨm. In the absence of UCP2, proliferating endothelial cells had higher ΔΨm and excess mitochondrial superoxide that prevented their proliferation, migration, and participation in angiogenesis. Similarly, endothelium lacking mitochondrial superoxide dismutase had excess mitochondrial superoxide and mimicked the UCP2-null phenotype. This excess mitochondrial superoxide led 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.

What New Information Does This Article Contribute?

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

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Supplemental Material

Supplemental Experimental Procedures

*Bioinformatics* – The gene expression profiles of human UCP1, 2, and 3 in various tissues were examined using the BioGPS database from microarrays (Genomics Institute of the Novartis Research Foundation; San Diego, CA).

*Capillary Sprouting Assay in Adipose Tissues* – Freshly harvested epididymal fat pads were placed in Krebs-Ringer solution buffered with HEPES (KRH) as described,¹ digested for 30 min at 37°C in KRH, pH 7.4, containing 1 mg/ml collagenase type I (Worthington Biochemical; Lakewood, NJ) and 2.5% BSA Fraction V (Sigma-Aldrich). Digested tissue was then filtered through a 100 µm cell strainer (Fisher Scientific BD Falcon; Pittsburgh, PA) and the captured stromal vascular fraction was cut into small (1 mm³) pieces that were 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 adipose tissue was examined daily and capillary sprouts counted along the sample perimeter under 100 and 200 x magnification. Vascular sprouts were distinguished from fibroblasts via morphology as described¹,² and CD31 staining.

*Vascular Ring Relaxation* – To study NO• bioactivity, mice aged 8-12 weeks were euthanized and thoracic aortae were isolated, cleaned of connective tissue, and cut into 2 mm segments. The vessel segments were mounted on stainless-steel holders in organ baths (Danish Myo Technology, Denmark) containing physiological saline solution (PSS; 119 mM NaCl, 4.69 mM KCl, 1.17 mM MgSO₄, 1.18 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, 0.03 mM EDTA, 5.5
mM Glucose) and aerated with 95% O$_2$ / 5% CO$_2$ for isometric force recording. Preparations were allowed to equilibrate for 30 min under constant passive force of (~1 mN) and synchronized with KCl and phenylephrine. Basal NO• bioactivity was estimated as the difference in contraction to phenylephrine with or without 300 µM L-NAME to inhibit endothelial nitric oxide synthase. Evoked NO• bioactivity was estimated as the relaxation response to acetylcholine (ACh) in phenylephrine-contracted segments. Data were analyzed with PowerLab software (AD Instruments).
Online Figure I. Uncoupling protein mRNA expression as a function of cell type.

Gene expression profiles of human UCP1, 2, and 3 as a function of cell type examined with the BioGPS online database. Expression levels are indicated as a multiple of the median expression level.
Online Figure II. c-Jun, N-terminal kinase and UCP2 upregulation. (A) Wild-type (WT) and UCP2-null endothelium was exposed to 10% serum for the indicated number of minutes, lysed, and immunoblots performed for actin or phosphorylated (activated) forms of c-Jun (Ser63) or p38 MAP kinase (Thr180/Tyr182). (B) BAECs in the presence or absence of vehicle alone (CTL), the JNK inhibitor SP600125 (0.5 µM), or the p38 MAP kinase inhibitor SB203580 (10 µM) were exposed to 10% serum for the indicated time. Cells were then lysed and examined for UCP2 or activation of JNK or p38 MAP kinase as in (A). (C) BAECs were exposed to vehicle (DMSO) or VEGF (25 ng/mL) with or without SP600125 as indicated for 24h followed by assessment of proliferation by [³H]-thymidine incorporation. N=4; *P<0.05 vs DMSO; †P<0.05 vs. VEGF/DMSO by one-way ANOVA with Tukey post hoc test.
Online Figure III. Uncoupling protein manipulation and angiogenesis.

(A) UCP2 and 3 mRNA levels in mouse lung endothelial cells. Dagger = not detectable. (B) Representative phase contrast images of capillary sprouts from UCP2/- adipose tissue explants taken with a 40 x objective on the day 4 culture. Scale bar, 500 µm. (C) Ex vivo capillary sprouting in fat pad explants from the indicated genotype embedded in collagen gel 5d after in vivo transfection with UCP2 or LacZ adenovirus. N=5/group; *P < 0.05 vs. UCP2/- with Ad-LacZ by two-way ANOVA. (D) Blood flow recovery in UCP2/- mice with unilateral hindlimb ischemia and treatment with control (Ad-LacZ) or UCP2 adenovirus. N=5; *P < 0.05 vs. LacZ by two-way ANOVA.
Online Figure IV. UCP2, antioxidant enzymes, and NO• bioactivity.

(A) BAECs were treated with CTL or UCP2 siRNA as indicated for 24h followed by immunoblotting for the indicated antioxidant enzymes. (B) Acetylcholine-induced relaxation of aortic segments of the indicated genotype contracted with phenylephrine. N=6; P = NS. (C) Phenylephrine-induced contraction of aortic segments by genotype. N=6; P < 0.05 vs. WT by two-way repeated measures ANOVA. (D) Phenylephrine-induced contraction of aortic rings by genotype in the presence of 300 µM L-NAME (P = NS).