The Mitochondrial Permeability Transition Pore Regulates Endothelial Bioenergetics and Angiogenesis

Raluca Marcu, Surya Kotha, Zhongwei Zhi, Wan Qin, Christopher K. Neeley, Ruikang K. Wang, Ying Zheng, Brian J. Hawkins

Rationale: The mitochondrial permeability transition pore is a well-known initiator of cell death that is increasingly recognized as a physiological modulator of cellular metabolism.

Objective: We sought to identify how the genetic deletion of a key regulatory subunit of the mitochondrial permeability transition pore, cyclophilin D (CypD), influenced endothelial metabolism and intracellular signaling.

Methods and Results: In cultured primary human endothelial cells, genetic targeting of CypD using siRNA or shRNA resulted in a constitutive increase in mitochondrial matrix Ca\(^{2+}\) and reduced nicotinamide adenine dinucleotide (NADH). Elevated matrix NADH, in turn, diminished the cytosolic NAD\(^+/\)NADH ratio and triggered a subsequent downregulation of the NAD\(^+/\)dependent deacetylase sirtuin 1 (SIRT1). Downstream of SIRT1, CypD-deficient endothelial cells exhibited reduced phosphatase and tensin homolog expression and a constitutive rise in the phosphorylation of angiogenic Akt. Similar changes in SIRT1, phosphatase and tensin homolog, and Akt were also noted in the aorta and lungs of CypD knockout mice. Functionally, CypD-deficient endothelial cells and aortic tissue from CypD knockout mice exhibited a dramatic increase in angiogenesis at baseline and when exposed to vascular endothelial growth factor. The NAD\(^+\) precursor nicotinamide mononucleotide restored the cellular NAD\(^+\)/NADH ratio and normalized the CypD-deficient phenotype. CypD knockout mice also presented accelerated wound healing and increased neovascularization on tissue injury as monitored by optical microangiography.

Conclusions: Our study reveals the importance of the mitochondrial permeability transition pore in the regulation of endothelial mitochondrial metabolism and vascular function. The mitochondrial regulation of SIRT1 has broad implications in the epigenetic regulation of endothelial phenotype. (Circ Res. 2015;116:1336-1345. DOI: 10.1161/CIRCRESAHA.116.304881.)

Key Words: acetylation ■ angiogenesis effect ■ cyclophilin D ■ mitochondria

Once overlooked as merely a passive lining of blood vessels, endothelial cells (ECs) are increasingly recognized as active participants in vascular homeostasis. A particularly important responsibility of the endothelium is to form new blood vessels in a complex and energy-intensive process known as angiogenesis. To fuel angiogenesis, ECs are adept at using glucose as an energy source,\(^1\) which allows ECs to proliferate and vascularize hypoxic tissue, whereas preserving oxygen for perivascular cells. Despite a reliance on glycolysis, ECs contain functional mitochondria that are active during angiogenesis.\(^2\) Endothelial mitochondria are, therefore, considered signaling organelles that modulate the angiogenic process\(^3\) or supply biosynthetic molecules required for growth.\(^4\) Precisely how mitochondrial metabolism affects endothelial function and angiogenesis is virtually unknown.

An intriguing means to manipulate angiogenesis may involve the mitochondrial permeability transition pore (mPTP). Arsenic-based compounds that target a purported component of the mPTP have an uncanny ability to inhibit endothelial mitochondrial function and angiogenesis in solid tumors\(^5\) and are being developed as anticancer therapies in patients.\(^6\) Pathologically, mPTP activation terminates mitochondrial function and triggers cell death.\(^7\) However, accumulating evidence support a nonlethal role for the mPTP in mitochondrial Ca\(^{2+}\) homeostasis, bioenergetics, and redox signaling.\(^8\) The mitochondrial matrix protein cyclophilin D (CypD), which is encoded by the nuclear gene Ppif and inhibited by cyclosporine...
A (CsA), is a key regulator of Ca\textsuperscript{2+}-induced mPTP opening.\textsuperscript{9} Indeed, mitochondria isolated from CypD knockout mice have an increased Ca\textsuperscript{2+} retention capacity compared with wild-type (WT) counterparts.\textsuperscript{10} As a result, CypD knockout mice possess alterations in glucose oxidation that render cardiomyocytes metabolically inflexible and prone to heart failure.\textsuperscript{11} CypD knockout mice also exhibit changes in branch chain amino acid, pyruvate, and Krebs cycle metabolism.\textsuperscript{12}

Given that ECs are predominately glycolytic and inherently metabolically inflexible, it is unclear whether the mPTP plays a physiological role in the vascular intima. Our studies demonstrate a fundamental role for the endothelial mPTP in matrix Ca\textsuperscript{2+} homeostasis and mitochondrial bioenergetics. Genetic CypD targeting in both ECs and mice enhances mitochondrial Ca\textsuperscript{2+} loading and triggers a persistent alteration in cellular NAD+/NADH homeostasis. Phenotypically, the reduction in the cytosolic NAD+/NADH ratio reduces SIRT1 expression, as well as define a mitochondria-SIRT1 signaling axis which downregulates phosphatase and tensin homolog (PTEN)

Nonstandard Abbreviations and Acronyms

\begin{tabular}{|l|l|}
\hline
\textbf{CsA} & cyclosporine A \\
\textbf{CypD} & cyclophilin D \\
\textbf{EC} & endothelial cells \\
\textbf{HPAEC} & human pulmonary artery endothelial cells \\
\textbf{mPTP} & mitochondrial permeability transition pore \\
\textbf{NADH} & nicotinamide adenine dinucleotide \\
\textbf{NMN} & nicotinamide mononucleotide \\
\textbf{PTEN} & phosphatase and tensin homolog \\
\textbf{SIRT1} & sirtuin 1 \\
\textbf{VEGF} & vascular endothelial growth factor \\
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\textbf{Methods}

\textbf{Cell Culture}

Primary human pulmonary artery endothelial cells (HPAECs; Invitrogen) were cultured according to the manufacturer’s instructions. Human pulmonary microvascular ECs were cultured as described previously.\textsuperscript{13}

\textbf{Animals}

CypD-null and strain-matched controls were obtained from the Jackson Laboratory. All experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Ca\textsuperscript{2+} Measurement

Mitochondrial Ca\textsuperscript{2+} was measured by confocal microscopy using the fluorescence resonance energy transfer (FRET)-based mitochondrial Ca\textsuperscript{2+} indicator Cameleon D3cpv\textsuperscript{14} (Addgene 36324). Cytosolic Ca\textsuperscript{2+} was measured by fluorescence microscopy using Fura-2 AM (Molecular Probes).

NADH and NAD+/NADH Ratio Measurements

Mitochondrial NAD(P)H autofluorescence was measured by fluorescence microscopy with an ultraviolet filter. Cytosolic NAD+/NADH ratio was measured using Peredox\textsuperscript{15} (Addgene 32383). Total intracellular NAD+/NADH ratio was measured using the NAD+/NADH Quantitation Kit (BioVision) and the EnzyChrom NAD+/NADH Assay Kit (BioAssay Systems). Lactate and pyruvate concentrations were measured using Lactate and Pyruvate Assay Kits (Cayman Chemical).

Mitochondrial Mass, Membrane Potential, and Superoxide Production

Mitochondrial mass, membrane potential, and superoxide were quantified by flow cytometry using MitoTracker Green, tetramethylrhodamine methyl ester, 5,5′,6,6′-tetraethylbenzimidazolylcarbocyanine iodide and MitoSox Red (Invitrogen).

Cellular Respiration

Oxygen consumption rate and extracellular acidification rate were measured using the XF24 Analyzer (Seahorse Bioscience).

Invasion Assay

HPAECs were seeded on collagen and provided vascular endothelial growth factor (VEGF) every third day. After 7 days, the collagen layer was fixed and stained with primary antibodies against CD31 and Ki67. Image stacks (5 μm) were acquired with an LSM510 META Zeiss confocal microscope and endothelial invasion was quantified by CD31 positivity >5 μm from the plane of focus.

Aortic Rings Angiogenesis Assay

Aortic rings embedded in collagen type I matrix were stimulated with 30 or 90 ng/mL VEGF every third day for 9 days, fixed, stained with 4′,6-diamidino-2-phenylindole and imaged with a Zeiss Axiovert200 fluorescence microscope. The number of primary and secondary sprouts was quantified in a blinded manner.

Ear Wound Healing Assay

Mouse ear pinna was excised using a 0.5-mm biopsy punch and pictures of the wound were taken biweekly to measure wound size. Microvascular changes during wound healing were visualized and quantified by optical microangiography.\textsuperscript{16}

Statistical Analysis

Data are shown as mean±SEM of ≥3 independent experiments. Statistical significance was assessed using Student t test or 2-way ANOVA test with Tukey post hoc analysis at P<0.05 a priori.

Results

Genetic Inhibition of the mPTP Increases Mitochondrial Calcium Levels

Genetic targeting of CypD (Ppif) in primary pulmonary artery endothelial cells (HPAECs) did not dramatically alter either the expression of mitochondrial proteins involved in electron transport and antioxidant defense (Figure 1A) or the overall cellular mitochondrial content (Figure 1B). CypD knockdown did, however, cause an increase in the length and degree of mitochondrial branching as measured by the mitochondrial form factor (Figure 1C; Online Figures IA and IB), a morphological change reflected by a more complex and reticulated mitochondrial network in Ppif siRNA HPAECs (Figure 1D). Because the mPTP is a known regulator of mitochondrial matrix Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{m}), ECs were transfected with the FRET-based mitochondrial Ca\textsuperscript{2+} sensor Cameleon D3cpv\textsuperscript{14} 48 hr prior to measurement. CypD knockdown significantly increased [Ca\textsuperscript{2+}]\textsubscript{m} at baseline (Figure 1E), suggesting constitutive mPTP activation in ECs similar to that observed in cardiomyocytes.\textsuperscript{11} Although we anticipated elevated [Ca\textsuperscript{2+}]\textsubscript{m} in the presence of Ca\textsuperscript{2+}-mobilizing agonists, such as VEGF or histamine, a significant rise in baseline [Ca\textsuperscript{2+}]\textsubscript{m} was unexpected given that ECs are nonexcitable cells without
First paragraph:

the excitation–contraction coupling present in cardiomyocytes. Further analysis revealed sporadic Ca$^{2+}$ transients in serum-containing media in the absence of additional agonists (Figure 1F), with similar oscillatory pattern on Cyp D deletion or mPTP inhibition by CsA (Online Figures IC and ID), which would be sufficient to load Ca$^{2+}$ into mitochondria. Indeed, Cameleon D3cpv [Ca$^{2+}$]m measurements revealed similar Ca$^{2+}$ transients in mitochondrial matrix of both control and Ppif siRNA HPAECs in the presence of serum (Online Figure IE). In total, these findings indicate that the mPTP is an important regulator of endothelial mitochondrial Ca$^{2+}$ homeostasis.

CypD Influences Endothelial Mitochondrial Functions

The elevated basal [Ca$^{2+}$]m in CypD-deficient ECs would probably elicit mitochondrial functional alterations. Indeed, CypD deletion evoked a significant rise in mitochondrial membrane potential as detected by the cationic fluorescent indicators tetramethylrhodamine methyl ester (Figure 2A) and 5,5',6,6'-tetrachloro-1',1',3',3'-tetracythylbenzimidazolylcarbocyanine iodide (Figure 2B). The rise in membrane potential corresponded with elevated basal respiration: 59.95±2.24 pmol/min O$_2$ for control cells (Figure 2C and 2D). Inhibition of ATP synthase by oligomycin-lowered oxygen consumption rate to similar levels in both CypD-deficient and control ECs, indicating that the elevation in respiration was coupled to ATP production. However, there was no effect of CypD targeting on overall cellular ATP levels in either proliferating or confluent ECs (Figure 2E). Moreover, lactate production (as detected by extracellular acidification rate) was similar between CypD-deficient and control ECs (Figure 2F), indicating that CypD knockdown did not simply shift energy production from the cytosol to the mitochondria. Rather, this data suggest increased mitochondrial ATP generation paired with ATP consumption, resulting in a net neutral ATP level. Increased endothelial respiration also evoked mitochondrial reactive oxygen species generation (Figure 2G) that would imply CypD-deficient ECs might be less metabolically robust and more prone to dysfunction and death. Paradoxically, CypD-deficient ECs seem to be better able to respond to increases in energy demand as denoted by a significant increase in spare respiratory capacity (Figure 2H) suggesting that CypD targeting conveys a protective cellular phenotype.

Endothelial mPTP Inactivation Triggers NADH Accumulation and Alters Cytosolic NAD$^+/$/NADH Homeostasis

Mitochondrial Ca$^{2+}$ activates Ca$^{2+}$-sensitive dehydrogenases that increase NADH production$^1$ to drive respiration. Consistent with the measured increase in [Ca$^{2+}$]m and oxygen consumption rate, CypD-deficient ECs exhibited a 27.5±0.032% rise in basal mitochondrial NAD(P)H autofluorescence versus controls (Figure 3A). Recent data from our laboratory have shown that histamine stimulation of ECs results in mitochondrial Ca$^{2+}$ loading and persistent accumulation of NAD(P)H in the matrix.$^{18}$ However, histamine stimulation of CypD-deficient ECs did not further increase mitochondrial NAD(P)H (Figure 3B), suggesting that the basal increase in [Ca$^{2+}$]m maximally activates the Ca$^{2+}$-dependent dehydrogenases. Approximately 75% of cellular NAD$^+/$/NADH is localized within mitochondria.$^{19}$ As such, direct measurement of total cellular NADH revealed that mPTP inhibition with CsA-stimulated NADH production (Online Figure IF) and a reciprocal reduction in the NAD$^+/$/NADH ratio to a similar degree as acute mitochondrial Ca$^{2+}$ loading using histamine (Online Figure IH). No significant changes in NAD$^+$ levels were
detected (Online Figure IG). To recapitulate these results in vivo, the NAD+/NADH ratio was measured in tissues obtained from CypD knockout mice, the mitochondria from which exhibit an enhanced ability to sequester Ca2+ (Figure 3C). Similar to ECs, CypD knockout mice had a decreased NAD+/NADH ratio in both the aorta and the endothelial-rich lung tissue when assessing total cellular NAD+ and NADH (Figure 3D). Intraperitoneal injection of nicotinamide mononucleotide (NMN), a cell-permeable

Figure 2. Cyclophilin D (CypD) regulates endothelial mitochondrial function. Fluorescence-activated cell sorter (FACS) measurement of mitochondrial membrane potential in scrambled and Ppif siRNA human pulmonary artery endothelial cells (HPAECs) stained with (A) tetramethylrhodamine methyl ester (TMRM; 30 nmol/L) and (B) 5,5′,6,6′-tetramethyl-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; 0.5 µg/mL; mean±SEM; n=3). C, Oxygen consumption rate (OCR) of control, scrambled siRNA, and Ppif siRNA HPAECs on addition of oligomycin (1 µmol/L), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; 1 µmol/L), rotenone, and antimycin A (1 µmol/L) measured with Seahorse XF24 analyzer. D, Quantitation of basal OCR of control, scrambled siRNA, and Ppif siRNA HPAECs (mean±SEM; n=3). E, Normalized baseline ATP content of scrambled and Ppif siRNA HPAECs in proliferating and confluent cultures (mean±SEM; n=3). F, Seahorse X24 extracellular acidification rate (ECAR) measurement of control, scrambled siRNA, and Ppif siRNA HPAECs on addition of oligomycin (1 µmol/L), FCCP (1 µmol/L), rotenone and antimycin A (1 µmol/L). G, FACS measurement of mitochondrial superoxide production using MitoSOX Red (5 µmol/L) in control, scrambled siRNA, and Ppif siRNA HPAECs at baseline and after antimycin A (5 µmol/L; mean±SEM; n=3). H, Quantification of spare respiratory capacity of control, scrambled siRNA, and Ppif siRNA HPAECs (mean±SEM; n=3).

Figure 3. Genetic targeting of cyclophilin D (CypD) influences cellular NAD+/NADH homeostasis. A, Fluorescence microscopy measurement of mitochondrial NAD(P)H autofluorescence in control (n=106), scrambled siRNA (n=215), and Ppif siRNA (n=195) human pulmonary artery endothelial cells (HPAECs) at baseline and on rotenone (5 µmol/L; mean±SEM). B, Normalized mitochondrial NAD(P)H autofluorescence in Ppif siRNA HPAECs at baseline and on histamine (100 nmol/L) and rotenone (5 µmol/L; mean±SEM; n=27). C, Spectrofluorometric measurement of calcium retention capacity of wild-type (WT) and CypD knockout (KO) liver mitochondria (1 mg/mL and 2 mmol/L succinate and 20 µmol/L CaCl2 pulses every 1.5 minutes) using Fura-FF as an indicator of extramitochondrial Ca2+. D, Total intracellular NAD+/NADH ratio of aorta and lung tissues from WT and CypD KO mice (nicotinamide mononucleotide (NMN; 100 mg/kg, 1 hour) normalized to the WT mice ratio values (mean±SEM; n=6 mice per group). E, Cytosolic NAD+/NADH ratio calculated from the measured lactate/pyruvate ratio of control, scrambled siRNA, and Ppif siRNA HPAECs at baseline and control cells treated with cyclosporine A (1 µmol/L) and histamine (100 nmol/L; mean±SEM; n=3). F, Cytosolic NAD+/NADH ratio in scrambled (n=72), Ppif siRNA (n=65), and NMN (100 µmol/L)-treated Ppif siRNA (n=19) HPAECs calculated from the Peredox green-to-red fluorescence ratio after sensor calibration (mean±SEM).
NAD+ precursor, completely restored the NAD+/NADH ratio in CypD knockout mice.

To counter the buildup of NADH within mitochondria, our group recently discovered that matrix-reducing equivalents trans-mit to the cytosol.18 CsA evoked a significant reduction in the cytosolic NAD+/NADH ratio ([NAD+/NADH]cyt) as calculated by the cytosolic concentrations of NADH-linked lactate and pyruvate (Figure 3E). Control ECs had a similar reduction in [NAD+/NADH]cyt when mitochondria were acutely loaded with Ca2+ using histamine. In contrast, CypD-deficient ECs exhibited a significant basal reduction in the [NAD+/NADH]cyt (28%±0.01) that was unaltered by histamine (Figure 3E). To confirm these results, [NAD+/NADH]cyt was directly measured by confocal microscopy using the genetically encoded ratiometric sensor Peredox.15 Similar to the lactate/pyruvate measurements, Peredox measurements revealed a significant reduction in the [NAD+/NADH]cyt from 102±9.2 in scrambled siRNA cells to 77.63±4.9 in Ppif siRNA cells, which was normalized in the presence of NMN (Figure 3F, Online Figure II). Thus, genetic mPTP inactivation evokes an elevation in [Ca2+]m that enhances mitochondrial NADH production and alters cellular NAD+/NADH metabolism.

CypD-Mediated [NAD+/NADH]cyt Alterations Influence SIRT1 Expression

The NAD+/NADH ratio regulates the activity20 and expression21 of the sirtuin family of NAD+-dependent deacetylases. CypD knockout lung and aortic tissues displayed a significant decrease in the expression of SIRT1 (Figures 4A and 4B) similar to that of CypD-deficient ECs (Figure 4C and 4D). Immortalized human pulmonary microvascular ECs in which CypD was targeted using shRNA also exhibited reduced SIRT1 protein levels (Figure 4C and 4D), demonstrating that the CypD-mediated regulation of SIRT1 is not exclusive to the aorta and pulmonary artery. Indeed, follow-up analysis revealed a strong positive correlation between CypD and SIRT1 expression in both HPAECs and human pulmonary microvascular ECs (Figure 4E). Pharmacological inhibition of mitochondrial Ca2+ export through the mitochondrial Na+/Ca2+ exchanger using CGP-37157 also reduced SIRT1 protein levels similar to CypD knockdown (Online Figure IIA), effectively linking SIRT1 expression to [Ca2+]m.

A reduction in SIRT1 expression concomitant with a decrease in NAD+/NADH ratio would predictably trigger an increase in protein acetylation. Indeed, overall protein acetylation was elevated in both CypD knockout aortic and lung tissues (Figure 4F and 4G). No difference in the protein levels of the acetyl transferase CBP was noted between control and CypD-deficient cells (Online Figure IIB and IIC), implying that acetylation was because of diminished deacetylation. SIRT1 expression was restored by NMN supplementation, confirming that SIRT1 levels were responsive to [NAD+/NADH]cyt (Figure 4H). We hypothesized that SIRT1 regulation in CypD-deficient ECs may be tied to the NAD+/NADH ratio by the actions of the NADH-sensitive transcription factor C-terminal–binding protein.21 However, we found no significant difference in C-terminal–binding protein expression between control and CypD-deficient ECs (Online Figure IIB and IIC), suggesting an alternative mechanism for SIRT1 regulation.
CypD-Mediated SIRT1 Alterations Influence PTEN Expression and Akt Phosphorylation

PTEN is indispensable for angiogenesis and has been implicated in vascular function in diabetes mellitus but has no known role in PTEN expression. A reduction in both CypD and SIRT1 corresponded to diminished PTEN expression in ECs (Figure 5A). Inhibition of Ca\(^{2+}\) efflux through the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger (CGP-37157) also resulted in decreased PTEN expression (Online Figure IIA), demonstrating that [Ca\(^{2+}\)]\(_{\text{m}}\) is a key regulator of PTEN protein levels. Normalization of the [NAD\(^+\)/NADH]\(_{\text{cyt}}\) with NMN increased PTEN protein levels in CypD-deficient ECs (Figure 5B). In addition to expression, PTEN exhibited enhanced acetylation in CypD-deficient ECs (Online Figure IIIA and IIIB) that may further inhibit enzymatic activity. PTEN protein levels were lower in the lung, but not aorta, of CypD knockout mice compared with controls (Online Figure IIIC and IIID). Although PTEN immunoprecipitation could not be performed in aortic homogenate, CypD knockout aortic tissue did exhibit increased acetylation that corresponded to PTEN via Western blotting (Online Figure IIIE). Liver tissue obtained from CypD knockout mice exhibited an overall increase in acetylation (Online Figure IIIF) similar to the aorta and lung. PTEN immunoprecipitated from CypD knockout liver homogenate revealed significant acetylation compared with the WT control (Online Figure IIIG and IIIH). Therefore, genetic inactivation of CypD in ECs and tissues results in either PTEN downregulation or increased acetylation that would diminish enzyme activity.

Akt is an important downstream target of PTEN that is phosphorylated by numerous agonists, such as histamine and VEGF. Consistent with a reduction in PTEN activity or expression, CypD genetic knockdown effectively increased Akt phosphorylation in unstimulated HPAECs and human pulmonary microvascular ECs (Figure 5A), as well as in aortic and lung tissue from CypD knockout mice (Figure 5C). Genetic targeting of SIRT1 by siRNA resulted in significant decrease in PTEN and a coincident increase in Akt phosphorylation that was unresponsive to NMN (Figure 5E). Conversely, PTEN overexpression in CypD-deficient HPAECs reduced basal Akt phosphorylation without altering SIRT1 levels (Figure 5F; Addgene plasmid 10750). Taken together, these findings demonstrate that PTEN is downstream of SIRT1 and its expression is governed by the [NAD\(^+\)/NADH]\(_{\text{cyt}}\) in CypD-deficient ECs. Pharmacological inhibition of SIRT1 deacetylase activity with nicotinamide also resulted in increased Akt activation (Figure 5D).
CypD Deletion Increases Endothelial Proliferation and Angiogenesis

CypD-deficient ECs displayed a significant increase in cell number that calculated as 23.4±1.02% reduction in doubling time and was normalized by NMN (Figure 6A). Challenging CypD-deficient HPAECs with VEGF evoked an even further increase in Akt phosphorylation versus controls (Figure 6B), suggesting that CypD targeting may increase the inherent angiogenic potential of ECs. CypD-deficient HPAECs were, therefore, plated on collagen matrix and endothelial invasion evaluated after 7 days in culture (Figure 6C) both in the absence and presence of VEGF and NMN. Western blotting was used to confirm that CypD expression remained depressed during the time course of the invasion assay (Online Figure IV A). Consistent with decreased doubling time CypD-deficient ECs exhibited an increased invasion frequency both at baseline and in response to VEGF (Figure 6D). NMN strongly reduced endothelial invasion in CypD-deficient but not control ECs, supporting the primacy of the [NAD+/NADH]cyt in the CypD-deficient phenotype. Further analysis revealed that CypD-deficient ECs experienced deeper basal invasion depth (SEM, 25.37±0.58 μm; n=278 processes) with early lumen formation versus control ECs (SEM, 12.3±7.38 μm; n=26 processes) that in many cases colocalized with the proliferation marker Ki-67 (Figure 6C).

Aortas from WT and CypD knockout mice were also evaluated for ex vivo vascular remodeling using the aortic ring angiogenesis assay. In response to VEGF (30 ng/mL), CypD knockout aortic rings experienced significantly increased vessel formation (primary sprouts and secondary branches) compared with WT counterparts that was normalized by NMN (Figures 6E and 6G). NMN did not diminish sprout formation in WT aortas in which vessel formation was induced by elevated VEGF (90 ng/mL; Online Figure IVB). No differences in VEGF receptor expression were noted between WT and CypD knockout aorta and lung tissues (Online Figure IVC), excluding the possibility that CypD knockout mice were simply more responsive to agonist stimulation. To recapitulate our findings in vivo, new vessel formation was monitored during tissue injury by optical microangiography during ear wound healing. CypD knockout mice showed earlier onset and significantly accelerated wound closure compared with WT counterparts (Figure 7A), which correlated with increased vascular distribution around the wound at 14 days after ear excision (Figure 7B). Angiographic quantification of vessel area density revealed a significant increase in wound area vessel density in CypD knockout animals: 0.218±0.05 knockout versus 0.048±0.02 WT (mean±SEM; n=3) after wounding but not at baseline (Figure 7C). In total, our results support the mitochondrial regulation of angiogenesis through the NAD+/NADH–dependent regulation of SIRT1 and PTEN (Figure 7D).

Discussion

The discovery that CypD knockout mice have a propensity for heart failure has accelerated interest in the physiological actions of the mPTP. In this study, ECs in which the regulatory subunit CypD was genetically targeted exhibited an increase in both membrane potential and matrix Ca²⁺ that are indicative of constitutive low-level mPTP opening. Elevated matrix
Ca\textsuperscript{2+} was likely because of the presence of variable Ca\textsuperscript{2+} transients at baseline. Our results, therefore, support the regulatory role of constitutive mPTP activity in EC Ca\textsuperscript{2+} homeostasis in agreement with documented data in metabolically active cardiomyocytes, hepatocytes, and neurons. CypD-deficient ECs exhibited a striking mitochondrial functional phenotype as indicated by increased NADH production and respiration, which we attribute to Ca\textsuperscript{2+} stimulation of matrix dehydrogenases. Similar to the heart, ECs also seem to exhibit a Ca\textsuperscript{2+}-mediated increase in ATP synthase activity as witnessed by the dramatic reduction in respiration using oligomycin. However, unlike cardiomyocytes where NADH production is effectively paired with consumption, the low energetic requirements of ECs apparently allow mitochondrial NADH production to outpace consumption and accumulate. We noted a similar rise in matrix NADH in response to repetitive Ca\textsuperscript{2+} oscillations, further supporting that Ca\textsuperscript{2+} is the driving force behind endothelial mitochondrial NADH accumulation, and a reduction in the cellular and the cytosolic NAD\textsuperscript{+}/NADH ratios. A similar buildup of matrix NADH and a reduction in the cellular NAD\textsuperscript{+}/NADH ratio was observed in cardiac tissue in which NADH oxidation was perturbed by genetic deletion of the mitochondrial complex I subunit NADH dehydrogenase iron-sulfur protein 4 (NDUFS4). As we recently discovered, a buildup of NADH in endothelial mitochondria can transmit to the cytosol, possibly via the reverse activation of the malate-aspartate shuttle, which can transmit reducing equivalents from the mitochondria to the cytosol in liver mitochondria.

The [NAD\textsuperscript{+}/NADH]\textsubscript{cyt} is a key determinant of cytosolic Ca\textsuperscript{2+} availability per se but also by the mitochondrial integration of Ca\textsuperscript{2+} signals. ECs seem to interpret a chronic Ca\textsuperscript{2+}-stimulated increase in mitochondrial bioenergetics and the resultant reduction in the [NAD\textsuperscript{+}/NADH]\textsubscript{cyt} as an imbalance between energy production and energy demand similar to that triggered by nutrient excess and, as a result, repress SIRT1 expression. SIRT1 expression is depressed in diabetic mice and in patients with chronic obstructive pulmonary disease. On the basis of our findings, it is interesting to speculate whether endothelial mitochondrial bioenergetics may contribute to SIRT1 downregulation and the vascular complications present in these inflammatory-related diseases. In muscle tissue, SIRT1 diminishes the transcription and activity of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1\textalpha), which reduces the expression of genes involved in mitochondrial electron transport, transcription, and fatty acid metabolism. However, we detected no changes in PGC1-\alpha protein and mRNA levels and mitochondrial biogenesis markers (Online Figures VA and VC), excluding the role of PGC-1\textalpha in the CypD endothelial phenotype.

Pharmacological mPTP blockade protects from ischemia/reperfusion injury by activating prosurvival Akt. Our data not only support this finding but also define a linear pathway in which an mPTP-mediated reduction in [NAD\textsuperscript{+}/NADH]\textsuperscript{cyt} downregulates SIRT1, resulting in PTEN inhibition and Akt phosphorylation. As SIRT1 can directly target Akt, lowered SIRT1 expression would theoretically enhance Akt acetylation and diminish its phosphorylation. In contrast, our study indicates that SIRT1 affects Akt indirectly through PTEN
inactivation in ECs. A similar relationship between SIRT1 expression and Akt activation was noted in cancer cells,9 because of an increase in PTEN acetylation. Enhanced oxidant generation can also directly inactivate PTEN by triggering both the intramolecular cysteine bonding and the S-glutathionylation. Although we cannot completely exclude the possibility that CypD-deficient ECs also inhibit PTEN via oxidation, any oxidant-mediated enzymatic inactivation is secondary to the reduction in PTEN expression, as NMN completely normalizes SIRT1 levels, PTEN expression, and Akt phosphorylation in CypD-deficient ECs without influencing oxidant generation (Online Figure VD). This data strongly suggest that it is the NAD/NADH ratio–mediated regulation of SIRT1 and PTEN expression, and not the increase in mitochondrial oxidant generation, which ultimately drives the CypD-deficient phenotype.

Although incredibly varied in clinical presentation, a common thread among patients with cardiovascular disease is endothelial dysfunction and impaired angiogenesis either as a primary pathology or as collateral damage. Previous work indicates a positive interaction between SIRT1 and angiogenesis, as genetic SIRT1 deletion retards vessel growth in vivo.41

In contrast, our results demonstrate a negative relationship between SIRT1 expression and angiogenesis when genetic CypD targeting only diminishes SIRT1 levels, which is more biologically relevant than complete SIRT1 deletion. CypD knockout mice typically do not present with an overt pathology, but rather as a latent phenotype that manifests only in the presence of an additional stressor.42 Indeed our in vivo data show increased neovascularization in CypD knockout mice after tissue wounding, but no significant vascular phenotype at baseline. Thus, CypD knockout animals may more closely mimic the endothelial dysfunction that occurs in humans during the progression of chronic disease. Further investigation is required to determine the therapeutic implications of our findings, and whether CypD-mediated angiogenesis is restricted to oxygenated tissues that can support increased mitochondrial respiration.

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Disclosures

B.J. Hawkins is currently employed by BioLife Solutions, Bothell, WA. The other authors report no conflicts.

References


In ECs, the mitochondrial permeability transition pore plays an important role in regulating blood vessel function and endothelial dysfunction. Mitochondria can generate and release reactive oxygen species to initiate intracellular signaling.

What New Information Does This Article Contribute?

- In ECs, the mitochondrial permeability transition pore plays a critical role in regulating blood vessel function and endothelial dysfunction.
- Mitochondria contribute to reactive oxygen species metabolism for their energetic requirements.
- Endothelial mitochondria can generate and release reactive oxygen species to initiate intracellular signaling.

Vascular ECs are critical for normal blood vessel function but their dysfunction also contributes to cardiovascular disease, diabetes mellitus, aging, and cancer. A common characteristic of dysfunctional ECs is excessive generation of mitochondrial reactive oxygen species and impaired vessel growth (angiogenesis). In this study, we examined the signaling pathways by which EC mitochondria affect angiogenesis. We found that genetic deletion of cyclophilin D inactivates the mitochondrial permeability transition pore and triggered a constitutive increase in mitochondrial calcium levels and NADH generation. As a result, cyclophilin D-deficient ECs and mice exhibited an imbalance in NAD+/NADH metabolism that influenced the expression of the NAD+-dependent deacetylase SIRT1 and the angiogenic molecule phosphatase and tensin homolog. These genetic changes led to the constitutive activation of Akt, resulting in enhanced angiogenesis both in vitro and in vivo.
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Supplemental Material

Detailed Methods

Cell culture
Primary human pulmonary artery endothelial cells (HPAEC) (Invitrogen) were cultured in M200 medium supplemented with Low Serum Growth Supplement (Invitrogen), 50 U/ml penicillin and 50 µg/ml streptomycin and used between passages 5 and 10. Human pulmonary microvascular endothelial cells (HPMVEC) were cultured in M199 medium supplemented with 20% FBS, 30 µg/ml Endothelial Cell Growth Supplement (Millipore), 2 mmol/L L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. For RNA interference and plasmid expression experiments, HPAEC were transfected using the NEON electroporation system (Invitrogen) with 50 nmol/L siRNA or 0.5 -1 µg DNA/shRNA. Human Ppif siRNA and SIRT1 siRNA were purchased from Ambion and human Ppif shRNA was purchased form Origene. Cells were assayed for protein expression 72 h after transfection. Unless otherwise stated, all experiments were performed on confluent cells plated 2 - 3 days before assays.

Animals
CypD-null (B6/129-Ppiftm1Jmol/J homozygote) and strain-matched controls (B6/129SF2/J wild type) were obtained from the Jackson Laboratory. Male mice between 8-14 weeks of age were anaesthetized with pentobarbital (80 mg/kg body weight, IP) and euthanized by severing the abdominal aorta prior to tissue harvest. NMN (100 mg/kg, IP) was delivered 1 hr prior to terminal surgery. All experimental protocols were approved by the University of Washington Institutional Animal Care and Use Committee.

Western blot and Immunoprecipitation
Cells were lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Thermo Scientific), phosphatase inhibitors cocktail (Roche), 10 mmol/L nicotinamide and 10 µmol/L trichostatin A. For Western blot, 10 µg proteins were electrophoresed on NuPAGE 4-12% Bis-Tris Acrylamide gels (Invitrogen), transferred on PVDF membrane and probed with primary antibodies at 4°C overnight. For immunoprecipitation, 2 mg cell or tissue lysates were incubated with primary antibody overnight at 4°C followed by incubation with A/G Plus agarose beads (Santa Cruz). HRP-conjugated secondary antibodies (Thermo Scientific) were used for detection.

[Ca^{2+}] measurement
Mitochondrial [Ca^{2+}] was measured using the FRET-based genetically encoded mitochondrial Ca^{2+} indicator Cameleon D3cpv (Palmer and Tsien, 2006) (Addgene plasmid 36324). HPAEC were transfected by electroporation with the D3cpv sensor, plated on MatTek dishes and imaged by confocal microscopy 72 h post transfection in ECM buffer (120 mmol/L NaCl, 5 mmol/L NaHCO_{3}, 10 mmol/L Na-HEPES, 4.7 mmol/L KCl, 1 mmol/L KH_{2}PO_{4}, 1.2 mmol/L MgSO_{4}, 2 mmol/L CaCl_{2}, 10 mmol/L glucose, and 2.0% BSA, pH 7.4), at room temperature. Baseline images were acquired every 10 seconds for 3 min with a LSM510 META Zeiss confocal microscope using a Zeiss Fluar 40x/1.3 oil objective at 405/488 nm excitation wavelengths. Ratio images (R) were obtained by dividing the intensity of the FRET channel to the intensity of the CFP channel after background correction. To obtain baseline mitochondrial calcium concentration the sensor response was calibrated at the end of the experiment for each cell by measuring R_{min} (in the presence of 5 µmol/L Ionomycin and 5 mmol/L EGTA) and R_{max} (in the presence of 5 µmol/L Ionomycin and 5 mmol/L CaCl_{2}). Mitochondrial [Ca^{2+}] values were obtained by substituting measured R, R_{min}, and R_{max} in the following equation, assuming K_{d}=0.76
Changes in cytosolic $[\text{Ca}^{2+}]$ were measured by wide field fluorescence microscopy using the Fura-2 AM dye (Molecular Probes). HPAEC plated on MatTek dishes were loaded with 5 $\mu$mol/L Fura-2 AM in ECM buffer supplemented with 100 $\mu$mol/L sulfinpyrazone and 0.003% pluronic acid, for 30 min at room temperature. After dye loading cells were washed and imaged in ECM buffer with 0.25% BSA and 100 $\mu$mol/L sulfinpyrazone using a Nikon Eclipse Ti microscope equipped with a xenon arc lamp and DeltaRamX monochromator (Photon Technology International), an Evolve 512 EMCCD camera (Photometrics), a 40x/1.30 oil Nikon Plan Fluor objective and using a UV filter. The microscope was calibrated for Fura-2 fluorescence (Molecular Probes) and Ca$^{2+}$ concentrations were obtained using the EasyRatioPro software (Photon Technology International). Calcium retention capacity assay was performed on isolated liver mitochondria as described previously (Marcu et al., 2012) using 800 nmol/L Fura FF (Invitrogen) to measure changes in extramitochondrial Ca$^{2+}$ upon pulsing mitochondria with CaCl$_2$ at fixed time intervals.

**NADH and NAD$^+$/NADH ratio measurements**

Mitochondrial NADH fluorescence was measured with a Nikon Eclipse Ti microscope equipped with a xenon arc lamp and DeltaRamX monochromator (Photon Technology International) and an Evolve 512 EMCCD camera (Photometrics) with the assistance of EasyRatioPro software using a UV filter and a 40x/1.30 oil Nikon Plan Fluor objective. Specificity for mitochondrial NADH was determined by colocalization with the mitochondrial dye MitoTracker Green (Invitrogen). Cytosolic NAD$^+$/NADH ratio was measured using the genetically encoded ratiometric fluorescence indicator Peredox (Hung et al., 2011) (Addgene plasmid 32383). Baseline green and red fluorescence images were acquired every 30 seconds for 5 min with a LSM510 META Zeiss confocal microscope using a Zeiss Fluar 40x/1.3 oil objective at an excitation/emission of 405/505-530 nm and 543/650 nm. Images were background corrected and green-to-red ratio images were obtained using ImageJ software. For each cell, ratio data were normalized to the minimal green-to-red ratio signal obtained with 10 mmol/L pyruvate. The Peredox sensor was calibrated in ECM buffer without glucose by varying extracellular concentrations of lactate and pyruvate as previously described (Hung and Yellen, 2014). Total intracellular NAD$^+$/NADH ratio was measured using the NAD$^+$/NADH Quantitation Kit (BioVision) for tissue extracts and the EnzyChrom NAD$^+$/NADH Assay Kit (BioAssay Systems) for cell extracts. Intracellular lactate and pyruvate concentrations were measured using the Lactate Assay Kit and Pyruvate Assay Kit (Cayman Chemical) and used to calculate the cytosolic NAD$^+$/NADH ratio (Williamson et al., 1967).

**Mitochondrial mass, membrane potential and superoxide production**

Mitochondrial mass, membrane potential and superoxide production were quantified in live cells by flow cytometry using a FACS CantoII cells sorter (BD Bioscience). To measure mitochondrial mass, cells were stained with 50 nmol/L MitoTracker Green (Invitrogen) and analyzed by FACS using an excitation/emission of 488/530 nm. To measure mitochondrial membrane potential cells were stained with either 30 nmol/L tetramethylrhodamine methyl ester (TMRM, Invitrogen) or 0.5 $\mu$g/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen) and analyzed by FACS at an excitation/emission of 488/585 nm and respectively 488/530 nm (JC-1$_{\text{monomer}}$) and 488/585 nm (JC-1$_{\text{aggregate}}$). For JC-1 measurements, changes in mitochondrial membrane potential were expressed as changes in the JC-1$_{\text{aggregate}}$/JC-1$_{\text{monomer}}$ fluorescence ratio. To measure mitochondrial superoxide production cells were stained with 5

\[
[Ca^{2+}] = \frac{K_d^{0.74} (R - R_{\text{min}})}{(R - R_{\text{max}})^{1/0.74}}
\]
µmol/L MitoSox Red (Invitrogen) and analyzed by FACS using an excitation/emission of 488/585 nm.

**Mitochondrial morphology**
Quantitative analysis of mitochondrial morphology was performed on confocal images of MitoTracker Green fluorescence (LSM510 META Zeiss confocal microscope, Zeiss Fluar 40x/1.3 oil objective, 488/520 nm excitation/emission). Image processing was performed using the Fiji/ImageJ software (Schindelin et al., 2012) as previously described (Koopman et al., 2005, Yu et al., 2008). Gray scale images were processed with a convolve filter followed by a median filter and thresholding and subjected to particle analysis to generate mitochondrion area (A), perimeter (P) and aspect ratio (AR, the ratio between the major and minor axis of the ellipse equivalent to the mitochondrion). The form factor (F) was calculated using the following equation:

\[ F = \frac{P^2}{4\pi A} \]

**Cellular respiration**
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF24 Analyzer (Seahorse Bioscience). HPAECs were transfected with either Ppif siRNA or scrambled siRNA and were plated in Seahorse 24 well assay plates, 30,000 cells/well, in M200 growth medium, 24 hours after transfection. OCR and ECAR measurements were performed in XF Assay medium (Seahorse Bioscience) at 10 min intervals 72 hours after transfection.

**ATP content**
ATP levels were measured using the EnzyLight Assay Kit (BioAssay Systems).

**Invasion assay**
Invasion assay was performed in polydimethylsiloxane wells, corona treated and coated with polyethyleneimine (bottom chamber: 4 mm diameter, 2 mm height, reservoir: 8 mm diameter, 2 mm height). HPAECs were seeded on top of collagen type I matrix, 40,000 cells/well, and were fed with 50 ng/ml VEGF every 3rd day. Cells were allowed to invade the collagen layer for 7 days after which they were fixed with 3.7% formaldehyde, permeabilized and blocked with 2% BSA and 0.2% Triton-X, and incubated overnight at 4°C with primary antibodies against CD31 and Ki67. DAPI (3 µmol/L) was used to visualize cell nuclei. Alexa 488 and Alexa 567 secondary antibodies were used for detection. Image stacks (5 µm interval) were acquired with a LSM510 META Zeiss confocal microscope using a Zeiss Fluar 10x/0.5 objective at 405/488/543 nm excitation. Image stacks were processed and analyzed using Fiji/ImageJ (Schindelin et al., 2012). Prior to analysis, noise was removed from the image stacks using the despeckle function. Image stacks were then subjected to Gaussian smoothing and endothelial invasion (CD31 staining) was quantified by manual counting of CD31 positivity >5 µm from the plane of focus. Depth of invasion was quantified using View5D.

**Aortic rings angiogenesis assay**
Mouse aortic ring angiogenesis assay was performed as described previously (Baker et al., 2012). Briefly, thoracic aortae were excised from WT and CypD KO mice, cleaned from extraneous fat and dissected into 0.5-1 mm thickness rings. Aortic rings were serum-starved overnight in Opti-MEM reduced serum medium (Invitrogen) and embedded in collagen type I matrix in 96 well plates, 1 ring/well. Rings were fed with 30 or 90 ng/ml VEGF every 3rd day for 9 days, fixed in formalin, stained with DAPI and imaged with a Zeiss Axiovert200 fluorescence microscope.
equipped with an Olympus Color3 camera using a Zeiss Fluar 5x/0.25 objective. The number of primary and secondary sprouts was quantified in a blinded manner for each ring.

**Wound healing assay**

To induce tissue wounding, mouse ear pinna was excised using a 0.5 mm biopsy punch. 3 WT mice and 3 Ppif KO mice were used per group, and the punch was performed on both ears. Pictures of the wound were taken biweekly in order to quantify the wound area. Microvascular changes during wound healing were visualized and quantified weekly by optical microangiography (OMAG) (Jung et al., 2013), a recently developed non-invasive optical coherence tomography technique capable of producing 3-D images of dynamic blood perfusion within microcirculatory tissue beds with capillary resolution (Wang et al., 2007, Zhi et al., 2011). Before and during the OMAG imaging mice were anesthetized with isoflurane (0.2 L/min oxygen and 0.8 L/min air) by applying a face mask. Hair on the ear pinna was removed by application of Nair (Church and Dwight Co., Inc, Princeton, New Jersey) and the pinna was immobilized with double-sided tape onto a glass slide to minimize motion artifacts. For each animal, a series of three-dimensional OMAG data volumes were acquired around the punch hole immediately after the punch (baseline), and at 7 and 14 days after the punch. The OMAG data were post-processed to provide microvasculature maps using OMAG algorithms as described (An et al., 2011, Zhi et al., 2011). To obtain vessel area density, OMAG images were processed in Fiji/ImageJ (Schindelin et al., 2012) using a low pass filter followed by adaptive thresholding to generate binary images (Reif et al., 2012). Vessel area density was calculated by dividing the number of white pixels representing the vessels in the binary image to the total number of pixels in the image. To estimate neovascularization during wound healing, the area depleted of vessels at day 1 after the tissue excision was delineated and vessel area density was measured within the same region at day 14 after wounding.

**Statistical analysis**

The data are shown as mean ± SEM of 3 or more independent experiments. Statistical significance was assessed using Student's t test or two-way ANOVA test with Tukey post-hoc analysis, and p values <0.05 were considered statistically significant.

**Reagents**

Pan-Acetylated lysine, PTEN, GAPDH, Akt and pAkt antibodies were from Cell Signaling. SIRT1 and MnSOD antibodies were from Invitrogen, VEGFR was from Fisher Thermo Scientific. CD31, Ki-67, Cyclophilin D, complex I, II, III and IV antibodies were from Abcam. NMN and nicotinamide were from Sigma-Aldrich and trichostatin A from Cayman Chemical.
Supplemental Figures and Figure Legends

Online Figure I. (A), (B) Mitochondrial morphology description by the aspect ratio (AR) plotted as a function of the form factor (F) for HPAECs expressing either scrambled siRNA or Ppif siRNA (n=189 scrambled siRNA, n=137 Ppif siRNA). (C) Percentage of control, CsA-treated (2 μmol/L), scrambled siRNA and Ppif siRNA HPAECs displaying intracellular Ca\(^{2+}\) oscillatory patterns in the presence of FBS (5%) (mean ± SEM, n=63 control, n=48 CsA, n=95 scrambled siRNA, n=92 Ppif siRNA). (D) Mean oscillatory frequency of control, CsA-treated (2 μmol/L), scrambled siRNA and Ppif siRNA HPAECs in the presence of FBS (5%) (mean ± SEM, n=35 control, n=32 CsA, n=64 scrambled siRNA, n=58 Ppif siRNA). (E) Representative oscillatory [Ca\(^{2+}\)]\(_m\) transients assessed by confocal microscopy with the genetically-encoded mitochondrial
Ca\textsuperscript{2+} sensor Cameleon D3cpv and presented as CFP/FRET ratio of the sensor in scrambled and Ppif siRNA HPAECs stimulated with FBS (5%). (F), (G) Total intracellular NADH and NAD\textsuperscript{+} concentrations measured in HPAECs at baseline and after incubation with CsA (1 \textmu mol/L, 6 hr) (mean ± SEM, n=3). (H) Total cellular NAD\textsuperscript{+}/NADH ratio calculated from the measured NAD\textsuperscript{+} and NADH in HPAECs at baseline (± 1 \textmu mol/L CsA) and after stimulation with histamine (100 nmol/L) (mean ± SEM, n=3). (I) Confocal microscopy measurements of the Peroxidox sensor green-to-red fluorescence ratio in scrambled siRNA (mean ± SEM, n=72), Ppif siRNA (mean ± SEM, n=65) and NMN (100 mmol/L) treated Ppif siRNA HPAECs (mean ± SEM, n=19).
Online Figure II. (A) Western blot analysis of SIRT1 and PTEN expression in control HPAECs and HPAECs treated with 10 µmol/L and 20 µmol/L of the mitochondrial Na⁺/Ca²⁺ exchanger inhibitor CGP-37157 (20 hr). (B), (C), (D) Western blot analysis and densitometry quantification of CBP and CtBP1 protein expression in HPAECs transfected with either scrambled siRNA or Ppif siRNA. GAPDH was used as a loading control (mean ± SEM, n=6).
**Online Figure III.** (A), (B) Western blot analysis and densitometry quantification (mean ± SEM, n=6) of PTEN acetylation in HPAECs transfected with scrambled siRNA or Ppif siRNA. Cell extracts were probed by western blotting with a pan-Acetylated lysine antibody. The membrane was re-probed with a PTEN antibody and the corresponding acetylation band is shown. (C), (D) Western blot analysis and densitometry quantification (mean ± SEM, n=6 mice/group) of PTEN protein levels in lung and aortic tissue from WT and CypD KO mice. GAPDH was used as a loading control. (E) Western blot analysis of PTEN acetylation in aorta of WT and CypD KO mice. Aorta extracts were first probed with a pan-Acetyl lysine antibody and subsequently with a PTEN antibody. (F) Western blot analysis of liver lysates from WT and CypD KO mice probed with a pan-Acetylated lysine antibody and a GAPDH antibody as loading control. (G), (H) Western blot analysis and densitometry quantification of PTEN acetylation immunoprecipitated from liver of WT and CypD KO mice (mean ± SEM, n=3).
Online Figure IV. (A) Western blot analysis of CypD expression in HPAECs transfected with scrambled siRNA or Ppif siRNA at 3, 5 and 7 days after transfection. GAPDH was used as a loading control. (B) Quantification of primary sprouts formation during aortic ring angiogenesis assay of WT aortic rings, at baseline and upon treatment with VEGF (90 ng/ml) ± NMN (100 µmol/L) (mean ± SEM, n= 3). (C). Western blot analysis of VEGFR expression in aorta and lung tissue lysates from WT and CypD KO mice using GAPDH as loading control.
Online Figure V. (A), (B) Western blot analysis and densitometry quantification of PGC1α protein levels in scrambled siRNA and Ppif siRNA-expressing HPAECs, using GAPDH as loading control (mean ± SEM, n=6). (C) Relative mRNA levels of PGC1α and PGC1α targets TFAM, NRF1 and ERRα in HPAECs expressing scrambled siRNA and Ppif siRNA, normalized to 18S mRNA levels (mean ± SEM, n=3). (D) FACS measurement of mitochondrial superoxide production using MitoSOX Red (5 µmol/L) in scrambled siRNA and Ppif siRNA HPAECs at baseline and after treatment with NMN (100 µmol/L, 48 hr) (mean ± SEM, n=3).
Supplemental References

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