Mitochondrial Permeability Transition Pore Regulates Endothelial Bioenergetics and Angiogenesis

Raluca Marcu1,2, Surya Kotha2, Zhongwei Zhi2, Wan Qin2, Christopher K. Neeley1,4, Ruikang K. Wang2,3, Ying Zheng2 and Brian J. Hawkins1

1Mitochondria and Metabolism Center, Department of Anesthesiology and Pain Medicine, University of Washington, Seattle, WA 98109, USA; 2Department of Bioengineering, University of Washington, Seattle, WA 98109, USA; 3Department of Ophthalmology, University of Washington, Seattle, WA 98195, USA, and; 4General Surgery, University of Michigan, Ann Arbor, USA.

Running title: The Permeability Transition Pore and Angiogenesis

Subject codes:
[95] Endothelium/vascular type
[129] Angiogenesis
[140] Energy metabolism
[145] Genetically altered mice

Address correspondence to:

Dr. Brian J. Hawkins  Dr. Raluca Marcu
850 Republican Street  850 Republican Street
Seattle, WA 98109-8057  Room Brotman 403G
USA  Seattle, WA 98109-8057
Tel: 206-543-6140  USA
Fax: 206-616-4819  Tel: 206-685-4208
bhawkins@uw.edu  Fax: 206-616-4819
bhawkins@biolifesolutions.com  rmarcu@uw.edu

In January 2015, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.7 days.
ABSTRACT

Rationale: The mitochondrial permeability transition pore (mPTP) 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 mPTP, Cyclophilin D (CypD), influenced endothelial metabolism and intracellular signaling.

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

Conclusion: Our study reveals the importance of the mPTP 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.

Keywords: Mitochondria, cyclophilin D, SIRT1, acetylation, NAD$^+$/NADH ratio, angiogenesis, endothelial function.

Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA</td>
<td>Cyclosporine A</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cells</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular acidification rate</td>
</tr>
<tr>
<td>HPAEC</td>
<td>Human pulmonary artery endothelial cells</td>
</tr>
<tr>
<td>HPMVEC</td>
<td>Human pulmonary microvascular endothelial cells</td>
</tr>
<tr>
<td>mNCLX</td>
<td>Mitochondrial Na$^+$/Ca$^{2+}$ exchanger</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NMN</td>
<td>Nicotinamide mononucleotide</td>
</tr>
<tr>
<td>NAM</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td>OMAG</td>
<td>Optical microangiography</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen consumption rate</td>
</tr>
<tr>
<td>ROSE</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
INTRODUCTION

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 utilizing glucose as an energy source\(^1\), which allows ECs to proliferate and vascularize hypoxic tissue while 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 impacts 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\(^6\) and are being developed as anticancer therapies in patients\(^7\). Pathologically, mPTP activation terminates mitochondrial function and triggers cell death\(^8\). However, accumulating evidence support a non-lethal role for the mPTP in mitochondrial Ca\(^{2+}\) homeostasis, bioenergetics and redox signaling\(^9\). 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\(^{2+}\)-induced mPTP opening\(^10\). Indeed, mitochondria isolated from CypD KO mice have an increased Ca\(^{2+}\)-retention capacity compared to WT counterparts\(^11\). As a result, CypD KO mice possess alterations in glucose oxidation that render cardiomyocytes metabolically inflexible and prone to heart failure\(^12\). CypD KO mice also exhibit changes in branch chain amino acid, pyruvate, and Krebs cycle metabolism\(^13\).

Given that ECs are predominately glycolytic and inherently metabolically inflexible, it is unclear whether the mPTP plays a physiologic role in the vascular intima. Our studies demonstrate a fundamental role for the endothelial mPTP in matrix Ca\(^{2+}\) homeostasis and mitochondrial bioenergetics. Genetic CypD targeting in both ECs and mice enhances mitochondrial Ca\(^{2+}\) loading and triggers a persistent alteration in cellular NAD\(^+\)/NADH homeostasis. Phenotypically, the reduction in the cytosolic NAD\(^+\)/NADH ratio reduces SIRT1 expression, which downregulates PTEN and results in constitutive Akt phosphorylation. As a result, both CypD-deficient ECs and CypD KO mice exhibit enhanced angiogenesis in vitro, ex vivo and in vivo upon tissue injury. In total, these findings elucidate a novel pathway by which mitochondrial bioenergetics influence vascular function, as well as define a Mitochondria-SIRT1 signaling axis that may have broad implications in cell biology.

METHODS

Cell culture.
Primary human pulmonary artery endothelial cells (HPAEC) (Invitrogen) were cultured according to the manufacturer’s instructions. Human pulmonary microvascular endothelial cells (HPMVEC) were cultured as described previously\(^14\).

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 FRET-based mitochondrial Ca\textsuperscript{2+} indicator Cameleon D3cpv\textsuperscript{15} (Addgene 36324). Cytosolic [Ca\textsuperscript{2+}] was measured by fluorescence microscopy using Fura-2 AM (Molecular Probes).

NAD\textsuperscript{+} and NAD\textsuperscript{+}/NADH ratio measurements.
Mitochondrial NAD(P)H autofluorescence was measured by fluorescence microscopy with an UV filter. Cytosolic NAD\textsuperscript{+}/NADH ratio was measured using Peredox\textsuperscript{17} (Addgene 32383). Total intracellular NAD\textsuperscript{+}/NADH ratio was measured using the NAD\textsuperscript{+}/NADH Quantitation Kit (BioVision) and the EnzyChrom NAD\textsuperscript{+}/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 (TMRM), 5,5\textprime,6,6\textprime-tetrachloro-1,1\textprime,3,3\textprime-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and MitoSox Red (Invitrogen).

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

Invasion assay.
HPAECs were seeded on collagen and provided VEGF every 3\textsuperscript{rd} day. After 7 days, the collagen layer was fixed and stained with primary antibodies against CD31 and Ki67. Image stacks (5 \textmu m) were acquired with a LSM510 META Zeiss confocal microscope and endothelial invasion was quantified by CD31 positivity >5 \textmu m 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 3\textsuperscript{rd} day for 9 days, fixed, stained with DAPI 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 (OMAG)\textsuperscript{20}.

Statistical analysis.
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 at p <0.05 \textit{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, IB), a morphological change reflected by a more complex and reticulated mitochondrial network in Ppif siRNA HPAECs (Figure 1D). As the mPTP is a known regulator of mitochondrial matrix Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_m\)), ECs were transfected with the FRET-based mitochondrial Ca\(^{2+}\) sensor Cameleon D3cpv\(^{15}\). CypD knockdown significantly increased [Ca\(^{2+}\)]\(_m\) at baseline (Figure 1E), suggesting constitutive mPTP activation in ECs similar to that observed in cardiomyocytes\(^{12}\). While we anticipated elevated [Ca\(^{2+}\)]\(_m\) in the presence of Ca\(^{2+}\)-mobilizing agonists such as VEGF or histamine, a significant rise in baseline [Ca\(^{2+}\)]\(_m\) was unexpected given that ECs are non-excitable cells without 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 upon Cyp D deletion or mPTP inhibition by CsA (Online Figures IC, 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 likely elicit mitochondrial functional alterations. Indeed, CypD deletion evoked a significant rise in mitochondrial membrane potential as detected by the cationic fluorescent indicators TMRM (Figure 2A) and JC-1 (Figure 2B). The rise in membrane potential corresponded with elevated basal respiration: 59.95 ± 2.24 pmol/min O\(_2\) for Ppif siRNA ECs compared to 30.98 ± 1.34 pmol/min for control cells (Figure 2C, 2D). Inhibition of ATP synthase by oligomycin lowered OCR 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 ECAR) 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 suggests increased mitochondrial ATP generation paired with ATP consumption, resulting in a net neutral ATP level. Increased endothelial respiration also evoked mitochondrial ROS 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 appear 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\(^{21}\) in order to drive respiration. Consistent with the measured increase in [Ca\(^{2+}\)]\(_m\) and OCR, 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 has shown that histamine stimulation of ECs results in mitochondrial Ca\(^{2+}\) loading and persistent accumulation of NAD(P)H in the matrix\(^{22}\). 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\(^{23}\). 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 KO mice, the mitochondria from which exhibit an enhanced ability to sequester Ca\(^{2+}\) (Figure 3C). Similar to ECs, CypD KO mice had a decreased NAD\(^{+}\)/NADH ratio in both the aorta and endothelial-rich lung tissue.

DOI: 10.1161/CIRCRESAHA.116.304881
when assessing total cellular NAD⁺ and NADH (Figure 3D). Intraperitoneal injection of nicotinamide mononucleotide (NMN), a cell-permeable NAD⁺ precursor, completely restored the NAD⁺/NADH ratio in CypD KO mice.

To counter the buildup of NADH within mitochondria, our group recently discovered that matrix reducing equivalents transmit to the cytosol. CsA evoked a significant reduction in the cytosolic NAD⁺/NADH ratio ([NAD⁺/NADH]₉₀) as calculated by the cytosolic concentrations of NADH-linked lactate and pyruvate (Figure 3E). Control ECs had a similar reduction in [NAD⁺/NADH]₉₀ when mitochondria were acutely loaded with Ca²⁺ using histamine. In contrast, CypD-deficient ECs exhibited a significant basal reduction in the [NAD⁺/NADH]₉₀ (28% ± 0.01) that was unaltered by histamine (Figure 3E). To confirm these results, [NAD⁺/NADH]₉₀ was directly measured by confocal microscopy using the genetically encoded ratiometric sensor Peredox. Similar to the lactate/pyruvate measurements, Peredox measurements revealed a significant reduction in the [NAD⁺/NADH]₉₀ 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 I-I). Thus, genetic mPTP inactivation evokes an elevation in [Ca²⁺]ₙₐₚ that enhances mitochondrial NADH production and alters cellular NAD⁺/NADH metabolism.

CypD-mediated [NAD⁺/NADH]₉₀ alterations influence SIRT1 expression.

The NAD⁺/NADH ratio regulates the activity and expression of the sirtuin family of NAD⁺-dependent deacetylases. CypD KO lung and aortic tissues displayed a significant decrease in the expression of SIRT1 (Figures 4A, 4B) similar to that of CypD-deficient ECs (Figures 4C, 4D). Immortalized human pulmonary microvascular ECs (HPMVECs) in which CypD was targeted using shRNA also exhibited reduced SIRT1 protein levels (Figures 4C, 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 HPMVECs (Figure 4E). Pharmacologic inhibition of mitochondrial Ca²⁺ export through the Na⁺/Ca²⁺ exchanger (mNCLX) using CGP-37157 also reduced SIRT1 protein levels similar to CypD knockdown (Online Figure IIA), effectively linking SIRT1 expression to [Ca²⁺]ₙₐₚ.

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 KO aortic and lung tissues (Figures 4F, 4G). No difference in the protein levels of the acetyl transferase CBP was noted between control and CypD-deficient cells (Online Figures IIB, IIC), implying that acetylation was due to diminished deacetylation. SIRT1 expression was restored by NMN supplementation, confirming that SIRT1 levels were responsive to [NAD⁺/NADH]₉₀ (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 CtBP (C-terminal binding protein). However, we found no significant difference in CtBP expression between control and CypD-deficient ECs (Online Figures IIB, IID), suggesting an alternative mechanism for SIRT1 regulation.

CypD-mediated SIRT1 alterations influence PTEN expression and Akt phosphorylation.

Phosphatase and tensin homolog (PTEN) is indispensable for angiogenesis and has been implicated in vascular function in diabetes. SIRT1 regulates PTEN acetylation and enzymatic activity, 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 Ca²⁺ efflux through the mNCLX (CGP-37157) also resulted in decreased PTEN expression (Online Figure IIA), demonstrating that [Ca²⁺]ₙₐₚ is a key regulator of PTEN protein levels. Normalization of the [NAD⁺/NADH]₉₀ 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 Figures IIIA, IIIB) that may further inhibit enzymatic activity. PTEN protein levels
were lower in the lung, but not aorta, of CypD KO mice compared with controls (Online Figures IIIC, IIID).

While PTEN immunoprecipitation could not be performed in aortic homogenate, CypD KO aortic tissue did exhibit increased acetylation that corresponded to PTEN via western blotting (Online Figure IIIE). Liver tissue obtained from CypD KO mice exhibits an overall increase in acetylation (Online Figure IIIF) similar to the aorta and lung. PTEN immunoprecipitated from CypD KO liver homogenate revealed significant acetylation compared to the WT control (Online Figures IIG, IIGH). Therefore, genetic inactivation of CypD in ECs and tissues results in PTEN downregulation and/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 and/or expression, CypD genetic knockdown effectively increased Akt phosphorylation in unstimulated HPAECs and HPMVECs (Figure 5A) as well as in aortic and lung tissue from CypD KO 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 \([\text{NAD}^+/\text{NADH}]_\text{cyt}\) in CypD-deficient ECs. Pharmacologic 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 IVA). 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 \([\text{NAD}^+/\text{NADH}]_\text{cyt}\) in CypD-deficient phenotype. Further analysis revealed that CypD-deficient ECs experienced deeper basal invasion depth (25.37 ± 0.58 \(\mu\)m SEM, \(n=278\) processes) with early lumen formation versus control ECs (12.3 ± 7.38 \(\mu\)m SEM, \(n=26\) processes) that in many cases colocalized with the proliferation marker Ki-67 (Figure 6C).

Aortas from WT and CypD KO mice were also evaluated for ex vivo vascular remodeling using the aortic ring angiogenesis assay. In response to VEGF (30 ng/ml), CypD KO aortic rings experienced significantly increased vessel formation (primary sprouts and secondary branches) compared to WT counterparts that was normalized by NMN (Figures 6E-G). 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 KO aorta and lung tissues (Online Figure IVC), excluding the possibility that CypD KO 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 (OMAG) during ear wound healing. CypD KO mice showed earlier onset and significantly accelerated wound closure compared to WT counterparts (Figure 7A), which correlated with increased vascular distribution around the wound at 14 days after ear excision (Figure 7B). Angiographic quantitation of vessel area density revealed a significant increase in wound area vessel density in CypD KO animals: 0.218±0.05 KO vs 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 \([\text{NAD}^+/\text{NADH}]_\text{cyt}\) dependent regulation of SIRT1 and PTEN (Figure 7D).
DISCUSSION

The discovery that CypD KO mice have a propensity for heart failure\textsuperscript{12} has accelerated interest in the physiological actions of the mPTP\textsuperscript{35}. In the present study, ECs in which the regulatory subunit CypD was genetically targeted exhibited an increase in both membrane potential and matrix Ca\textsuperscript{2+} that are indicative of constitutive low-level mPTP opening. Elevated matrix Ca\textsuperscript{2+} was likely due to 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\textsuperscript{12}, hepatocytes\textsuperscript{36} and neurons\textsuperscript{37}.

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\textsuperscript{21}. Similar to the heart\textsuperscript{38}, ECs also appear 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\textsuperscript{22}, 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 Ndufs4\textsuperscript{39}. 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\textsuperscript{22}, which can transmit reducing equivalents from the mitochondria to the cytosol in liver mitochondria\textsuperscript{40}.

The [NAD\textsuperscript{+}/NADH\textsubscript{cyt}] is a key determinant of cytosolic protein acetylation via sirtuins. Both CypD-deficient ECs and CypD KO mice exhibited a significant decline in SIRT1 expression, which is regulated largely by nutrient status and metabolic transcription factors. Our study strengthens the relationship between SIRT1 expression and the [NAD\textsuperscript{+}/NADH\textsubscript{cyt}], but is unique in that all experiments were performed under normal nutrient availability. This distinction is important, and reveals that SIRT1 regulation is impacted not only by nutrient availability per se, but also by the mitochondrial integration of Ca\textsuperscript{2+} signals. ECs appear 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\textsuperscript{41} and in patients with chronic obstructive pulmonary disease\textsuperscript{42}. Based on 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 PGC1-\(\alpha\), which reduces the expression of genes involved in mitochondrial electron transport, transcription, and fatty acid metabolism\textsuperscript{43}. However, we detected no changes in PGC1-\(\alpha\) protein and mRNA levels and mitochondrial biogenesis markers (Online Figures VA-C), excluding the role of PGC-1\(\alpha\) in the CypD endothelial phenotype.

Pharmacologic mPTP blockade protects from ischemia/reperfusion injury by activating pro-survival Akt\textsuperscript{44}. Our data support this finding, but also define a linear pathway in which an mPTP-mediated reduction in [NAD\textsuperscript{+}/NADH\textsubscript{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\textsuperscript{45}. 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\textsuperscript{46} due to an increase in PTEN acetylation. Enhanced oxidant generation can also directly inactivate PTEN by triggering both intramolecular cysteine bonding\textsuperscript{47} and S-glutathionylation. While 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 suggests 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.

While incredibly varied in clinical presentation, a common thread among cardiovascular disease patients 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 vivo⁴⁹. 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 KO mice typically do not present with an overt pathology, but rather as a latent phenotype that manifests only in the presence of an additional stressor¹². Indeed our in vivo data show increased neovascularization in CypD KO mice following tissue wounding, but no significant vascular phenotype at baseline. Thus, CypD KO 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.

ACKNOWLEDGMENTS

We thank Seahorse Bioscience for reagents and technical instruction.

SOURCES OF FUNDING

This study was supported by R01HL093140, R01EB009862 to R.K.W., 12SDG9230006, DP2DK102258 to Y.Z., HL094536, UW RRF award to B.J.H..

DISCLOSURES

B.J.H. is currently employed by BioLife Solutions, Bothell, WA.
REFERENCES


FIGURE LEGENDS

**Figure 1.** CypD knockdown increases $[\text{Ca}^{2+}]_m$ in ECs. (A) Representative immunoblot of CypD and mitochondrial proteins MnSOD, NADH dehydrogenase iron-sulfur protein 3 (NDUFS3), Succinate dehydrogenase flavoprotein subunit (SDHA Fp), Ubiquinol-Cytochrome c reductase core protein I (CP I), Cytochrome c oxidase subunit Vb (COXVb) in HPAECs transfected with scrambled or Ppif siRNA, normalized to GAPDH. (B) FACS measurement of cellular mitochondrial content using MitoTracker Green (50 nmol/L) (mean ± SEM, n=3). (C) Form factor quantification of mitochondrial morphology for scrambled (n=189) and Ppif (n=137) siRNA HPAECs (mean ± SEM). (D) Representative confocal images of mitochondrial morphology in scrambled and Ppif siRNA HPAECs stained with MitoTracker Green (50 nmol/L) and Hoechst 33342 (10 µg/ml). (E) Mean calibrated basal $[\text{Ca}^{2+}]_m$ concentration of scrambled (n=137) and Ppif (n=12) siRNA HPAECs measured with Cameleon D3cpv sensor. (F) Microscopic analysis of intracellular $\text{Ca}^{2+}$ concentration measured with Fura-2 AM in scrambled and Ppif siRNA HPAECs in the presence of 5% FBS.

**Figure 2.** CypD regulates endothelial mitochondrial function. FACS measurement of mitochondrial membrane potential in scrambled and Ppif siRNA HPAECs stained with (A) TMRM (30 nmol/L) and (B) JC-1 (0.5 µg/µl) (mean ± SEM, n=3). (C) OCR of control, scrambled siRNA and Ppif siRNA HPAECs upon addition of oligomycin (1 µmol/L), FCCP (1 µmol/L), rotenone and antimycin (1 µmol/L) measured with Seahorse XF24 analyzer. (D) Quantification of basal OCR of control, scrambled siRNA and Ppif siRNA HPAECs in proliferating and confluent cultures (mean ± SEM, n=3). (F) Seahorse X24 ECAR measurement of control, scrambled siRNA and Ppif siRNA HPAECs upon 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, scramble siRNA and Ppif siRNA HPAECs (mean ± SEM, n=3).

**Figure 3.** Genetic targeting of 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) HPAECs at baseline and upon rotenone (5 µM) (mean ± SEM). (B) Normalized mitochondrial NAD(P)H autofluorescence in Ppif siRNA HPAECs at baseline and upon histamine (100 nmol/L) and rotenone (5 µmol/L) (mean ± SEM, n=27). (C) Spectrofluorometric measurement of calcium retention capacity of WT and CypD KO liver mitochondria (1 mg/ml, 2 mmol/L succinate, 20 µmol/L CaCl$_2$ pulses every 1.5 min) using Fura-FF as an indicator of extramitochondrial Ca$^{2+}$. (D) Total intracellular NAD+/NADH ratio of aorta and lung tissues from WT and CypD KO mice ± NMN (100 mg/kg, 1hr) normalized to the WT mice ratio values (mean ± SEM, n=6 mice/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 CsA (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).

**Figure 4.** CypD-mediated [NAD$^+/\text{NADH}$]$_{cyt}$ alterations influence SIRT1 expression. (A), (B) Western blot analysis and densitometry quantification of SIRT1 expression in lung and aorta extracts from WT and CypD KO mice (mean ± SEM, n=8 mice/group lung, n=5 mice/group aorta) normalized to GAPDH. (C), (D) Western blot analysis and densitometry quantification of SIRT1 expression in HPAECs (n=6) and HPMVECs (n=3) in which CypD was genetically targeted using siRNA and shRNA normalized to GAPDH.
Figure 5. CypD-mediated SIRT1 alterations influence PTEN expression and Akt phosphorylation.
(A) Western blot analysis and densitometry quantification of PTEN and pAkt(Ser473) expression in scrambled and Ppif siRNA HPAECs (n=6) and scrambled and Ppif shRNA HPMVECs (n=3) normalized to total Akt and GAPDH (mean ± SEM). (B) Western blot analysis and densitometry quantification of PTEN expression in scrambled Ppif siRNA HPAECs ± NMN (100 μmol/L, 48 hr) normalized to GAPDH (mean ± SEM, n=3). (C) Western blot analysis and densitometry quantification of pAkt(Ser473) expression and Akt and lung from WT and CypD KO mice normalized to total Akt (mean ± SEM, n=3). (D) Western blot analysis and densitometry quantification of PTEN and pAkt(Ser473) levels in HPAECs expressing scrambled siRNA and SIRT1 siRNA ± NMN (100 μmol/L, 48 h) normalized to GAPDH and total Akt. (E) Western blot analysis and densitometry quantification of PTEN, PTEN and pAkt(Ser473) levels in Ppif siRNA HPAECs expressing the empty vector PSG5L and Ppif siRNA HPAECs expressing PSG5L PTEN, normalized to GAPDH (mean ± SEM, n=3).

Figure 6. CypD deletion increases endothelial proliferation and angiogenesis.
(A) Doubling time of scrambled and Ppif siRNA HPAECs ± NMN (100 μmol/L, 48 hr) (mean ± SEM, n=3). (B) Western blot analysis of SIRT1 and pAkt(Ser473) levels in serum-starved scrambled and Ppif siRNA HPAECs ± VEGF (50 ng/ml, 15 min) normalized to GAPDH and total Akt. (C) Representative confocal images of scrambled and Ppif siRNA HPAECs ± VEGF (50 ng/ml) invading on collagen matrix. Cells were stained for endothelial marker CD31 (magenta), proliferation marker Ki-67 (green) and nuclei with DAPI (blue). (D) Quantification of invasion events for scrambled and Ppif siRNA HPAECs at baseline and upon VEGF (50 ng/ml) ± NMN (100 μmol/L) (mean ± SEM, n=3). (E) Confocal images of angiogenic vessel growth in WT and CypD KO aortic rings after VEGF treatment (30 ng/ml), stained with DAPI. (F), (G) Quantitation of primary sprouts and secondary branches of WT and CypD KO aortic rings at baseline and upon VEGF (30 ng/ml) ± NMN (100 μmol/L) (mean ± SEM, n=3).

Figure 7. CypD knockdown accelerates wound healing and neovascularization in vivo.
(A) Wound area progression in WT and CypD KO mice after skin excision of mouse pinnae with a biopsy punch. Measurements were normalized to the day 1 wound size (mean ± SEM, n=6/group). (B) Representative OMAG images of microvascular changes during wound healing in WT and CypD KO mice, at days 1 and 14 after wounding (structure images of pinna cross-section overlaid with 3D view of OMAG vasculature, red, and projection view images of OMAG vasculature, yellow). (C) Vessel area density in WT and CypD KO mice at baseline and wound location at 14 days post wounding calculated from projection OMAG images (mean ± SEM, n=3 mice/group). (D) Genetic or pharmacological inhibition of mPTP in ECs increases matrix Ca2+ and stimulates NADH accumulation. Simultaneously a decrease in cytosolic NAD+/NADH ratio downregulates SIRT1 and PTEN resulting in Akt activation and increased angiogenesis. NMN supplementation restores NAD+/NADH ratio and recovers the phenotype.
Novelty & Significance

What Is Known?

- Vascular endothelial cells regulate blood vessel function and endothelial dysfunction contributes to cardiovascular disease.

- Endothelial cells contain functional mitochondria but rely primarily on cytosolic glucose metabolism for their energetic requirements.

- Endothelial mitochondria can generate and release reactive oxygen species to initiate intracellular signaling.

What New Information Does This Article Contribute?

- In endothelial cells, the mitochondrial permeability transition pore (mPTP) plays an important physiological role in matrix calcium homeostasis and NAD⁺/NADH metabolism.

- Genetic inactivation of mPTP by deletion of Cyclophilin D led to an imbalance in NAD⁺/NADH metabolism, which was transmitted to the nucleus to regulate gene expression independent of reactive oxygen species.

- Endothelial cells and mice deficient in Cyclophilin D exhibit constitutive activation of pro-angiogenic Akt that enhances vessel formation in response to wounding.

Vascular endothelial cells (ECs) are critical for normal blood vessel function but their dysfunction also contribute to cardiovascular disease, diabetes, aging, and cancer. A common characteristic of dysfunctional ECs is excessive generation of mitochondrial reactive oxygen species and impaired vessel growth (angiogenesis). In the present study we examined the signaling pathways by which endothelial cell mitochondria impact angiogenesis. We found that genetic deletion of Cyclophilin D (CypD) inactivated the mitochondrial permeability transition pore (mPTP) and triggered a constitutive increase in mitochondrial calcium levels and NADH generation. As a result, CypD-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 PTEN. These genetic changes led to the constitutive activation of Akt, resulting in enhanced angiogenesis both in vitro and in vivo. These results elucidate a novel link between mitochondrial bioenergetics and the regulation of gene expression, and highlight the importance of mitochondrial metabolism in modulating endothelial-mediated vessel formation.
Figure 7

(A) Wound area (%) over time (days) for WT and KO groups. 
(B) Images of vessels at Day 1 and Day 14 for WT and KO groups. 
(C) Bar graph showing vessel area density at baseline and wound stages for WT and KO groups. 
(D) Diagram illustrating the regulation of Ca^{2+}, NADH, and various molecules including mPTP, [NAD^+/NADH]_cyl, NAD, SIRT1, PTEN, pAkt, and ANGIOGENESIS.
Mitochondrial Permeability Transition Pore Regulates Endothelial Bioenergetics and Angiogenesis
Raluca Marcu, Surya Kotha, Zhongwei Zhi, Wan Qin, Christopher K Neeley, Ruikang Wang, Ying Zheng and Brian J Hawkins

Circ Res. published online February 26, 2015;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2015/02/26/CIRCRESAHA.116.304881

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2015/02/26/CIRCRESAHA.116.304881.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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 Acrilamide 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.

\([\text{Ca}^{2+}]\) measurement
Mitochondrial \([\text{Ca}^{2+}]\) was measured using the FRET-based genetically encoded mitochondrial \(\text{Ca}^{2+}\) 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 NaHCO₃, 10 mmol/L Na-HEPES, 4.7 mmol/L KCl, 1 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 2 mmol/L CaCl₂, 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 \(R_{\text{min}}\) (in the presence of 5 µmol/L Ionomycin and 5 mmol/L EGTA) and \(R_{\text{max}}\) (in the presence of 5 µmol/L Ionomycin and 5 mmol/L CaCl₂). Mitochondrial \([\text{Ca}^{2+}]\) values were obtained by substituting measured R, \(R_{\text{min}}\), and \(R_{\text{max}}\) in the following equation, assuming \(K_d = 0.76\)
(McCombs and Palmer, 2008):

\[
[Ca^{2+}] = \frac{K_d^{0.74} (R - R_{\text{min}})}{(R - R_{\text{max}})}]
\]

Changes in cytosolic [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 µmol/L Fura-2 AM in ECM buffer supplemented with 100 µmol/L sulfipyrazone 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 µmol/L sulfipyrazone 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 µg/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyld carbocyanine iodide (JC-1, Invitrogen) and analyzed by FACS at an excitation/emission of 488/585 nm and respectively 488/530 nm (JC-1_monomer) and 488/585 nm (JC-1_aggregate). For JC-1 measurements, changes in mitochondrial membrane potential were expressed as changes in the JC-1_{aggregate}/JC-1_{monomer} fluorescence ratio. To measure mitochondrial superoxide production cells were stained with 5
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 aortas 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.
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 D3epv 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 µmol/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 µmol/L CsA) and after stimulation with histamine (100 nmol/L) (mean ± SEM, n=3). (I) Confocal microscopy measurements of the Peredox 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

An L, Qin J, and Wang RK. Ultrahigh sensitive optical microangiography for in vivo imaging of microcirculations within human skin tissue beds, *Opt Express* 2010, 18, 8220-8228.


