Mitochondrial Cyclophilin D in Vascular Oxidative Stress and Hypertension

Hana A. Itani,* Anna E. Dikalova,* William G. McMaster, Rafal R. Nazarewicz, Alfiya T. Bikineyeva, David G. Harrison, Sergey I. Dikalov

Abstract—Vascular superoxide (O$_2^-$) and inflammation contribute to hypertension. The mitochondria are an important source of O$_2^-$; however, the regulation of mitochondrial O$_2^-$ and the antihypertensive potential of targeting the mitochondria remain poorly defined. Angiotensin II and inflammatory cytokines, such as interleukin 17A and tumor necrosis factor-α (TNF-α) significantly contribute to hypertension. We hypothesized that angiotensin II and cytokines cooperatively induce cyclophilin D (CypD)-dependent mitochondrial O$_2^-$ production in hypertension. We tested whether CypD inhibition attenuates endothelial oxidative stress and reduces hypertension. CypD depletion in CypD$^{-/-}$ mice prevents overproduction of mitochondrial O$_2^-$ in angiotensin II-infused mice, attenuates hypertension by 20 mmHg, and improves vascular relaxation compared with wild-type C57Bl/6J mice. Treatment of hypertensive mice with the specific CypD inhibitor Sanglifehrin A reduces blood pressure by 28 mmHg, inhibits production of mitochondrial O$_2^-$ by 40%, and improves vascular relaxation. Angiotensin II–induced hypertension was associated with CypD redox activation by S-glutathionylation, and expression of the mitochondria-targeted H$_2$O$_2$ scavenger, catalase, abolished CypD S-glutathionylation, prevented stimulation mitochondrial O$_2^-$, and attenuated hypertension. The functional role of cytokine–angiotensin II interplay was confirmed by co-operative stimulation of mitochondrial O$_2^-$ by 3-fold in cultured endothelial cells and impairment of aortic relaxation incubated with combination of angiotensin II, interleukin 17A, and tumor necrosis factor-α which was prevented by CypD depletion or expression of mitochondria-targeted SOD2 and catalase. These data support a novel role of CypD in hypertension and demonstrate that targeting CypD decreases mitochondrial O$_2^-$, improves vascular relaxation, and reduces hypertension. (Hypertension. 2016;67:00-00. DOI: 10.1161/HYPERTENSIONAHA.115.07085.)

Key Words: angiotensin II • cyclophilin D • endothelial oxidative stress • hypertension • inflammatory cytokines • mitochondria • vasorelaxation

Hypertension is a multifactorial disorder involving perturbations of the vasculature, the kidney, and the central nervous system.1 This disease represents a major risk factor for stroke, myocardial infarction, and heart failure.2 Despite treatment with multiple drugs, 24% of hypertensive patients remain hypertensive,3 likely because of the mechanisms contributing to blood pressure elevation that are not affected by current treatments. In almost all experimental models of hypertension, production of reactive oxygen species (ROS: O$_2^-$ and H$_2$O$_2$) is increased in multiple organs.4 In the vasculature, ROS promote vasoconstriction and remodeling, thus increasing systemic vascular resistance.5 Systemic vascular resistance is increased in virtually all cases of adult hypertension,6 and thus, understanding alterations of vascular function in hypertension remains of utmost importance.

The mitochondria are an important source of O$_2^-$,7 and we have recently shown that overexpression of mitochondrial superoxide dismutase (SOD2) or scavenging of mitochondrial ROS with mitochondria-targeted antioxidants attenuate hypertension.8 Regulation of mitochondrial O$_2^-$ and therapeutic potential of targeting the mitochondria, however, is poorly defined.9

We previously reported that inhibition of mitochondrial cyclophilin D (CypD) in isolated endothelial mitochondria reduces O$_2^-$ production, and recent studies have shown that CypD deficiency reduced O$_2^-$ production in leukocytes.10,11 CypD is a regulatory subunit of the mitochondrial permeability transition pore and acts as Ca$^{2+}$ sensitizer for mitochondrial permeability transition pore opening,12 which is implicated in the regulation of cell death.13,14 CypD has been targeted for reducing ischemic heart injury and transplant rejection using the nonspecific CypD inhibitor cyclosporine A.15 However, the off-target effects of cyclosporine A paradoxically lead to increased sympathetic outflow, endothelin production, vasoconstriction, and hypertension, which is likely associated with calcineurin inhibition.16 Indeed, genetic CypD depletion did not alter the basal blood pressure.17 We therefore hypothesized that CypD contributes to O$_2^-$ overproduction in mitochondria,

Received December 31, 2015; first decision January 19, 2016; revision accepted March 8, 2016.
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This article was sent to Theodore A. Kotchen, Guest Editor, for review by expert referees, editorial decision, and final disposition.
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© 2016 American Heart Association, Inc.
Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.115.07085
vascular oxidative stress, and hypertension and that targeting CypD decreases mitochondrial $O_2^-$ and reduces hypertension. To test this hypothesis, we studied CypD-depleted human aortic endothelial cells and CypD−/− mice. We also studied redox activation of CypD and the potential interplay in this process between angiotensin II (Ang II), interleukin 17A (IL17A), and tumor necrosis factor-α (TNFα) which significantly contribute to hypertension. Finally, we examined the therapeutic potential of targeting CypD in hypertension by treatment of hypertensive mice with the CypD inhibitor Sanglifehrin A.

**Materials and Methods**

**Reagents**

Ang II, TNFα, and IL17A were purchased from Sigma (St. Louis, MO), Thermo Scientific (Rockford, IL), and Ebioscience (San Diego, CA). Mitochondria-targeted analog of dihydroethidium, fluorescent $O_2^-$ probe (MitoSOX), and dihydroethidium were supplied by Invitrogen (Grand Island, NY). CypD, SOD2, and GSH antibodies were obtained from Abcam (San Francisco, CA), mitoEbselen was purchased from Enzo Life Sciences (San Diego, CA).

**Cell Culture**

Human aortic endothelial cells (HAECs) were purchased from Lonza (Chicago, IL) and cultured in EGM-2 medium supplemented with 2% fetal bovine serum but without antibiotics. On the day before the study, the fetal bovine serum concentration was reduced to 1%. Potential interplay between Ang II, IL17A, and TNFα was tested in HAECs treated for 24 hours with Ang II (Sigma, A9525), TNFα (Thermo Scientific, 1857619), and IL17A (Ebioscience, 14–8171).

**Animal Experiments**

Hypertension was induced by Ang II infusion (0.7 mg/kg per day; Sigma A6402) in 2- to 3-month-old male mice as described previously using mice expressing mitochondria targeted catalase (mCAT; Stock# 016197), transgenic mice that overexpress mitochondrial superoxide dismutase (TgSOD2), and CypD−/− (Stock No 022308) mice which have C57Bl/6J genetic background (Jackson Laboratories). In some experiments, 6 days after saline or Ang II minipump placement, C57Bl/6J mice received a second minipump for infusion with saline as vehicle or the CypD inhibitor Sanglifehrin A (IP 10 mg/kg/d). To test the prohypertensive role of IL17A, some mice were infused with recombinant mouse IL17A (IP 1.5 μg/day; Ebioscience, 14–8171). The role of TNFα was tested by IP treatment with TNFα blocker Etanercept (IP 0.2 mg/day; Amgen Inc). Blood pressure was monitored by the telemetry and tail cuff methods as previously described.

**Superoxide Measurements Using High-Performance Liquid Chromatography**

Cells were cultured ≤80% confluence. Stock solutions of MitoSOX (4 mmol/L) and dihydroethidium (10 mmol/L) were dissolved in DMSO and were diluted in KHB buffer to a final concentration of 2 μmol/L MitoSOX and 10 μmol/L dihydroethidium. Cells loaded with dye were incubated in a tissue culture incubator for 20 minutes. Next, buffer was aspirated, and scraped cells were mixed with methanol (300 μL) and homogenized with a glass pestle. The cell homogenates were passed through a 0.22 μm syringe filter, and methanol filtrates were analyzed by high-performance liquid chromatography according to previously published protocols. Dihydroethidium and MitoSOX oxidation products, 2-hydroxyethidium and ethidium, were separated using a C-18 reverse-phase column (Nucleosil 250–4.5 mm) and a mobile phase containing 0.1% trifluoroacetic acid and an acetonitrile gradient (from 37% to 47%) at a flow rate of 0.5 mL/min. Ethidium and 2-hydroxyethidium were detected with a fluorescence detector using an emission wavelength of 580 nm and an excitation of 480 nm. Production of cytoplasmic and mitochondrial $O_2^-$ was measured as accumulation of 2-hydroxyethidium and mito-2-hydroxyethidium in dihydroethidium or mitSOX supplemented samples as described previously.

**Nitric Oxide Measurements by Electron Spin Resonance**

$NO^-$ levels in endothelial cells and vessels were quantified by electron spin resonance and colloid Fe(IV)TC, as described previously. Electron spin resonance spectra were recorded by EMX electron spin resonance spectrometer (Bruker Biospin Corp, Billerica, MA) with super high Q microwave cavity using suprasil nitrogen Dewar flask (Wilmad-Labglass, Vineland, NJ). The electron spin resonance settings for NO measurements were as follows: field sweep, 100 Gauss; microwave frequency, 9.43 GHz; microwave power, 10 milliwatts; modulation amplitude, 2 Gauss; conversion time, 70 ms; time constant, 5.24 s; scan number, 4.

**Vascular Relaxation Study**

Isometric tension studies were performed on 2 mm mouse aortic rings dissected free of perivascular fat from C57Bl/6J, mCAT, TgSOD2, and CypD−/− mice (Jackson Laboratory). Studies were performed in a horizontal wire myograph (DMT, Aarhus, Denmark; models 610 and 620 mol/L) containing physiological salt solution with the composition of 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgSO4, 1.2 mmol/L KH2PO4, 25 mmol/L NaHCO3, 11 mmol/L glucose, and 1.8 mmol/L CaCl2. The isometric tone of each vessel was recorded using LabChart Pro v7.3.7 (AD Instruments, Australia). The aortic rings were equilibrated over a 2-hour period by heating and stretching the vessels to an optimal baseline tension of 36 millinewtons before contracting them with 3 cycles of 60 mmol/L KCl physiological saline solution. Endothelium-dependent and independent vascular relaxation was tested after preconstriction with 1 μM phenylephrine. Once the vessels reached a steady state contraction, increasing concentrations of acetylcholine were administered, and the response to each concentration of drug was recorded.

**Statistics**

Experiments were analyzed using the Student–Neuman–Keuls post hoc test and analysis of variance (ANOVA). $P$ levels <0.05 were considered significant.

**Results**

**Effect of CypD Depletion on Angiotensin II–Induced Hypertension, Vascular $O_2^-$, and Vasodilation**

In initial studies, we sought to determine whether CypD deletion in the CypD−/− mice decreases vascular oxidative stress, improves vascular relaxation, and attenuates Ang II–induced hypertension compared with wild-type C57Bl/6J mice. Indeed, CypD−/− mice infused with Ang II (0.7 mg/kg per day) had lower blood pressure compared with wild-type mice while basal blood pressure was not different (Figure 1A). After 14 days of Ang II infusion, mice were euthanized and aortas were isolated for the measurement of mitochondrial $O_2^-$ and the analysis of vascular relaxation. As expected, CypD deficiency prevented overproduction of mitochondrial $O_2^-$ in Ang II infused mice (Figure 1B) and improved endothelium-dependent and endothelium-independent relaxation (Figure 1C and 1D) compared with Ang II–infused wild-type mice.

**Therapeutic Potential of Targeting CypD After Onset of Hypertension**

Based on the above results with genetic depletion of CypD, we tested whether the specific CypD inhibitor Sanglifehrin
A can lower blood pressure, reduce mitochondrial \( \text{O}_2^- \), and improve vascular relaxation. To accomplish this, we implanted wild-type mice with osmotic minipump containing Ang II and started treatment with Sanglifehrin A after the onset of Ang II–induced hypertension (Figure 2A). Indeed, treatment of hypertensive mice with CypD inhibitor Sanglifehrin A reduced blood pressure (Figure 2A), normalized mitochondrial \( \text{O}_2^- \) production (Figure 2B), and improved endothelium-dependent and endothelium-independent relaxation (Figure 2C and 2D). These data support the therapeutic potential of targeting CypD in hypertension.

**Role of CypD S-Glutathionylation in Stimulation of Mitochondrial \( \text{O}_2^- \) and Hypertension**

CypD is exquisitely \( \text{H}_2\text{O}_2 \)-sensitive via its cysteine 203 residue, which acts as a redox switch when it is S-glutathionylated. \( \text{H}_2\text{O}_2 \) activates CypD by S-glutathionylation, and this induces overproduction of mitochondrial \( \text{O}_2^- \) in the electron transport chain. The potential role of mitochondrial \( \text{H}_2\text{O}_2 \) in hypertension, CypD S-glutathionylation, and stimulation of mitochondrial \( \text{O}_2^- \) was studied in transgenic mCAT and aortic vessels treated with \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{mol/L} \), Krebs–Hepes buffer, 60 minutes at \( 37^\circ \text{C} \)) or treated with the specific CypD inhibitor Sanglifehrin A (1 \( \mu \text{mol/L} \)) or treatment with the complex I inhibitor rotenone blocked \( \text{H}_2\text{O}_2 \)-induced mitochondrial \( \text{O}_2^- \) production (Figure 3A). Scavenging mitochondrial \( \text{H}_2\text{O}_2 \) using mitoEbselen or mitochondrial-targeted catalase in aorta isolated from mCAT mice completely prevented CypD S-glutathionylation and reduced mitochondrial \( \text{O}_2^- \).

A potential role of CypD S-glutathionylation in hypertension was studied in Ang II–infused mCAT and wild-type mice. Ang II–induced hypertension was inhibited in mCAT mice compared with C57Bl/6J wild-type mice (Figure 3C). After 14 days of Ang II infusion, mice were euthanized and we analyzed CypD S-glutathionylation, SOD2 expression, and mitochondrial \( \text{O}_2^- \) production. Western blot analysis revealed significant CypD S-glutathionylation in tissue isolated from wild-type mice infused with Ang II, while expression of mitochondrial-targeted \( \text{H}_2\text{O}_2 \) scavenger catalase in mCAT mice significantly attenuated CypD S-glutathionylation (Figure 3D). Furthermore, production of mitochondrial \( \text{O}_2^- \) was substantially increased in aorta from Ang II–infused wild-type mice but not in aorta from Ang II–infused mCAT mice (Figure 3E). Of note, Western blot showed no change in mitochondrial superoxide dismutase level between wild-type and mCAT mice; therefore, the lack of mitochondrial \( \text{O}_2^- \) overproduction in mCAT mice is not because of elevated SOD2 level (Figure 3D). These data support a potential role of CypD S-glutathionylation in stimulation of mitochondrial \( \text{O}_2^- \) and hypertension.

**Interplay Between Angiotensin II and Inflammatory Cytokines IL17A and TNFα in Hypertension**

Given that Ang II and inflammatory cytokines IL17A and TNFα increase vascular ROS, we hypothesized that Ang II, IL17A, and TNFα may co-operatively activate...
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redox-dependent CypD and increase mitochondrial O$_2^-$ production. To test this hypothesis, we infused mice with IL17A or IL17A+TNFα blocker, Etanercept (Amgen Inc). IL17A increased blood pressure to 133 mm Hg and vascular mitochondrial O$_2^-$, whereas Etanercept attenuated hypertensive response to IL17A and inhibited IL17A-induced mitochondrial O$_2^-$ (Figure 4A and 4B). These data support the important role of IL17A and TNFα in the stimulation of mitochondrial O$_2^-$ and hypertension.

In additional experiments, we tested the potential interplay between Ang II and inflammatory cytokines in hypertension in mice infused with low dose of Ang II (0.3 mg/kg per day) and IL17A (1 µg/day). In wild-type mice, infusion of low doses of either Ang II or IL17A caused small increases of blood pressure, but combined treatment with IL17A and low dose Ang II co-operatively induced severe hypertension. SOD2 overexpression abrogated this hypertensive response to Ang II and IL17A (Figure 4C). These data support an important role of the interplay between Ang II and cytokines in stimulation of mitochondrial O$_2^-$ in hypertension.

Co-Operative Stimulation of CypD-Dependent Mitochondrial O$_2^-$ by Angiotensin II, IL17A, and TNFα

The above studies in intact mice demonstrate that Ang II and inflammatory cytokines act in concert to increase blood pressure and that this is likely mediated by mitochondrial O$_2^-$ (Figure 4). We therefore hypothesized that Ang II, IL17A, and TNFα co-operatively induce CypD-dependent production of mitochondrial O$_2^-$ in human cells, we studied mitochondrial O$_2^-$ in HAECs treated with low doses of Ang II, IL17A, TNFα either separately or in combination using high-performance liquid chromatography analysis of O$_2^-$-specific MitoSOX product 2-OH-Mito-E+ (Figure 5A, inset). Treatment of endothelial cells with Ang II and cytokines induced mitochondrial O$_2^-$ in a dose-dependent manner. The lowest concentration of TNFα that increased mitochondrial O$_2^-$ was 1 nmol/L, whereas Ang II and IL17A required 10 nmol/L. We then tested whether the combination of these low doses of Ang II, IL17A, and TNFα co-operatively increased mitochondrial O$_2^-$ production. We found that the combinations of Ang II+IL17A, Ang II+TNFα, and IL17A+TNFα modestly increased mitochondrial O$_2^-$ above the basal level; however, the triple combination of Ang II, IL17A, and TNFα co-operatively increased mitochondrial O$_2^-$ above maximal levels compared with each single stimulation (Figure 5A).

To directly confirm a role of CypD in stimulation of mitochondrial O$_2^-$ in human cells, we used siRNA against CypD. Transfection of HAECs with CypD siRNA reduced CypD expression and abolished the stimulation of mitochondrial O$_2^-$ (Figure 5B). These data support the interplay between Ang II and cytokines in the stimulation of CypD-dependent mitochondrial O$_2^-$ overproduction and show that CypD has...
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a role in the production of mitochondrial $O_2^-$ by human endothelial cells.

Role of CypD and Mitochondrial ROS in Impaired Vasodilation by Angiotensin II, IL17A, and TNFα

We have previously shown that Ang II stimulates cytokine production and that Ang II causes blunted hypertension in RAG1 KO mice lacking lymphocytes, mice deficient in the cytokine IL17, or in mice cotreated with the TNFα antagonist Etanercept. We hypothesized that inflammatory cytokines, such as TNFα and IL17A, act in concert with Ang II to stimulate $O_2^-$ production in vessels, leading to inactivation of endothelial nitric oxide and impaired vascular relaxation. Indeed, ex vivo incubation of aorta with Ang II did not significantly affect aortic $O_2^-$ or NO levels, whereas the combination of Ang II, IL17A, and TNFα caused a 2-fold increase in $O_2^-$ and a 3-fold reduction of NO (Figure 6). We directly tested the role of mitochondrial $O_2^-$ in cytokine-induced vascular oxidative stress and impaired NO production using TgSOD2. SOD2 overexpression completely abolished vascular oxidative stress and prevented loss of endothelial NO (Figure 6).

We further examined the functional role of mitochondrial CypD-dependent oxidative stress in response to Ang II, IL17A, and TNFα in aortic sections isolated from CypD−/− mice and compared them to wild-type littermates (C57Bl/6J). As we expected, treatment of aortic vessels with Ang II+IL17A+TNFα significantly increased production of mitochondrial $O_2^-$ and impaired endothelium-dependent relaxation which were prevented by CypD deletion (Figure 7A and 7B).

We have examined the functional role of CypD-dependent oxidative stress in aortic sections isolated from TgSOD2 or catalase (mCAT). Our data show that treatment of aortic vessels with Ang II+IL17A+TNFα lead to severe impairment of endothelium-dependent relaxation. Interestingly, SOD2 overexpression or expression of mitochondria-targeted catalase significantly attenuated the impairment of relaxation similar to the protection afforded by CypD deletion (Figure 7B–7D).

These data show that the pro-oxidant milieu of Ang II, IL17A, and TNFα leads to severe vascular oxidative stress, reduces endothelial NO, and impairs vascular relaxation, which is prevented by the scavenging of mitochondrial $O_2^-$ and $H_2O_2$ in vessels from TgSOD2 and mCAT mice. Furthermore, our data confirm an important role of CypD in the regulation of vascular oxidative stress.
The present study provides the first evidence that CypD facilitates production of mitochondrial $\text{O}_2^-$ by intact vessels and in cultured human endothelial cells. Our data show that inflammatory cytokines and Ang II co-operatively induce CypD-dependent mitochondrial $\text{O}_2^-$ in HAECs and aortic vessels (Figures 5 and 7). In this work, we found that CypD depletion diminished vascular production of mitochondrial $\text{O}_2^-$, improved relaxation, and attenuated the development of hypertension in Ang II–infused mice (Figure 1). Furthermore, treatment of hypertensive mice with CypD inhibitor Sanglifehrin A diminished vascular oxidative stress and reduced blood pressure (Figure 2). These data suggest that CypD has a previously unidentified role in hypertension and support the therapeutic potential of targeting CypD in this disease.

CypD-mediated overproduction of mitochondrial ROS contributes to impaired vascular relaxation and overexpression of mitochondrial SOD2 and catalase protect endothelium-dependent relaxation (Figure 7). These data support an important role of mitochondrial oxidative stress in impairment of vascular relaxation. We would stress that systemic vascular resistance is increased in virtually all cases of adult hypertension, and thus understanding alterations of vascular function in hypertension is important. We propose that CypD can also contribute to other vascular conditions associated with oxidative stress, such as atherosclerosis.

It should be noted that resistance arteries contribute more significantly to blood pressure changes than large arteries, such as aorta; however, hypertension is associated with impaired vascular relaxation both in resistance and conduit arteries. In our study, we used aorta because it provides sufficient material from a single mouse to perform several critical measurements: high-performance liquid chromatography analysis of $\text{O}_2^-$-specific products 2-OH-Mito-E + and 2HO-E+;32 specific EPR analysis of endothelial NO; 27 and vascular relaxation. The mechanisms of impaired vasodilatation may differ in resistance and large arteries; therefore, future studies have to confirm that relaxation of resistance vessels can be rescued by CypD inhibition or depletion. Our data showed that CypD inhibition improved endothelium-dependent and endothelium-independent relaxation (Figure 2B), implicating CypD in endothelial and smooth cells dysfunction. The cell-specific role of CypD in vascular impairment; however, remain unclear.

We have previously shown that Ang II stimulates the production of mitochondrial $\text{O}_2^-$ in endothelial cells which is blocked by nonspecific CypD inhibitor, cyclosporine A, or depletion of NADPH oxidase.9,11 In this work, we further examined the mechanisms of mitochondrial $\text{O}_2^-$ overproduction and found that stimulation of mitochondrial $\text{O}_2^-$ in mice and isolated aortic vessels is associated with CypD S-glutathionylation and scavenging of mitochondrial $\text{H}_2\text{O}_2$. 

Figure 4. Angiotensin II (Ang II)–cytokine interplay in hypertension. Blood pressure (A) and aortic mitochondrial $\text{O}_2^-$ (B) in C57Bl/6J mice infused with interleukin 17A (IL17A) (1.5 μg/day) and Etanercept (ETN, IP 0.2 mg/day). *P<0.05 vs Sham, **P<0.01 vs Ang II (N=8). C, Tail-cuff blood pressure measurements in C57Bl/6J and Tg SOD2 mice infused for 7 days with Ang II (0.8 mg/kg per day), IL17A (1 μg/day) or both. *P<0.05 vs Sham, **P<0.001 vs Ang II (N=6). Tg SOD2 indicates transgenic mice that overexpress mitochondrial superoxide dismutase.

Discussion

The present study provides the first evidence that CypD facilitates production of mitochondrial $\text{O}_2^-$ by intact vessels and in cultured human endothelial cells. Our data show that inflammatory cytokines and Ang II co-operatively induce CypD-dependent mitochondrial $\text{O}_2^-$ in HAECs and aortic vessels (Figures 5 and 7). In this work, we found that CypD depletion diminished vascular production of mitochondrial $\text{O}_2^-$, improved relaxation, and attenuated the development of hypertension in Ang II–infused mice (Figure 1). Furthermore, treatment of hypertensive mice with CypD inhibitor Sanglifehrin A diminished vascular oxidative stress and reduced blood pressure (Figure 2). These data suggest that CypD has a previously unidentified role in hypertension and support the therapeutic potential of targeting CypD in this disease.

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by mitoEbselen, or mitochondria-targeted catalase prevents CypD S-glutathionylation and diminishes CypD-dependent production of mitochondrial $O_2^\cdot$ (Figures 3, 5, and 7). These data implicate a redox-sensitive activation of CypD in regulation of vascular mitochondrial $O_2^\cdot$.

Inflammation is commonly associated with hypertension and contributes to pathogenesis of this disease. $1,3,5$ TNF$\alpha$ and IL17 are increased in hypertensive subjects by 4-fold, $18,19$ and TNF$\alpha$ is an independent risk factor for hypertension. $18$ Our previous animal studies showed an important role of T cell activation in IL17 and TNF$\alpha$ production in hypertension; $19,34$ however, specific targets of cytokines in hypertension are not clear. It is understood that multiple cytokines interact to promote other established immune and inflammation-based diseases; however, the potential role of cytokine–angiotensin II interplay in hypertension has not been studied. In this work, we have shown that Ang II, TNF$\alpha$, and IL17A co-operatively induced CypD-dependent overproduction of mitochondrial ROS, which contributes to impaired vasodilatation in hypertension.

Previous studies have shown that CypD deficiency and Sanglifehrin A therapy reduced ROS production in leukocytes of Ang II–infused mice. $12$ It was suggested that ROS overproduction by phagocytic cells contributes to vascular dysfunction and that blockade of CypD diminishes ROS production by leukocytes and reduces oxidative stress. This work adds to the previous studies in showing an important role of this mitochondrial protein in endothelial cells and its role in intact

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Figure 5. Production of mitochondrial $O_2^\cdot$ in human aortic endothelial cells (HAECs). A, HAECs were treated with angiotensin II (Ang II), interleukin 17A (IL17A), and tumor necrosis factor-$\alpha$ (TNF$\alpha$) (1–100 ng/mL) or their combinations for 24 hours before measurements of mitochondrial $O_2^\cdot$ by MitoSOX and high-performance liquid chromatography (HPLC; A). *$P<0.05$ vs control, **$P<0.01$ vs control, ***$P<0.001$ vs double treatments (N=4–6). B, Measurements of mitochondrial $O_2^\cdot$ in cyclophilin D (CypD)–depleted cells. HAECs were transfected with nonsilencing (NS) or CypD siRNA before stimulation with Ang II (10 nmol/L), IL17A (10 ng/mL), and TNF$\alpha$ (1 ng/mL). CypD depletion was confirmed by Western blot analysis (inset). *$P<0.05$ vs Control, **$P<0.01$ vs Ang II+IL17A+TNF$\alpha$ (ATI; N=6).

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Figure 6. Angiotensin II (Ang II)–cytokine interplay in vascular oxidative stress. in C57Bl/6J (WT) or TgSOD2 mouse aorta isolated 3 mm aortic segments were placed in Dulbecco’s Modified Eagle Medium tissue culture and treated ex vivo with Ang II (10 nmol/L) or Ang II + IL17A (10 ng/mL) + TNF$\alpha$ (1 ng/mL) for 24 hours. After incubation, aortic segments were used for $O_2^\cdot$ and nitric oxide analysis. A, Vascular $O_2^\cdot$ was measured by dihydroethidium (DHE) probe and high-performance liquid chromatography (HPLC). $8$ *$P<0.05$ vs control, **$P<0.01$ vs Ang II+ATI (N=6).

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vessels. In this work, we have investigated CypD-dependent stimulation of mitochondrial $\text{O}_2^-$ in endothelial cells and intact aortic vessels. We defined a novel role of inflammatory cytokines in redox-dependent CypD activation and directly demonstrated the role of vascular mitochondrial $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ in impaired endothelium-dependent relaxation in response to Ang II, TNF-$\alpha$, and IL17A using vessels isolated from transgenic mice overexpressing mitochondrial SOD2 and catalase (Figure 7). This work therefore provides direct evidence for CypD-mediated endothelial dysfunction.

The precise molecular mechanism of CypD-dependent stimulation of mitochondrial $\text{O}_2^-$ in endothelial cells is not clear. It likely occurs at mitochondrial complex I because treatment with complex I inhibitor rotenone significantly inhibited CypD-dependent mitochondrial $\text{O}_2^-$ production (Figure 3A). We have previously shown an important role of reverse electron transport and complex I in mitochondrial $\text{O}_2^-$ overproduction and hypertension, and now we found that CypD depletion prevents stimulation of mitochondrial $\text{O}_2^-$ in response to Ang II, TNF-$\alpha$, and IL17A.

It is understood that multiple cytokines interact to promote other established immune and inflammation-based diseases; however, the potential role of cytokine–angiotensin II interplay in hypertension has not been studied. Inflammation is commonly associated with hypertension and contributes to the pathogenesis of this disease. TNF-$\alpha$ and IL17 are increased in hypertensive subjects by 4-fold, and TNF-$\alpha$ is an independent risk factor for hypertension. In this work, we have shown that Ang II, TNF-$\alpha$, and IL17A co-operatively induced CypD-dependent overproduction of mitochondrial ROS which contributes impaired vasodilatation in hypertension.

Previous studies showed an important role of cytokines in vascular dysfunction and hypertension both in Ang II– and salt-induced hypertension models. In this work, we show that Ang II alone did not increase $\text{O}_2^-$ or decrease NO in cultured aortic tissue, yet Ang II alone caused a modest blood pressure elevation to the prehypertensive level of 130 mm Hg likely because of increased water intake (thirst) and volume retention directly regulated by Ang II. The increased shear stress and salt accumulation subsequently causes local inflammation and mitochondrial ROS overproduction, leading to end-organ damage, such as endothelial dysfunction which drives the development of hypertension. Our data showed that CypD inhibition reduced endothelial dysfunction in response to inflammatory cytokines; therefore, therapeutic targeting of CypD can be potentially beneficial in reducing end-organ damage in hypertension.

In addition to hypertension, there are many other common conditions, including aging, atherosclerosis, diabetes mellitus,

Figure 7. Role of cyclophilin D (CypD) and mitochondrial reactive oxygen species (ROS) in impaired relaxation in vessels treated with angiotensin II (Ang II), interleukin 17A (IL17A), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$). Aortas were isolated from wild-type, CypD$^{-/-}$, mCAT, or TgSOD2 mice and were placed in tissue culture with Dulbecco’s Modified Eagle Medium for 24 hours. Aortas were supplemented with vehicle or combination of Ang II+TNF-$\alpha$+IL17A (ATI). Mitochondrial $\text{O}_2^-$ (A) and endothelium-dependent relaxation (B–D) in vessels treated with combination of Ang II (10 nmol/L), IL17A (10 ng/mL), and TNF-$\alpha$ (1 ng/mL) for 24 hours. Mitochondrial $\text{O}_2^-$ was measured by MitoSOX and HPLC. Results are mean±SEM (n=8). *P<0.05 vs WT, **P<0.01 vs WT+ATI. mCAT indicates mice expressing mitochondria targeted catalase; MitoSOX, mitochondria-targeted analog of dihydroethidium, fluorescent $\text{O}_2^-$ probe; and TgSOD2, transgenic mice that overexpress mitochondrial superoxide dismutase.
and degenerative neurological disorders, in which mitochondrial oxidative stress seems to play a role.\(^\text{42,43}\) Of note, large clinical trials have failed to show a benefit of often-used antioxidants, such as vitamin E and vitamin C, in many of these conditions\(^\text{44,45}\) and have paradoxically shown deleterious effects in some trials.\(^\text{46}\) There are many potential explanations why these antioxidants have proven ineffective in these studies, but one relates to the fact that agents, such as vitamin E and vitamin C, are not targeted to sites of ROS generation that are most important in pathological conditions. It is conceivable that CypD inhibitors or mitochondria-targeted antioxidants would be more effective in these conditions. The ability to achieve these effects in relatively low doses might also limit potential untoward effects of antioxidant therapy observed with other agents.

### Perspectives

The present studies show that CypD plays an important role in mitochondrial O\(_\text{2}^-\) overproduction in endothelial cells and vasculature in response to inflammatory cytokines and Ang II. These studies revise the traditional concept that CypD mainly acts as Ca\(^{2+}\) sensitizer for mitochondrial permeability transition pore opening, leading to mitochondrial swelling and cell death. Our studies demonstrate that Ang II, TNFα, and IL17A co-operatively induced CypD-dependent mitochondrial O\(_\text{2}^-\) production, implicating CypD in development of vascular oxidative stress, impaired vascular relaxation and hypertension. Our data suggest that redox activation of CypD by S-glutathionylation stimulates and IL17A co-operatively induced CypD-dependent mitochondrial oxidative stress. Int J Mol Sci. 2015;16:823–839. doi: 10.3390/ijms16010823.


### Acknowledgments

We thank Dr Richard Sedrani (Novartis Pharma AG) and Dr Andreas Daiber (Mainz University Medical Center) for providing CypD inhibitor Sanglifehrin A for our animal studies. We thank AMGEN Inc for providing TNFα blocker Etanercept for our work.

### Sources of Funding

This work was supported by funding from National Institute of Health (R01HL124116-01A1), Vanderbilt University (VR7040 and UL1 RR024975-01), and the American Heart Association (15RZ22730049). Dr Itani was funded by the National Institute of Health (1F32HL124972-01).

### Disclosures

None.

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Hypertension. published online April 11, 2016;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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