

**Mitochondrial Isolevuglandins Contribute to Vascular Oxidative Stress and Mitochondria-
Targeted Scavenger of Isolevuglandins Reduces Mitochondrial Dysfunction and Hypertension**

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Short title: Mitochondrial Isolevuglandins Contribute to Hypertension

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Abstract

Hypertension remains a major health problem in Western Societies, and blood pressure is poorly controlled in a third of patients despite use of multiple drugs. Mitochondrial dysfunction contributes to hypertension and mitochondria-targeted agents can potentially improve treatment of hypertension. We have proposed that mitochondrial oxidative stress produces reactive dicarbonyl lipid peroxidation products isolevuglandins (isoLGs) and that scavenging of mitochondrial isoLG improves vascular function and reduces hypertension. To test this hypothesis, we have studied the accumulation of mitochondrial isoLGs-protein adducts in patients with essential hypertension and angiotensin II model of hypertension using mass spectrometry and Western blot analysis. The therapeutic potential of targeting mitochondrial isoLG was tested by the novel mitochondria-targeted isoLG scavenger, mito2HOBA. Mitochondrial isoLGs in arterioles from hypertensive patients were 250% greater than in arterioles from normotensive subjects, and ex vivo mito2HOBA treatment of arterioles from hypertensive subjects increased deacetylation of a key mitochondrial antioxidant, superoxide dismutase 2 (SOD2). In human aortic endothelial cells stimulated with angiotensin II plus TNF α , mito2HOBA reduced mitochondrial superoxide and cardiolipin oxidation, a specific marker of mitochondrial oxidative stress. In angiotensin II-infused mice, mito2HOBA diminished mitochondrial isoLGs-protein adducts, raised Sirt3 mitochondrial deacetylase activity, reduced vascular superoxide, increased endothelial nitric oxide, improved endothelium-dependent relaxation, and attenuated hypertension. Mito2HOBA preserved mitochondrial respiration, protected ATP production, and reduced mitochondrial permeability pore opening in angiotensin II-infused mice. These data support the role of mitochondrial isoLGs in endothelial dysfunction and hypertension. We conclude that scavenging of mitochondrial isoLGs may have therapeutic potential in treatment of vascular dysfunction and hypertension.

Keywords: hypertension; mitochondria; isolevuglandins; superoxide dismutase 2, Sirtuin 3.

Introduction

By recent guidelines, almost one-half of adults have hypertension, and an estimated 1.4 billion people have hypertension worldwide.^{1, 2} This disease represents a major risk factor for stroke, myocardial infarction, and heart failure.³ Despite treatment with multiple drugs, a third of hypertensive patients remain hypertensive,⁴ likely due to the mechanisms that are not affected by current treatments. New classes of antihypertensive agents could therefore improve treatment of hypertension. Hypertension is a multifactorial disorder⁵ and oxidative stress is increased in multiple organs in hypertension. Oxidative stress contributes to hypertension by increasing sympathetic outflow,^{6, 7} promoting kidney dysfunction,⁸ and increasing systemic vascular resistance.⁹ Meanwhile, common antioxidants, like ascorbate and vitamin E, are ineffective in the treatment of cardiovascular diseases and hypertension, and in some studies have worsen the outcome.^{10, 11} Intrinsic enzymatic antioxidants are much more effective against oxidative stress compared with low molecular weight antioxidants, but these intrinsic antioxidants can be inactivated in hypertension. Essential hypertension is associated with inactivation of a key mitochondrial antioxidant, superoxide dismutase 2 (SOD2), by acetylation of lysine residues at the catalytic center due to reduced activity of mitochondrial deacetylase sirtuin 3 (Sirt3),^{12, 13} however the precise mechanism of Sirt3 inactivation and molecular consequences of SOD2 inhibition are not known.

One potential mechanism involves lipid peroxidation, particularly the formation of mitochondrial isolevuglandins (isoLGs). IsoLGs are highly reactive and harmful dicarbonyl lipid peroxidation products.¹⁴ They are produced by peroxidation of arachidonic acid by oxidizing species such as the protonated form of superoxide, the hydroperoxyl radical.¹⁵ IsoLGs rapidly adduct to primary amines such as protein lysine residues¹⁶ promoting cell dysfunction. In dendritic cells, isoLGs promote modification of self-proteins, which can act as neoantigens driving an adaptive immune response.¹⁷ Treatment with the isoLG scavenger, 2-hydroxybenzylamine (2HOBA), reduces dendritic and T cell activation and attenuates angiotensin II- and DOCA-salt induced hypertension. Of note, 2HOBA is not an antioxidant but it reduces superoxide production in dendritic cells by scavenging the reactive isoLGs and decreasing the dendritic cells activation.¹⁷ The specific sources and the targets of isoLGs, however, remain elusive. The pathophysiological role of isoLGs is not limited to

dendritic cells as isoLGs can be produced in vascular tissue, endothelial, epithelial, and other cells.¹⁷⁻¹⁹ Hypertension is associated with mitochondrial oxidative stress,²⁰ and the mitochondria can be a potential source of isoLGs, but the role of mitochondrial isoLGs in hypertension has not been studied. We suggest that mitochondrial isoLGs may contribute to Sirt3 inactivation and mitochondrial dysfunction in hypertension.

Treatment of isolated mitochondria with bolus isoLGs disrupts mitochondrial respiration and promotes mitochondrial permeability transition pore (mPTP) opening.^{21, 22} To study the specific role of intra-mitochondrial isoLGs *in vivo*, we have developed the mitochondria-targeted isoLG scavenger mito2HOBA by conjugating 2HOBA to the lipophilic cation triphenylphosphonium.²² Mito2HOBA does not scavenge the reactive oxygen species.¹⁷ It can potentially react with different gamma-ketoaldehydes,²³ but it is particularly reactive with isoLGs.²² Mito2HOBA accumulates in mitochondria and mito2HOBA supplementation in a lipopolysaccharide mouse model of sepsis increases animal survival by 3-fold, increases complex I-mediated respiration, and prevents renal cortical injury²² supporting the role of mitochondrial isoLGs in mitochondrial dysfunction.

Mitochondrial dysfunction contributes to the pathogenesis of hypertension and cardiovascular disease;^{24, 25} however, despite the central role of mitochondria in human health and disease, there are no approved drugs that directly target mitochondria.²⁶ Mitochondrial dysfunction is characterized by reduced ATP levels and increased oxidative stress leading to cell dysfunction and apoptosis.²⁷ Opening of the mitochondrial permeability transition pore (mPTP) plays a key role in mitochondrial dysfunction²⁸ and end-organ-damage in hypertension.^{20, 27} We discovered that depletion or inhibition of Cyclophilin D (CypD), a regulatory subunit of mPTP opening,²⁹ improves vascular function and attenuates hypertension.³⁰ Interestingly, CypD acetylation at lysine 166 promotes mPTP opening and mitochondrial Sirt3 deacetylates CypD-K166.³¹ The specific alterations of CypD in hypertension and the potential role of CypD acetylation in this disease remains unclear.

Sirt3 is key node in regulation of metabolic and antioxidant mitochondrial functions.³² Sirt3 depletion promotes endothelial dysfunction, vascular hypertrophy, vascular inflammation and end-organ damage.¹³ Clinical studies show that cardiovascular disease risk factors are associated with reduced Sirt3 level and activity.^{33, 34} We propose Sirt3 impairment as a new convergent mechanism underlying the interplay of major cardiovascular

risk factors. We hypothesized that mitochondrial isoLGs inactivate Sirt3, and scavenging of mitochondrial isoLGs protects Sirt3 activity, improves vascular function and reduces hypertension.

To test the role of mitochondrial isoLGs in hypertension, we studied the accumulation of mitochondrial isoLG-protein adducts in normotensive and hypertensive human subjects and in angiotensin II mouse model of hypertension using mass spectrometry and Western blot analysis. The therapeutic potential of targeting mitochondrial isoLGs was tested by novel mitochondria-targeted isoLG scavenger mito2HOBA.²² The results of our study showed substantial accumulation of mitochondrial isoLG protein adducts in vascular and kidney tissues in hypertension. Furthermore, mito2HOBA treatment of arterioles from hypertensive subjects increases SOD2 deacetylation and reduces mitochondrial superoxide in human aortic endothelial cells. In mice, mito2HOBA prevents accumulation of mitochondrial isoLG-protein adducts, reduces acetylation of SOD2 and CypD, protects mitochondrial respiration, preserves ATP production, blocks mitochondrial permeability pore opening, reduces vascular superoxide, protects endothelial NO, improves endothelium-dependent relaxation, and attenuates hypertension. These data support the hypothesis that mitochondrial isoLGs promote mitochondrial and endothelial dysfunction and scavenging of mitochondrial isoLGs may have therapeutic potential in treatment of vascular dysfunction and hypertension.

Materials and Methods

The authors declare that all supporting data are available within the article and its online supplementary files. All methods have the corresponding literature reference. Additional protocol information is available from the corresponding author upon reasonable request.

Reagents

Dihydroethidium (DHE) and MitoSOX superoxide probes were supplied by Invitrogen (Grand Island, NY). Sirt3 (54905) antibodies were from Cell Signaling. Acetyl-K68-SOD2 (ab137037), complex I NDUFS1 75 kDa subunit (ab22094) and CypD (ab110324) and GAPDH (ab8245) antibodies were from Abcam. SOD2 (sc30080) antibodies were obtained from Santa Cruz Biotechnology. Acetyl-lysine antibodies (ab3839) were provided by Millipore-Sigma. D11, an isoLG-lysyl adducts-specific scFv antibody, has been previously

characterized.³⁵ All antibodies were used at 1000-fold dilution. 2-Hydroxybenzylamine (2HOBA), mitochondria-targeted isoLG scavenger mito2HOBA and isoLG inactive analog 4-hydroxybenzylamine (4HOBA) were synthesized as described previously.^{22, 36, 37} All other reagents were obtained from Sigma (St Louis, MO).

Animal experiments

Hypertension was induced by angiotensin II (0.7 mg/kg/min) as described previously.³⁸ To test the therapeutic potential of scavenging of mitochondrial isoLGs, wild-type C57Bl/6J male and female mice (Jackson Labs) received saline or angiotensin II minipump placement, and provided with the plain water (vehicle) or mito2HOBA in the drinking water (0.1 g/liter). Blood pressure was monitored by the telemetry and tail-cuff measurements as previously described.^{39, 40} The Vanderbilt Institutional Animal Care and Use Committee approved the procedures (Protocol M1700207). Simple randomization was used to select animals for sham, angiotensin II or mito2HOBA groups for equal chance of being allocated to treatment groups.

Kidney mitochondria isolation

C57Bl/6J mice were sacrificed by carbon dioxide and kidneys removed, cleaned from fat tissue and placed in the ice-slurry cold isolation medium. In a cold room, kidneys were minced, washed with the isolation medium and homogenized using a Polytron disintegrator with two pulses of 2 seconds each. Homogenate was diluted 7-fold (w/v) and mitochondria were isolated by differential centrifugation and purified with Percoll gradient.⁴¹ Isolation medium contained 75 mM mannitol, 175 mM sucrose, 20 mM MOPS, pH 7.2, 1 mM EGTA. Mitochondrial protein concentration was measured by Bradford method.

Measurements of mitochondrial isoLGs by Mass Spectrometry

IsoLG-Lysyl-Lactam adducts were measured in mitochondria isolated from the kidneys of sham or angiotensin II-infused mice using Mass Spectrometry. Mitochondrial proteins were subjected to complete enzymatic digestion to individual amino acids.⁴² A [¹³C₆] internal standard was added and the isoLG-lysyl adducts were purified by solid phase extraction and HPLC before being quantified by Liquid Chromatography-tandem Mass Spectrometry assay (LC/ESI/MS/MS) using isotopic dilution as described previously.⁴³

Measurements of cardiolipin oxidation

Cardiolipin oxidation in human aortic endothelial cells was measured by Liquid Chromatography-Mass Spectrometry (LC/MS) as described previously.⁴⁴ The extracted lipid fraction was separated online by UPLC using a Waters Acquity UPLC system (Waters Corp., Milford, MA). Mass Spectrometry analysis was performed on a Thermo Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific Inc., San Jose, CA, USA).

Measurement of mitochondrial respiration

Respiration of the kidney mitochondria was measured as described previously²² using Fluorescence Lifetime Micro Oxygen Monitoring System (Instech Laboratories, Inc.). The following respiration medium was used (in mM): 125 mM KCl, 10 mM MOPS (pH 7.2), 2 mM MgCl₂, 2 mM KH₂PO₄, 10 mM NaCl, 1 mM EGTA, 0.7 mM CaCl₂, 10 mM glutamate and 2 mM malate. Kidney mitochondria (0.2 mg/ml), ADP (125 μM) and CCCP (0.2 μM) were consequently added to the respiration chamber. Respiratory control ratio was calculated as the rate ratio State 3 to State 4, where State 4 is the rate after ADP phosphorylation.

ATP levels in kidney tissue

ATP concentration in the kidney tissue was measured by luminescent ATP detection assay kit (Abcam; Cat # ab113849). Luminescence signals were read using a Biotek Synergy H1 plate reader. Luminescence units were calculated as μmol/mg protein based on the ATP calibration curve and protein concentration measured by the Bradford method.

Estimation of calcium retention capacity

Calcium retention capacity (CRC) is the amount of calcium that can be loaded into mitochondria until the permeability transition pore opens. CRC is expressed as nanomol Ca²⁺ per mg of kidney mitochondrial protein. We used the pH method as described previously.⁴¹ This method is based on the fact that in the presence of 1mM Pi, the H⁺/Ca²⁺ ratio is relatively stable, and the pH shift clearly shows the moment when added Ca²⁺ was consumed. Mitochondrial CRC values were estimated in a medium containing 210 mM sucrose, 20 mM KCl, 3 mM glycyl-glycine (pH 7.2), 1 mM KH₂PO₄, and 0.5 mg/m mitochondria, final volume 2.0 ml. Substrates were 10 mM glutamate and 2mM malate. Titration with CaCl₂ was performed by addition to mitochondria 5 μl aliquots of 10 mM CaCl₂.

Cell culture

Human aortic endothelial cells (HAEC) were purchased from Lonza (Chicago, IL) and cultured in EGM-2 medium supplemented with 2% FBS but without antibiotics. On the day before the study, the FBS concentration was reduced to 1%.

Superoxide measurements using HPLC

Mouse aortic segments were loaded with DHE (50 μ M) or mitochondria-targeted mitoSOX (1 μ M) in KHB buffer by 30-minute incubation in a tissue culture incubator at 37 °C. Next, aortic segments were placed in methanol (300 μ l) and homogenized with a glass pestle. The tissue homogenate was passed through a 0.22 μ m syringe filter and methanol filtrates were analyzed by HPLC according to previously published protocols.⁴⁵ The superoxide specific product 2-hydroxyethidium was detected using a C-18 reverse-phase column (Nucleosil 250 to 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. 2-Hydroxyethidium was quantified by fluorescence detector using an emission wavelength of 580 nm and an excitation of 480 nm as described previously.⁴⁶

Nitric oxide measurements by Electron Spin Resonance (ESR)

Nitric oxide production in aortas was quantified by ESR and colloid Fe(DETC)₂ as we have described previously.⁴⁷ All ESR samples were placed in quartz Dewar (Corning, New York, NY) filled with liquid nitrogen. ESR spectra were recorded using an EMX ESR spectrometer (Bruker Biospin Corp., Billerica, MA) and a super high Q microwave cavity. The ESR settings were as follows: field sweep, 160 Gauss; microwave frequency, 9.42 GHz; microwave power, 10 milliwatts; modulation amplitude, 3 Gauss; scan time, 150 msec; time constant, 5.2 sec; and receiver gain, 60 dB ($n = 4$ scans).

Vasodilatation Study

Isometric tension studies were performed on 2 mm mouse aortic rings dissected free of perivascular fat from C57B/6J mice. Studies were performed in a horizontal wire myograph (DMT, Aarhus, Denmark, models 610M and 620M) containing physiological salt solution with the composition of 130 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 15 mM NaHCO₃, 5.5 mM glucose, and 1.6 mM 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 mNewtons before contracting them with three cycles of 60 mM KCl physiological saline solution. Endothelial dependent and independent vascular relaxation was tested after pre-constriction with 1 μ M phenylephrine. Once the vessels reach a steady state contraction, increasing concentrations of acetylcholine were administered, and the response to each concentration of drug was recorded.

Human Studies

Arterioles (100 to 200- μ m diameter) were harvested from human mediastinal fat obtained from patients during cardiac surgery enrolled in the Risk of Oxygen during Cardiac Surgery (ROCS) randomized clinical trial with essential hypertension (BP>140/90 mmHg) and normotensive subjects as previously described ⁴⁸ for Western blot analysis of SOD2 and SOD2 acetylation. Full informed consent was obtained for all tissue samples.

Statistics

Data are expressed as mean \pm SEM. To compare the responses to angiotensin II infusion with or without mito2HOBA, two-way analysis of variance (ANOVA) was used followed by a Bonferroni post hoc test. For comparisons between more than two groups, one-way ANOVA followed by a Bonferroni post hoc test was used. For telemetry blood pressure measurements over time, two-way ANOVA with repeated measures was employed using GraphPad Prism 7. P values < 0.05 were considered significant.

Results

Accumulation of mitochondrial isoLGs in arterioles from hypertensive subjects and SOD2 acetylation

Mitochondria are a major source of superoxide radicals and are rich in polyunsaturated fatty acids. Peroxidation of arachidonic acid can produce highly reactive isoLGs which quickly form protein adducts with lysine residues. To test the accumulation of mitochondrial isoLGs, we performed Western blots using the antibody D11, which detects isoLG-adducted proteins independent of the amino acid sequence,³⁵ in mitochondria isolated from human arterioles. We observed a 250% increase in the mitochondrial isoLG-

Lysyl-Lactam protein adducts in mitochondria isolated from hypertensive patients compared with normotensive subjects (Figure 1A).

Hypertension is associated with inactivation of mitochondrial deacetylase Sirt3 and hyperacetylation of mitochondrial superoxide dismutase (SOD2).¹³ To study the potential role of mitochondrial isoLGs in Sirt3 inactivation, we developed the mitochondria-targeted isoLG scavenger mito2HOBA (Figure 1B).²² Mito2HOBA selectively accumulates in the mitochondria due to its lipophilic cation triphenylphosphonium.^{22, 49} We tested if treatment of human arterioles in organoid cultures with low dose of mito2HOBA stimulates SOD2 deacetylation. Indeed, supplementation with mito2HOBA (0.5 μ M, 24 hours, DMEM) significantly reduced SOD2 acetylation (Figure 1C, D). Because SOD2 is deacetylated by Sirt3, these data suggest that mitochondrial isoLGs inhibit Sirt3 function. Moreover, because SOD2 acetylation inactivates SOD2 and contributes to mitochondrial oxidative stress, scavenging mitochondrial isoLGs may reduce mitochondrial oxidative stress.

Mitochondria-targeted isoLG scavenger mito2HOBA reduces oxidative stress in endothelial cells

We previously showed that angiotensin II (Ang II) and TNF α promote hypertension and reduce endothelial Sirt3 activity.¹² We tested if mito2HOBA reduces mitochondrial oxidative stress in human aortic endothelial cells (HAEC) stimulated with angiotensin II plus TNF α for 4-hours (Figure 2). Indeed, supplementation of HAEC with mito2HOBA (50 nM) reduced mitochondrial superoxide production stimulated by TNF α and Ang II as measured by accumulation of specific superoxide-MitoSOX product, 2-OH-Mito-Ethidium (Figure 2A).⁵⁰ Importantly, supplementation of cells with an identical concentration of the untargeted isoLG scavenger 2HOBA (50 nM) did not affect mitochondrial superoxide levels. Treatment with isoLGs-inactive analog, 4HOBA, which due to rearrangement of the hydroxyl group site cannot scavenge isoLGs,¹⁷ did not protect endothelial cells from mitochondrial oxidative stress.

Cardiolipin is selectively localized to the matrix side of the mitochondrial inner membrane and cardiolipin oxidation is a specific marker of mitochondrial oxidative stress.⁵¹ We tested if mito2HOBA reduces the cardiolipin oxidation in human aortic endothelial cells stimulated with angiotensin II plus TNF α . Indeed,

supplementation of HAEC with low dose of mito2HOBA (50 nM) inhibited cardiolipin oxidation while the untargeted isoLG scavenger 2HOBA was not effective (Figure 2B). These data support the role of mitochondrial isoLG in development of mitochondrial oxidative stress associated with SOD2 acetylation.

Effects of mito2HOBA on mitochondrial isoLG protein adduct accumulation and hypertension

To test a functional role of mitochondrial isoLGs in hypertension, we used an Ang II model of hypertension, and monitored blood pressure by tail-cuff (Figure 3A) and telemetry (Figure 3B). Mito2HOBA alone did not affect the blood pressure in control mice. Infusion of wild-type C57Bl/6J mice with Ang II (0.7 mg/kg/day) increased systolic blood pressure to 162 mm Hg. Treatment of mice with mito2HOBA in the drinking water (0.1 g/L) significantly attenuated the Ang II-induced hypertension to 140 mm Hg as measured by both tail-cuff and telemetry. It is important to note that supplementation of mice with the same molar dose of the untargeted analog 2HOBA did not attenuate Ang II-induced hypertension (Figure 3A).

To provide unambiguous evidence for scavenging of mitochondrial isoLG, we measured isoLG-Lysyl-Lactam adducts accumulation by Liquid Chromatography Tandem Mass Spectrometry (LC/MS) after proteolytic digestion of extracted proteins from isolated mitochondria. Hypertension was associated with 4-fold increase in the mitochondrial isoLG-Lysyl-Lactam protein adducts and mito2HOBA abolished isoLG-Lysyl-Lactam adducts formation in kidney mitochondria (Figure 3C, D).

Effects of mito2HOBA on mitochondrial CypD and SOD2 deacetylation in angiotensin II-infused mice

In additional experiments, we found that Ang II-induced hypertension is linked to a striking hyperacetylation of mitochondrial proteins (420%) in aortas and that this is normalized by co-treatment of animals with mito2HOBA (Figure 4A, B). Since Sirt3 is the predominant, if not the only deacetylase in the mitochondria, this suggests that mitochondrial isoLGs reduce activity of Sirt3. Sirt3 activates SOD2 by deacetylation of specific lysine residues¹² and hypertension is linked to SOD2 hyperacetylation.¹³ We tested if scavenging of mitochondrial isoLGs reduces SOD2 acetylation. Indeed, SOD2 acetylation in aortas isolated from hypertensive mice was increased by 260% while mito2HOBA supplementation significantly reduced SOD2 acetylation (147% compared to control mice) (Figure 4C, D).

We have reported that deletion of Cyclophilin D (CypD), a regulatory subunit of the mitochondrial permeability transition pore (mPTP),²⁹ improves vascular function and attenuates hypertension.³⁰ Sirt3-mediated deacetylation of CypD attenuates mPTP opening.⁵² We sought to determine if Ang II-induced hypertension induces CypD hyperacetylation and if mito2HOBA would attenuate CypD acetylation. Indeed, CypD acetylation was increased by 356% in aortas isolated from hypertensive mice and mito2HOBA supplementation significantly reduced CypD acetylation (156% compared to control) (Figure 4E).

Hypertension was associated with accumulation of isoLG-Lysyl-Lactam protein adducts in aortic mitochondria. Mito2HOBA inhibited formation of mitochondrial isoLG adducts and reduced isoLG-complex I NDUFS1 subunit adduct levels which was accompanied with the reduction of mitochondrial acetylation (Figure 4).

Effect of mito2HOBA on aortic superoxide, endothelial nitric oxide, and endothelial-dependent relaxation

Mito2HOBA prevented SOD2 hyperacetylation suggesting that mito2HOBA can reduce mitochondrial superoxide. Indeed, Ang II-infused hypertension was associated with 2-fold increase in aortic mitochondrial superoxide which was completely prevented by mito2HOBA supplementation (Figure 5A). Hypertension is associated with an increase in vascular superoxide both in the mitochondria and cytoplasm which is facilitated by crosstalk between the mitochondria and the NADPH oxidase.^{53, 54} We tested if mito2HOBA reduces cytoplasmic superoxide level in Ang II-infused mice measured by untargeted cellular superoxide probe DHE.⁴⁶ Ang II-infused hypertension was associated with 217% increase in aortic cellular superoxide which was substantially reduced by mito2HOBA supplementation (152% compared to Sham control, Figure 5B).

Increased vascular superoxide contributes to endothelial dysfunction in hypertension.^{55, 56} It reduces endothelial nitric oxide levels promoting vasoconstriction and increasing systemic vascular resistance.⁵⁷ Decreased nitric oxide bioavailability is therefore a hallmark of endothelial oxidative stress in hypertension. We tested if treatment of mice with mitochondria-targeted isoLG scavenger mito2HOBA protects endothelial nitric oxide and improves endothelial dependent relaxation. Aortic nitric oxide production was quantified by electron spin resonance and specific nitric oxide spin trap Fe(DETC)₂.⁴⁷ As shown in Figure 5, Ang II-induced hypertension was associated with 2-fold decrease in endothelial nitric oxide and impaired endothelial-

dependent relaxation. Notably, supplementation of mito2HOBA completely prevented the decline in nitric oxide and preserved endothelial-dependent relaxation in Ang II-infused mice (Figure 5C, D). These data demonstrate a previously unrecognized role of mitochondrial isoLGs in endothelial dysfunction.

Effect of mito2HOBA on mitochondrial respiration, renal ATP level and Ca²⁺ retention capacity

Hypertension is associated with mitochondrial dysfunction characterized by impaired respiration and reduced ATP production which can be mediated by mPTP opening²⁸ and contributes to end-organ damage in hypertension.^{20, 27} In the current study, mito2HOBA reduced acetylation of CypD, a Ca²⁺ dependent regulatory subunit of mPTP. We have tested if mito2HOBA supplementation reduces mPTP opening as measured by mitochondrial Ca²⁺ retention capacity, improves mitochondrial respiration and ATP production. Indeed, Ang II-induced hypertension was associated with 50% decrease in renal mitochondrial Ca²⁺-retention capacity and this was normalized by ex vivo supplementation with CypD inhibitor Cyclosporin A. Treatment of Ang II-infused mice with mito2HOBA completely prevented decline in Ca²⁺-retention capacity (Figure 6A). Furthermore, mito2HOBA also preserved mitochondrial respiration supported by glutamate plus malate as substrates (Figure 6B). Ang II-induced hypertension was also associated with a 50% decrease in renal ATP levels and this was prevented by mito2HOBA (Figure 6C). These data implicate mitochondrial isoLGs in CypD-dependent mPTP opening in hypertension that can inhibit mitochondrial respiration, reduce ATP level, and promote end-organ-damage in hypertension.

Discussion

This study provides the first evidence that mitochondrial isoLGs accumulate in arterioles of patients with essential hypertension and in mice with Ang II-induced hypertension. Mitochondrial isoLGs were significantly increased in mitochondria isolated from arterioles of hypertensive patients compared with normotensive subjects and in mitochondria isolated from aorta and kidney in mice after onset of Ang II-induced hypertension. The formation of mitochondrial isoLG-Lysyl-Lactam protein adducts was confirmed by two independent methods, D11-antibody assay and mass spectroscopy. These methods were previously vigorously validated and provide unambiguous support for accumulation of isoLG-protein adducts in the

mitochondria. In addition, the mitochondria-targeted isoLG scavenger mito2HOBA prevented accumulation of isoLG-protein adducts in mitochondria and mito2HOBA increased SOD2 deacetylation in human arterioles from hypertensive patients, reduced mitochondrial superoxide in human aortic endothelial cells, inhibited vascular oxidative stress, improved endothelial function, and reduced Ang II-induced hypertension. Furthermore, mito2HOBA supplementation of Ang II-infused mice raised kidney ATP level, protected mitochondrial respiration, and attenuated mPTP opening, supporting the role of mitochondrial isoLGs accumulation in the development of mitochondrial dysfunction in hypertension. Western blot studies revealed that hypertension was associated with reduced Sirt3 deacetylase activity and mitochondrial hyperacetylation, while mito2HOBA increased Sirt3-mediated deacetylation of mitochondrial proteins, particularly SOD2 and CypD. These findings support the role of mitochondrial isoLGs in SOD2 inactivation and CypD-dependent mPTP opening (see Graphic Abstract).

Hypertension is a multifactorial disorder associated with mitochondrial oxidative stress; however, the precise targets of mitochondrial oxidative stress in hypertension are not clear. We have previously shown an increased production of mitochondrial superoxide and reduced activity of mitochondrial SOD2 in animal models of hypertension.¹² The imbalance between increased mitochondrial superoxide and reduced SOD2 activity leads to mitochondrial oxidative stress. Mitochondria are the major source of superoxide radicals⁵⁸ and they are rich in unsaturated fatty acids such as arachidonic acid.⁵⁹ Free radical oxidation of arachidonic acid produces the highly reactive lipid dicarbonyls including isoLGs.⁶⁰ They rapidly adduct to protein lysine residues and can induce cellular dysfunction.⁶¹ Our data show a substantial accumulation of isoLG-Lysyl-Lactam protein adducts in mitochondria isolated from vascular and kidney tissue in hypertension. Supplementation with low dose of the mitochondria-targeted isoLG scavenger mito2HOBA (50 nM) prevents mitochondrial oxidative stress in human aortic endothelial cells while untargeted analog 2HOBA is not effective. It is important to note that 2HOBA and mito2HOBA does not react with superoxide, peroxynitrite or hydrogen peroxide and therefore does not exert its effect directly by ROS scavenging.¹⁷ In contrast, the mito2HOBA-mediated reduction in mitochondrial, cellular and aortic superoxide observed in Ang II-infused mice and in HAEC is likely due to enhanced scavenging of this radical by SOD2. This is a reasonable interpretation of

our findings because we observed a dramatic reduction in Ang II-induced SOD2 hyperacetylation in mito2HOBA-treated animals and SOD2 is the only mitochondrial superoxide dismutase.

Endothelial dysfunction is linked to increased vascular superoxide which leads to nitric oxide inactivation, reduced endothelial nitric oxide production and impaired endothelial dependent relaxation. Mito2HOBA reduces vascular superoxide, protects endothelial nitric oxide and improves endothelial dependent relaxation. In endothelial cells, mito2HOBA inhibits superoxide production and reduces oxidative stress. These effects of mito2HOBA were associated with increased Sirt3-mediated deacetylation of SOD2 and CypD. Sirt3 impairment contributes to vascular inflammation, hypertrophy and endothelial dysfunction.¹³ Our new data support an important role of mitochondrial isoLGs in Sirt3 inactivation, endothelial and vascular dysfunction.

Mitochondrial dysfunction contributes to target-organ-damage in hypertension. Hypertension is a leading cause of kidney disease which is linked to metabolic and mitochondrial dysfunction.²⁷ In this work, we found that Ang II-induced hypertension is associated with 4-fold increase in renal mitochondrial isoLGs, increased mPTP opening and impaired respiration in kidney mitochondria. These events were associated with a 2-fold decrease in kidney ATP levels. Remarkably, mito2HOBA supplementation prevents accumulation of mitochondrial isoLG in kidney, attenuates mPTP opening, preserves mitochondrial respiration, and protects kidney ATP production. These data strongly support a role of mitochondrial isoLG in hypertensive renal injury. These data are in line with our previous finding showing that mito2HOBA supplementation in lipopolysaccharide treated mice improves respiration of kidney mitochondria and protects the renal cortex from cell injury.²²

We propose Sirt3 inactivation as a new convergent mechanism underling the interplay of major cardiovascular risk factors. Sirt3 impairment inhibits fatty metabolism⁶² and inactivates a key mitochondrial antioxidant, superoxide dismutase 2 (SOD2), due to hyperacetylation of specific lysine residues.¹² Therefore, Sirt3 inactivation increases levels of polyunsaturated fatty acids and superoxide which react together producing highly reactive isoLGs in the mitochondria. IsoLGs covalently bind to lysine residues making cytotoxic and pro-inflammatory isoLGs adducts.⁶¹ We discovered 4-fold increase in mitochondrial

isoLGs in hypertension. Mitochondrial isoLGs are emerging as a mechanistic link between mitochondrial oxidative stress and disease progression. Previous studies have identified isoLGs adduct with the F1Fo subunit of complex V,⁶³ and we report formation of isoLG adduct with NDUFS1 subunit of mitochondrial complex I. It is conceivable that mitochondrial isoLGs causes Sirt3 inactivation by direct⁶⁴ and indirect interactions. Meanwhile, the cause-and-effect relationship between mitochondrial isoLGs and Sirt3 inactivation remains elusive. It is clear that isoLGs exposure inhibits Sirt3, however, it is also possible that Sirt3 impairment promotes mitochondrial isoLGs formation. Indeed, treatment of human arteriole isolated from hypertensive patients rescues Sirt3 activity and increases Sirt3-mediated SOD2 deacetylation. We propose that a feed-forward cycle between Sirt3 inactivation and mitochondrial isoLGs promotes vascular dysfunction and that scavenging mitochondrial isoLGs will break this cycle and improve vascular function (see Graphic Abstract). It is, therefore, possible that isoLGs are both upstream and downstream of Sirt3 inactivation.

Pathophysiological role of isoLGs has been reported in various conditions including vascular inflammation, hypertension, and heart failure.^{17, 65, 66} Supplementation with untargeted isoLG scavenger 2HOBA reduces vascular inflammation, diminishes tissue fibrosis, decreases aortic stiffening, abates cardiac hypertrophy, attenuates hypertension and heart failure. In these conditions, stimulation of NADPH oxidases can promote formation of isoLGs in cytoplasm where they can be eliminated by 2HOBA. Meanwhile, mitochondria are both the source and the potential target for isoLGs, therefore, isoLGs produced in the cytoplasm may also contribute to mitochondrial dysfunction. Indeed, our experiments showed that 2HOBA partially attenuates mitochondrial superoxide overproduction in cultured human aortic endothelial cells (Figure 2) suggesting that both intramitochondrial and extramitochondrial isoLGs promote mitochondrial oxidative stress.

In this work we tested the effect of mito2HOBA in cultured endothelial cells, in organoid culture with human arterioles and whole animal supplementation. Further studies are needed to determine the specific role of mitochondrial isoLGs in endothelial, smooth muscle, and other cells. We propose that blocking of mitochondrial isoLGs will rescue Sirt3 deacetylase activity which recover the metabolic and antioxidant mitochondrial functions reducing vascular oxidative stress and improving the endothelial function, therefore, mito2HOBA can potentially improve the treatment of vascular dysfunction and hypertension.

Hypertension is highly prevalent with aging, and 75% of adults are hypertensive at age of 70 and over.² Sirt3 function declines with age³³ and Sirt3 depletion accelerates vascular aging and induces age-dependent hypertension associated with mitochondrial oxidative stress.¹³ Sirt3 expression is associated with human longevity⁶⁷ and Sirt3 overexpression protects from vascular dysfunction and hypertension.¹³ It is intriguing to speculate that Sirt3 impairment and mitochondrial isoLGs can promote age-dependent vascular alterations and hypertension,⁶⁸ and scavenging of mitochondrial isoLG, therefore, can slow down and reverse these age-related alterations. Indeed, our human tissue study suggest that mito2HOBA partially rescues Sirt3 activity in the patients with essential hypertension. Of note, most of the oxidants have a very short lifetime (seconds) but isoLGs produce rather stable adducts (lifetime days)²³ which can accumulate with age and, therefore, contribute to the development of age-associated conditions.

In addition to hypertension, mitochondrial oxidative stress likely contributes to many other conditions including aging, atherosclerosis, diabetes, inflammation, and degenerative neurological disorders.^{69, 70} Accumulation of mitochondrial isoLGs may impact these conditions. It is conceivable that the use of mitochondria-targeted isoLG scavengers such as mito2HOBA would be beneficial in these conditions. The ability to protect mitochondria in relatively low doses might also limit potential untoward effects compared to untargeted agents such as 2HOBA.

Perspectives

Hypertension is associated with inactivation of mitochondrial deacetylase Sirt3 promoting oxidative stress and vascular dysfunction; however, the mechanism of Sirt3 inactivation is not clear. These studies provide a novel concept that mitochondrial accumulation of reactive dicarbonyl lipid peroxidation products isolevuglandins (isoLGs) contribute to Sirt3 inactivation and hypertension. Mitochondrial superoxide dismutase 2 (SOD2) is hyperacetylated in arterioles isolated from hypertensive patients and *ex vivo* treatment of arterioles with mitochondria-targeted isoLG scavenger mito2HOBA reduced SOD2 acetylation and reduced mitochondrial oxidative stress in human aortic endothelial cells. Mito2HOBA attenuated angiotensin II-induced hypertension, reduced acetylation of mitochondrial CypD and SOD2, inhibited vascular oxidative stress, preserved endothelial-dependent relaxation, and prevented mitochondrial

dysfunction. Our data suggest that inactivation of Sirt3 by mitochondrial isoLGs promotes mitochondrial and vascular dysfunction. Targeting mitochondrial isoLGs could be used for development of future pharmacological interventions to improve treatment of vascular dysfunction, reduce hypertension, and attenuate end organ damage associated with hypertension.

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Disclosures

None.

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Novelty and Significance

What Is New?

- This study provides first evidence of mitochondrial isolevuglandins in human subjects with essential hypertension and animal models of hypertension. We showed that mitochondria-targeted isolevuglandins scavenger, mito2HOBA, protects mitochondrial function, rescues Sirt3 and SOD2 activity, inhibits vascular oxidative stress and attenuates hypertension.

What Is Relevant?

- Hypertension is associated with mitochondrial dysfunction; however, the precise pathophysiological role of mitochondria remains unclear. This study provides evidence for therapeutic potential of targeting harmful mitochondrial isolevuglandins in hypertension.

Summary

Hypertension is linked to Sirt3 inactivation which leads to hyperacetylation of mitochondrial proteins such as cyclophilin D (CypD) and mitochondrial superoxide dismutase (SOD2). CypD acetylation stimulates mPTP opening which increases the production of mitochondrial superoxide while SOD2 acetylation inactivates SOD2. This results in imbalance between the increased superoxide production and diminished superoxide dismutation leading to mitochondrial oxidative stress and oxidation of polyunsaturated fatty acids (PUFA) to reactive gamma-ketoaldehydes, isolevuglandins (isoLGs), in the mitochondria. Mitochondrial isoLGs promote vascular and mitochondrial dysfunction while treatment with mitochondria-targeted isoLG scavenger mito2HOBA reduces Sirt3 inactivation, improves mitochondrial and vascular function, and attenuates hypertension. We propose that targeting mitochondrial isoLGs prevents Sirt3 inactivation and can improve the treatment of vascular dysfunction in human subjects.

Figure legends

Figure 1. Western blots of mitochondrial isoLGs (**A**) in human arterioles from normotensive and hypertensive subjects (n=5), (**B**) development of mitochondria-targeted isoLG scavenger mito2HOBA and (**C, D**) SOD2 acetylation in human arterioles isolated from normotensive and hypertensive subjects and treated *ex vivo* with mito2HOBA (0.5 μ M, 24 hours, DMEM). Data were normalized by Complex I levels (Sham is 100%). Data are mean \pm SEM. *P<0.01 vs Normotensive Sham, **P<0.01 vs Hypertensive (n=5).

Figure 2. Effect of mito2HOBA on mitochondrial superoxide and cardiolipin oxidation in human aortic endothelial cells induced by angiotensin II plus TNF α . (A) Mitochondrial superoxide was measured by HPLC analysis of mitoSOX-superoxide specific product, mito-2-hydroxyethidium (Mito-2OH-Et⁺).^{46, 50} Mito2HOBA (50 nM) abolishes stimulation of mitochondrial superoxide while similar dose of untargeted isoLG scavenger 2HOBA (50 nM) or high dose of isoLG-inactive analog 4HOBA (10 μ M) are not protective. *P<0.01 vs control, **P<0.001 vs Angiotensin II + TNF α . (B) Cardiolipin oxidation induced by Angiotensin II + TNF α ¹² measured by LC/MS.²⁰ Cardiolipin oxidation is significantly attenuated by mito2HOBA (50 nM) while untargeted 2HOBA (10 μ M) is not effective. Supplemental figure S1 shows typical chromatograms. Data are mean \pm SEM. *P<0.01 vs Control, **P<0.01 vs Angiotensin II + TNF α (n=4).

Figure 3. Effect of mito2HOBA on angiotensin II-induced hypertension and accumulation of mitochondrial isoLGs protein adducts. (**A**) Blood pressure tail-cuff measurements in male Sham or angiotensin II-infused mice supplied with mito2HOBA in drinking water (0.1 g/L) or equimolar amount of untargeted analog 2HOBA (0.17 mmol/L). (**B**) Telemetry studies of blood pressure in angiotensin II-infused mice supplied with mito2HOBA or plain water as a vehicle. (**C**) Representative LC/MS/MS chromatograms of isoLG-Lysyl-Lactam adduct; (**D**) isoLG-Lys-Lactam levels in kidney mitochondria isolated from Sham or angiotensin II-infused mice supplied with mito2HOBA. Results are mean \pm SEM. *P<0.01 vs Sham, **P<0.01 vs Ang II (n=8).

Figure 4. Western blot analysis of mitochondrial acetylation in aortas isolated from Sham and angiotensin II-infused mice treated with mito2HOBA. (**A**) Typical Western blots isoLG-protein adducts (D11 ab), Sirt3,

Acetyl-Lysine, SOD2-K68-Acetylation, CypD Acetylation, isoLG adduct with complex I NDUFS1 75 KDa subunit and mitochondrial complex I ; **(B)** Sirt3 levels; **(C)** mitochondrial protein lysine acetylation; **(D)** SOD2-K68-Acetyl levels; and **(E)** CypD-Acetyl levels. Mice supplied with mito2HOBA (m2H) in drinking water (0.1 g/L) and angiotensin II (osmotic pump, 0.7 mg/kg/day) for 14 days. Data were normalized by Complex I levels (Sham is 100%). Results are mean \pm SEM (n=5). * P <0.01 vs Sham, ** P <0.01 vs angiotensin II (AngII).

Figure 5. Effect of mito2HOBA supplementation on mitochondrial superoxide **(A)**, vascular superoxide **(B)**, endothelial nitric oxide **(C)** and endothelial-dependent relaxation **(D)** in angiotensin II-infused mice. Mitochondrial and vascular O_2^{\bullet} was measured by mitochondria-targeted superoxide probe mitoSOX (1 μ M) or untargeted superoxide probe DHE (50 μ M) using HPLC.⁴⁶ Endothelial nitric oxide was analyzed by NO spin trap Fe(DETC)₂ and ESR.⁴⁷ C57Bl/6J mice were infused with Ang II and mito2HOBA was provided in the drinking water (0.1 g/L). Supplemental figure S2 shows typical HPLC chromatograms. Supplemental figure S3 shows representative ESR spectra of nitric oxide measurements. Results are mean \pm SEM. * P <0.01 vs Sham, ** P <0.01 vs Ang II (n=6).

Figure 6. Mito2HOBA reduces mPTP opening and prevents mitochondrial dysfunction. C57Bl/6J mice were infused with Ang II (0.7 mg/kg/ml) and mito2HOBA in the drinking water (0.1 g/L). Following 14 days of Ang II infusion the animals were sacrificed and kidneys were isolated for mitochondrial studies. Addition of $CaCl_2$ to mitochondria above Ca^{2+} retention capacity led to mPTP opening and mitochondria swelling.⁷¹ Mitochondria isolated from Ang II-infused mice had significant reduction in Ca^{2+} capacity due to increased mPTP opening and CypD inhibitor Cyclosporine A (CsA) rescued Ca^{2+} retention capacity **(A)**. Respiratory control ratio (State 3/State 4) was measured in isolated kidney mitochondria with glutamate and malate **(B)**. Control level is 100%. **(C)** Renal ATP was measured in freshly isolated tissue by luciferase-based luminescent assay.⁷² Results are mean \pm SEM. * P <0.01 vs Sham, ** P <0.01 vs Angiotensin II (n=5).