Flow-Mediated Vasodilation

PGC-1α (Peroxisome Proliferator–Activated Receptor γ Coactivator 1-α) Overexpression in Coronary Artery Disease Recruits NO and Hydrogen Peroxide During Flow-Mediated Dilation and Protects Against Increased Intraluminal Pressure

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Abstract—Blood flow through healthy human vessels releases NO to produce vasodilation, whereas in patients with coronary artery disease (CAD), the mediator of dilation transitions to mitochondria-derived hydrogen peroxide (mtH2O2). Excessive mtH2O2 production contributes to a proatherosclerotic vascular milieu. Loss of PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α) is implicated in the pathogenesis of CAD. We hypothesized that PGC-1α suppresses mtH2O2 production to reestablish NO-mediated dilation in isolated vessels from patients with CAD. Isolated human adipose arterioles were cannulated, and changes in lumen diameter in response to graded increases in flow were recorded in the presence of PEG (polyethylene glycol)–catalase (H2O2 scavenger) or L-NAME (Nω-nitro-l-arginine methyl ester; NOS inhibitor). In contrast to the exclusively NO- or mtH2O2-mediated dilation seen in either non-CAD or CAD conditions, respectively, flow-mediated dilation in CAD vessels was sensitive to both L-NAME and PEG-catalase after PGC-1α upregulation using ZLN005 and α-lipoic acid. PGC-1α overexpression in CAD vessels protected against the vascular dysfunction induced by an acute increase in intraluminal pressure. In contrast, downregulation of PGC-1α in non-CAD vessels produces a CAD-like phenotype characterized by mtH2O2-mediated dilation (no contribution of NO). Loss of PGC-1α may contribute to the shift toward the mtH2O2-mediated dilation observed in vessels from subjects with CAD. Strategies to boost PGC-1α levels may provide a therapeutic option in patients with CAD by shifting away from mtH2O2-mediated dilation, increasing NO bioavailability, and reducing levels of mtH2O2. Furthermore, increased expression of PGC-1α allows for simultaneous contributions of both NO and mtH2O2 to flow-mediated dilation. (Hypertension. 2017;70:166-173. DOI: 10.1161/HYPERTENSIONAHA.117.09289.) ● Online Data Supplement

Key Words: arterioles ■ catalase ■ coronary artery disease ■ microcirculation ■ nitric oxide

Cardiovascular disease remains a pressing global health issue. One unifying pathogenic factor in the development of cardiovascular disease is endothelial dysfunction, manifest as an impaired vasodilatory response to increased blood flow (ie, shear stress), or pharmacological agonists, an abnormality typically associated with endothelial inflammation and oxidative stress.1,2 Although most interventions are aimed at mitigating the influence of a single risk factor pathway associated with endothelial dysfunction, such as diabetes mellitus or hypercholesterolemia, targeting the participating mechanisms of endothelial dysfunction itself is an exciting approach to combat cardiovascular diseases, such as atherosclerosis.3

Microvascular dysfunction is strongly prognostic for cardiovascular events,4 suggesting that endothelial mechanisms in the microcirculation carry disease significance as either indicators of or underlying contributors to cardiovascular disease. However, microvascular dysfunction in humans has not been extensively examined. We have reported that the mediator of microcirculatory dilation to shear stress, the most physiologically important mechanism of endothelium-dependent dilation, is different between healthy and diseased vessels. NO elicits flow-mediated dilation (FMD) in vessels from subjects without cardiovascular disease. In contrast, in vessels from subjects with coronary artery disease (CAD), NO bioavailability is reduced as NO reacts with rising endothelial superoxide levels, and microvascular dilation is maintained by compensatory release of mitochondria-derived hydrogen peroxide (mtH2O2).5,6 Excessive mtH2O2 production can contribute to an inflammatory vascular milieu and may be a key early pathogenic step in the progression of CAD. Pathological stimuli associated with CAD, including telomerase inhibition,7 an acute increase in intraluminal pressure,8 and exogenous administration of the sphingolipid ceramide,9 can induce a switch to mtH2O2-mediated dilation, but the endogenous regulator of this shear-sensitive switch is not known.

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166
In this study, we sought to elucidate a novel role for the transcriptional coactivator PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α) in regulating the mechanism of dilation in the human microcirculation. Although largely studied in relation to obesity, skeletal muscle, and diabetes mellitus, interest in the vascular effects of PGC-1α and its protective role in atherogenesis has risen in the past several years.\textsuperscript{10–13} Given the shear-sensitive,\textsuperscript{14} redox-modulating\textsuperscript{15} vascular properties of PGC-1α, as well as its known interaction with NO in cultured endothelial cells and animal vessels,\textsuperscript{16,17} we considered whether PGC-1α could be an endogenous switch determining the mechanism of FMD between health and disease. We hypothesized that loss of PGC-1α in non-CAD arterioles produces a CAD phenotype, characterized by a switch from NO-mediated to αv vessels from subjects with CAD.

We further hypothesized that PGC-1α upregulation in microvessels from subjects with CAD will restore a non-CAD vascular phenotype characterized by a return to NO-mediated dilation. We discovered that PGC-1α upregulation uniquely recruits both NO and H₂O₂ during FMD, and protects against acute increases in intraluminal pressure, in vessels from subjects with CAD.

Methods

Materials

ZL005 (Sigma), a known small-molecule activator of PGC-1α,\textsuperscript{18,19} was prepared in dimethyl sulfoxide. Bio-Enhanced Na-Rala (GeroNova Research) was dissolved in distilled water. Endothelin-1 (Sigma) was prepared in 1% bovine serum albumin. Lentiviral constructs (GFP [green fluorescent protein] and PGC-1α siRNA) were produced by the Blood Center of Wisconsin Hybridoma Core Laboratory and dissolved in distilled water. mitoPBA/mitoB (Cayman) was prepared in ethanol. Rotenone (Sigma) and MitoPY1 (Cayman) were prepared in DMSO.

Statistical Analysis

Data are expressed as mean±SEM. FMD is expressed as a percent change of maximal dilation to papaverine after endothelin-1 constriction. To compare flow–response relationships, a 2-way repeated-measures ANOVA was used with pressure gradient and intervention as parameters. When a significant difference was observed between control and inhibitor curves, responses at individual concentrations were compared using a Holm–Sidak multiple comparison test. An unpaired Student t test was used to compare baseline characteristics for patients with and without CAD. Differences in Western blot protein levels and H₂O₂ production in human umbilical vein endothelial cells (HUVECs) were also assessed with an unpaired Student t test. Analyses were performed using SigmaPlot and GraphPad. Statistical significance was defined as \( P<0.05 \).

Results

Subject Demographics

Discarded adipose tissue was obtained from 49 patients. Twenty-eight of those patients had a clinical diagnosis of CAD. Detailed patient demographics are summarized in Table I (online-only Data Supplement).

PGC-1α Protein Levels Are Decreased in Heart Tissue From Subjects With CAD

To determine whether a decline in PGC-1α levels occurs in the presence of CAD, we compared PGC-1α protein expression in non-CAD and CAD human left ventricular tissue. Western blotting revealed that PGC-1α protein content is lower in human CAD tissue (Figure I in the online-only Data Supplement). In human microvessels, we performed immunohistochemistry, which, although not quantitative, also suggests a reduction in staining for PGC-1α in the microvasculature of subjects with CAD (Figure IIA versus Figure IIB in the online-only Data Supplement). Therefore, a diagnosis of CAD is associated with a relative decline of PGC-1α in both the human heart and microcirculation.

PGC-1α Levels in the Human Microcirculation

Forty-eight-hour incubation with lentiviral PGC-1α siRNA decreased PGC-1α levels in non-CAD vessels relative to untreated control (Figure IIA versus Figure IIB in the online-only Data Supplement). Overnight treatment (16–24 hours) with either α-lipoic acid (ALA) or ZLN005, both activators of PGC-1α, increased PGC-1α levels in vessels from subjects with CAD (Figure SIIE and SIIF in the online-only Data Supplement).

Downregulation of PGC-1α in Non-CAD Vessels Produces a CAD Phenotype

Treatment of non-CAD vessels with lentiviral PGC-1α siRNA established a CAD phenotype characterized by normal magnitude H₂O₂-mediated dilation to shear (inhibited by PEG [polyethylene glycol]–catalase) (% max diameter at 100 cm H₂O: vehicle 81.3±4.3, PEG-catalase 0.7±9.6). In contrast, L-NAME (N\textsuperscript{\textbeta}-nitro-L-arginine methyl ester), which abolishes dilation in untreated non-CAD vessels, had no effect on FMD after PGC-1α downregulation (Figure 1C) (% max diameter at 100 cm H₂O: vehicle 81.3±4.3, L-NAME 71.9±6.3). These data suggest that loss of PGC-1α in non-CAD vessels shifts away from NO-mediated dilation and exposes H₂O₂ as the compensatory vasodilator.

Because a relative reduction in PGC-1α levels is observed in CAD vessels, and CAD vessels dilate to αH₂O₂, we anticipated that a forced downregulation of PGC-1α in non-CAD arterioles using siRNA would also result in the unmasking of compensatory H₂O₂ production from the mitochondria. Indeed, after PGC-1α knockdown, rotenone (inhibitor of electron transport chain complex 1) and mitoPBA (mitochondria-targeted H₂O₂ scavenger) both inhibited FMD (Figure 1D) (% max diameter at 100 cm H₂O: vehicle 81.3±4.3, rotenone –6.3±9.6, mitoPBA 4.8±12.5).

To control for possible off-target effects of the lentivirus, we also treated non-CAD vessels with a lentivirus harboring a GFP construct. Because a relative reduction in PGC-1α levels is observed in CAD vessels, and CAD vessels dilate to αH₂O₂, we anticipated that a forced downregulation of PGC-1α in non-CAD arterioles using siRNA would also result in the unmasking of compensatory H₂O₂ production from the mitochondria. Indeed, after PGC-1α knockdown, rotenone (inhibitor of electron transport chain complex 1) and mitoPBA (mitochondria-targeted H₂O₂ scavenger) both inhibited FMD (Figure 1D) (% max diameter at 100 cm H₂O: vehicle 81.3±4.3, rotenone –6.3±9.6, mitoPBA 4.8±12.5).

Overexpression of PGC-1α Confers Vasodilatory Plasticity in CAD Vessels

After observing that the loss of PGC-1α in non-CAD vessels produces a switch to αH₂O₂-mediated dilation, we hypothesized...
168 Hypertension July 2017

that, conversely, upregulation of PGC-1α levels in CAD vessels, which normally dilate to increased shear via H₂O₂, might reverse the disease phenotype and restore NO-mediated dilation. To test this hypothesis, we treated vessels with 2 chemically distinct compounds to increase PGC-1α expression: ALA, an over-the-counter supplement known to increase PGC-1α, and ZLN005, a novel small molecule transcriptional activator of PGC-1α. Before treatment, PEG-catalase blocked dilation in CAD vessels (Figure 2A) (% max diameter at 100 cm H₂O: vehicle 75.3±11.1, PEG-catalase 24.2±5.6), as we have previously observed. Unexpectedly, PGC-1α overexpression in CAD vessels exposed a novel phenotype characterized by contributions of both NO and H₂O₂ to FMD. After treatment with either ALA or ZLN005, dilation was only partly inhibited by either L-NAME or PEG-catalase. Instead, dilation was abolished after combined coincubation with both L-NAME and PEG-catalase after PGC-1α upregulation with these 2 distinct compounds (Figure 2B and 2C) (ZLN treatment, % max diameter at 100 cm H₂O: vehicle 80.7±4.1, L-NAME+PEG-catalase 19.0±6.5; ALA treatment: vehicle 79.4±4.4, L-NAME+PEG-catalase 14.0±7.7). We also compared the responses between PGC-1α overexpressing and control CAD vessels (Figure 2D), which indicates that PGC-1α overexpression using ZLN005 or ALA renders dilation in CAD vessels less susceptible to inhibition by catalase (% maximal dilation at 100 cm H₂O flow in vessels that are: untreated: 24.2±5.6; versus ALA-treated: 56.3±11.0, and ZLN-treated: 54.4±8.5). Thus, although a component of NO-mediated dilation was restored in CAD vessels,
we observed a maintained contribution of H$_2$O$_2$ to dilation, highlighting that forced PGC-1α overexpression allows for more than one dilator mechanism to be present and that inhibition of dilation can only be achieved by simultaneously interfering with NO production and scavenging H$_2$O$_2$.

**Source of H$_2$O$_2$ After Overexpression of PGC-1α in CAD Vessels**

We next examined whether the mitochondria remain the subcellular source of H$_2$O$_2$ after PGC-1α upregulation in CAD vessels. FMD in CAD vessels is mediated entirely by mtH$_2$O$_2$ and can be inhibited by either PEG-catalase or specific mitochondrial H$_2$O$_2$ inhibitors or scavengers. In contrast, after PGC-1α overexpression in CAD vessels, these mitochondrial inhibitors (in combination with L-NAME) no longer influenced FMD (Figure 3B). These findings suggest that, despite the continued presence of H$_2$O$_2$ as a vasodilator, there is a transition away from the mtH$_2$O$_2$ production observed in untreated CAD vessels.

To further investigate these findings using a complementary technique, we assessed _mtH$_2$O$_2$ production using a mitochondria-targeted H$_2$O$_2$ probe (MitoPY1) in untreated and PGC-1α-overexpressing CAD vessels. Exposure of these vessels to shear stress revealed that PGC-1α overexpression suppresses _mtH$_2$O$_2_ production in ALA-treated vessels from subjects with CAD relative to untreated CAD vessels (Figure 3A).

**Overexpression of PGC-1α Leads to Simultaneous Release of Both NO and H$_2$O$_2$ in HUVECs in Response to Shear**

To confirm the dual release of NO and H$_2$O$_2$, PGC-1α-overexpressing HUVECs were exposed to shear stress (15 dynes/cm$^2$) for 1 hour, and levels of NO and H$_2$O$_2$ were determined relative to untreated, sheared HUVEC controls. DAF-2 (4,5-diaminofluorescein diacetate) fluorescence signal (NO) increased in ALA-treated versus untreated sheared cells (Figure 4A), illustrating the increase in NO production in response to shear stress.

**Figure 3.** Source of H$_2$O$_2$ after PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α) overexpression on flow-mediated dilation (FMD) in coronary artery disease (CAD) vessels. A, Reduction in MitoPY1 fluorescence after 24-hour α-lipoic acid (ALA) treatment (250 µmol/L). Changes in fluorescence intensity in response to shear stress were evaluated in untreated or ALA-treated CAD vessels. n=6 to 7 per treatment group. *P<0.05 ALA vs control; (B) mitoPBA had no additional effect on dilation after incubation with the endothelial nitric oxide synthase inhibitor L-NAME (N$^\text{G}$-nitro-L-arginine methyl ester) after 24-hour ALA treatment (250 µmol/L) in CAD vessels. $P$=not significant vs control between flow curves.

**Figure 4.** Assessment of nitric oxide (NO) and H$_2$O$_2$ production after shear and antioxidant levels after PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α) overexpression in human umbilical vein endothelial cells (HUVECs). A, PGC-1α overexpression using 250 µmol/L α-lipoic acid (ALA) treatment increased NO production during 1-hour shear stress (15 dynes/cm$^2$), as determined by DAF-2A (4,5-diaminofluorescein diacetate)/HPLC (high-performance liquid chromatography); (B) PGC-1α overexpression using 250 µmol/L ALA treatment increased global H$_2$O$_2$ production during 1-hour shear stress (15 dynes/cm$^2$), as determined by Amplex Red fluorescence. Inhibition of NO production (L-NAME, N$^\text{G}$-nitro-L-arginine methyl ester) did not further increase H$_2$O$_2$ release. n=4 to 6 per treatment group, *P<0.05 ALA vs vehicle control. Data reported as mean±SEM. C and D, Western blot evaluation of the antioxidant levels of MnSOD and catalase after overnight treatment with ALA and ZLN in HUVECs. $P$=not significant ALA/ZLN vs control.
shear stress as a result of increasing endothelial PGC-1α levels. Amplex Red fluorescence signal (H₂O₂) also increased after shear in ALA-treated versus untreated cells, indicating that H₂O₂ release is elevated in response to shear stress after PGC-1α overexpression (Figure 4B). To determine whether shear-induced H₂O₂ release occurs tonically (basal release) or only after inhibition of NO production (compensatory release), we repeated the Amplex Red measurements after 30-minute incubation with L-NAME to block NO production. No change in the Amplex Red fluorescence signal was observed when NO was inhibited (Figure 4B), suggesting that H₂O₂ coexists alongside NO during shear and is not acting as a compensatory vasodilator that only emerges when NO bioavailability is reduced.

**Overexpression of PGC-1α Does Not Reduce Antioxidant Levels**

Because endogenous antioxidants modulate H₂O₂ levels in the vasculature, we evaluated whether the appearance of H₂O₂ as a vasodilator was associated with a loss of antioxidant defense mechanisms in HUVECs. Interestingly, PGC-1α upregulation did not lead to a decrease in either catalase or MnSOD levels in ALA-treated HUVECs (Figure 4C and 4D), revealing maintained antioxidant defense mechanisms alongside the increase in H₂O₂ production.

**Overexpression of PGC-1α Protects Against Acute Increases in Intraluminal Pressure**

After observing this novel phenotype wherein more than one vasodilator contributes to dilation in the presence of chronic disease (CAD), we sought to determine the functional relevance of this discovery. We questioned whether this additional vasodilatory plasticity confers a broader increased protection against acute vascular insults. Adipose microvessels from subjects with CAD experienced a severely impaired dilation to flow after 30-minute exposure to increased intraluminal pressure (IILP; 150 mm Hg) (% max diameter at 100 cm H₂O: vehicle 75.3±4.3, IILP 22.7±10.7). In contrast, PGC-1α upregulation with ALA fully prevented IILP-induced vascular dysfunction, showing no reduction in maximal FMD after exposure to IILP (Figure 5A).

Endothelium-independent dilation to papaverine was not different between the treatment groups (Figure 5B), highlighting that our results are related to endothelial-dependent mechanisms.

![Figure 5](http://hyper.ahajournals.org/)

**Figure 5.** Effect of increased intraluminal pressure after PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α) overexpression in coronary artery disease (CAD) vessels. A, PGC-1α overexpression using 250 μmol/L α-lipoic acid (ALA) preserved the magnitude of flow-mediated dilation (FMD) in response to acute increases in intraluminal pressure (IILP; 150 mm Hg, 30 minutes), whereas acute hypertension severely impaired the overall magnitude of dilation in untreated CAD vessels; *P<0.05 IILP vs control at specific pressure gradients. B, Endothelium-independent, papaverine-induced dilation is not altered by overnight treatment with lipoic acid. N=4 per treatment group. P=not significant vs control.

![Figure 6](http://hyper.ahajournals.org/)

**Figure 6.** Proposed schematic of the regulation of flow-mediated dilation by PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α). A, Basal PGC-1α in non–coronary artery disease (CAD) vessels allows for nitric oxide (NO)–mediated dilation (inhibitable by L-NAME [N^G-nitro-L-arginine methyl ester] alone). B, PGC-1α overexpression provides plasticity in CAD vessels, resulting in contributions of both NO and H₂O₂ to dilation.
**Discussion**

There are several major findings of this study. First, we have identified a functionally relevant effect of PGC-1α on FMD, adopting a reverse translational approach in human tissue to explore the reported link between the loss of PGC-1α and the development of CAD. As hypothesized, the loss of PGC-1α in non-CAD arterioles produced a diseased (CAD) phenotype characterized by a shift from NO- to mtH₂O₂-mediated dilation to flow, positioning PGC-1α as a key factor in microvascular atherosclerotic disease development. Second and unexpectedly, restoring PGC-1α in vessels from subjects with CAD produced a novel phenotype wherein both NO and H₂O₂ contribute to dilation. Third, the source of H₂O₂ was no longer mitochondrial in CAD vessels after PGC-1α upregulation. Fourth, the maintained, nonmitochondrial H₂O₂ release was accompanied by conserved mitochondrial and cytosolic antioxidant expression. From a therapeutic perspective, we have identified 2 distinct compounds, ALA and ZLN005, that can produce upregulation of PGC-1α in the human microcirculation. We have uncovered a novel phenotype characterized by the simultaneous presence of NO and H₂O₂, lending plasticity to the mechanism of dilation to shear. We also report that microvascular FMD in CAD vessels is severely compromised after increased intraluminal pressure (IILP) (more so than non-CAD vessels) and that PGC-1α provides protection against this IILP-induced vascular dysfunction. These findings position PGC-1α as a promising therapeutic target for the microvascular complications of CAD via attenuation of mtH₂O₂ release during shear, restoration of antiatherogenic NO as a vasodilator, and added resistance to acute barotrauma in CAD vessels.

That PGC-1α overexpression results in the coexistence of 2 vasodilators, NO and H₂O₂, which are canonically viewed as antagonistic, warrants additional discussion. H₂O₂ is traditionally viewed as a prothrombotic and proinflammatory vasoactive substance that contributes to atherosclerotic disease burden. However, recent evidence suggests that NO and H₂O₂ may act in a synergistic fashion in certain circumstances, such as H₂O₂-induced activation of endothelial NOS20,21 and that H₂O₂ may even function as a primary mediator of dilation in healthy animal models.22–24 In addition, the detrimental effects of H₂O₂ in relation to cardiovascular disease are mainly attributable to the source (mitochondria localized) and local concentrations of H₂O₂.25–27 For example, recent evidence suggests that NOX4-derived H₂O₂ is atheroprotective28,29 and can improve vasodilation without harmful oxidative effects on the vascular wall.30 Data from other laboratories illustrate that excessive NO, and deficient H₂O₂, may increase, rather than decrease, endothelial dysfunction and that achieving moderate levels of these 2 vasodilators simultaneously is most cardioprotective.31,32 Although recent studies provide evidence to support this stance, few provide therapeutic strategies or molecular mechanisms to achieve this homeostatic balance between these 2 vasodilators. Given our results, PGC-1α may serve as a fundamental “molecular switch” that unlocks this compensatory pathway wherein NO and H₂O₂ contribute to dilation, providing a window to advance our understanding surrounding this issue.

Our data support the protective role of H₂O₂ by demonstrating that the presence of >1 vasodilator (NO and H₂O₂) in CAD vessels fully preserves the overall magnitude of FMD after exposure to acute hypertension, a stimulus known to precipitate endothelial damage,33 impair microvascular FMD,3 and worsen development of CAD.34,35 This endothelial resilience after PGC-1α upregulation is not observed after acute hypertension in vessels relying on a single mediator, including both CAD vessels (H₂O₂) and non-CAD vessels (NO).36 This observation underscores that PGC-1α upregulation not only restores NO bioavailability and reduces H₂O₂ (ie, reversal of the CAD phenotype) but also confers additional protection against barotrauma within the context of CAD. This finding corroborates the antihypertensive effects of PGC-1α described in a recent mouse model of angiotensin II infusion.37

Given the known relationship between PGC-1α and mitochondrial dynamics,38–40 and the results from previous studies in our laboratory indicating a central role for H₂O₂, we chose to examine the contribution of mtH₂O₂ during FMD during PGC-1α knockdown in non-CAD vessels and PGC-1α upregulation in CAD vessels. We further demonstrate the continued presence of H₂O₂ but no longer of mitochondrial H₂O₂ in PGC-1α-overexpressing CAD vessels, emphasizing that one must look beyond the mere presence of H₂O₂ and focus on its more specific subcellular source. It is also possible that there is a concentration-dependent effect of H₂O₂, with low levels serving a physiologically and MitoQ, blocks pathological reactive oxygen species (ROS) in addition to the physiological ROS needed for proper cellular signaling. As a result, recent attempts to develop and clinically test more targeted antioxidants (MitoQ, MitoVitE) that specifically limit mitochondrial—rather than total cellular—free radical production have been reported,44,45 with preliminary preclinical results describing the beneficial effects of these compounds within the context of cardiovascular disease.46,47 Our results suggest that PGC-1α upregulation may be an effective strategy to specifically dampen mtROS production, in the same manner as MitoQ and MitoVitE, while also preserving endogenous antioxidant levels and promoting release of cellular, nonmitochondrial H₂O₂. The mechanism underlying this relationship between PGC-1α and mtROS production warrants continued investigation.

We do not identify the molecular event caused by PGC-1α overexpression that results in dual contributions of NO and H₂O₂ to FMD. We speculate that several possibilities exist based on past literature. Caveolin-1 may act as a mechano-sensor regulating this pathway, given previous reports from the Shimokawa laboratory indicating that caveolin-1 may be a key mechanism responsible for setting the balance between NO and H₂O₂ in resistance vessels.32 Concerning potential intracellular mediators of this pathway, PKG-1α is downstream of both NO and H₂O₂, and establishes equilibrium between NO and H₂O₂ during dilation.48–50 One pathway responsible for the decreased mtROS production after PGC-1α overexpression may relate to the increase in NO bioavailability. Because NO inhibits mtROS in vessels,51 restoring NO in CAD vessels may decrease mtROS during FMD without impairing ROS formation at other sites.

We offer both practical and mechanistic rationales for the use of ALA, a compound that can be administered orally, as an adjunct treatment in cardiovascular disease via upregulation of NO bioavailability and downregulation of mtROS. Several
studies have noted broad associations between ALA supplementation and an improvement in cardiovascular parameters, such as brachial artery FMD in patients with CAD, and our data both support and extend these previous findings. Of note, the concentration of lipic acid that we used for incubation studies (250 μmol/L) is consistent with therapeutically achievable plasma concentrations of ALA found in human studies.

**Limitations**

Please see online-only Data Supplement for a complete list of study limitations.

**Perspectives**

This study suggests that the loss of PGC-1α is sufficient to shift the mechanism of FMD from NO to H₂O₂, thus establishing a link between decreased PGC-1α and CAD pathogenesis in the microcirculation. Overexpression of PGC-1α has a beneficial effect in vessels from subjects with CAD by restoring a component of NO-mediated dilation and conferring protection against acute increases in intraluminal pressure. Lipic acid supplementation has the potential to produce therapeutically advantageous effects in patients with CAD, but in-human clinical trials are needed to advance this concept.

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**Disclosures**

None.

**References**

Novelty and Significance

What Is New?
- Loss of PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α) contributes to the microvascular phenotype observed in subjects with coronary artery disease (CAD).
- PGC-1α upregulation allows both NO and hydrogen peroxide to contribute to flow-mediated dilation and decreases mitochondrial reactive oxygen species production in CAD vessels.
- Microvessels from subjects with CAD experience severe dysfunction after acute increase in intraluminal pressure.

What Is Relevant?
- PGC-1α protects against acute increases in intraluminal pressure–induced vascular dysfunction and may provide a therapeutic target to combat CAD.

Summary
This study demonstrates the functional significance of targeting PGC-1α in the human microcirculation and that loss of PGC-1α can expose a CAD phenotype. For the first time, to our knowledge, we show that overexpressing an endogenous molecule can provide plasticity during flow-mediated dilation that is preserved in the presence of CAD. We also provide evidence for the therapeutic potential of lipoic acid and ZLN005 within the setting of CAD (chronic) and acute hypertension.
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SUPPLEMENTAL METHODS

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were obtained from local hospitals and cultured in endothelial cell growth medium (EGM®-2-MV; Lonza) containing 5% fetal calf serum, subcultures were plated onto 100mm culture plates in order to study the effects of shear stress on vasodilator production. Cells are grown in EGM®-2 containing growth factors, cytokines, and supplements and fetal bovine serum (FBS) of 5%. Cells at passages 3-5 with >85% viability were used.

Tissue Acquisition
Fresh human adipose tissue was obtained from the discarded specimens of patients undergoing surgical procedures. Tissues were placed in cold HEPES buffer immediately following the surgical procedure and promptly transported to our laboratory. Arterioles were isolated from the adipose tissue and then placed into EGM®-2-MV media. De-identified patient demographic data were collected using the Generic Clinical Research Database at the Medical College of Wisconsin. All protocols were approved by the local Institutional Review Board at the Medical College of Wisconsin and the Zablocki VA Medical Center.

Immunohistochemistry
Following treatment with compounds to up- or down-regulate PGC-1α, fresh adipose arterioles were placed in zinc formalin buffer and subsequently processed for paraffin embedding. Immunohistochemistry was performed by the Children’s Hospital of Wisconsin Histology Core. Samples were sectioned and immunolabeled using a rabbit antihuman antibody to PGC-1α (Abcam; 1:1000). Immunostaining was performed using a Leica Bond MAX Immunostainer. Slides were de-paraffinized and subject to heat-induced epitope retrieval for 10 minutes at pH 6.0. The primary antibody was optimized using the Bond Refine-HRP detection system. Slides were scanned with a NanoZoomer HT slide scanner (Hamamatsu, Japan).

Western Blotting
After scraping cells from culture dishes and centrifuging them to form a cell pellet, cells were homogenized in cold lysis buffer (50 mmol/L Tris, pH 7.4, 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% NP40) supplemented with a protease and phosphatase inhibitor cocktail (Roche), and centrifuged at 12,000 g for 10 min (4oC). Total protein amount was quantified using a BCA protein assay. Protein samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies to catalase (Cell Signaling; 1:5000), manganese superoxide dismutase (Cell Signaling; 1:5000), beta-actin (β-actin; 1:1000), and GAPDH (1:10000), followed by peroxidase-conjugated secondary antibodies, were used for membrane blotting. All protein levels were normalized to levels of β-actin. Images were quantified with ImageJ.

Fluorescence Detection of Extracellular H2O2 Production in HUVECs
To assess H₂O₂ release in response to increases in PGC-1α levels following lipoic acid treatment, cultured HUVECs were exposed to shear stress (15 dyn/cm²) for 60 minutes at 37°C in the dark and in the presence of Amplex Red (2 mmol/L, Thermofisher) diluted in the appropriate buffer (NaCl 140mM, KCl 4.86mM, MgSO4.7H2O 1.22mM, NaH2PO4·H2O 1.08mM, NaH2PO4·7H2O·4.62mMEDTA 0.026mM, CaCl2·2H2O 0.54-0.1mM, Glucose 5.5mM, pH 7.35-7.40). While continuing to limit light exposure, the supernatant was collected and a volume of 100 µmol/L was loaded into a 96-well plate and fluorescence was measured relative to standard controls generated by serial dilutions of H2O2 on a SpectraMax M5 using excitation and emission levels of 490 nanometers and 585 nanometers, respectively. To correct for background fluorescence, measurements were compared to a no-H₂O₂ control. All fluorescence values were normalized to total protein from each dish using a BCA protein assay.
FMD and Videomicroscopy
Arterioles were carefully isolated from human adipose tissue acquired from discarded surgical specimens under a dissecting microscope, placed into endothelial cell growth medium containing 5% serum (Lonza) along with treatments that increased (15 µmol/L ZLN005 and 250 µmol/L ALA for 16-24 hours) or decreased (lentiviral siRNA for 40-48 hours; final titer concentration 1x105 transfection units/mL) PGC-1α levels in a 37° C incubator. Vessels were allowed to equilibrate at 37° C for 30 minutes prior to incubation. Fluorescence was recorded before and after initiation of flow allowed for determination of the contribution of NO and H₂O₂ to flow-mediated dilation, respectively. To determine the subcellular source of H₂O₂, we assessed dilation after addition of the mitochondria-targeted pharmacological agents mitoPBA (a mitochondrial-specific H₂O₂ scavenger; 5 µM) or rotenone (mitochondrial complex I inhibitor; 1 µM).

Fluorescence Detection of mH₂O₂ Production in Human Arterioles
To assess microvascular mH₂O₂ production in response to increases in PGC-1α levels following lipoic acid treatment, non-CAD and CAD arterioles were removed from media and cannulated in a warmed organ chamber containing HEPES buffer. Intraluminal perfusion of arterioles with the fluorescent probe Mito Peroxy Yellow 1 (MitoPY1; 10 µmol/L) allowed for detection of mH₂O₂. Fluorescence was recorded before and after initiation of flow (100 cmH₂O) using a krypton/argon lamp fluorescent microscope (model TE 200 Nikon Eclipse) and an excitation/emission wavelength of 488 nm/530-590 nm. Following initiation of flow, images were captured at one-minute intervals for 5 minutes. Relative fluorescence intensity (vessel fluorescence minus background fluorescence; arbitrary units) was analyzed with Metamorph software (Universal Imaging Corp.). Data were recorded as percent change in fluorescence after initiation of flow (i.e., no flow vs flow). Percent change in MitoPY1 fluorescence in CAD vessels treated with lipoic acid were compared to untreated CAD vessels.

Detection of NO production in HUVECs
Nitric oxide production was determined by the conversion of DAF2 to DAF2-triazole (DAF2-T) and detected via HPLC. Briefly DAF2-DA (5 µmol/L) was added to cells for 1 hour. Cells were harvested and lysed and supernatants were filtered through a 5Kd cut-off microcon filter. Eluent (80 µl) was injected onto a C18 reverse-phase HPLC column and separated using a mobile phase of sodium phosphate (10 mmol/L, pH 7.5) and acetonitrile (5%) with a flow-rate of 1 ml/min. DAF2-T was detected by fluorescence (excitation/emission wavelength of 490 nm/515 nm) and quantified by area under the curve. HPLC was performed by MCW’s Redox Biology Core.

SUPPLEMENTAL STUDY LIMITATIONS
Our tissue acquisition process results in several study limitations. First, we cannot control for differing subject characteristics. There is a notable difference between the mean age between the CAD and non-CAD subjects. However, we have recently demonstrated that presence of age itself does not influence the mechanism of FMD in adults. Second, subjects taking medications cannot be excluded due to the inability to obtain these clinical data. However, we utilize an extensive washout protocol that has been shown to minimize effects of medications. Arterioles harvested from subcutaneous or pericardial adipose tissue were used in this study, whereas many of our previous studies have used coronary arterioles from atrial appendages. Differences in the reactivity of vessels in different vascular beds may exist, but previous work from our lab demonstrates that the responses are largely similar in vessels harvested at different sides in the human body, particularly in relation to FMD in subjects with and without CAD. Most of the non-CAD arterioles were isolated from female subjects. Sex-based differences in the magnitude of arteriolar FMD has been described in mice but not in humans. The number of cases tested in this study is not sufficient to exclude an influence of sex on vascular reactivity in these subjects. Finally, it is possible that maximal dilation is different between groups or interventions, and that the degree of pre-constriction affected our results. No differences in maximal dilation to papaverine or degree of pre-constriction were found between groups.
Our HUVEC data in Figure 4 are not completely reflective of the complexity of findings generated using intact human vessels. However, the question we wanted to answer was, what changes, if any, in endothelial redox genes might reveal why we observe a maintained contribution of H$_2$O$_2$ as an endothelium-derived vasodilator? In addition, since we use an endothelium-dependent stimulus, we consider the PGC-1α overexpression phenotype to be reflective of redox dynamics in the endothelium, which is why we studied redox genes in the endothelium alone. Still, we cannot exclude that the nearby SMCs affect the vasodilator response. Unfortunately, it is technically difficult to assess protein and mRNA levels in the small sample sizes provided by the arterioles we study.

Alpha-lipoic acid (ALA) is a non-specific agent for upregulating PGC-1α. Therefore, we cannot exclude the possibility that the results we obtained are the result of additional, off-target effects. To address this concern, we used the more selective small-molecule transcriptional activator of PGC-1α, ZLN005. Results using either compound were strikingly similar, bolstering the idea that the novel phenotype we observed was attributable to PGC-1α upregulation. Unfortunately, we did not determine the exact level of PGC-1α upregulation at which this vasodilatory plasticity is produced; however, given that we used two different concentrations of these compounds, we anticipate that such plasticity is achievable over a range of upregulated PGC-1α levels in both healthy and diseased tissue.

Future studies will decipher the upstream and downstream mechanisms responsible for the simultaneous contribution of NO and H$_2$O$_2$ to FMD in human microvessels. These data support a promising approach using PGC-1α upregulation as a therapeutic target in CAD through reversal of the disease phenotype (i.e., reduction in mtH$_2$O$_2$ and restoration of NO-mediated dilation). Future studies will also address the source of H$_2$O$_2$ following PGC1a overexpression.

SUPPLEMENTAL REFERENCES


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Table S1. Patient demographics for adipose microvessels. n, number of patients. *P<0.05 non-CAD patients vs patients with CAD.
Figure S1. PGC-1α levels in human left ventricular heart tissue. Protein levels of PGC-1α in left ventricular tissue homogenates were analyzed via western blot. n=7-9. *P < 0.05 non-CAD vs CAD.
Figure S2. Immunohistochemical staining for PGC-1α and CD31 in human arterioles. A) 15 µM DMSO (16-24 hr); B) PGC-1α siRNA (48 hr); C) endothelial cell marker CD31; D) 15 µM DMSO (16-24 hr); E) 15 µM ZLN005 (16-24 hr); F) 250 µM ALA (16-24 hr). Vessel size range: 100-200 µM.