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 (\(\text{H}_2\text{O}_2\)). Excessive \(\text{H}_2\text{O}_2\) production contributes to a proatherosclerotic vascular milieu. Loss of PGC-1\(\alpha\) (peroxisome proliferator–activated receptor \(\gamma\) coactivator 1\(\alpha\)) is implicated in the pathogenesis of CAD. We hypothesized that PGC-1\(\alpha\) suppresses \(\text{H}_2\text{O}_2\) 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 (\(\text{H}_2\text{O}_2\) scavenger) or L-NAME (\(\text{N}^\text{\#}-\text{nitro-\text{L-arginine methyl ester; NOS inhibitor}\)). In contrast to the exclusively NO- or \(\text{H}_2\text{O}_2\)-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-1\(\alpha\) upregulation using ZLN005 and \(\epsilon\)-lipoic acid. PGC-1\(\alpha\) overexpression in CAD vessels protected against the vascular dysfunction induced by an acute increase in intraluminal pressure. In contrast, downregulation of PGC-1\(\alpha\) in non-CAD vessels produces a CAD-like phenotype characterized by \(\text{H}_2\text{O}_2\)-mediated dilation (no contribution of NO). Loss of PGC-1\(\alpha\) may contribute to the shift toward the \(\text{H}_2\text{O}_2\)-mediated dilation observed in vessels from subjects with CAD. Strategies to boost PGC-1\(\alpha\) levels may provide a therapeutic option in patients with CAD by shifting away from \(\text{H}_2\text{O}_2\)-mediated dilation, increasing NO bioavailability, and reducing levels of \(\text{H}_2\text{O}_2\). Furthermore, increased expression of PGC-1\(\alpha\) allows for simultaneous contributions of both NO and \(\text{H}_2\text{O}_2\) 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. 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.

Microvascular dysfunction is strongly prognostic for cardiovascular events, 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 (\(\text{H}_2\text{O}_2\)). Excessive \(\text{H}_2\text{O}_2\) 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, an acute increase in intraluminal pressure, and exogenous administration of the sphingolipid ceramide, can induce a switch to \(\text{H}_2\text{O}_2\)-mediated dilation, but the endogenous regulator of this shear-sensitive switch is not known.

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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.10–13 Given the shear-sensitive,14 redox-modulating15 vascular properties of PGC-1α, as well as its known interaction with NO in cultured endothelial cells and animal vessels,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 \( \text{H}_2\text{O}_2 \)-mediated dilation. 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 \( \text{H}_2\text{O}_2 \) during FMD, and protects against acute increases in intraluminal pressure, in vessels from subjects with CAD.

**Methods**

**Materials**

ZLN005 (Sigma), a known small-molecule activator of PGC-1α,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 (GeroNova Research) was dissolved in distilled water. Endothelin-1 (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 percentage 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's t test was used to compare baseline characteristics for patients with and without CAD. Differences in Western blot protein levels and \( \text{H}_2\text{O}_2 \) production in human umbilical vein endothelial cells (HUVECs) were also assessed with an unpaired Student's 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 \( \text{H}_2\text{O}_2 \)-mediated dilation to shear (inhibited by PEG [polyethylene glycol]–catalase) (% max diameter at 100 cm \( \text{H}_2\text{O} \): vehicle 81.3±4.3, PEG-catalase 0.7±9.6). In contrast, L-NAME (N\(^\circ\) nitro-\( \text{L} \)-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 \( \text{H}_2\text{O} \): 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 \( \text{H}_2\text{O}_2 \) as the compensatory vasodilator.

Because a relative reduction in PGC-1α levels is observed in CAD vessels, and CAD vessels dilate to \( \text{H}_2\text{O}_2 \), we anticipated that a forced downregulation of PGC-1α in non-CAD arterioles using siRNA would also result in the unmasking of compensatory \( \text{H}_2\text{O}_2 \) production from the mitochondria. Indeed, after PGC-1α knockdown, rotenone (inhibitor of electron transport chain complex 1) and mitoPBA (mitochondria-targeted \( \text{H}_2\text{O}_2 \) scavenger) both inhibited FMD (Figure 1D) (% max diameter at 100 cm \( \text{H}_2\text{O} \): 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 segment. The mechanism of dilation in these non-CAD vessels was unchanged (ie, FMD was still inhibited by L-NAME; Figure 1A) (% max diameter at 100 cm \( \text{H}_2\text{O} \): vehicle 77.2±10.1, L-NAME –10.9±10.8), and successful transfection of GFP into arterioles was confirmed (Figure 1B). These findings indicate that non-CAD vessels dilate to NO, consistent with our previous reports.6,7 Moreover, the switch from NO- to \( \text{H}_2\text{O}_2 \)-mediated dilation that we observed in non-CAD vessels after PGC-1α downregulation originates from mitochondrial sources and is attributable to changes in PGC-1α levels, not the lentivirus itself.

**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 \( \text{H}_2\text{O}_2 \)-mediated dilation, we hypothesized...
that, conversely, upregulation of PGC-1α levels in CAD vessels, which normally dilate to increased shear via H$_2$O$_2$, 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,$^{11}$ and ZLN005, a novel small molecule transcriptional activator of PGC-1α.\textsuperscript{18,19} Before treatment, PEG-catalase blocked dilation in CAD vessels (Figure 2A) (% max diameter at 100 cm H$_2$O: vehicle 75.3±11.1, PEG-catalase 24.2±5.6), as we have previously observed.\textsuperscript{6} Unexpectedly, PGC-1α overexpression in CAD vessels exposed a novel phenotype characterized by contributions of both NO and H$_2$O$_2$ 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 H$_2$O flow in vessels that are: untreated: 24.2±5.6; versus ALA-treated: 56.3±11.0, and ZLN-treated: 54.4±8.5). 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 H$_2$O flow in vessels that are: untreated: 24.2±5.6; versus ALA-treated: 25.3±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,

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Effect of PGC-1α (peroxisome proliferator–activated receptor γ coactivator 1α) downregulation on flow-mediated dilation (FMD) in non–coronary artery disease (CAD) vessels. **A**, The magnitude of FMD is preserved in human arterioles after 48-hour treatment with lentiviral GFP (green fluorescent protein). L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester; endothelial nitric oxide synthase inhibitor) acts to inhibit FMD. **B**, Confirmation of lentiviral GFP uptake in non-CAD vessel via immunohistochemistry. **C**, The magnitude of FMD is preserved in human arterioles after 48-hour treatment with PGC-1α siRNA. PEG (polyethylene glycol)–catalase (H$_2$O$_2$ scavenger) acts to inhibit FMD, whereas L-NAME has no effect. **D**, To determine the source of H$_2$O$_2$ following PGC-1α knockdown, vessels were incubated with mitochondria-targeted inhibitors rotenone (1 μmol/L) and mitoPBA (5 μmol/L) for 30 minutes before initiation of flow. n=4 to 7 per treatment condition. *P<0.05 vs control curves at specific pressure gradients.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Effect of peroxisome proliferator–activated receptor γ coactivator 1α overexpression on flow-mediated dilation (FMD) in coronary artery disease (CAD) vessels. Mechanism of FMD in CAD vessels relies on H$_2$O$_2$ at (**A**) baseline, n=4 (control) and 5 (PEG-catalase), *P<0.05 Peg-CAT vs control curve at specific pressure gradients; or both nitric oxide and H$_2$O$_2$ after treatment with (**B**) 250 μmol/L ALA (16–24 hours) and (**C**) 15 μmol/L ZLN005 (16–24 hours). n=5 to 8 per curve. *P<0.05 Peg-CAT+L-NAME vs control curve at specific pressure gradients. **D**, Effect of Peg-CAT on FMD between different treatment groups. *P<0.05 ALA/ZLN+Peg-CAT vs control+Peg-CAT curve at specific pressure gradients. L-NAME indicates N\textsuperscript{G}-nitro-L-arginine methyl ester; and PEG, polyethylene glycol.
we observed a maintained contribution of H2O2 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 H2O2.

**Source of H2O2 After Overexpression of PGC-1α in CAD Vessels**

We next examined whether the mitochondria remain the subcellular source of H2O2 after PGC-1α upregulation in CAD vessels. FMD in CAD vessels is mediated entirely by mtH2O2 and can be inhibited by either PEG-catalase or specific mitochondrial H2O2 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 H2O2 as a vasodilator, there is a transition away from the mtH2O2 production observed in untreated CAD vessels.

To further investigate these findings using a complementary technique, we assessed mtH2O2 production using a mitochondria-targeted H2O2 probe (MitoPY1) in untreated and PGC-1α-overexpressing CAD vessels. Exposure of these vessels to shear stress revealed that PGC-1α overexpression suppresses mtH2O2 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 H2O2 in HUVECs in Response to Shear**

To confirm the dual release of NO and H2O2, PGC-1α-overexpressing HUVECs were exposed to shear stress (15 dynes/cm²) for 1 hour, and levels of NO and H2O2 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 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.
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 NOX4-derived H2O2, which is atheroprotective, and can improve vasodilation without harmful oxidative effects on the vascular wall. 

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. 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, impair microvascular FMD, and worsen development of CAD. 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). 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. 

Given the known relationship between PGC-1α and mitochondrial dynamics, and the results from previous studies in our laboratory indicating a central role for mtH2O2, 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 physiological role while high levels are detrimental. Our data highlight that H2O2 production may exist alongside conserved antioxidant defense mechanisms. This is a particularly intriguing finding, considering the high-profile failure of several global exogenous antioxidant trials in the past, believed to be the result of blocking 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, with preliminary preclinical results describing the beneficial effects of these compounds within the context of cardiovascular disease. Our results suggest that PGC-1α upregulation may be an effective strategy to specifically dampen ROS 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 ROS 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. 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. One pathway responsible for the decreased mtROS production after PGC-1α overexpression may relate to levels and promoting release of cellular, nonmitochondrial H2O2. The mechanism underlying this relationship between PGC-1α and mtROS production warrants continued investigation.

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
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. Lipoic 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.
- Microvesicles from subjects with CAD experience severe dysfunction after acute increases in intraendothelial pressure.

What Is Relevant?

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

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 lipidic 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 (4°C). 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 H$_2$O$_2$ release in response to increases in PGC-1α levels following lipoic acid treatment, cultured HUVECs were exposed to shear stress (15 dyn/cm$^2$) for 60 minutes at 37°C in the dark and in the presence of Ampex Red (2 mmol/L, Thermofisher) diluted in the appropriate buffer (NaCl 140mM, KCl 4.86mM, MgSO4.7H2O 1.22mM, NaH2PO4.H2O 1.08mM, NaH2PO4.7H2O4.62mMEDTA 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-H$_2$O$_2$ 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 with a flow to 30 cmH2O between groups or a scavenger; 5 µM) or rotenone (mitochondrial complex I inhibitor; 1 µM). Fluorescence was recorded before or flow).

To assess microvascular mtH2O2 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 mtH2O2. 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.