Resveratrol induces mitochondrial biogenesis in endothelial cells

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Departments of 1Physiology, 2Biochemistry, and 3Cell Biology and Anatomy and Pediatrics, New York Medical College, Valhalla, New York; 4Department of Internal Medicine, Medical Pharmacology and Physiology and Nutritional Sciences, University of Missouri, Columbia, Missouri; 5Department of Pulmonology, Semmelweis University, Hungary; 6Laboratory of Experimental Gerontology, National Institute on Aging, National Institutes of Health, Baltimore; and 7Laboratory of Physiological Studies, Section on Oxidative Stress Tissue Injury, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, Maryland; and 8Reynolds Oklahoma Center on Aging, Department of Geriatric Medicine, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma

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Csiszar A, Labinskyy N, Pinto JT, Ballabh P, Zhang H, Losonczy G, Pearson K, de Cabo R, Pacher P, Zhang C, Ungvari Z. Resveratrol induces mitochondrial biogenesis in endothelial cells. Am J Physiol Heart Circ Physiol 297: H13–H20, 2009. First published May 8, 2009; doi:10.1152/ajpheart.00368.2009.—Pathways that regulate mitochondrial biogenesis are potential therapeutic targets for the amelioration of endothelial dysfunction and vascular disease. Resveratrol was shown to impact mitochondrial function in skeletal muscle and the liver, but its role in mitochondrial biogenesis in endothelial cells remains poorly defined. The present study determined whether resveratrol induces mitochondrial biogenesis in cultured human coronary arterial endothelial cells (CAECs). In CAECs resveratrol increased mitochondrial mass and mitochondrial DNA content, upregulated protein expression of electron transport chain constituents, and induced mitochondrial biogenesis factors (proliferator-activated receptor-coactivator-1α, nuclear respiratory factor-1, mitochondrial transcription factor A). Sirtuin 1 (SIRT1) was induced, and endothelial nitric oxide (NO) synthase (eNOS) was upregulated in a SIRT1-dependent manner. Knockdown of SIRT1 (small interfering RNA) or inhibition of NO synthesis prevented resveratrol-induced mitochondrial biogenesis. In aortas of type 2 diabetic (db/db) mice impaired mitochondrial biogenesis was normalized by chronic resveratrol treatment, showing the in vivo relevance of our findings. Resveratrol increases mitochondrial content in endothelial cells via activating SIRT1. We propose that SIRT1, via a pathway that involves the upregulation of eNOS, induces mitochondrial biogenesis. Resveratrol induced mitochondrial content in the aortas of type 2 diabetic mice, suggesting the potential for new treatment approaches targeting endothelial mitochondria in metabolic diseases.

vasoprotection; histone deacetylation; endothelial dysfunction; diabetes; obesity

ENDOTHELIAL MITOCHONDRIA have a crucial role in vascular pathophysiology (1, 12, 25, 34). Mitochondria are highly dynamic organelles, and their biogenesis is likely to be involved in the regulation of endothelial cell metabolism, redox regulation, and signal transduction. Impairment of mitochondrial biogenesis is frequently observed in diabetes and the metabolic syndrome (28) and is thus likely to contribute to cellular energetic imbalance, oxidative stress, and endothelial dysfunction in these pathological conditions (16). Previous studies show that dysregulation of mitochondrial biogenesis represents an early manifestation of endothelial dysfunction, shifting cell metabolism toward metabolic hypoxia in animals with impaired nitric oxide (NO) bioavailability (1). Since increased mitochondrial production of reactive oxygen species (ROS) due to impaired mitochondrial biogenesis also appears to be a key event in the development of aging-induced vascular pathologies (6, 41, 42), identification of mechanisms that promote mitochondrial biogenesis in the endothelial cells may contribute to the development of improved pharmacological approaches to promote vascular health in both patients with diabetes (20) and the elderly.

In mice treatment with resveratrol, (3,5,4’-trihydroxy stilbene), a diet-derived polyphenol, improved mitochondrial function and biogenesis in the skeletal muscle (21) and the liver (3). Studies in diabetic mice demonstrated that resveratrol treatment improves endothelial function and attenuates vascular inflammation in diabetes mellitus (32, 35, 37, 38, 46) and extends longevity (3, 32). Similar protective effects of resveratrol treatment were observed in aged mice (32, 42). As noted above, both diabetes and aging are characterized by impaired mitochondrial biogenesis, yet the effects of resveratrol on mitochondria in the endothelial cells remain incompletely understood.

The present study was conducted to determine whether resveratrol induces mitochondrial biogenesis in endothelial cells. The effects of resveratrol treatment on mitochondrial mass and the induction of factors regulating mitochondrial biogenesis were assessed in primary human coronary arterial endothelial cells (HCAECs). We focused on the mechanistic role of sirtuin 1 (SIRT1) activation and endothelial NO synthase (eNOS) induction in the effects of resveratrol. The relevance of the effects of resveratrol in vivo was tested on vascular mitochondrial content in type 2 diabetic (db/db) mice.

METHODS

Cell cultures and SIRT1 knockdown. Primary HCAECs (purchased from Cell Applications) in culture were treated with resveratrol (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol; 1–10 μmol/l; for 24–48 h) as described (8, 39). Resveratrol (purity ≥98%) was purchased from Cayman Chemical Company (Ann Arbor, MI). To disrupt SIRT1 signaling, downregulation of SIRT1 was achieved by RNA interference using proprietary small interfering RNA (siRNA) sequences (Superarray) and the Amazza Nucleasector technology (Amazza, Gaithersburg, MD), as our laboratory has previously reported (5). Specific gene silencing was verified with quantitative QRT-PCR and Western blotting (at the mRNA and protein level,

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respectively) as described (7). The SIRT1 siRNA used in our studies did not affect the expression of the reference genes β-actin, HPRT, and YWHAZ. Cell density at transfection was 30%. Experiments were performed on day 2 after the transfection, when gene silencing was optimal. Specific gene silencing was verified with Western blotting (5, 7). N°-nitro-L-arginine methyl ester (L-NAME; 3 × 10⁻⁴ M) was used to inhibit NO synthesis.

SIRT1 activity assay. Nuclear SIRT1 activity was measured in cells treated with resveratrol. In brief, cells were suspended in lysis buffer containing 10 mM Tris·HCl (pH 7.5), 10 mM NaCl, 15 mM MgCl₂, 250 mM sucrose, 0.5 mM Nonidet P-40, and 0.1 mM EGTA and were centrifuged through 4 ml of sucrose cushion [30% sucrose, 10 mM Tris·HCl, 10 mM NaCl, 0.5% Nonidet P-40, and 0.1 mM EGTA and 10% glycerol; sonicated for 30 s, and incubated on ice for 30 min, followed by centrifugation (15,000 rpm for 10 min) The nuclear extract was collected, and the protein concentration was determined by the Bradford method. SIRT1 was immunoprecipitated from the samples using a rabbit polyclonal antibody directed against the COOH terminus of SIRT1 (Abcam no. ab28170). SIRT1 activity in the samples was measured using the Cyclex SIRT1 Deacetylase Fluorometric Assay Kit according to the manufacturer’s protocols (CycLex, Nagano, Japan). In brief, this assay is based on the principle that upon NAD-dependent deacetylation of the specific substrate by SIRT1 (in the presence of trichostatin A, a potent inhibitor of SIRT1-independent histone deacetylases), the fluorosubstrate peptide is cleaved by a lysyl endopeptidase, separating the quencher from the fluorophore. Specific activity of SIRT1 was assessed by measuring time-dependent changes in fluorescence intensity, normalized to protein concentration. Standard assay controls included the use of a fluoresceinated peptide (to control for lysyl endopeptidase activity), no enzyme control, no NAD⁺ control, and no inhibitor control. We also assessed resveratrol-induced increases in the specific activity of recombinant SIRT1 in the presence and absence of the specific SIRT1 inhibitor sirtinol (10⁻⁴ M).

Measurement of mitochondrial mass in coronary arterial endothelial cells using Mitotracker red staining. Coronary arterial endothelial cells (CAECs) were treated with resveratrol (10 μmol/l for 48 h) in the absence or presence of SIRT1 knockdown (siRNA). Mitochondrial mass in CAECs was determined by selectively loading the mitochondria with the red fluorescent dye Mitotracker red (Invitrogen, Carlsbad, CA). The fluorescent dyes calcein (green) and Hoechst 33258 (blue) were used to stain the cytoplasm and nuclei, respectively. Optical sections of CAECs were captured at ×60 magnification, and the ratio of mitochondrial area densities to cytoplasmic volume was calculated using the Zeiss Axiovision imaging software. Only cells with intact cytoplasmic calcein staining were included in the analysis. In separate experiments the mitochondria were loaded with Mitotracker red and fluorescence signal intensities were assessed by flow cytometry. The impact of pretreatment with L-NAME (3 × 10⁻⁴ mol/l) or SIRT1 siRNA on the effects of resveratrol treatment was assessed.

Measurement of mitochondrial DNA content in CAECs. Total DNA was isolated from CAECs (DirectPCR; Viagen Biotech). Mitochondrial DNA (mtDNA) copy number was determined by QRT-PCR as described (1), using cytochrome oxidase III and β-actin as markers for the copy numbers of mtDNA and genomic DNA, respectively. The impact of pretreatment with L-NAME (3 × 10⁻⁴ mol/l) or SIRT1 siRNA on the effects of resveratrol treatment was assessed.
RESULTS

Resveratrol induces mitochondrial biogenesis in endothelial cells. Mitotracker staining showed that mitochondria are localized in the perinuclear region in CAECs (Fig. 1, A and B). The ratio of mitochondrial area densities to cytoplasmic volume in Mitotracker-labeled endothelial cells was significantly increased by resveratrol treatment (Fig. 1, B and C). Analysis of Mitotracker fluorescence intensities in CAECs confirmed that resveratrol treatment increased mitochondrial mass significantly in treated compared with untreated animals (Fig. 1D). Knockdown of SIRT1 (siRNA; Fig. 3C) and pretreatment with i-NAMe prevented resveratrol-induced increase in mitochondrial mass (Fig. 1D).

Resveratrol induces SIRT1 and eNOS in endothelial cells. Resveratrol upregulated protein expression of SIRT1 and increased SIRT1 enzymatic activity in endothelial cells (Fig 2). Resveratrol significantly increased mRNA and protein expression of eNOS, which were prevented by knockdown of SIRT1 (Fig. 3, A and B).

Resveratrol upregulates electron transport chain constituents in endothelial cells. Western blotting revealed that when compared with untreated CAECs, expression of porin was significantly increased in resveratrol-treated cells (Fig. 4A). Levels of porin (which controls the diffusion of small metabolites through the outer membrane) correlate with the cellular mitochondrial volume. Expression of complex I, complex II, complex III, complex IV, and complex V in CAECs (Fig. 4, B–F) was also increased by resveratrol treatment. Pretreatment with i-NAMe prevented resveratrol-induced increases in mitochondrial protein expression (Fig. 4).

Resveratrol upregulates mitochondrial biogenesis factors. Mitochondrial biogenesis involves the integration of multiple transcriptional pathways controlling both nuclear and mitochondrial gene expression. The PPAR coactivator PGC-1α, Nrf-1, and Tfam are considered key regulators of mitochondrial biogenesis in multiple tissues. QRT-PCR measurements revealed that the expression of the mitochondrial biogenesis factors PGC-1α, Nrf-1, and Tfam in CAECs (Fig. 5, A–C) was significantly increased by resveratrol.

Fig. 1. When compared with untreated controls (A), resveratrol (Res; 10 μM for 48 h) significantly increased the number of mitochondria in cultured coronary arterial endothelial cells (CAECs). Relative mitochondrial mass was estimated by using Mitotracker red staining. The cell body was visualized by differential interference contrast and by green calcein fluorescence (blue: nuclei). Only cells with intact calcium staining were analyzed. Summary data for mitochondrial surface area are shown in C. D: Mitotracker Fluorescent intensities in CAECs were measured to assess mitochondrial biogenesis. Knockdown of siRNA (SIRT1) [small interfering RNA (siRNA)] or 1-NAMe prevented resveratrol-induced mitochondrial biogenesis. E: resveratrol increased mitochondrial DNA (mtDNA) content in CAECs, which was prevented by i-NAMe or SIRT1 siRNA. Data are means ± SE. *P < 0.05; #P < 0.05 vs. resveratrol only. au, Arbitrary units.

Fig. 2. Treatment of CAECs with resveratrol (Res; 10 μmol/l) upregulated SIRT1 protein expression (A; Western blotting) and activity (B; data from fluorimetric SIRT1 activity assay). C: time course for siRNA knockdown of SIRT1 in CAECs (Western blotting). OD, optical density; AFU, arbitrary fluorescence units; c, control; sc, scrambled siRNA control. *P < 0.05 vs. control.
We found that in aortas of db/db mice relative mtDNA content (Fig. 6A) and induced the expression of mitochondrial biogenesis factors (Fig. 6, B and C) thus eliminating the difference between the two groups.

DISCUSSION

Pathways that regulate mitochondrial biogenesis have recently emerged as potential therapeutic targets for the amelioration of endothelial dysfunction and vascular disease observed in metabolic diseases (34). Our studies show that resveratrol induces mitochondrial biogenesis in CAECs. Formation of new mitochondria is associated with activation of SIRT1, upregulation of eNOS, and induction of specific mitochondrial biogenesis factors. We also show that resveratrol treatment induces mitochondrial biogenesis in aortas of type 2 diabetic mice.

Resveratrol is a prototype of a new class of drugs referred to as caloric restriction mimetics (4), which are being developed to reverse organ pathologies associated with aging and metabolic diseases. Resveratrol was shown to exert diverse antiaging effects (3, 18, 19, 32, 44). Previous studies focused on the effects of resveratrol on proinflammatory pathways and antioxidant defense mechanisms in endothelial cells (7, 8, 39) but provided little information on its effects on endothelial mitochondria. Our data support the finding that resveratrol increases mitochondrial content in CAECs (Fig. 1, A–D). Increased mitochondrial biogenesis in resveratrol-treated cells is also indicated by increased cellular mtDNA content (Fig. 1E) and increased protein expression of respiratory chain components (Fig. 4). Multiple mechanisms may explain resveratrol-induced mitochondrial biogenesis and its contribution to vascular health. Impaired mitochondria (e.g., in diabetes and in aging) may diminish ATP production, thereby impairing the synthesis and secretion of endothelium-derived factors that serve as paracrine signals in the vascular wall. Lack of ATP also affects transport functions of the vascular endothelium. Resveratrol-induced mitochondrial biogenesis would correct this impairment. Furthermore, because mitochondrial proliferation reduces the flow of electrons per unit mitochondria, resveratrol-induced mitochondrial biogenesis may reduce mitochondrial ROS production in endothelial cells. Indeed, our recent studies suggest that resveratrol, at physiologically relevant concentrations, attenuates mitochondrial oxidative stress in endothelial cells (7).

To determine whether the increased number of mitochondria in resveratrol-treated endothelial cells is a consequence of the induction of mitochondrial biogenesis factors, we used QRT-PCR to examine the expression of Nrf-1, Tfam, and PGC-1α. Nrf-1 activates the transcription of a number of the nuclear-encoded components of the electron transport chain and also regulates Tfam, which is responsible for the transcription of mtDNA-encoded genes. The regulatory function of Nrf-1 and other mitochondrial biogenesis factors is modulated by PGC-1α. We found that in CAECs, resveratrol induced Nrf-1, Tfam, and PGC-1α (Fig. 5, A–C). It has been established that the altered expression of these factors modulates mitochondrial biogenesis activity (27, 29–31).

The NAD+-dependent protein deacetylase SIRT1 plays a critical role in resveratrol-induced effects in endothelial cells.
Accordingly, resveratrol induces SIRT1 in endothelial cells (Fig. 2), thus extending previous observations in other cell types (18, 21). Resveratrol also lowers the $K_m$ of SIRT1 for the acetylated substrate and for NAD$^+$ (18). Knockdown of SIRT1 prevented resveratrol-induced mitochondrial biogenesis (Fig. 1, D and E) and the induction of mitochondrial biogenesis factors (Fig. 5). Overexpression of SIRT1 also induces mitochondrial biogenesis in CAECs, mimicking the effects of resveratrol (unpublished observations). These findings are in accord with previous studies, which showed that resveratrol and SIRT1 regulate mitochondrial function and mitochondrial biogenesis in skeletal muscle and the liver (15, 21). SIRT1 likely regulates multiple pathways involved in mitochondrial biogenesis in the endothelial cells, among which NO-dependent pathways appear to play a key role. In addition, SIRT1 may also directly deacetylate PGC-1α, increasing its activity (21, 27).

We demonstrated that inhibition of NO synthesis prevents resveratrol-induced mitochondrial biogenesis in CAECs (Fig. 1, D and E, and Fig. 4), suggesting that NO has an autocrine function in mediating the effects of resveratrol/SIRT1 in endothelial cells. Accordingly, we also found that genetic lack of eNOS prevents resveratrol-induced mitochondrial biogenesis and the upregulation of mitochondrial biogenesis factors in cultured mouse arteries (Fig. 3, C–F). In that regard, it is significant that resveratrol in a SIRT1-dependent manner upregulates eNOS in both cultured arteries and CAECs (Fig 2, A and B), confirming results of previous studies (26, 39, 43). Overexpression of SIRT1 also induces eNOS in cultured rat aortas and CAECs, mimicking the effects of resveratrol (unpublished observations). Although the precise role of eNOS in the SIRT1-induced signaling pathway is unclear, recent evidence supports that NO plays a critical role in initiating and integrating signaling events underlying mitochondrial biogenesis in various tissues. Importantly, inhibition of NO synthesis significantly decreases mitochondrial content in the vasculature (1). Treatment with NO donors also increases mitochondrial mass in various cell types, including brown adipocytes and 3T3-L1, U937, and HeLa cells (29–31). Previous studies also suggest that expression of mitochondrial biogenesis factors may be directly regulated by bioavailability of NO (2, 31).

The association between the pathogenesis of diabetes and its complications and mitochondrial biogenesis has been recently reported (28, 33). Because resveratrol has been reported to exert a possible additional benefit in preventing diabetes complications, we investigated the effect of resveratrol on vascular mitochondrial biogenesis in type 2 diabetes mellitus. In db/db mice, type 2 diabetes diminishes mitochondrial biogenesis in the aorta, as indicated by the decreased mtDNA content (Fig. 6A) and downregulation of mitochondrial biogenesis factors (Fig. 6, B and C). Resveratrol treatment significantly increased vascular mtDNA content (Fig. 6A) and induced the expression of mitochondrial biogenesis factors (Fig. 6, B and C) in both control and db/db mice, eliminating the difference between the two groups. These results extend previous findings that showed chronic resveratrol treatment in type 2 diabetic mice induces mitochondrial biogenesis in the liver and skeletal muscle (3, 21), demonstrating the in vivo relevance of our in vitro find-
Type 2 diabetes mellitus is associated with both macrovascular and microvascular dysfunction, which is characterized by impaired bioavailability of NO in the coronary arteries and other vascular beds (13, 14, 45), which is thought to lead to macrovascular complications of diabetes. We have recently demonstrated that resveratrol induces eNOS and significantly increases NO bioavailability in animal models of type 2 diabetes, including mice fed a high-fat diet (32) and db/db mice (47). Because NO appears to be a key regulator of endothelial mitochondrial content (1), we attribute the resveratrol-induced mitochondrial biogenesis in diabetic mice to the restoration of NO bioavailability. A recent study utilizing a systems biology approach showed that resveratrol can recapitulate many of the molecular events downstream of caloric restriction in vivo (36). In that context it is significant that caloric restriction mimetics to target endothelial mitochondria in metabolic diseases.

In conclusion, resveratrol, at physiologically relevant concentrations (4), increases mitochondrial content in endothelial cells via activating SIRT1. We propose that SIRT1, via a pathway that involves upregulation of eNOS, induces mitochondrial biogenesis factors (including Nrf1, Tfam, and PGC-1α), promoting mitochondrial biogenesis. The finding that resveratrol treatment induces mitochondrial biogenesis in the aortas of type 2 diabetic mice suggests the potential for caloric restriction mimetics to target endothelial mitochondria in metabolic diseases.

Fig. 5. Effect of resveratrol (Res) on mRNA expression of PGC-1α (A), Nrf-1 (B), and Tfam (C) in CAECs. Knockdown of SIRT1 (siRNA) prevented resveratrol-induced upregulation of mitochondrial biogenesis factors. *P < 0.05 vs. control; #P < 0.05 vs. resveratrol only; n = 5 in each group.

Fig. 6. In aortas of type 2 diabetic db/db mice, relative mtDNA content (A) was significantly decreased compared with vessels of control mice. In aortas of db/db mice, expression of PGC-1α and Nrf-1 was downregulated (B). Resveratrol (Res) treatment significantly increased vascular mtDNA content (A) and induced the expression of mitochondrial biogenesis factors (B and C) in aortas of db/db mice, eliminating the difference between the 2 groups. Data are means ± SE. *P < 0.05 vs. no resveratrol; #P < 0.05 vs. control mice. wt, Wild-type.
REFERENCES


