Resveratrol attenuates mitochondrial oxidative stress in coronary arterial endothelial cells

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ENDOTHELIAL MITOCHONDRIA have a crucial role in vascular pathophysiology (1, 16, 27, 39). Mitochondrial oxidative stress is frequently observed in diabetes and the metabolic syndrome and is thus likely to contribute to cellular energetic imbalance, activation of inflammatory processes, and endothelial dysfunction in these pathological conditions (19). Since increased mitochondrial production of reactive oxygen species (ROS) appears to be a key event in the development of vascular pathologies both in diabetes and aging (10, 46, 47), an identification of the mechanisms that regulate mitochondrial ROS (mtROS) generation in the endothelial cells may contribute to the development of improved pharmacological approaches to promote vascular health both in patients with diabetes and the elderly.

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring polyphenol found in more than 70 species of plants, including grapevines (Vitis vinifera). Since the original observation that resveratrol prolongs the life span in lower organisms, mimicking the antiaging effects of caloric restriction (49), it became the prototype of a new class of drugs termed caloric restriction mimetics, which are being developed to delay/reverse organ pathologies associated with aging and metabolic diseases (3). Resveratrol was recently shown to extend the life span (2) and to confer vasoprotection in animal models of diabetes mellitus, improving endothelial function and attenuating vascular inflammation (35, 40, 42, 43, 50). Similar protective effects of resveratrol treatment were observed in aged mice (35, 47). Moreover, the consumption of Mediterranean-style diets, which are rich in resveratrol, are associated with a reduced risk of cardiovascular mortality in humans (17, 23). As noted at the outset, both diabetes and aging are characterized by increased mtROS production, yet the effects of resveratrol on mitochondria in the endothelial cells remain incompletely understood.

The present study was conducted to determine whether resveratrol attenuates steady-state mtROS production in primary human coronary arterial endothelial cells (CAECs). The effects of resveratrol treatment on high glucose-induced mitochondrial oxidative stress were also assessed. Since resveratrol activates the NAD+-dependent protein deacetylase silent information regulator 2/sirtuin 1 (SIRT1) (21, 25) and SIRT1 regulates numerous proteins [including peroxisome proliferator-activated receptor coactivator-1α (PGC-1α)] implicated in the regulation of cellular energetics and mitochondrial function in various cell types (5, 18, 25, 30, 33, 41), this study focused on the mechanistic role of SIRT1 in mediating the mitochondrial protective effects of resveratrol in endothelial cells.

METHODS

Cell cultures, SIRT1 knockdown, and SIRT1 overexpression. Primary human CAECs (purchased from Cell Applications) in culture were treated with resveratrol (purchased from Sigma-Aldrich) as described (15, 44). To disrupt SIRT1 signaling, the downregulation of
SIRT1 was achieved by RNA interference using proprietary small-interfering RNA (siRNA) sequences (Superarray) and the electroporation-based Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported (9). 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 blot analysis as described (9). SIRT1 overexpression was achieved in CAECs by transfection with a SIRT1 full-length cDNA encoding plasmid (Stratagen) as described (13).

Measurement of resveratrol-induced changes in mtROS production in CAECs. The effect of resveratrol on steady-state mitochondrial O$_2^-$ production in endothelial cells was assessed by flow cytometry (FACsalibur; BD Bioscience, San Jose, CA) using MitoSox red (Invitrogen, Carlsbad, CA), a mitochondrion-specific hydroethidine-derivative fluorescent dye, as previously reported (11, 31, 32). Cell debris (low forward and side scatter), dead cells (Sytox green and annexin V positive), and apoptotic cells (annexin V positive) were gated out for analysis (31, 32). The data are presented as fold changes in the mean intensity of MitoSox fluorescence when compared with the respective controls. Also, CAECs were treated with high glucose (30 mM for 24 h) to assess the protective effect of resveratrol on mtROS production. In separate experiments, cellular H$_2$O$_2$ production was measured fluorometrically in CAECs using the Amplex red/horseradish peroxidase assay as described (14). The H$_2$O$_2$ generation rate was compared by measuring the time course of the buildup of resorufin fluorescence for 60 min by a Tecan Infinite M200 plate reader. In other experiments, cytoplasmic peroxide levels were measured fluorometrically using the 5(6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence assay, as reported (11, 14). Controls included measurements of cellular autofluorescence, time course measurements of dye-only controls, and polyethylene glycol-catalase controls. Calibration curves were generated with exogenous additions of H$_2$O$_2$. The effects of SIRT1 overexpression and SIRT1 knockdown on mtROS production were also determined using both the MitoSox, Amplex red, and C-H$_2$DCFDA assays.

In further experiments, to detect increases in mitochondrial H$_2$O$_2$ generation at the single cell level, CAECs were transfected with mitochondria-targeted HyPer-Mito (Evrogen, www.evrogen.com), which is a fully genetically encoded fluorescent sensor capable for highly specific detection of mitochondrial H$_2$O$_2$ (4). This probe consists of circularly permuted yellow fluorescent protein inserted into the regulatory domain of the prokaryotic O$_2$-sensing protein, OxyR (4). The cells were pretreated with resveratrol (10 μmol/l, for 24 h) or vehicle and then exposed to high glucose (30 mM for 24 h) to assess the protective effect of resveratrol on mtROS production. The data are presented as fold changes in fluorescence intensity, normalized to protein concentration. 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 fluoro-deacetylated 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 ($10^{-7}$ mol/l).

Measurement of resveratrol-induced changes in MnSOD expression in CAECs. To determine the effects of resveratrol on the expression of MnSOD protein in CAECs, Western blot analysis was performed as described using a primary antibody directed against MnSOD (11, 14). The impact of SIRT1 siRNA and SIRT1 overexpression on the expression of MnSOD was also determined. Anti-β-actin (No. 6276, Abcam) was used for normalization purposes.

Determination of glutathione levels in CAECs using HPLC electrochemical detection. Concentrations of redox-active GSH were measured in homogenates of CAECs pretreated with resveratrol (10$^{-7}$ to $10^{-7}$ mol/l, for 24 h) using a Perkin-Elmer HPLC equipped with an eight-channel coulometric array detector (ESA, Chelmsford, MA) as described (6). In brief, 10-mg aliquots of the samples were washed with ice-cold PBS and homogenized in 5% (wt/vol) metaphosphoric acid. Samples were centrifuged at 10,000 g for 10 min to sediment protein, and the supernatant fraction was saved for the analysis of redox sensitive compounds. Precipitated proteins were dissolved in 0.1 N NaOH and saved for protein determinations by a spectrophotometric quantitation method using BCA reagent (Pierce Chemical, Rockford, IL). Concentrations of GSH in saved supernatant fractions were determined by injecting aliquots onto an Ultrasphere 5 μ, 4.6 × 250 mm, C18 column and eluting with mobile phase of 50 mM NaH$_2$PO$_4$, 0.05 mM octane sulfonic acid, and 1.5% acetonitrile (pH 2.62) at a flow rate of 1 mL/min. The eight-channel CouArray detectors were set at 200, 350, 400, 450, 500, 550, 600, and 700 mV, respectively. Peak areas were analyzed using ESA software, and concentrations of GSH are reported (as nmol/mg protein).

Data analysis. Data were normalized to the respective control mean values and are expressed as means ± SE. Statistical analyses of data were performed by Student’s t-test or by two-way ANOVA followed by the Tukey post hoc test, as appropriate. *P < 0.05 was considered statistically significant.

Fig. 1. Resveratrol (for 48 h) significantly decreases steady-state mitochondrial O$_2^-$ production (A; assessed by MitoSox fluorescence) in cultured coronary arterial endothelial cells (CAECs). Overexpression of silent information regulator 2 (SIRT1) mimics the effects resveratrol. In contrast, resveratrol treatment after SIRT1 small-interfering RNA (siRNA) pretreatment fails to decrease mitochondrial reactive oxygen species generation (B). AU, arbitrary units. *P < 0.05 vs. untreated.
RESULTS

Resveratrol decreases mtROS production in endothelial cells: role of SIRT1. To test the effect of resveratrol on steady-state mtROS generation and cellular H2O2 levels, cultured CAECs were treated with resveratrol (10^{-6} to 10^{-5} mol/l). The analysis of flow cytometry data showed that resveratrol significantly attenuated basal MitoSox fluorescence in CAECs (Fig. 1A).

In CAECs, the treatment with resveratrol significantly increased the specific activity of SIRT1 (by ~50%). Resveratrol (from 10^{-6} to 10^{-5} mol/l) also significantly increased the activity of recombinant SIRT1, which could be prevented by sirtinol (not shown), extending our recent findings (12). We found that SIRT1 overexpression effectively attenuated both mtROS production (Fig. 1A) and decreased cellular peroxide levels, as shown by a decreased resorufin fluorescence (fold change: control, 100 ± 2%; and SIRT1 overexpression, 87 ± 1%) and C-H2DCFDA fluorescence (not shown). Endothelial SIRT1 expression was effectively downregulated by siRNA (by ~90%), which prevented a resveratrol-induced attenuation of mtROS production (Fig. 1B). To test the protective effect of resveratrol against metabolic stress-induced mitochondrial oxidative stress, CAECs were treated with high glucose. The analysis of flow cytometry data showed that high glucose significantly increased mitochondrial O2_{2} production, whereas mannitol was without effect (Fig. 2A). Resveratrol treatment, in a concentration-dependent manner, attenuated high glucose-induced mitochondrial oxidative stress (Fig. 2A).

For the specific detection of intramitochondrial H2O2, CAECs were transfected with HyPer-Mito, a genetically encoded fluorescent sensor. The transfection efficiency was ~80%. Fluorescent microscopy showed the perinuclear localization (Fig. 2B) of the HyPer-Mito fluorescence. High-glucose treatment resulted in a significant increase in the green fluorescent HyPer-Mito signal, which was prevented by resveratrol (10 μmol/l) treatment (Fig. 2C). We found that in CAECs resveratrol also significantly attenuated high glucose-induced cellular H2O2 production as shown by a decreased resorufin fluorescence (Fig. 2D). In CAECs, in which SIRT1 was knocked down by siRNA, resveratrol failed to significantly attenuate high glucose-induced increases in both mitochondrial (Fig. 2C) and cellular (Fig. 2D) H2O2 production.
Resveratrol upregulates MnSOD and increases GSH levels in cultured endothelial cells: role of SIRT1. Western blot analysis showed that in CAECs, resveratrol elicited significant increases in the expression of MnSOD (Fig. 3A). A knockdown of SIRT1 prevented the resveratrol-induced induction of MnSOD (Fig. 3A), whereas an overexpression of SIRT1 significantly potentiated the effect of resveratrol on cellular MnSOD expression (Fig. 3A). HPLC coulometric analysis revealed that resveratrol elicited concentration-dependent increases in GSH content in CAECs (Fig. 3B). A knockdown of SIRT1 significantly attenuated resveratrol-induced increases in cellular GSH levels (Fig. 3C). By contrast, an overexpression of SIRT1 elicited significant increases in cellular GSH content (Fig. 3C).

DISCUSSION

Pathways that regulate mitochondrial function and ROS production have recently emerged as potential therapeutic targets for the amelioration of endothelial dysfunction and prevention of vascular disease in diabetes and other pathological conditions (39). Our studies show that resveratrol attenuates mitochondrial oxidative stress in CAECs. A reduction of mtROS production is associated with the activation of SIRT1 and the induction of mitochondrial antioxidant systems.

Resveratrol is a promising new therapeutic approach for preventing cardiovascular diseases in type 2 diabetes and aging (3, 35). Previous studies focused on the direct effects of resveratrol on proinflammatory pathways and antioxidant defense mechanisms in endothelial cells (13, 15, 44) but provided little information on its effects on endothelial mitochondria. Our data support the finding that resveratrol significantly attenuates steady-state levels of mitochondrial $O_2^-$ production in CAECs (Fig. 1A).

To test whether resveratrol treatment can also prevent mitochondrial oxidative stress induced by metabolic stress, we exposed endothelial cells to high glucose to mimic diabetic conditions. In CAECs, high glucose in vitro is known to elicit substantial increases in mitochondrial $O_2^-$ generation (32, 34, 36). We found that resveratrol attenuates high glucose-induced mitochondrial oxidative stress in the endothelial cells (Fig. 2, A–C), suggesting that it effectively increases cellular metabolic stress resistance. The concentrations of resveratrol required to reduce mtROS generation are achievable in the plasma in vivo when resveratrol is used as a dietary supplement (3), suggesting that the attenuation of mitochondrial oxidative stress may contribute to the vasoprotective effects of resveratrol treatment under pathophysiological conditions (35). We recently demonstrated that in mice with type 2 diabetes, resveratrol treatment effectively attenuated oxidative stress in the aorta, protecting endothelial function (35). Mitochondria-derived $O_2^-$ is membrane impermeable (except in the protonated perhydroxyl radical form, which represents only a small fraction of total $O_2^-$ produced), whereas $H_2O_2$ easily penetrates the mitochondrial membranes. As shown in Fig. 2D, resveratrol prevents high glucose-induced increases in resorufin fluorescence in the Amplex red assay, suggesting that decreased mtROS production also results in lower cytoplasmic $H_2O_2$ levels in resveratrol-treated cells.

There are multiple mechanisms by which resveratrol-induced reduction of mitochondrial oxidative stress may promote vascular health. Mitochondrion-derived ROS have important signaling functions, such as the activation of NF-$\kappa$B-dependent inflammatory pathways in aging (47) and metabolic diseases. Thus resveratrol-induced attenuation of mitochondrial oxidative stress is likely to confer anti-inflammatory effects. Indeed, we have found that resveratrol treatment significantly decreases NF-$\kappa$B-dependent gene expression in aortas of aged mice and mice with type 2 diabetes (35). Resveratrol also inhibits NF-$\kappa$B-driven gene expression in vessels of aged rats (47). Furthermore, resveratrol effectively inhibits high glucose-induced NF-$\kappa$B activation and inflammatory gene expression in cultured endothelial cells (A. Csiszar and Z. Ungvari, unpublished observation). Recent studies also suggest that a link exists between mitochondrial oxidative stress, mtDNA depletion, and development of pathological vascular phenotypes in diabetes (37). Increased ROS levels in the mitochondria are known to inactivate critical enzymes involved in mitochondrial metabolism (e.g., $\alpha$-ketoglutarate dehydrogenase and aconitase). Dysfunctional mitochondria may diminish ATP production, thereby impairing the synthesis and secretion of endothelium-derived factors that serve as paracrine signals in the vascular wall and affecting the transport functions of the vascular endothelium. A Resveratrol-induced attenuation of

Fig. 3. A: original Western blot and densitometric results show that resveratrol (RES) induces MnSOD expression in CAECs. Knockdown of SIRT1 (siRNA) prevents the effect of resveratrol, whereas SIRT1 overexpression (Overexp) substantially augments expression. Data are means ± SE. *P < 0.05; #P < 0.05 vs. untreated. B: summary data for HPLC coulometric analysis of glutathione (GSH) content in homogenates of CAECs. Resveratrol in concentration-dependent manner elicits significant increases in cellular GSH content. C: resveratrol increases cellular content of GSH vs. control, whereas knockdown of SIRT1 using siRNA blocks resveratrol-enhancing effect. By contract, overexpression of SIRT1 ameliorates endogenous GSH levels. Data are means ± SE. *P < 0.05 vs. control; #P < 0.05 vs. no resveratrol.
mitochondrial oxidative stress would correct these impairments.

The NAD⁺-dependent protein deacetylase SIRT1 plays a critical role in resveratrol-induced effects in endothelial cells. Accordingly, resveratrol, similar to that observed in other cell types (21, 25), induces SIRT1 in endothelial cells (13). Resveratrol also lowers the Km of SIRT1 for the acetylated substrate and for NAD⁺ (21). A knockdown of SIRT1 prevents a resveratrol-induced reduction in mtROS production (Fig. 1B) and cellular H2O2 levels (Fig. 2D). An overexpression of SIRT1 also attenuates mtROS generation (Fig. 1A) and cellular H2O2 levels in CAECs, mimicking the effects of resveratrol. These findings are in accord with previous studies that showed that resveratrol and SIRT1 regulate mitochondrial function in skeletal muscle and liver (18, 25). SIRT1 likely regulates multiple pathways involved in mtROS generation in the endothelial cells, among which the upregulation of mitochondrial antioxidant systems appear to play a key role. Accordingly, resveratrol and SIRT1 overexpression increases MnSOD expression in CAECs (Fig. 3A). We attribute the resveratrol-induced reduction of mitochondrial O2⁻ levels, at least in part, to this effect. Furthermore, resveratrol also significantly increases cellular GSH levels (Fig. 3B). In addition, resveratrol was previously shown to upregulate glutathione peroxidase and catalase in the endothelial cells (44). GSH, glutathione peroxidase, and catalase are important components in the cellular antioxidant system involved in the detoxification of H2O2, which play an important role in oxidative stress resistance in the mitochondria. Because increased MnSOD per se would increase mitochondrial H2O2 release, we attribute the reduction of H2O2 levels in resveratrol-treated cells to the upregulation of the aforementioned H2O2 detoxification systems. The effects of resveratrol on MnSOD and GSH are prevented by knockdown of SIRT1 (Fig. 3, A and B) and mimicked/potentiated by SIRT1 overexpression (Fig. 3, A and C), suggesting that SIRT1 activation plays a key role in inducing mitochondrial antioxidant defense mechanisms in endothelial cells. In addition, resveratrol, via a SIRT1-dependent pathway, increases mitochondrial proliferation. This work was supported by grants from the American Federation for Aging Research (to A. Csizsar) and the American Diabetes Association (to Z. Ungvari); by American Heart Association Grant 110530047A to (C. Zhang) and National Institutes of Health (NIH) Grants HL-077256 and HL-43023 (to Z. Ungvari and A. Csizsar), CA-111842 (to J. P. Pinto), and ROI-HL-077566 and RO1-HL-085119 (to C. Zhang); and by the Intramural Research Program of the NIH (to P. Pacher).

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