Resveratrol Reverses Endothelial Nitric-Oxide Synthase Uncoupling in Apolipoprotein E Knockout Mice

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ABSTRACT

A crucial cause of the decreased bioactivity of nitric oxide (NO) in cardiovascular diseases is the uncoupling of the endothelial NO synthase (eNOS) caused by the oxidative stress-mediated deficiency of the NOS cofactor tetrahydrobiopterin (BH4). The reversal of eNOS uncoupling might represent a novel therapeutic approach. The treatment of apolipoprotein E knockout (ApoE-KO) mice with resveratrol resulted in the up-regulation of superoxide dismutase (SOD) isoforms (SOD1–SOD3), glutathione peroxidase 1 (GPx1), and catalase and the down-regulation of NADPH oxidases NOX2 and NOX4 in the hearts of ApoE-KO mice. This was associated with reductions in superoxide, 3-nitrotyrosine, and malondialdehyde levels. In parallel, the cardiac expression of GTP cyclohydrolase 1 (GCH1), the rate-limiting enzyme in BH4 biosynthesis, was enhanced by resveratrol. This enhancement was accompanied by an elevation in BH4 levels. Superoxide production from ApoE-KO mice hearts was reduced by the NOS inhibitor L-N³-nitro-arginine methyl ester, indicating eNOS uncoupling in this pathological model. Resveratrol treatment resulted in a reversal of eNOS uncoupling. Treatment of human endothelial cells with resveratrol led to an up-regulation of SOD1, SOD2, SOD3, GPx1, catalase, and GCH1. Some of these effects were preventable with sirtinol, an inhibitor of the protein deacetylase sirtuin 1. In summary, resveratrol decreased superoxide production and enhanced the inactivation of reactive oxygen species. The resulting reduction in BH4 oxidation, together with the enhanced biosynthesis of BH4 by GCH1, probably was responsible for the reversal of eNOS uncoupling. This novel mechanism (reversal of eNOS uncoupling) might contribute to the protective effects of resveratrol.

Introduction

Nitric oxide (NO) produced by endothelial NO synthase (eNOS) is an antihypertensive, antithrombotic, and antiatherosclerotic principle (Li and Förstermann, 2000). Cardiovascular diseases such as hypertension, atherosclerosis, and diabetes mellitus are associated with decreased NO bioactivity caused by reduced NO production by eNOS and/or the increased inactivation of NO after reaction with superoxide (Li and Förstermann, 2009a).

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The reduction in the production of NO by eNOS is a result of the inhibition of eNOS enzymatic activity and/or dysfunction of the enzyme (i.e., eNOS uncoupling). The expression of eNOS, at least in early atherosclerosis, is unchanged or even augmented (Li et al., 2002). The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia (Feron et al., 1999) and endogenous NOS inhibitors such as asymmetrical dimethyl-arginine (Cooke, 2004).

Under a number of pathological conditions, the enzymatic reduction of molecular oxygen by eNOS is no longer coupled to L-arginine oxidation, resulting in the production of superoxide rather than NO. This phenomenon is referred to as eNOS uncoupling (Förstermann and Münzel, 2006). Evidence for the uncoupling of eNOS has been obtained in different hyperten-

ABBREVIATIONS: NO, nitric oxide; eNOS, endothelial NO synthase; SOD, superoxide dismutase; ApoE-KO, apolipoprotein E knockout; BH4, 7,8-dihydrobiopterin; BH2, tetrahydrobiopterin; GCH1, GTP cyclohydrolase 1; HPLC, high-performance liquid chromatography; L-NAME, L-N³-nitro-arginine methyl ester; RT-PCR, reverse transcription polymerase chain reaction; ROS, reactive oxygen species; MDA, malondialdehyde; PBS, phosphate-buffered saline; GPx, glutathione peroxidase; NOX, NADPH oxidase; TBS, Tris-buffered saline; 2-HE, 2-hydroxyethidium; DHE, dihydroethidium; SIRT1, sirtuin 1.

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sion models including spontaneously hypertensive rats, angiotensin II-induced hypertension, and hypertension induced by the mineralocorticoid deoxycorticosterone acetate. Animal models of diabetes, nitroglycerin tolerance, and atherosclerosis are also associated with eNOS uncoupling. It is noteworthy that eNOS uncoupling has also been seen in patients with hypercholesterolemia, diabetes mellitus, and essential hypertension, chronic smokers, and nitroglycerin-treated patients (reviewed in Li and Förstermann, 2009a).

A number of potential mechanisms have been reported to contribute to eNOS uncoupling. Among these, the NOS cofactor tetrahydrobiopterin (BH₄) is likely to act as the major “coupling switch” (Wei et al., 2008), and BH₄ deficiency seems to be the primary cause for eNOS uncoupling in pathophysiology (Thum et al., 2007; Li and Förstermann, 2009a). In animal models of diabetes, angiotensin II-induced hypertension and nitrate tolerance, and in spontaneously hypertensive rats, we showed that eNOS uncoupling was associated with NADPH oxidase-mediated oxidative stress. The pharmacological suppression of NADPH oxidase expression resulted in a reduction in BH₄ oxidation, a reversal of eNOS uncoupling, and improved endothelial function (Li et al., 2006a).

Resveratrol is a polyphenol phytoalexin that is present in a variety of plant species (Bradamante et al., 2004; Baur and Sinclair, 2006; Li and Förstermann, 2009b). Studies have demonstrated that resveratrol can increase the expression of antioxidative enzymes including superoxide dismutase (SOD1 and SOD2), catalase, and glutathione peroxidase (GPx). We demonstrated that resveratrol also reduces the expression of the NADPH oxidase NOX4 (Spanier et al., 2009).

In the present study, we show that resveratrol also regulates NOX2, SOD3, and GTP cyclohydrolase 1 (GCH1). The latter is the rate-limiting enzyme in BH₄ biosynthesis. By elevating tissue BH₄ levels, resveratrol reverses eNOS uncoupling.

**Materials and Methods**

**Animals and Treatment.** Male ApoE-KO mice (Charles River Laboratories, Sulzfeld, Germany) aged 6 months were used. Resveratrol (trans-3,4’,5-trihydroxy stilbene; empirical formula C₁₅H₁₄O₄; CAS 501-36-0) was obtained from Cayman Chemical (Ann Arbor, MI). Mice were treated with resveratrol at doses of 30 or 100 mg/kg via gavage for 7 days and then killed with an overdose of pentobarbital. The heart was taken for analyses of mRNA expression or measurement of superoxide production. All animal experiments were performed in accordance with German animal protection law and the guidelines for the use of experimental animals as stipulated by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Real-Time RT-PCR Analyses.** Total RNA was isolated from mouse heart by using the E.Z.N.A. total RNA kit (Omega Bio-Tek, Norcross, GA). Total RNA (1 µg) was reverse-transcribed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative real-time RT-PCR amplification was performed in an iCycler iQ System (Bio-Rad Laboratories, Munich, Germany) using the ABsolute QPCR SYBR Green Mix Kit (Thermo Fisher Scientific, Surrey, UK). The primers used are shown in Table 1. The comparative threshold cycle (Ct) method was used for relative mRNA quantification (Livak and Schmittgen, 2001). Gene expression was normalized to TATA box-binding protein mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of the control.

**Detection of 3-Nitrotyrosine and Malondialdehyde.** Total protein samples (5 µg each) from each mouse heart were dotted onto nitrocellulose membranes, and dot blot analyses were performed. In brief, the membranes were blocked for 1 h at room temperature with 5% powdered milk in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) with 0.1% Tween 20 and then incubated with the primary antibodies [a rabbit polyclonal antibody against 3-nitrotyrosine (Millipore Corporation, Billerica, MA) or a rabbit polyclonal against MDA (Academy Biomedical, Houston, TX)] in 5% powdered milk in TBS with 0.1% Tween 20 overnight at 4°C. Membranes were washed three times in TBS/Tween 20 (0.1%) and then incubated with a horseradish peroxidase-conjugated secondary antibody in 5% powdered milk and 0.1% Tween 20 in TBS for 1 h at room temperature. After washing, immunocomplexes were developed by using an enhanced horseradish peroxidase/luminal chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) according to the manufacturer’s instructions.

**Measurement of Superoxide.** Oxidative stress and superoxide were measured by using a modified high-performance liquid chromatography (HPLC)-based method to quantify ethidium and 2-hydroxyethidium (2-HE) levels as described previously (Wenzel et al., 2008). In brief, heart tissue was incubated with 50 µM dihydroethidium (DHE; 5-ethyl-6-phenyl-6'H-phenanthridine-3,8-diamine; empirical formula C₁₂H₁₁N₂O; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C in PBS buffer. Heart pieces were snap-frozen and stored at −80°C until they were homogenized in 50% acetonitrile/50% PBS and centrifuged. Fifty microliters of the supernatant was subjected to HPLC analysis. The system consisted of a control unit, two pumps, a mixer, detectors, a column oven, a degasser, and an autosampler (AS-2057 plus) from Jasco (Groß-Umstadt, Germany), and a C₁₈-Nucleosil 100-3 (125 × 4) column from Macherey-Nagel (Düren, Germany). A high-pressure gradient was used with acetonitrile and 25 mM citrate buffer, pH 2.2, as the mobile phases with the following percentages of organic solvent: 0 min, 36%; 7 min, 40%; 8 to 12 min, 95%; and 13 min, 36%. The flow was 1 ml/min, and DHE was detected by its absorption at 355 nm; 2-HE and ethidium were detected by fluorescence (excitation 480 nm/emission 580 nm). The

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Results

Effects of Resveratrol on the Expression of Redox Genes in the Heart of ApoE-KO Mice. Treatment of ApoE-KO mice for 7 days with 30 and 100 mg/kg resveratrol resulted in the dose-dependent up-regulation of SOD isoforms 1 to 3 (Fig. 1A). The mRNA expression of catalase and GPx1 was also increased by resveratrol (Fig. 1B). Resveratrol had no effect on the expression of NOX1. At 100 mg/kg, resveratrol significantly decreased the expression of NOX2 and NOX4 (Fig. 1C).

Resveratrol Reduces Reactive Oxygen Species/Superoxide Levels in the Heart of ApoE-KO Mice. ApoE-KO mice were treated orally for 7 days with resveratrol at doses of 30 or 100 mg/kg. Reactive oxygen species (ROS)/superoxide levels were determined in cardiac tissues by using an HPLC-based method for the measurement of DHE oxidation products. Both the superoxide-specific oxidation product 2-HE and the nonspecific oxidation product ethidium were significantly reduced by resveratrol treatment (Fig. 1A). The treatment of ApoE-KO mice with resveratrol led to the enhanced expression of GCH1, the rate-limiting enzyme in BH4 biosynthesis (Fig. 1B). In control ApoE-KO mice, superoxide production was reduced by the NOS inhibitor L-NAME, indicating eNOS uncoupling in this pathophysiological model. The treatment of ApoE-KO mice with resveratrol resulted in a significant reduction in superoxide production (Fig. 1C). Under these conditions, L-NAME did not further reduce superoxide production in resveratrol-treated animals (Fig. 1C), indicating the recoupling of oxygen reduction and NO synthesis in eNOS.

Discussion

Resveratrol is a polyphenol phytoalexin that is present in a variety of plant species, including white hellebore (Veratrum grandiflorum O. Loes), Polygonum cuspidatum, grapes, peanuts, and mulberries (Bradamante et al., 2004; Baur and Sinclair, 2006; Li and Förstermann, 2009b). Resveratrol has been postulated to explain some of the cardioprotective effects of red wine. Recently, accumulating evidence has shown that resveratrol can prevent or slow the progression of a wide variety of diseases including cancer and cardiovascular dis-
As a polyphenolic compound, resveratrol has been shown to be a scavenger of hydroxyl, superoxide, and metal-induced radicals (Bradamante et al., 2004). However, the direct antioxidant effects of resveratrol are rather poor; resveratrol is less potent than other well established antioxidants such as ascorbate and cysteine (Bradamante et al., 2004). Thus, the protective effects of resveratrol against oxidative injury are likely to be attributed to the up-regulation of the endogenous cellular antioxidant system rather than its direct ROS scavenging activity.

Living organisms have evolved a number of antioxidant defenses to protect themselves against oxidative stress. The major cardiovascular enzymatic antioxidants are SOD, catalase, and GPx (Paravicini and Touyz, 2008). SOD catalyzes the dismutation of superoxide into hydrogen peroxide. There are three mammalian isoforms: copper/zinc SOD (SOD1), mitochondrial SOD (SOD2), and extracellular SOD (SOD3). GPx proteins consume reduced glutathione to convert hydrogen peroxide to water and lipid peroxides to their respective alcohols. GPx1 is the most abundant selenoperoxidase and is a key antioxidant enzyme in many cell types. Catalase is an intracellular antioxidant enzyme that is located mainly in cellular peroxisomes and to some extent in the cytosol and catalyzes the reaction of hydrogen peroxide to water (Paravicini and Touyz, 2008).

The induction of antioxidant enzymes by resveratrol has been reported in cardiovascular tissues, although some controversy remains. Resveratrol increases the activity of SOD, catalase, and GPx in cardiac H9C2 cells (Cao and Li, 2004). However, gene expression was not examined in that study; it was therefore unclear which isoforms of SOD or GPx were responsible for the observed effects. SOD activity was also increased by resveratrol in aortic smooth muscle cells (Li et al., 2006b). Another study, however, found no changes in the protein levels of SOD1 or SOD2 in rat aortic segments ex vivo-treated with resveratrol up to 100 μg/kg (Ungvari et al., 2007). We have shown the up-regulation of SOD1 by resveratrol in endothelial cells (Spanier et al., 2009). A recent study also demonstrated the effect of resveratrol on SOD2 in cardiac myoblasts (Tanno et al., 2010). However, the effect of resveratrol on SOD3 has not yet been reported. The incubation of aortic segments or cultured aortic smooth muscle cells leads to the up-regulation of GPx1 and catalase (Li et al., 2006b; Ungvari et al., 2007).

In the present study, we show that resveratrol increases the expression of SOD1, SOD2, SOD3, GPx1, and catalase.
compared with control.

Biopterin, BH4, and BH2 was measured by HPLC. B, GCH1 mRNA determined in the presence or absence of the NOS inhibitor L-NAME in the heart. C, the superoxide content (DHE oxidation product 2-HE) was determined. A, the cardiac content of total biopeterin, BH4, and BH2 was measured by HPLC. B, GCH1 mRNA expression in mice hearts was analyzed by quantitative real-time RT-PCR. C, the superoxide content (DHE oxidation product 2-HE) was determined in the presence or absence of the NOS inhibitor L-NAME in the heart. Columns represent mean ± S.E.M. n = 8, *P < 0.05; **P < 0.01, compared with control.

Fig. 4. Effects of resveratrol on BH4 synthesis and eNOS functionality. ApoE-KO mice were treated orally for 7 days with resveratrol at doses of 30 mg/kg (Res 30) or 100 mg/kg (Res 100). A, the cardiac content of total biopeterin, BH4, and BH2 was measured by HPLC. B, GCH1 mRNA expression in mice hearts was analyzed by quantitative real-time RT-PCR. C, the superoxide content (DHE oxidation product 2-HE) was determined in the presence or absence of the NOS inhibitor L-NAME in the heart. Columns represent mean ± S.E.M. n = 8, *P < 0.05; **P < 0.01, compared with control.

(Fig. 1, A and B) in the heart of ApoE-KO mice, thereby enhancing ROS inactivation.

ROS can be produced by several enzyme systems in the vascular wall, including NADPH oxidases, xanthine oxidase, uncoupled eNOS, enzymes of the respiratory chain, and cytochrome P450 monoxygenases (Förstermann, 2008; Paravicini and Touyz, 2008). Although all of these enzymes contribute to the oxidative burden, evidence is accumulating that the initial generation of ROS by NADPH oxidases triggers the release of ROS by the other enzymes (e.g., xanthine oxidase and eNOS) (Griendling, 2004; Brandes and Kreuzer, 2005). Moreover, NADPH oxidases are likely to be the predominant source of ROS in the vasculature. Several homologous proteins of the NADPH oxidase catalytic subunit (gp91phox/NOX2) are expressed in the cardiovascular tissues of rodents, NOX1, NOX2, and NOX4 (Griendling, 2004; Brandes and Kreuzer, 2005). We have previously shown that resveratrol treatment decreases NOX4 expression in human umbilical vein endothelial cells and human umbilical vein endothelial cell-derived EA.hy 926 endothelial cells (Spanier et al., 2009), which is the most predominant NOX isoform in these cell types (Xu et al., 2008). In the present study, we demonstrate that resveratrol reduces the expression of NOX2 and NOX4 in the mouse heart (Fig. 1C).

The hypercholesterolemic, atherosclerosis-prone ApoE-KO mice were used in this study as a model of oxidative stress and eNOS uncoupling. Untreated ApoE-KO mice showed an increased oxidative degradation of BH4 (Alp et al., 2004) and significant ROS production in their aortas (Alp et al., 2004; Wohlfart et al., 2008) and hearts (Fig. 4C). Both the aortic (Alp et al., 2004; Wohlfart et al., 2008) and cardiac (Fig. 4C) superoxide production could be reduced by the NOS inhibitor L-NAME, indicating that eNOS was in an uncoupled state and that it was producing ROS in this pathological model. The present study reports for the first time that resveratrol enhances the expression of GCH1 (Fig. 4B) and elevates levels of BH4 (Fig. 4A). Cardiac superoxide production in resveratrol-treated mice was markedly reduced to a level that could not be lowered any further by L-NAME (Fig. 4C). These data suggest that eNOS was no longer producing superoxide in resveratrol-treated ApoE-KO mice, i.e., resveratrol could reverse eNOS uncoupling.

The major cause of eNOS uncoupling under pathological conditions is a deficiency of the essential eNOS cofactor BH4. BH4 supplementation is capable of correcting eNOS dysfunction in several types of pathophysiology (Förstermann and Münzel, 2006; Li and Förstermann, 2009a).

The tissue levels of BH4 depend on its biosynthesis and its degradation/oxidation (Schmidt and Alp, 2007; Crabtree et al., 2009). BH4 is synthesized from GTP via a de novo pathway, with GCH1 acting as the rate-limiting enzyme. BH4 can be rapidly oxidized by peroxynitrite (Laursen et al., 2001; Landmesser et al., 2003). In deoxycorticosterone acetate-salt hypertensive mice, superoxide produced by NADPH oxidase leads to the formation of peroxynitrite in reaction with NO and induces the uncoupling of eNOS (Landmesser et al., 2003). In the present study, we show that resveratrol decreases the cardiac content of superoxide (Fig. 2) and 3-nitrotyrosine (Fig. 3). The latter indicates reduced levels of peroxynitrite and thereby decreased BH4 oxidation (Fig. 4A).

Resveratrol is a putative activator of the class III histone deacetylase sirtuin 1 (SIRT1) (Milne et al., 2007), which regulates a variety of cellular functions such as genome maintenance, longevity, and metabolism (Michan and Sinclair, 2007; Milne and Denu, 2008). We are currently studying the molecular mechanisms of resveratrol’s effects in cell culture experiments. It is noteworthy that the effects of resveratrol on the genes investigated in this study in vivo can also be observed in human endothelial cells in vitro (Supplemental Fig. 1). Results obtained using the SIRT1 inhibitor sirtinol and SIRT1 siRNA demonstrate that some of the effects of resveratrol on gene expression are indeed SIRT1-dependent (Supplemental Fig. 1). It is highly likely that this also applies to the in vivo effect of resveratrol.

Although it has been identified as the most potent SIRT1 activator among natural polyphenolic plant compounds, the SIRT1-activating potency of resveratrol is rather limited. A resveratrol concentration of 46.2 µM is required to increase SIRT1 enzymatic activity by 50% (Milne and Denu, 2008). To study the role of SIRT1, we used relatively high doses/concentrations of resveratrol in the present study. The maximal tolerated dose of resveratrol has not been thoroughly determined. A dose of 300 mg/kg showed no detrimental effects in rats, and doses up to 100 mg/kg have been used routinely in studies on rodents (Baur and Sinclair, 2006).

Previous studies have demonstrated that resveratrol stimulates endothelial NO production. Multiple mechanisms have been implicated in this action (Li and Förstermann, 2009b), including 1) the enhancement of eNOS expression (Wallerath et al., 2002; Csiszar et al., 2009), 2) the reduction of eNOS acetylation (Mattagajasingh et al., 2007), and 3) the stimulation of eNOS phosphorylation at serine 1177 (Klinge
et al., 2005, 2008). SIRT1 seems to be involved in resveratrol-induced eNOS up-regulation and eNOS deacetylation. eNOS phosphorylation by resveratrol, however, is likely to be mediated by the estrogen receptor ERα in caveolae, leading to eNOS activation via the Gα, Cav-1, Src, and extracellular signal-regulated kinase 1/2 pathways (Klinge et al., 2005, 2008). For the latter, only nanomolar concentrations of resveratrol are required.

In summary, this study demonstrates that resveratrol reverses eNOS uncoupling in ApoE-KO mice. The underlying mechanism is likely to be the elevation of BH4 levels. By up-regulating GCH1 expression, resveratrol enhances BH4 biosynthesis. By reducing superoxide production (suppression of NOX2 and NOX4) and enhancing ROS inactivation (up-regulation of SOD1, SOD2, SOD3, catalase, and GPx1), resveratrol decreases BH4 oxidation. This novel mechanism (reversal of eNOS uncoupling) might contribute to the protective effects of resveratrol.

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References


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