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RESVERATROL REDUCES ENDOTHELIAL OXIDATIVE STRESS BY MODULATING THE GENE EXPRESSION OF SUPEROXIDE DISMUTASE 1 (SOD1), GLUTATHIONE PEROXIDASE 1 (GPX1) AND NADPH OXIDASE SUBUNIT (NOX4)

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Resveratrol, an important antioxidant found in grapes and wine, is likely to contribute to red wine's potential to prevent human cardiovascular disease. In addition to its known (direct) antioxidant effect, we have found that resveratrol also regulates the gene expression of pro-oxidative and anti-oxidative enzymes in human endothelial cells. NADPH oxidases (Nox) are the predominant producers of superoxide in the vasculature, whereas superoxide dismutase (SOD) and glutathione peroxidase 1 (GPx1) are the major enzymes responsible for the inactivation of superoxide and hydrogen peroxide, respectively. Incubation of human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells with resveratrol resulted in a concentration- and time-dependent downregulation of Nox4, the most abundant NADPH oxidase catalytic subunit (quantitative real-time RT-PCR). The same resveratrol regimen upregulated the mRNA expression of SOD1and GPx1. The addition the protein levels of SOD1 and GPx1 were enhanced by resveratrol in a concentration-dependent manner (Western blot analyses). Pretreatment of EA.hy 926 cells with resveratrol completely abolished DMNQ-induced oxidative stress. Thus, the expressional suppression of pro-oxidative genes (such as NADPH oxidase) and induction of anti-oxidative enzymes (such as SOD1 and GPx1) might be an important component of the vascular protective effect of resveratrol.

Key words: *resveratrol, oxidative stress, NADPH oxidase, superoxide dismutase, glutathione peroxidase, endothelial cells*

INTRODUCTION

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a polyphenol phytoalexin present in a variety of plant species, including white hellebore (*Veratrum grandiflorum O. Loes*), *Polygonum cuspidatum*, grapes, peanuts and mulberries (1-3). Resveratrol attracted little interest until 1992, when it was postulated to explain some of the cardioprotective effects of red wine (4). Since then, accumulating reports have shown that resveratrol can prevent or slow the progression of a wide variety of diseases, including cancer (5), cardiovascular disease (2), ischaemic injuries (6, 7), and Alzheimer's disease (8), as well as enhance stress resistance (9) and extend the lifespans of various organisms from yeast to vertebrates (1).

Resveratrol is a polyphenolic compound and has been shown to be a scavenger of hydroxyl, superoxide, and metal-induced radicals (2). However, the direct antioxidant effects of resveratrol are rather poor; resveratrol is less potent than other well-established antioxidants, such as ascorbate and cysteine (2). A 50% scavenging (EC_{50}) of superoxide anion produced the xanthine/xanthine oxidase (XXO) system has been observed at a resveratrol concentration of 245 μM (10). The superoxide-scavenging activity of resveratrol at a concentration of 10 μM is only 2.8% (10).

Pretreatment with 100 μM of resveratrol for 2 h failed to protect rat cardiac H9C2 cells from XXO-induced cytotoxicity. A resveratrol treatment for 72 h, however, produced a marked cytoprotection against the same injury, even at lower concentrations (11). These data suggest that the protective effects of resveratrol against oxidative injury are likely to be attributed to the upregulation of endogenous cellular antioxidant systems rather than the direct reactive oxygen species (ROS) scavenging activity of the compound. Indeed, long-term (>48 h) treatment of H9C2 cells with resveratrol increases the activity of cellular antioxidant systems including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and the content of glutathione (GSH) (11).

It is well established that resveratrol protects against cardiac ischemia/reperfusion injury (2). In addition to adenosine receptor activation (2) and stimulation of nitric oxide (NO) release (12), induction of antioxidative enzymes is also implicated in this resveratrol-mediated pharmacologic preconditioning effect. Both *in vivo* and *in vitro* treatment with resveratrol leads to an induction

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of cardiac thioredoxin-1 (Trx-1), Trx-2, and heme oxygenase-1 (HO-1) (13-17). The cardioprotective effect of resveratrol can be abolished by cisplatin, a blocker of the Trx family (17), or by tin-protoporphyrin IX (SnPP), an HO-1 inhibitor (13).

Induction of antioxidative enzymes by resveratrol has also been reported for vascular tissues. Incubation of rat aortic segments leads to an upregulation of catalase, and HO-1 (9). In cultured aortic smooth muscle cells, resveratrol increases the mRNA expression of catalase, glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase-1 (NOQ1) (18). Treatment of endothelial cells with resveratrol prevents apoptotic cell death induced by TNF-α or by ox-LDL. The protective effect of resveratrol is attenuated by inhibition of GPx, suggesting a role for antioxidant systems in the antiapoptotic action of resveratrol (9). In human coronary arteriolar endothelial cells, resveratrol enhances angiogenesis by upregulation of Trx-1, which sequentially induces HO-1 as well as VEGF (13).

In the present study, we provide evidence that resveratrol downregulates NADPH oxidase subunit Nox4, and upregulates SOD1 and GPx1in human endothelial cells.

MATERIAL AND METHODS

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion as described (19) and cultured in M199 medium containing 1% FCS and ECGS/H-2 (Promocell, Heidelberg, Germany). HUVEC-derived EA.hy 926 cells were kindly provided by Dr. Cora-Jean Edgell (Chapel Hill, NC, USA). EA.hy 926 cells were grown under 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Deisenhofen, Germany) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1x HAT (hypoxanthine, aminopterin and thymidine) (Invitrogen, Karlsruhe, Germany) (20). Resveratrol (*trans*-3,4',-5-trihydroxystilebene; empirical formula $C_{14}H_{12}O_3$; CAS-Nummer 501-36-0) was obtained from Sigma-Aldrich. Cells were treated with resveratrol or its solvent control dimethyl sulfoxide (DMSO).

Real-time RT-PCR for mRNA expression analyses

Gene expression at mRNA level was analyzed with quantitative real-time RT-PCR using an iCyclerTM iQ System (Bio-Rad Laboratories, Munich, Germany). Total RNA was isolated from cultured endothelial cells using an E.Z.N.A. total RNA kit (Omega Bio-tek, Norcross, GA, USA). Total RNA (80 ng) was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen, Hilden, Germany). TaqMan Gene Expression Assays (pre-designed probe and primer sets) were obtained from Applied Biosystems (Foster City, CA, USA) for analyzing mRNA expression (assay ID Hs00276431_m1, Hs00166575_m1 and Hs00829989_gH for human Nox4, SOD1 and GPx1, respectively). The mRNA expression levels of the target genes were normalized to TATA box binding protein (TBP) mRNA (Applied Biosystems, assay ID Hs00427620_m1) (20).

Western blot for GPx1 protein analyses

Confluent EA.hy 926 cells were incubated with resveratrol for 24 h, and then total protein was isolated. Western blotting was performed using 30 µg of protein sample and antibodies against GPx1 (AF3798, R&D Systems, Wiesbaden, Germany; 1:1000); SOD1 (SOD-100, Stressgen, Hamburg, Germany; 1:2000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, California, USA; 1:5000). Briefly, protein samples were separated on 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked for 1 hour 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 in 5% powdered milk in TBS with 0.1% Tween 20 over night at 4°C. Blots 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 hour at room temperature. After washing, immunocomplexes were developed using an enhanced horseradish peroxidase/luminol chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA, USA) according to the manufacturer's instructions (21).

Detection of ROS

Cells were plated in 96-well plates (2.5 x 10^4 cells/well) and treated with 100 µM resveratrol for 24h. Then, cells were washed (HBSS) and incubated with 500 µM of the luminol derivative L-012 (Wako Chemicals, Germany) in HBSS at 37°C for 15 min before addition of 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, 10 µM, Calbiochem, Darmstadt, Germany). Then, ROS-induced chemiluminescence was determined every 5 min for a total of 60 min using a Centro LB960 plate luminometer (Berthold Technologies, Germany) (22-24).

Statistics

Statistical differences between mean values were determined by analysis of variance (ANOVA) followed by Fisher's protected least-significant-difference test for comparison of different means.

RESULTS

Resveratrol decreases Nox4 mRNA expression in human EA.hy 926 endothelial cells

Treatment of EA.hy 926 endothelial cells with resveratrol led to a concentration- and time-dependent decrease in Nox4 mRNA expression (*Fig. 1*). A downregulation of Nox4 could be observed with resveratrol at a concentration as low as 10 µM. At a resveratrol concentration of 60 µM, Nox4 mRNA was reduced to about 55% after 24 h. A maximal reduction of Nox4 mRNA (to about 33% of control) was seen with 100 µM resveratrol.

Resveratrol increases GPx1 mRNA expression in human EA.hy 926 endothelial cells

Treatment of EA.hy 926 endothelial cells with resveratrol led to a concentration- and time-dependent increase in GPx1 mRNA expression (*Fig. 2*). An upregulation of GPx1 mRNA could be observed as early as 6 h after incubation with 60 µM resveratrol. A maximal upregulation of GPx1 mRNA (about 230% of control) was reached after 24 h incubation with 60 µM resveratrol.

Resveratrol increases SOD1 mRNA expression in human EA.hy 926 endothelial cells

Treatment of EA.hy 926 endothelial cells with resveratrol led to an increase in SOD1 mRNA expression. At a concentration of 100 µM, SOD1 mRNA was upregulated to about 1.6-fold after 24 h (*Fig. 3*).

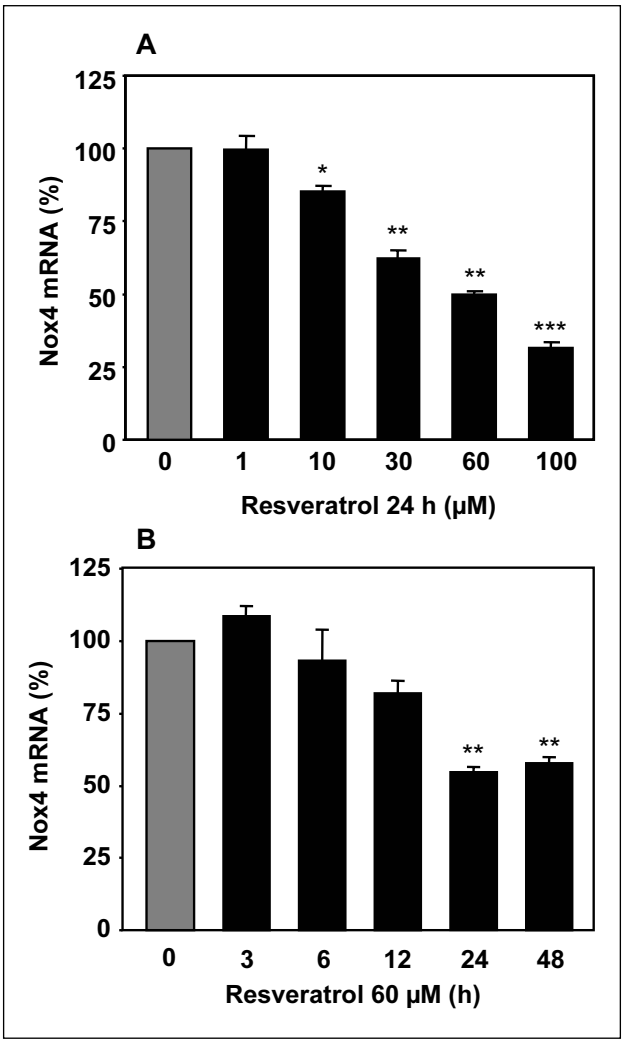

Fig. 1. Resveratrol decreases Nox4 mRNA expression in human EA.hy 926 endothelial cells in a concentration- (A) and time-dependent (B) manner. Cells were treated with resveratrol and Nox4 mRNA expression was analyzed by quantitative real-time RT-PCR. Columns represent mean ± SEM, n=9. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with control.

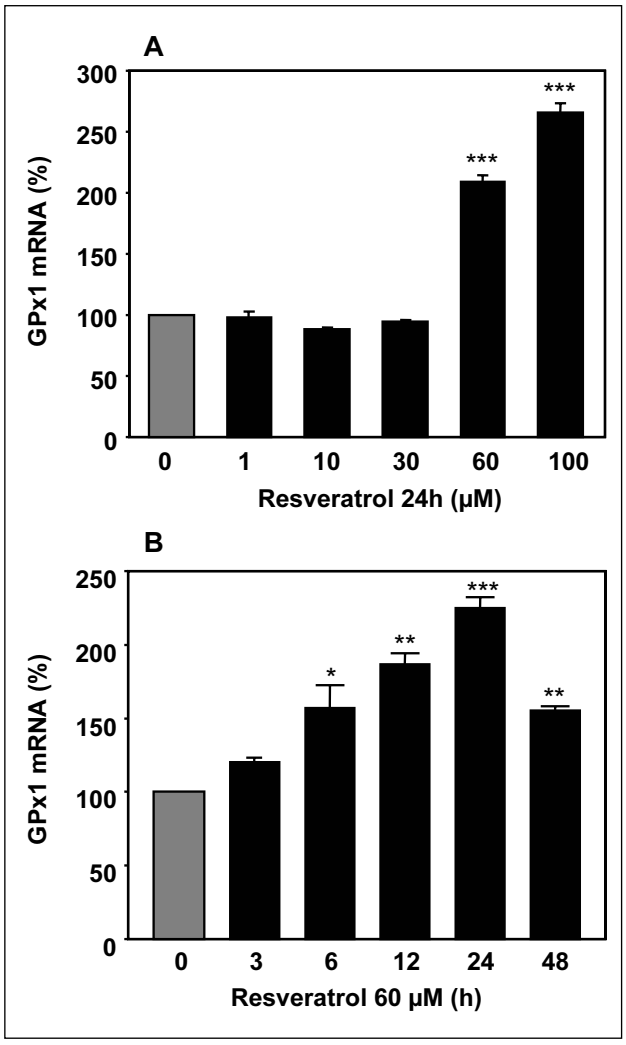

Fig. 2. Resveratrol increases GPx1 mRNA expression in human EA.hy 926 endothelial cells in a concentration- (A) and time-dependent (B) manner. Cells were treated with resveratrol and GPx1 mRNA expression was analyzed by quantitative real-time RT-PCR. Columns represent mean ± SEM, n=9. **P*<0.05, ***P*<0.01, ****P*<0.001, compared with control.

Effects of resveratrol on mRNA expression of redox genes in HUVEC

Native HUVEC were incubated with resveratrol at concentrations from 1 to 100 μM for 24 h and mRNA expression was analyzed with real-time RT-PCR. A concentration-dependent reduction of Nox4 mRNA was observed at resveratrol concentrations ≥ 10 μM. mRNA expression of GPx1 and SOD1 was upregulated in a concentration-dependent manner (*Fig. 4*).

Resveratrol increases SOD1 and GPx1 protein expression in human EA.hy 926 endothelial cells

EA.hy 926 cells were treated with resveratrol at concentrations from 10 to 100 μM for 24 h. Proteins expression of GPx1 and SOD1 was analyzed with Western blotting. As shown in *Fig. 5*, a concentration-dependent upregulation of GPx1 and SOD1 protein expression by resveratrol could be seen (*Fig. 5*). No reliable antibodies for human Nox4 were commercially available at this time. Therefore, we could not perform Western blot analyses for Nox4.

Resveratrol prevents DMNQ-induced oxidative stress in human EA.hy 926 endothelial cells

EA.hy 926 cells were pre-treated with 100 μM resveratrol or its solvent control DMSO for 24 h. Then, cells were stimulated with DMNQ (25) and ROS levels were measured by L-012 chemiluminescence. As shown in *Fig. 6*, resveratrol pretreatment completely abolished DMNQ-induced oxidative stress.

DISCUSSION

The present study demonstrates that resveratrol decreases the expression of the ROS-producing enzyme Nox4, and at the same time, increases the expression of ROS-inactivating enzymes, SOD1 and GPx1, in human endothelial cells.

Increased oxidative stress plays an important role in the pathogenesis of cardiovascular diseases such as hypertension, atherosclerosis or vascular diabetic complications (26). In the vascular wall, ROS can be produced by several enzyme systems, including NADPH oxidases, xanthine oxidase, uncoupled

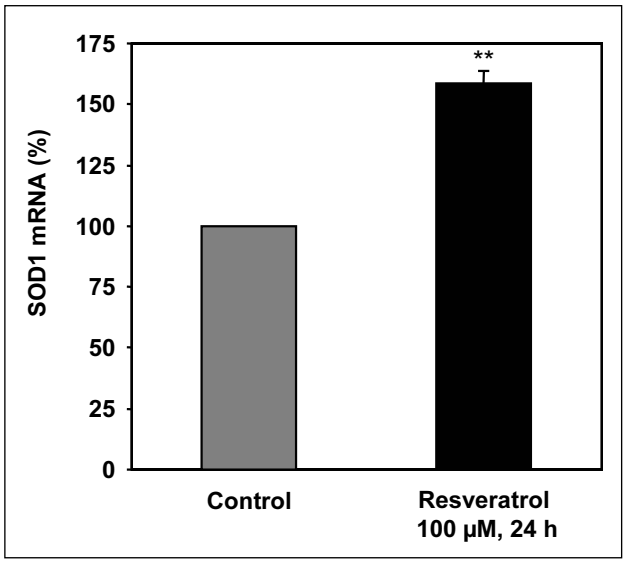

Fig. 3. Resveratrol increases SOD1 mRNA expression in human EA.hy 926 endothelial cells. Cells were treated with resveratrol and SOD1 mRNA expression was analyzed by quantitative real-time RT-PCR. Columns represent mean ± SEM, n 9. **P<0.01, compared with control.

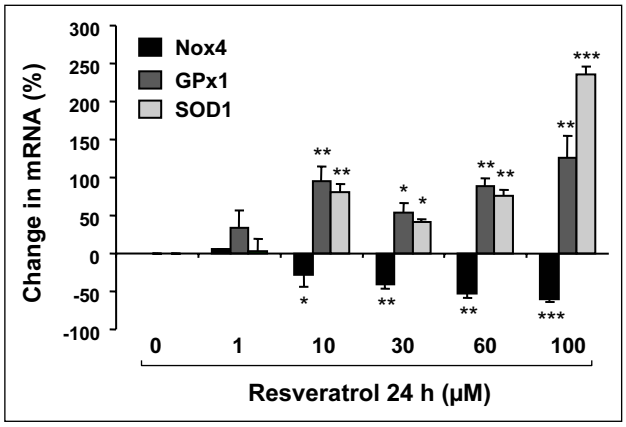

Fig. 4. Effects of resveratrol on mRNA expression of Nox4, GPx1 and SOD1 in HUVEC. Cells were treated with resveratrol and mRNA expression was analyzed by quantitative real-time RT-PCR. Columns represent mean ± SEM, n=9. *P<0.05, **P<0.01, ***P<0.001, compared with control.

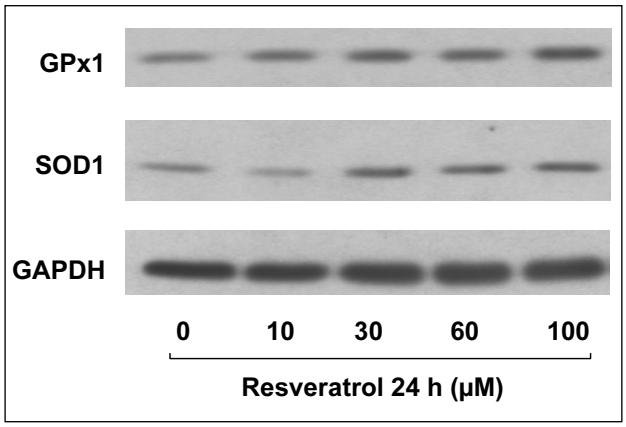

Fig. 5. Effects of resveratrol on protein expression of GPx1 and SOD1 in human EA.hy 926 endothelial cells. Cells were treated with resveratrol and protein expression was analyzed by Western blotting. GAPDH was shown as an internal control. The blots shown are representative of three independent experiments.

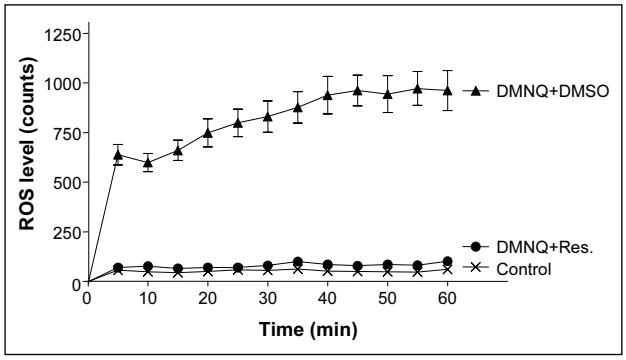

Fig. 6. Resveratrol prevents DMNQ-induced oxidative stress in human EA.hy 926 endothelial cells. Cells were pretreated with 100 μM resveratrol or its solvent control DMSO for 24 h. Then, cells were stimulated with DMNQ. ROS levels were determined with L-012 chemiluminescence. Control cells were without pretreatment and without DMNQ. Symbols represent mean ± SEM, n=6. P<0.001, DMNQ + DMSO versus control; P<0.001, DMNQ + Resveratrol *versus* DMNQ + DMSO.

endothelial NO synthase, enzymes of the respiratory chain, and cytochrome P450 monooxygenases (26). Although all these enzymes contribute to the oxidative burden, evidence is accumulating that an initial generation of ROS by NADPH oxidases triggers the release of ROS by the other enzymes (27, 28). Moreover, NADPH oxidases are likely to be the predominant source of ROS in the vasculature. In diseased human coronary arteries, about 60% of total vascular superoxide is derived from NADPH oxidases (29).

The NADPH oxidase enzyme complex consists of two essential membrane-bound subunits, gp91phox and p22phox, which compose flavocytochrome b558, and several cytosolic regulatory components (30). In the vasculature, several homologous proteins of the NADPH oxidase catalytic subunit gp91phox/Nox2 are expressed. These include Nox1, Nox2, Nox4, and Nox5 (30, 31). In HUVEC and HUVEC-derived EA.hy 926 cells, the expression level of Nox4 is 100-fold higher than that of Nox1, Nox2, or Nox5, suggesting that Nox4 is the major source of ROS in these cells (32).

NADPH oxidase-mediated ROS production is regulated at two levels: gene expression of the NADPH oxidase subunits and enzymatic activity. Activation of the NADPH oxidase enzyme complex requires the assembly of the cytosolic regulatory subunits (p47phox, p67phox, p40phox and Rac) with the membrane-bound cytochrome b558 (27). Previous studies have demonstrated that resveratrol diminishes endothelial ROS production, probably by inhibiting membrane translocation of the regulatory subunits (33, 34). Our study provides the first evidence that resveratrol also decreases the expression levels of Nox4, which is the predominant Nox isoform in endothelial cells. This represents a novel mechanism for the decrease in NADPH oxidase-mediated ROS production by resveratrol.

Reports on the effect of resveratrol on SOD isoforms are controversial. In cardiac H9C2 cells (11) and in aortic smooth muscle cells (18), resveratrol has been shown to increase the activity of SOD. A recent study, however, found no changes in protein levels of SOD1 or SOD2 in rat aortic segments *ex vivo*-treated with resveratrol up to 100 μM for 24 h (9). The effects of resveratrol on SOD could be cell- and isoform-specific. In human lung fibroblasts, resveratrol shows no significant effect

on the expression or activity of SOD1, whereas it dramatically and progressively induces mitochondrial SOD2 expression and activity (35). So far, there are no reports on the regulation of endothelial SOD1 by resveratrol. In the present study, we show for the first time that resveratrol upregulates the mRNA and protein expression of SOD1 in human endothelial cells.

GPx1 is the most abundant selenoperoxidase and a key antioxidant enzyme in many cell types including endothelial cells. GPx1 consumes reduced glutathione to convert hydrogen peroxide to water and lipid peroxides to their respective alcohols (36). It also acts as a peroxynitrite reductase (37). GPx1-overexpressing mice are more resistant, whereas GPx1 knockout mice are more susceptible to prooxidant-induced lethality than are wild-type mice (38).

Because of its major role in the prevention of oxidative stress, GPx1 is likely to be an important antiatherogenic enzyme. In fact, GPx1 activity is decreased or absent in carotid atherosclerotic plaques, and this reduced GPx1 activity has been linked to the development of atherosclerotic lesions in humans (39). In patients with coronary artery disease, the low activity of red blood cell GPx1 is associated with an increased risk of cardiovascular events independently from traditional risk factors, and an increase in GPx1 activity reduces cardiovascular risk (40). GPx1-deficient mice showed increased cell-mediated oxidation of LDL (41). Furthermore, GPx1 deficiency causes endothelial dysfunction (42, 43) and endothelial progenitor cell dysfunction (44) in mice. GPx1 deficiency is accompanied by increased periadventitial inflammation, neointima formation, and collagen deposition surrounding the coronary arteries (45). Finally, deficiency of GPx1 accelerates atherosclerotic lesion progression in apolipoprotein E-deficient mice (46, 47). These data demonstrate that GPx1 is a major intracellular antioxidant enzyme in protecting against vascular oxidative stress.

A recent study demonstrates that *ex vivo* treatment of rat aortic segments with resveratrol leads to upregulation of GPx1 protein expression (9). Our results show that resveratrol increases the expression of endothelial GPx1. Whether such a GPx1 upregulation also takes place in vascular smooth muscle cells remain unclear.

At present, the mechanisms by which resveratrol regulates gene expression are less understood. Resveratrol is a putative activator of sirtuin 1 (SIRT1) (48), a NAD^+-dependent histone deacetylase. SIRT1 facilitates the formation of heterochromatin, the more tightly packed form of chromatin associated with histone hypoacetylation and gene repression. Multiple non-histone targets have also been described for SIRT1. These include some transcription factors or cofactors such as the tumor suppressor p53, the Forkhead box class O (FOXO) transcription factors, nuclear factor κB (NF-κB) and peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) (49, 50). Whether these mechanisms are involved in the regulation of Nox4, GPx1 and SOD1 by resveratrol reported in the present study is still unknown.

In summary, the present study describes some novel effects of resveratrol. By decreasing the expression of Nox4 and by enhancing the expression of GPx1 and SOD1, resveratrol (and its derivatives) represents a unique approach to reducing endothelial oxidative stress.

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