Effects of resveratrol on eNOS in the endothelium and the perivascular adipose tissue

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Under physiological conditions, nitric oxide (NO) is produced in the vasculature mainly by the endothelial NO synthase (eNOS). Experiments using gene-disrupted mice have demonstrated that eNOS has antihypertensive, antithrombotic, and antiatherosclerotic effects. Recent studies show that eNOS is expressed not only in the endothelium but also in the perivascular adipose tissue (PVAT). Resveratrol prevents eNOS uncoupling and upregulates eNOS expression and activity. These effects of resveratrol are well established for the eNOS enzyme in the endothelium. Interestingly, resveratrol also improves PVAT function. However, a causal role for eNOS in the effects of resveratrol on PVAT function has not yet been verified and needs to be studied in the future.

Keywords: resveratrol; nitric oxide; nitric oxide synthase; endothelium; perivascular adipose tissue

Introduction

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenol phytoalexin present in various plant species, including Veratrum grandiflorum (white hellebore), Polygonum cuspidatum (Japanese knotweed), Vitis vinifera (grapes), Arachis hypogaea (peanuts), and Morus rubra (mulberries).¹⁻³ Preclinical studies have demonstrated protective effects of resveratrol in a variety of disease models, including cardiovascular diseases, diabetes, cancer, and neurodegenerative diseases.² Moreover, resveratrol is one of the major polyphenolic compounds in red wine and is likely to contribute to the protective effects of moderate wine consumption.⁴ This has been postulated as an explanation for the lower incidence of myocardial infarction in France compared with other countries, the so-called “French paradox.”⁵,⁶ In animal models of cardiovascular disease, resveratrol has been shown to improve vascular function, reduce blood pressure, and protect against atherosclerosis.⁷ At least some of the vasoprotective effects of resveratrol are attributable to an enhanced production of nitric oxide (NO) from endothelial NO synthase (eNOS).⁷⁻⁹

Endothelial NOS in the endothelium

NO can be produced by three isoforms of NO synthase (NOS): neuronal NOS (nNOS), inducible NOS (iNOS), and eNOS.¹⁰,¹¹ Vascular nNOS is expressed in perivascular nerve fibers and in the vascular wall.¹²,¹³ NO derived from nNOS contributes to vasodilation¹⁴⁻¹⁶ and can partly compensate the loss of eNOS in eNOS gene knockout mice.¹⁷⁻¹⁹ The expression of iNOS in the vasculature is induced under conditions of inflammation and sepsis.¹⁰,²⁰ Nevertheless, eNOS is the main source of vascular NO under physiological conditions.¹¹ Endothelial NOS is primarily expressed in endothelial cells.¹⁰ Endothelial eNOS is activated by shear stress produced by the flowing blood and by various agonists, such as bradykinin, acetylcholine, and vascular endothelial growth factor.²¹ NO produced by eNOS in the endothelium confers antihypertensive, antithrombotic, and antiatherosclerotic effects (Fig. 1).²²,²³ Once generated, NO can diffuse from the producing cell into the underlying smooth muscle cells and induce vasodilation.²⁴ NO derived from endothelial eNOS

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also diffuses into the blood and inhibits platelet aggregation and adhesion. In addition, eNOS-derived NO possesses multiple antiatherosclerotic properties, including prevention of leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall, inhibition of low-density lipoprotein oxidation, and inhibition of vascular smooth muscle cell proliferation.\(^{11,22,25,26}\) Mice with global disruption of the eNOS gene show elevated blood pressure\(^{27}\) and accelerated atherosclerosis.\(^{28,29}\)

Recent studies suggest that eNOS-derived NO also promotes mitochondrial biogenesis\(^ {30}\) and may contribute to the antiaging effects of calorie restriction.\(^ {31}\) Moreover, eNOS gene knockout mice show hyperinsulinemia and insulin resistance.\(^ {32}\) Overexpression of eNOS prevents weight gain in a mouse model of diet-induced obesity.\(^ {33}\)

The above-mentioned protective effects are usually attributed to eNOS in the endothelium. Although the importance of endothelial eNOS is beyond dispute, recent research results inspire us to rethink this concept. The expression of eNOS is not limited to endothelial cells; it is also expressed in other cell types, such as cardiac myocytes, platelets, certain neurons of the brain, syncytiotrophoblasts of the human placenta, and kidney tubular epithelial cells.\(^ {10}\) Importantly, abundant eNOS expression has been found in perivascular adipose tissue (PVAT) (Fig. 2).\(^ {34,35}\) Because PVAT is an integral part of the vascular wall,\(^ {35}\) it is conceivable that some of the vasoprotective effects of eNOS observed \textit{in vivo} are attributable to PVAT eNOS.

**Endothelial NOS in PVAT**

The role of PVAT in regulating vascular function was first discovered by Soltis and Cassis, who have observed a PVAT-mediated decrease in contractile responses to noradrenaline in rat aorta.\(^ {36}\) It is now known that PVAT regulates vascular tone by releasing a large number of bioactive molecules, including NO (Fig. 3).\(^ {35}\)

Within PVAT, both adipocytes and endothelial cells of the capillaries and vasa vasorum are stained positive for eNOS in immunohistochemistry analyses (Fig. 2).\(^ {34,37}\) Moreover, NO production in PVAT adipocytes can be directly visualized \textit{in situ} with fluorescence imaging techniques.\(^ {34,38,39}\)

In small arteries isolated from visceral fat of healthy individuals, basal NO production is reduced by PVAT removal,\(^ {40}\) indicating that PVAT contributes to vascular NO production. In PVAT-intact, endothelium-denuded rat mesenteric arteries, eNOS inhibition significantly enhances norepinephrine-induced contraction, indicating that PVAT-derived NO contributes to the anti-contractile effect of PVAT independently of the endothelium.\(^ {41,42}\) PVAT-derived NO may induce vasorelaxation through three major mechanisms (Fig. 3):\(^ {35}\) by diffusing into the adjacent smooth muscle cells and causing vasodilation, by stimulating adiponectin release from PVAT adipocytes,\(^ {43}\) or...
Figure 2. Expression of eNOS in endothelial cells and in PVAT adipocytes. Male C57BL/6J mice were put on high-fat diet (HFD) or normal control diet (NCD) for 20 weeks starting at the age of 8 weeks. Immunohistochemistry staining (A–C) was performed using an anti-eNOS antibody or IgG as a negative control. HFD feeding has no effect on eNOS expression. Reproduced from Ref. 34 (permission not required for authors). E, endothelium.

by modulating BKCa channels in smooth muscle cells and potentiating hyperpolarization.44

Moreover, PVAT eNOS plays a crucial role in obesity-induced vascular dysfunction.35 In a mouse model of diet-induced obesity, a vascular dysfunction of the thoracic aorta is only evident if PVAT is left intact. In contrast, the endothelium-dependent, NO-mediated vasodilator response to acetylcholine remains unchanged in PVAT-free aortas from mice fed a high-fat diet compared with control mice.34,45 Because the acetylcholine-induced vasodilation in the mouse aorta (either with or without PVAT) is completely dependent on NO, the reduced vasomotor function in the aorta of diet-induced obese mice is likely to result from eNOS dysfunction in the PVAT but not in the endothelium. Indeed, we found evidence for PVAT eNOS dysfunction in diet-induced obese mice.34 That PVAT eNOS dysfunction is causally linked to vascular dysfunction in diet-induced obesity is supported by findings that vascular function can be normalized by improving PVAT eNOS function, either by improving l-arginine availability34 or by restoring eNOS phosphorylation and acetylation.45

We therefore hypothesize that PVAT eNOS may be even more important than endothelium eNOS in obesity-induced vascular dysfunction under certain experimental settings.34,35,45

Resveratrol improves endothelial function

Endothelial dysfunction (characterized as an impairment of endothelium-dependent relaxation) is an early event in atherogenesis and occurs even before structural changes in the vasculature.7 Since most cardiovascular diseases are either related to or are a direct consequence of atherosclerosis, endothelial dysfunction is an early predictor of subsequent cardiovascular events or mortality.46

In preclinical studies, endothelial function is usually studied by assessing the acetylcholine-induced relaxation of PVAT-free blood vessels ex vivo in organ bath or myograph experiments.

Improvement of endothelial eNOS function

Oral treatment with resveratrol has been shown to improve endothelial function in animal models of cardiovascular disease.7 In particular, enhancement of agonist-stimulated, endothelium-dependent vasorelaxation by resveratrol has been shown in hypertensive rats,47 diabetic rats,48 diabetic mice,49 and hypercholesterolemic rabbits.50 Because vascular function was analyzed in these studies with PVAT-free vessels ex vivo, the observed effects of resveratrol on vascular function are clearly mediated by endothelial eNOS but not PVAT eNOS.

Resveratrol also improves vascular function in humans.51–54 Vascular function in human subjects is studied noninvasively by measuring the flow-mediated dilatation of the brachial artery. Because this method is based on the stimulating effect of the blood flow on the endothelium,46 it is likely that these reported effects of resveratrol are attributable to an activation of endothelial eNOS but not PVAT eNOS. This, however, does not exclude a possible effect of resveratrol on PVAT eNOS in vivo. The given experimental settings are not suitable for testing the role of PVAT eNOS.

Prevention of NO inactivation

Superoxide anion reacts with NO to form peroxynitrite. Therefore, enhanced oxidative stress in the endothelium has two major consequences on NO bioavailability: enhanced NO inactivation by superoxide and reduced NO production owing to eNOS uncoupling (see below). Resveratrol decreases

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Resveratrol and eNOS in the vasculature

Figure 3. PVAT-derived vasoactive factors, including NO. Methyl palmitate produced by PVAT adipocytes (ACs) causes vasodilation by opening the K_v channels on vascular smooth muscle cells (VSMCs). H2S is synthesized in PVAT by cystathionine-γ-lyase (CSE) and induces VSMC hyperpolarization by stimulating KCNQ-type Kv or K_ATP channels. Leptin induces endothelium-dependent vasodilatation by stimulating leptin receptor (LepR), which leads to activation of eNOS via a pathway involving AMP-activated protein kinase (AMPK) and Akt and to H2S production. This H2S functions as an endothelium-derived hyperpolarizing factor (EDHF) and activates endothelial small (SK_Ca) and intermediate (IK_Ca) conductance calcium-dependent K^+ channels via autocrine mechanisms. The resulting hyperpolarization of endothelial cells can be transmitted to VSMCs by electrical coupling through myoendothelial gap junctions (MEGJs). Leptin also causes endothelium-independent vasodilatation by inducing VSMC hyperpolarization through unknown mechanisms. NO and H2O2 released from PVAT can elicit vasodilatation by activating soluble guanylate cyclase (sGC), leading to the synthesis of cyclic guanosine monophosphate (cGMP). Adiponectin release from PVAT ACs can be enhanced by stimulation of β3-adrenoceptors and the NO–cGMP–PKG pathway. Adiponectin exerts multiple vascular effects: it stimulates NO production from PVAT and from endothelial cells and induces VSMC hyperpolarization by activating TRPM4 channels followed by opening BK_Ca channels. Ang1–7 produced by PVAT acts on endothelial Ang1–7 receptor (Mas; MAS1 receptor), thereby stimulating endothelial NO production. Besides stimulating sGC activity, NO from PVAT and endothelial cells can also induce/potentiate VSMC hyperpolarization through K_Ca or BK_Ca channels. Reproduced from Ref. 35, an open access article under the terms of the Creative Commons Attribution-NonCommercial License.

oxidative stress in vascular tissues, thereby preventing NO inactivation and eNOS uncoupling (Fig. 4).

Resveratrol reduces cellular levels of reactive oxygen species (ROS) through multiple mechanisms. As a polyphenolic compound, resveratrol has been shown to scavenge a variety of oxidants in in vitro systems, including hydroxyl radical, superoxide anion, hydrogen peroxide, and peroxynitrite. However, the direct scavenging activities of resveratrol are relatively poor. The antioxidant properties of resveratrol in vivo are more likely to be attributable to its effect as a gene regulator. Resveratrol reduces the expression and activity of NADPH oxidase in cardiovascular tissues, thereby inhibiting ROS production. The polyphenolic compound also reduces mitochondrial superoxide generation by stimulating...
Resveratrol and eNOS in the vasculature

Xia et al.

Figure 4. Resveratrol improves vascular NO bioavailability. Resveratrol can activate sirtuin 1 (SIRT1) directly (in a substrate-dependent manner) or indirectly (by either inhibiting phosphodiesterases or enhancing the effect of lamin A). SIRT1 stimulates endothelial NO synthase (eNOS) activity through deacetylation, enhances eNOS expression by deacetylating Forkhead box O (FOXO) transcription factors, and prevents eNOS uncoupling by upregulating GTP cyclohydrolase 1 (GCH1), the rate-limiting enzyme in tetrahydrobiopterin (BH4) biosynthesis. AMP-activated protein kinase (AMPK) and nuclear factor erythroid–derived 2-related factor-2 (NRF2) are indirect targets of resveratrol. AMPK phosphorylates eNOS at serine 1177. Endothelial NOS can also be phosphorylated by ERK1/2, which is stimulated by a pathway involving estrogen receptors (ER) and the tyrosine kinase Src. Caveolin-1 (Cav-1) is an eNOS-interacting protein that negatively regulates eNOS activity. Asymmetric dimethylarginine (ADMA) is an endogenous eNOS inhibitor that is degraded by dimethylarginine dimethylaminohydrolase (DDAH). The resveratrol targets for DDAH upregulation or for NADPH oxidase downregulation have not yet been identified. Reproduced from Ref. 8, an open access article distributed under the terms and conditions of the Creative Commons Attribution license.

Prevention of eNOS uncoupling
Under pathological conditions, eNOS may become dysfunctional, producing superoxide instead of NO. This phenomenon is referred to as eNOS uncoupling. Major mechanisms for eNOS uncoupling include deficiency of eNOS cofactor BH4, deficiency of eNOS substrate L-arginine, and eNOS S-glutathionylation. Peroxynitrite and superoxide can oxidize BH4, leading to BH4 deficiency and eNOS uncoupling. Therefore, the antioxidant effect of resveratrol prevents BH4 oxidation and eNOS uncoupling (i.e., resveratrol reduces ROS production from eNOS).

The second mechanism by which resveratrol prevents eNOS uncoupling is enhanced BH4 biosynthesis. As mentioned above, resveratrol upregulates the expression of GTP cyclohydrolase 1. This effect has been observed both in mice in vivo and in cultured endothelial cells. Uncoupling of eNOS is a crucial mechanism contributing to cardiovascular diseases. It not only reduces NO production but also potentiates the

mitochondria biogenesis. Transcriptional upregulation of guanine triphosphate (GTP) cyclohydrolase 1, the rate-limiting enzyme for tetrahydrobiopterin (BH4) biosynthesis, is a contributing mechanism for the reduced superoxide production from eNOS (i.e., prevention of eNOS uncoupling; see below). Moreover, resveratrol also accelerates ROS detoxification by enhancing the expression of a variety of antioxidant enzymes, including superoxide dismutases, glutathione peroxidase 1, catalase, NAD(P)H:quinone oxidoreductase, heme oxygenase 1, and γ-glutamylcysteine synthetase (GCLC), the rate-limiting enzyme for glutathione synthesis.
preexisting oxidative stress. The overproduction of ROS (e.g., superoxide and subsequently peroxynitrite) by uncoupled eNOS in turn enhances oxidation of BH4, creating a vicious circle.\textsuperscript{69–71} Conversely, prevention of eNOS uncoupling by resveratrol breaks this circle, thereby reducing ROS production and, at the same time, enhancing NO production from eNOS.

**Enhancement of eNOS activity**

The enzymatic activity of eNOS is regulated by different cellular events, such as intracellular calcium concentration; interaction with substrate, cofactors, adaptors, and regulatory proteins; and shuttling among distinct subcellular domains.\textsuperscript{21} In addition, eNOS activity is also regulated by posttranslational modification of the eNOS protein (e.g., phosphorylation and acetylation) and by methylarginines.\textsuperscript{21,76}

Serine 1177 of human eNOS is the best-studied phosphorylation site and its phosphorylation is associated with enhanced eNOS activity.\textsuperscript{21,76} Treatment of endothelial cells with resveratrol enhances eNOS serine 1177 phosphorylation and eNOS enzymatic activity.\textsuperscript{77,78} This effect can be achieved with nanomolar concentrations of resveratrol and is mediated by a signal cascade involving the estrogen receptor (ER)\textsubscript{α}, G protein G\textsubscript{α}, caveolin-1 (Cav-1), the tyrosine kinase c-Src, and the MAP kinase ERK1/2.\textsuperscript{77,78} At micromolar concentrations, resveratrol can also stimulate eNOS serine 1177 phosphorylation by activating AMP-activated protein kinase.\textsuperscript{79,80}

Acetylation of eNOS at lysines 494 and 504 (human eNOS sequence) in the calmodulin-binding domain decreases eNOS enzymatic activity.\textsuperscript{81} Treatment of endothelial cells with resveratrol leads to eNOS deacetylation at lysines 494 and 504 and enhanced eNOS activity, an effect that is mediated by the NAD\textsuperscript{+}-dependent deacetylase sirtuin 1 (SIRT1).\textsuperscript{81,82}

Asymmetric dimethylarginine (ADMA) is an endogenous eNOS inhibitor.\textsuperscript{83} It is degraded by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH). Treatment of endothelial cells with a high concentration of glucose leads to accumulation of intracellular ADMA owing to downregulation of DDAH expression and activity. These effects can be reversed by pretreatment with resveratrol,\textsuperscript{84} piceatannol (a resveratrol metabolite),\textsuperscript{84} or BTM-0512, a derivative of resveratrol.\textsuperscript{85}

Cav-1 is one of the proteins that negatively regulate eNOS activity through protein–protein interactions.\textsuperscript{86} Resveratrol modulates this interaction at two different levels: downregulation of Cav-1 expression and inhibition of Cav-1–eNOS association. These effects of resveratrol have been observed \textit{in vivo} in the rat heart\textsuperscript{87,88} and in cultured endothelial cells.\textsuperscript{78,89} The downregulation of Cav-1 (which takes hours to days) is likely to be mediated by HO-1,\textsuperscript{87} whereas the acute reduction of the Cav-1–eNOS association (which occurs within minutes) is likely to be due to Src kinase–mediated Cav-1 phosphorylation on Tyr14 and dependent on stimulation of estrogen receptors.\textsuperscript{78}

**Upregulation of eNOS expression**

Our previous studies demonstrated that both resveratrol\textsuperscript{90} and red wines rich in resveratrol\textsuperscript{91,92} upregulate the expression of eNOS in endothelial cells. This effect of resveratrol is independent of estrogen receptors and involves transcriptional (i.e., promoter activation) as well as posttranscriptional (i.e., mRNA stabilization) mechanisms.\textsuperscript{90}

Resveratrol-induced eNOS upregulation can be prevented by small interfering RNA (siRNA)-mediated SIRT1 knockdown in endothelial cells.\textsuperscript{30} Mice with selective SIRT1 overexpression in the endothelium show enhanced eNOS expression.\textsuperscript{93} These results suggest that resveratrol upregulates eNOS expression in a SIRT1-dependent manner. Our recent data indicate that Forkhead box O (FOXO) factors are likely to be the downstream SIRT1 targets for this effect of resveratrol.\textsuperscript{94}

**Resveratrol improves PVAT function**

Recent studies have provided evidence that resveratrol also improves PVAT function.\textsuperscript{95,96} The vasodilator response of normal rat aorta to acetylcholine is reduced by conditioned media derived from PVAT isolated from rats fed with fructose\textsuperscript{99} or high-fat diet (HFD)\textsuperscript{96} for 8 weeks, indicating that fructose and HFD feeding induce PVAT dysfunction. Interestingly, oral treatment of the fructose- and HFD-fed rats with resveratrol (20 mg/kg) by daily oral gavage during the 8-week feeding period leads to a marked improvement of PVAT function. Conditioned media derived from PVAT of resveratrol-treated rats had significantly less inhibitory effect on
the acetylcholine-induced vasodilation of normal rat aorta than PV AT from fructose- and HFD-fed rats. 
Because these are \textit{ex vivo} experiments using PV AT-derived conditioned media, the observed effects of resveratrol are attributable to PV AT, independent of the endothelium. However, it is yet unclear whether this improvement of PV AT function by resveratrol is related to PV AT eNOS.

\textbf{Conclusions}

Resveratrol improves eNOS function, which may be the molecular basis for many of the effects of resveratrol in the cardiovascular system. It prevents eNOS uncoupling and increases eNOS expression and activity, resulting in enhanced NO bioavailability in the vasculature. This has been well established for eNOS in the endothelium. Recent studies demonstrate that eNOS is also present in the PV AT and that resveratrol improves PV AT function. However, whether the improved PV AT function is due to an effect of resveratrol on PV AT eNOS is yet unclear. This warrants further studies in the future.

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\textbf{Competing interests}

The authors declare no competing interests.

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Resveratrol and eNOS in the vasculature

Xia et al.

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