Resveratrol Improves Vascular Function in Patients With Hypertension and Dyslipidemia by Modulating NO Metabolism

Albino Carrizzo, Annibale Puca, Antonio Damato, Marina Marino, Elio Franco, Franco Pompeo, Anna Traficante, Fabio Civitillo, Luigi Santini, Valentina Trimarco, Carmine Vecchione

Abstract—Epidemiological studies have demonstrated that the Mediterranean diet, which is rich in resveratrol, is associated with a significantly reduced risk of cardiovascular disease. However, the molecular mechanisms that underlie the beneficial effects of resveratrol on cardiovascular function remain incompletely understood. Therefore, we set out to identify the molecular target(s) mediating the protective action of resveratrol on vascular function. To this end, we performed vascular reactivity studies to evaluate the effects of resveratrol on superior thyroid artery obtained from 59 patients with hypertension and dyslipidemia. We found that resveratrol evoked vasorelaxation and reduced endothelial dysfunction through the modulation of NO metabolism via (1) an 5′ adenosine monophosphate–activated protein kinase–mediated increase in endothelial NO synthase activity; (2) a rise in tetrahydrobiopterin levels, which also increases endothelial NO synthase activity; and (3) attenuation of vascular oxidative stress, brought about by overexpression of manganese superoxide dismutase via an nuclear factor erythroid–derived 2-like 2–dependent mechanism. The effects of resveratrol on acetylcholine vasorelaxation were also tested in vessels from patients with nonhypertensive nondyslipidemia undergoing thyroid surgery. In this setting, resveratrol failed to exert any effect. Thus, our finding that resveratrol reduces endothelial dysfunction, an early pathophysiological feature and independent predictor of poor prognosis in most forms of cardiovascular disease, supports the concept that the risk of vascular events could be further reduced by adherence to a set of dietary and behavioral guidelines. (Hypertension. 2013;62:359-366.) Online Data Supplement

Key Words: endothelial cells ■ nitric oxide ■ nutrition assessment ■ oxidative stress ■ vessel

Dietary restriction delays the aging process, preventing the onset of aging-related diseases and extending life span.1,2 Research into the cardioprotective potential of dietary components supports the development of functional foods and nutraceuticals.3 On this point, resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural phenol that acts as a calorie-restriction mimetic and affects energy metabolism by improving insulin sensitivity, lowering plasma glucose, and increasing mitochondrial capacity.4,5 In experimental models, this stilbene has been reported to exert antioxidant, anticancer, antiproliferative, and antibacterial effects.6,7 Numerous experimental and clinical studies have suggested that endothelial dysfunction, frequently evaluated as impairment of endothelium-dependent vasodilation, is a phenomenon linking various cardiovascular risk factors8,9 and playing an important role in the development and progression of cardiovascular disease.10 Dietary restriction seems to protect against endothelial dysfunction and attenuate atherogenesis.11,12 Endothelial dysfunction, an early pathophysiological feature and independent predictor of poor prognosis in most forms of cardiovascular disease, supports the concept that the risk of vascular events could be further reduced by adherence to a set of dietary and behavioral guidelines.13,14 Importantly, resveratrol has effects also on vessels with endothelial dysfunction: in this setting, it induces vasorelaxation both directly and by enhancing endothelium-dependent vasodilation.15 However, the molecular mechanisms underlying these effects have been poorly explored in humans and remain, hitherto, unclear. A thorough understanding of the molecular mechanisms responsible for the beneficial effects of resveratrol could contribute to the development of therapies aimed at the slowing down or prevention of disease and to the development of approaches designed to enhance the effect of these therapies by coupling them with healthy lifestyle changes.15,16 In the present report, we characterize the pathways by which resveratrol produces benefits in dysfunctional vessels of patients with hypertension and dyslipidemia (HD). We show that resveratrol modulates NO metabolism at the vascular level, enhancing both its production and its bioavailability. Increased levels

Received January 7, 2013; first decision January 30, 2013; revision accepted May 17, 2013.

From the Vascular Physiopathology Unit (A.C., A.D., E.F., F.P., C.V.), and Experimental Medicine Unit (A.T.), IRCCS INM Neumorex, Pozzilli (IS), Italy; Department of Neuroscience (V.T.), and Department of Clinical Medicine, Cardiovascular and Immunological Sciences (M.M.), University of Naples Federico II, Naples, Italy; Department of Medicine and Surgery, Hospital G. Rummo, Benevento, Italy (E.F.); Department of General Surgery, Gepos Institute, Telese Terme, Benevento, Italy (F.C.); VII Division of General and Endocrine Surgery, Second University of Naples, Naples, Italy (L.S.); Cardiovascular Research Unit, IRCCS Multimedica, Milan, Italy (A.P.); and Department of Medicine and Surgery, University of Salerno, Salerno, Italy (A.P., C.V.).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.111.01009/-/DC1. Correspondence to Carmine Vecchione, Department of Medicine and Surgery, University of Salerno, Salerno 86077, Italy. E-mail cvecchione@unisa.it

Hypertension is available at http://hyper.ahajournals.org DOI: 10.1161/HYPERTENSIONAHA.111.01009

359
of tetrahydrobiopterin (BH₄), an essential cofactor for a set of enzymes that are of central importance for metabolism, stimulation of the 5’ adenosine monophosphate–activated protein kinase (AMPK) pathway, and modulation of the mitochondrially localized antioxidant enzyme manganese superoxide dismutase (MnSOD) through nuclear factor erythroid–derived 2-like 2 (NRF2) mediate the action of resveratrol on NO metabolism.

**Methods**

For detailed methodology, please see the online-only Data Supplement. In brief, we collected superior thyroid artery (STA) from patients with HD (Table). Each vessel was divided into rings for use in different experimental series. Some rings were treated with resveratrol at 37°C, and then proteins were rapidly extracted for molecular studies to evaluate total and phosphorylated endothelial NO synthase (eNOS) protein levels, eNOS dimerization, GTP cyclohydrolase 1, copper-zinc superoxide dismutase (Cu-ZnSOD) and MnSOD expression, and NRF2 translocation; other rings were kept in 4°C Krebs solution for vascular reactivity studies; some were used for high-performance liquid chromatography (HPLC) dosage of BH₄; and others were rapidly used for measurement of O₂⁻. Studies were also performed on vessels removed from patients with nonhypertensive, nondyslipidemia (nHD) undergoing thyroid surgery.

**Results**

**Resveratrol Exerts a Vasorelaxant Effect by Modulating the Phosphorylation Status of eNOS and NO Release**

Evaluation of the vascular reactivity of ex vivo STA from patients with HD revealed that endothelially mediated (acetylcholine-evoked) vasorelaxation was significantly blunted compared with relaxation induced by nitroglycerin (Figure 1A). However, when HD STA rings were preconstricted with phenylephrine, resveratrol was capable of evoking vasorelaxation (Figure 1B). This vasorelaxation was dose dependent and more pronounced than that elicited with the classical endothelial agonist acetylcholine (Figure 1A). Moreover, exposure to N⁶-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, consistently blunted resveratrol-mediated vasorelaxation, clearly indicating an involvement of the NO pathway in this phenomenon (Figure 1B). In contrast, pretreatment with indo-ethacin, an inhibitor of the Cox1/Cox2 pathway, did not affect resveratrol vasorelaxation (Figure 1B).

The activity of eNOS can be regulated by coordinated control of the phosphorylation status of this enzyme²¹; in particular, phosphorylation of Ser¹¹⁷⁷ increases NO production by facilitating the electron transport inside the enzyme, whereas Thr⁹⁵⁵ is basally phosphorylated and may be dephosphorylated in response to several stimuli.¹⁸ We found that under control conditions, HD STA expressed eNOS that was phosphorylated on Thr⁹⁵⁵ but not on Ser¹¹⁷⁷ (Figure 1D). Exposure to acetylcholine evoked a slight, but significant, increase in Ser¹¹⁷⁷ phosphorylation and a decrease in Thr⁹⁵⁵ phosphorylation, whereas resveratrol markedly increased Ser¹¹⁷⁷ phosphorylation and dephosphorylated Thr⁹⁵⁵ (Figure 1D).

Phosphatidylinositol-3-kinase (PI3K) and AMPK are 2 upstream modulators of NO release, which can act independently on their target.¹⁹,²⁰ We found that Compound C, an AMPK inhibitor, significantly blunted the vascular activity of resveratrol (Figure 1C). In contrast, exposure to LY292002, a PI3K inhibitor, did not modify resveratrol-mediated vasorelaxation (Figure 1C). Coherently, resveratrol phosphorylated AMPK on Thr¹⁷², an activation site of the enzyme (Figure 1D), and in the presence of Compound C failed to induce eNOS-Ser¹¹⁷⁷ phosphorylation (Figure 1E). Taken together, these data demonstrate that resveratrol-evoked vasorelaxation is mediated by an AMPK signal that induces phosphorylation of eNOS-Ser¹¹⁷⁷ and, hence, NO production.

**Resveratrol Increases the Level of Tetrahydrobiopterin (BH₄) in Human Dysfunctional Vessels**

Because BH₄ is an essential cofactor for the catalytic activity of eNOS²¹,²² and has important consequences for the structure of the enzyme,²³ we studied BH₄ levels in HD STA. We found that BH₄ was significantly more present in HD STA exposed to resveratrol (50 μmol/L; 30 minutes) than in those that were either untreated or stimulated with acetylcholine (Figure 2A). This suggested that the beneficial action of resveratrol on vascular function is mediated also through an effect on BH₄. Indeed, exposure of HD STA rings to exogenous BH₄ significantly enhanced acetylcholine-induced vasorelaxation (Figure 2B), increasing eNOS phosphorylation on Ser¹¹⁷⁷, whereas concomitantly dephosphorylating Thr⁹⁵⁵ (Figure 2C). Exposure to BH₄ did not influence nitroglycerin-evoked vasorelaxation (data not show). Thus, low levels of BH₄ may contribute to endothelial dysfunction in patients with HD.²⁴

These results, obtained in human vessels, are in agreement with those on an experimental model in which BH₄ depletion was responsible for endothelial dysfunction within a short timeframe.²² Furthermore, the loss of this critical eNOS cofactor is the most prominent cause of eNOS uncoupling, which promotes...
superoxide production at the expense of NO\textsuperscript{25,26}; in fact, oxidative stress contributes to BH\textsubscript{4} degradation and, thus, favors eNOS uncoupling, which is linked to a decreased eNOS dimer/monomer ratio.\textsuperscript{27} In line with this, we found that both the exposure to resveratrol and to exogenous BH\textsubscript{4} significantly reduced oxidative stress in pathological STA ex vivo (Figure 2D).

Taken together, these findings demonstrate that an inadequate level of BH\textsubscript{4} contributes to impaired eNOS-Ser\textsuperscript{1177} phosphorylation, increased oxidative stress, and hence, to endothelial dysfunction in vessels of patients with HD. Moreover, this first experimental series demonstrates that a window of \(\approx 30\) minutes is sufficient for direct induction of NO-mediated vasodilatation and stimulation of BH\textsubscript{4} levels and, consequently, reduction of oxidative stress in diseased vessels.

Resveratrol Induces Overexpression of the Mitochondrially Localized Antioxidant Enzyme MnSOD

After observing the results of the first phase of the study, we investigated the effects of resveratrol on endothelial dysfunction in an experimental condition characterized by its permanent presence for at least 6 hours. We found that resveratrol increased the tissue levels of BH\textsubscript{4} (Figure 3A) and GTP cyclohydrolase 1, the first enzyme of the BH\textsubscript{4} biosynthesis pathway (Figure 3D). In addition, resveratrol selectively potentiated endothelial vasorelaxation (Figure 3B) and completely abolished oxidative stress (Figure 3F). Extended incubation with exogenous BH\textsubscript{4} also produced improvements in oxidative stress mitigation (Figure 3F) and acetylcholine-evoked vasorelaxation.
Hypertension August 2013

neither resveratrol nor BH4 influenced nitroglycerin-evoked vasorelaxation (data not shown). These data suggested that resveratrol exerts a stronger antioxidant action than BH4, probably through the recruitment of additional mechanisms.

On this issue, it is well known that resveratrol potentiates mitochondrial activity.28 In agreement, we found in ex vivo HD STA that 6 hours of incubation with resveratrol induced significant overexpression of MnSOD, a main antioxidant defense mechanism of mitochondria, but did not alter Cu-Zn SOD, which is mainly localized in the cytoplasmic fraction (Figure 3E). In contrast, incubation with exogenous BH4 did not significantly influence MnSOD expression (data not shown).

Analysis of eNOS phosphorylation also revealed that in this experimental setting both BH4 and resveratrol increased phosphorylation on Ser 1177, whereas concomitantly dephosphorylating Thr495 (Figure S3 in the online-only Data Supplement). Moreover, resveratrol increased the eNOS dimer/monomer ratio, promoting the proper functioning of the enzyme (Figure 3C).

**Resveratrol Activates MnSOD Through NRF2**

The transcription factor NRF2 is held in the cytoplasm as an inactive complex bound to Kelch-like ECH-associated protein 1, the repressor molecule and sensor of intracellular redox state.29,30 We found that after incubation with resveratrol for 6 hours, NRF2 dissociated from Kelch-like ECH-associated protein 1 and translocated to the nucleus, where it became active (Figure 4A). This was associated with overexpression of MnSOD (Figure 3D). Coexposure to retinoic acid, an NRF2 inhibitor,31 hampered resveratrol-induced MnSOD overexpression (Figure 4B). In this experimental setting, resveratrol and BH4 exerted similar effects on oxidative stress (Figure 4C) and acetylcholine-evoked vasorelaxation (data not shown). These findings demonstrate that the NRF2-mediated mechanism is responsible, at least in part, for the stronger antioxidant effect of resveratrol compared with that of BH4.

**Treatment With Statins Is Not Responsible for the Effect Observed for Resveratrol**

It is well known that statins can influence eNOS phosphorylation,32 which represents the main target to improve endothelial dysfunction. Because HD STA were obtained from statin-treated patients, to rule out that treatment with statins might have influenced our results, we performed experiments on vessels removed from patients with HD who had not been treated with statins. We found that acetylcholine vasorelaxation was comparable in the 2 study groups, and that there was a similarly enhanced reaction to resveratrol (Figure S1A). Nitroglycerine-evoked vasorelaxation was also comparable in the 2 groups, and this was not affected by resveratrol (data not shown). Analysis at the molecular level revealed a slight, although nonsignificant,
increase in eNOS phosphorylation in vessels from statin-treated patients and, in agreement with data obtained at functional levels, resveratrol exerted a comparable effect on this parameter in the 2 groups (Figure S1B). Taken together, these data indicate that the beneficial effect of resveratrol in vessels from patients with HD is not influenced by statin treatment.

Lack of Resveratrol Efficacy on Acetylcholine-Evoked Vasorelaxation in Vessels From Patients With nHD

To test whether the beneficial action of resveratrol on acetylcholine-evoked vasorelaxation was present in healthy vessels, we performed experiments on vessels removed from patients with nHD undergoing thyroid surgery (Table S1). We found that the dose-dependent vasorelaxation evoked by acetylcholine in these vessels was significantly higher compared with that observed in HD vessels. However, in contrast to what was observed for HD STA, resveratrol did not alter acetylcholine-evoked vasorelaxation in nHD vessels (Figure S2A). Moreover, oxidative stress was lower in nHD vessels than in HD STA, and this was not affected by resveratrol (Figure S2C).

Our evidence showing that resveratrol enhances acetylcholine-evoked vasorelaxation in vessels from patients with HD suggests that the endothelial layer in whole
vessels represents its main target. In agreement with functional observations, analysis performed on isolated endothelial cells showed that acetylcholine-induced eNOS phosphorylation was higher in nHD vessels than in HD STA; moreover, resveratrol had an effect on eNOS phosphorylation only in the latter (Figure S2B).

**Discussion**

In this study, we describe for the first time the molecular mechanisms involved in the protective effects of resveratrol in diseased human vessels. In particular, resveratrol positively modulates NO metabolism in atherosclerotic vessels of patients with HD. The following 3 mechanisms seem to be involved: (1) increased eNOS activity stimulated by activation of the AMPK pathway; (2) increased eNOS activity subsequent to an augmented BH4 level; and (3) attenuation of vascular oxidative stress, brought about by overexpression of MnSOD through an NRF2-dependent mechanism.

Resveratrol is a natural compound that affects mitochondrial function and energy metabolism, and, at least in experimental models, serves as a calorie-restriction mimic. Diet and lifestyle affect the incidence of cardiovascular disease. On this issue, epidemiological studies have demonstrated that the Mediterranean diet, which is rich in resveratrol, is associated with a significantly reduced risk of cardiovascular disease. However, the molecular mechanisms that underlie the beneficial effects of resveratrol on cardiovascular function are incompletely understood.

In the first part of this study, we demonstrate the direct vasodilatory action of increasing doses of resveratrol: the stilbenoid induced vasodilation even in vessels in which the action of the classic vascular endothelial agonist acetylcholine was impaired. This can be explained by the finding that resveratrol induces an increase of BH4 levels, a cofactor necessary for the appropriate functioning of NO synthase, enhancing the expression of GTP cyclohydrolase 1. Moreover, we found that both resveratrol and exogenous BH4 increased eNOS activity through phosphorylation of Ser 1177 and concomitant dephosphorylation of Thr 495. This finding is fully supported by evidence that BH4 administration restores eNOS phosphorylation on Ser1177 in vessels of hypertensive rats. 

Cardiovascular risk factors, such as diabetes mellitus, hypertension, hypercholesterolemia, and cigarette smoke, reduce bioactive NO. These risk factors lead to an enhanced production of reactive oxygen species in vessel walls. BH4 is highly sensitive to oxidation and, with BH4 deficiency, oxygen reduction uncouples from NO synthesis, thereby converting eNOS into a superoxide-producing enzyme. In our ex vivo experiments on vessels from patients with HD, exposure to exogenous BH4 or to resveratrol restored eNOS functionality and, hence, reduced oxidative stress.

The action we observed on NO occurs through an AMPK-dependent mechanism. It was recently proposed that misregulation of AMPK is involved in the metabolic defects underlying progression to dysmetabolic syndrome. In fact, in experimental models of dysmetabolic syndrome, resveratrol improved dyslipidemia, hyperinsulinemia, and hyperleptinemia, and reduced blood pressure, through a mechanism involving activation of AMPK. Based on our findings, we speculate that the blood-pressure lowering effect of resveratrol could be related to an effect on NO release mediated through the AMPK pathway.
Resveratrol increased the level of BH₄ and, considering that this improved endothelial vasodilation in pathological vessels, we evaluated the effect of resveratrol on endothelial dysfunction. We found that exposure of diseased STA to resveratrol for 6 hours reduced the endothelial dysfunction of these vessels. Endothelial dysfunction was also reduced by exogenous BH₄. Because careful analysis of our data revealed that the effect on vascular function of resveratrol is greater than that of exogenous BH₄, and that this is coupled to abolition of oxidative stress, we hypothesized that other mechanisms are recruited by resveratrol other than an increase in BH₄.

On this point, it is well known that resveratrol potentiates the activities of mitochondrial complexes, therefore, we decided to study MnSOD, a primary mitochondrial antioxidant enzyme that is normally induced during calorie restriction. We found that resveratrol induces overexpression of MnSOD. The analysis of intracellular signals involved in the activation of antioxidant mechanisms by resveratrol pointed to a crucial role for the transcription factor NRF2. In fact, we found that the increase in oxidative stress occurring in HD STA results in dissociation of NRF2 from Kelch-like ECH-associated protein 1, and its translocation to the nucleus, where it binds to the regulatory sequences named antioxidant response elements. The finding that with NRF2 inhibition, resveratrol failed to activate MnSOD and protect against oxidative stress strongly demonstrates the critical role of this transcription factor in the antioxidant action of the stilbenoid. Moreover, with inhibition of NRF2 the enhancement of endothelial vasodilation evoked by resveratrol became similar to that observed with exogenous BH₄, indicating that activation of the NRF2/MnSOD pathway accounts for some of the greater protective effect induced by resveratrol.

Among target molecules mediating the vascular effects of resveratrol, estrogen receptors should be considered. On this issue, it was reported that resveratrol increases interaction between estrogen receptors and caveole in endothelial cells, thus promoting NO production.

The amount of resveratrol ingested from dietary sources, such as red wine and juices, often results in plasma levels that are either not detectable or several orders of magnitude below the micromolar concentrations that are used in experimentation in vitro, that is, =32 nmol/L to 100 μmol/L. However, because of its lipophilic character, which permits interaction with lipids and lipoproteins, such as those of cell membranes, tissue levels of resveratrol may be higher than those found in plasma. The data presented here describe acute beneficial effects of resveratrol in HD vessels, but because resveratrol can accumulate in tissue, more studies are needed to clarify whether effects can be extended also to chronic conditions. However, in agreement with our data, chronic resveratrol administration has been shown to enhance endothelium relaxation in vessels from hypertensive rats, improving NO bioavailability.

In conclusion, our findings on the ability of resveratrol to reduce endothelial dysfunction, an early pathophysiological feature and independent predictor of poor prognosis in most forms of cardiovascular disease, fully support the concept that the risk of vascular events could be further reduced by adherence to a set of dietary and behavioral guidelines.

**Perspectives**

The current study gives more specific information about the effects of resveratrol on vessels from patients with hypertension. Our findings strongly indicate that a diet rich in resveratrol and healthy lifestyle changes can be useful in patients with atherosclerosis and hypertension because resveratrol exerts a protective action on the vascular, regardless of concomitant classical drug therapy. Based on existing data, it is clear that grapes, and wine, should be considered an integral component of fruit- and vegetable-enriched diets that are recommended by health authorities and widely accepted as beneficial for human health and disease prevention. A proper understanding and appreciation of the beneficial effects of this polyphenol should place efficacy of resveratrol in an appropriate perspective. Finally, we have identified molecular mechanisms that could be therapeutically modulated to fight vascular dysfunction in hypertension.

**Acknowledgments**

We thank Manuel Casaburo and Maria Teresa Ambrosio for technical assistance.

**Disclosures**

None.

**References**

Resveratrol exerts beneficial effects on dysfunctional vessels from patients with hypertension, positively modulating NO metabolism via (1) an S’ adenosine monophosphate–activated protein kinase–mediated increase in endothelial NO synthase activity; (2) a rise in tetrahydrobiopterin levels, which also increases endothelial NO synthase activity; and (3) attenuation of vascular oxidative stress, brought about by overexpression of manganese superoxide dismutase via a nuclear factor erythroid–derived 2-like 2–dependent mechanism.
Resveratrol Improves Vascular Function in Patients With Hypertension and Dyslipidemia by Modulating NO Metabolism

Albino Carrizzo, Annibale Puca, Antonio Damato, Marina Marino, Elio Franco, Franco Pompeo, Anna Traficante, Fabio Civitillo, Luigi Santini, Valentina Trimarco and Carmine Vecchione

_Hypertension_. 2013;62:359-366; originally published online June 10, 2013; doi: 10.1161/HYPERTENSIONAHA.111.01009

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/62/2/359

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/06/09/HYPERTENSIONAHA.111.01009.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Resveratrol improves vascular function in patients with hypertension and dyslipidemia by modulating NO metabolism

Albino Carrizzo B.Sc., Annibale Puca, M.D., Antonio Damato, M.Sc., Marina Marino, M.D., Elio Franco, M.D., Franco Pompeo, M.D., Anna Traficante, M.Sc., Fabio Civitillo, M.D., Luigi Santini, M.D., Valentina Trimarco, Ph.D., Carmine Vecchione, M.D.

Running title: Resveratrol modulates eNOS metabolism

From the Vascular Physiopathology Unit (A.C., A.D., E.F., F.P., C.V.), IRCCS INM Neuromed, Pozzilli (IS), Italy; Cardiovascular Research Unit (A.P.), IRCCS Multimedica, Milan, Italy; Department of Clinical Medicine (M.M.), Cardiovascular and Immunological Sciences, University of Naples “Federico II”, Naples, Italy; Department of Medicine and Surgery (E.F.), Hospital “G. Rummo”, Benevento, Italy; Experimental Medicine Unit (A.T.), IRCCS INM Neuromed, Pozzilli (IS), Italy; Department of General Surgery (F.C.), Gepos Institute, Telese Terme, Benevento, Italy; VII Division of General and Endocrine Surgery (L.S.), Second University of Naples, Naples, Italy; Department of Neuroscience (V.T.), University of Naples “Federico II”, Naples, Italy; Department of Medicine and Surgery (A.P., C.V.), University of Salerno, Salerno, Italy.

Correspondence to Carmine Vecchione, Vascular Pathophysiology Unit, IRCCS INM Neuromed, Pozzilli (IS), Italy, or Department of Medicine and Surgery, University of Salerno, Salerno, Italy. Tel. +39 0865 915229; Fax. +39 0865 927575; E-mail: cvecchione@unisa.it

Supplemental Methods
Supplemental Tables: 1
Supplemental Figures: 3
Supplemental Methods

Tissues and Reagents
Superior thyroid artery (STA) were collected from male patients (n = 59) undergoing carotid revascularization (demographic characteristics are reported in Table 1). Five of these patients were untreated with statins. Some experiments were performed on STA removed from non-hypertensive non-dyslipidemic patients (n = 8) undergoing total thyroid excision (Table S2). The patients, in euthyroid state, were affected by multinodular goitre with a compressive effect on the trachea.

Fasting blood samples were collected by venipuncture at admission for routine examination. Hypertension was defined as DBP $\geq$ 90 mmHg and/or SBP $\geq$ 140 mmHg\(^1\) or on the basis of use of anti-hypertensive medication. For this study, elevated cholesterol was defined as total cholesterol $>$ 5.0 mmol/L and LDL $>$ 3 mmol/L.\(^2,3,4\) History of hyperlipidemia was defined as a documented past diagnosis of hyperlipidemia or reported use of lipid-lowering drugs. None of the patients died during the procedure. The experimental protocol was approved by the local Ethical Committee, research was carried out in accordance with the institute’s guidelines, and all patients gave their informed consent for excision of STA tissue. Antiplatelet therapy was ceased in all patients 5 days prior to surgical intervention.\(^5\)

Vessels were placed in cold (4°C) Krebs solution, and immediately transported to the laboratory. Each vessel was divided into rings for use in different experimental series. Some rings were treated with resveratrol at 37°C and then proteins rapidly extracted for molecular studies; other rings were kept in 4°C Krebs solution for vascular reactivity studies; some were used for HPLC dosage of BH4; and others were rapidly used for O$_2^-$ measurement. All reagents were purchased from Sigma. Antibodies were: anti-p-eNOS Ser$^{1177}$, anti-p-eNOS Thr$^{495}$, and anti-total eNOS (purchased from Cell Signaling Technology, UK), anti-NRF2, anti-Keap1, anti-$\beta$-tubulin, and anti-HDAC2 (purchased from Millipore, I), anti-MnSOD, anti-Cu-ZnSOD and anti GCH1 (purchased from Santa Cruz Biotechnology).

Evaluation of Vascular Reactivity
Vascular reactivity was evaluated in STA as previously described.\(^6\) All vessels were passively stretched to a resting tension of 4g, which is the optimal tension found to generate maximal isometric contractions.\(^7\) Precontraction was elicited with phenylephrine; the concentration (ranging from $10^{-9}$ to $10^{-6}$ mol/L) was adjusted to obtain identical preconstriction levels corresponding to $\sim$80% of the initial KCl constriction. Endothelial and smooth muscle function was tested by increasing concentrations of acetylcholine ($10^{-9}$ to $10^{-5}$ mol/L) and nitroglycerin ($10^{-9}$ to $10^{-6}$ mol/L), respectively.\(^6\)

First experimental series: Increasing doses of resveratrol (12.5–100 $\mu$mol/L) were tested on STA rings preconstricted with phenylephrine. Resveratrol’s vascular activity was also tested in the presence of the nitric oxide synthase inhibitor L-NAME (300 $\mu$mol/L), the Cox1/Cox2 inhibitor indomethacin (10 $\mu$mol/L), the AMPK inhibitor Compound C (40 $\mu$mol/L), and the PI3K inhibitor LY294002 (10 $\mu$mol/L). In some vessels, acetylcholine-evoked vasorelaxation was tested subsequently to exposure to exogenous BH$_4$ ($10^{-6}$ mol/L, 30 min).

Second experimental series: STA rings were incubated for six hours with resveratrol (50 $\mu$mol/L) or BH$_4$ ($10^{-6}$ mol/L) and dose–response curves to acetylcholine ($10^{-9}$ to $10^{-5}$ mol/L) and nitroglycerin ($10^{-9}$ to $10^{-5}$ mol/L) were then obtained. Some rings were preincubated with retinoic acid (15 $\mu$mol/L) and resveratrol (50 $\mu$mol/L) for six hours and then the response to acetylcholine evaluated.

Endothelial cells isolated from STA
Hanks’ balanced salt solution (HBSS), Dulbecco’s phosphate-buffered saline (DPBS), fetal calf serum (FCS), trypsin-EDTA solution, dispase II, fibronectin, MCDB131 medium, Trypan blue solution, and penicillin/streptomycin solution were acquired from Invitrogen; Endothelial Cell Growth Supplement (ECGS) and heparin (100 mg/ml) were acquired respectively from Biomedical...
Technologies and Sigma. A segment of STA was collected in a disposable container, clamped at both ends, severed, and stored at 4°C for a maximum of 24 h in sterile HBSS containing 100-unit/mL penicillin and 100 µg/mL streptomycin. To remove the blood remaining inside the vessels, the arteries were held vertically and perfused from one end with approximately 20–40 mL DPBS kept on ice, using a 20-mL syringe connected to the needle. To detach the endothelial cells, a 10-mL syringe was connected to the needle and the blood vessels perfused with 5 mL of a 1:1 (v:v) mixture, freshly prepared and kept on ice, of dispase II (2.4 unit/mL in DPBS, filtered sterile, and stored frozen at -20°C until use) and incubated for 30 min at 37°C. Subsequently, the dispase II solution was discarded and replaced with medium MCDB131 (with 8% FCS, 10mM L-glutamine, 50U/mL penicillin and 50 µg/mL streptomycin, 1ng/mL ECGS, and 0.4% heparin). The vessels were carefully massaged and the medium plus cells collected in a tube and centrifuged for 5 minutes at 100g. The resuspended pellet in culture medium was seeded on a fibronectin-coated (2.5 µg/cm2) 25 cm² cell culture flask and incubated at 37°C in an H2O-saturated 5% CO2/95% air atmosphere. The medium was changed after 24 hours to remove dead cells. After 2–4 days, the human endothelial cells formed a confluent monolayer. At this point, protein extraction was carried out for immunoblotting.

**Immunoblotting**

Immunoblots were performed as described elsewhere. Briefly, 50 µg tissue extract for each sample was separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Blocked membranes were incubated with primary antibodies in TBS-Tween and 5% milk (or 5% BSA for phospho-specific Abs) overnight. Blocked membranes were then incubated with anti-p-eNOS Ser1177 (1:1000), anti-p-eNOS Thr495 (1:1000), anti-total eNOS (1:1000), anti-CD31 (1:1000); anti-p-AMPK (1:800), anti-total AMPK (1:1000), anti-Mn-SOD (1:1500), anti-Cu-ZnSOD (1:1000), or anti-GCH1 (1:500).

Nuclear and cytoplasmic fractions, obtained as described elsewhere, were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blocked membranes were incubated with anti-NRF2 (1:2000), anti-Keap1 (1:1000), anti-β-tubulin (1:2000), and anti-HDAC2 (1:2000) overnight and then detected using appropriate horseradish peroxidise-coupled secondary antibody (Millipore) and visualized with enhanced chemiluminescence (Amersham). The purity of nuclear and cytoplasmic fractions was confirmed using anti-HDAC-2 and anti-β-tubulin, respectively. Immunoblotting data were analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, USA) to determine optical density (OD) of the bands. The OD reading was normalized to account for variations in loading.

**Detection of eNOS dimer and monomer**

Low-temperature SDS-PAGE (LT-PAGE) was performed for detection of SDS-resistant eNOS dimer and monomer, as described previously.

**High-performance liquid chromatography**

BH₄, BH₂, and biopterin levels in plasma or vessel tissue lysates were each determined separately from the same sample by high-performance liquid chromatography followed by serial electrochemical and fluorescent detection, as previously described. Levels of BH₄ are expressed as pmol/mg proteins.

**Evaluation of oxidative stress in vessels**

Vascular superoxide production was measured in paired segments of STA using lucigenin-enhanced chemiluminescence, as previously described. Vessels were opened longitudinally to expose the endothelial surface and equilibrated for 20 min in oxygenated (95% O₂/5% CO₂) Krebs-4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid buffer (pH = 7.4) at 37°C. Lucigenin-enhanced
chemiluminescence was measured using low-concentration lucigenin (5 μmol/l) because higher concentrations of lucigenin (up to 250 μmol/l) favor redox cycling.12

**Statistical Analysis**
Data are presented as mean±SEM. Statistical analysis was performed by 2-way ANOVA followed by Bonferroni post hoc test. Differences were considered to be statistically significant at P<0.05.

**References**


Figure S1. Effect of resveratrol in vessels from hypertensive and dyslipidemic patients not treated with statins

(A) Dose–response curves of STA from hypertensive and dyslipidemic (HD) patients treated (n=4) or not (n=5) with statins. STA were exposed to acetylcholine alone or co-exposed for six hours to Ach plus resveratrol (50 μmol/L, n=4). *P<0.005 vs Ach alone.

(B) Representative immunoblot for eNOS phosphorylated on Ser1177 and for total eNOS in STA from HD patients taking or not statins (Untr.). STA were exposed to acetylcholine alone (10^{-6} mol/L) or to acetylcholine plus resveratrol (50 μmol/L) for six hours. Columns are the means±SD of four independent experiments. *, P<0.005 vs Untr.; #, P<0.05 vs Ach.
Figure S2. Lack of resveratrol efficacy on acetylcholine evoked-vasorelaxation in vessels from non-hypertensive non-dyslipidemic patients.

(A) Dose–response curves of STA from hypertensive and dyslipidemic (HD) and non-hypertensive non-dyslipidemic (nHD) patients. STA were exposed to acetylcholine alone (n=5) or co-exposed for six hours to resveratrol (50 µmol/L, n=4). *, P<0.005 vs Ach.

(B) Representative immunoblot for eNOS phosphorylated on Ser1177 and Thr495 and for total eNOS in endothelial cells isolated from vessels of HD and nHD patients. Cells were not treated (Untr.), exposed to acetylcholine (10^{-6} mol/L), or exposed to resveratrol (50 µmol/L) for 30 minutes. Columns are the means±SD of three independent experiments. *, P< 0.01 vs Untr.; #, P< 0.05 vs Ach or Untr.

(C) O₂⁻ assessed by lucigenin-enhanced chemiluminescence in STA from non-hypertensive non-dyslipidemic (nHD, n=5) and from hypertensive and dyslipidemic (HD) patients. STA were exposed either to resveratrol (50 µmol/L, n=4) for six hours. *, P<0.05 vs all.
Figure S3. Phosphorylation of eNOS in HD STA

(A) Representative immunoblot for eNOS phosphorylation on Ser^{1177} and Thr^{495} and for total eNOS in STA that were not treated (Untr.), exposed to acetylcholine alone (10^{-6} mol/L), exposed to acetylcholine plus tetrahydrobiopterin (10^{-6} mol/L), or exposed to acetylcholine plus resveratrol (50 \mu mol/L) for six hours. Columns are the means±SD of five independent experiments. *, P<0.005 vs Untr; #, P<0.05 vs Ach.; §, P<0.05 vs Ach. plus BH4 (Upper graph). *, P<0.05 vs Untr; †, P<0.05 vs Ach (Bottom graph).
Table S1. Demographic Characteristics of the non-hypertensive non-dyslipidemic patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age y</td>
<td>54±6</td>
</tr>
<tr>
<td>Hypercholesterolemia (n)</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>0</td>
</tr>
<tr>
<td>Cigarette smoking (n)</td>
<td>5</td>
</tr>
<tr>
<td>Triglycerides mmol/L</td>
<td>1.45±0.10</td>
</tr>
<tr>
<td>Total cholesterol mmol/L</td>
<td>4.923±0.16</td>
</tr>
<tr>
<td>LDL-cholesterol mmol/L</td>
<td>2.822±0.09</td>
</tr>
<tr>
<td>HDL-cholesterol mmol/L</td>
<td>1.27±0.13</td>
</tr>
<tr>
<td>Glucose mmol/L</td>
<td>4.75±0.4</td>
</tr>
</tbody>
</table>

Values are unadjusted means ± standard deviation; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Total number of patients = 8, all males.