Red Wine Polyphenols Enhance Endothelial Nitric Oxide Synthase Expression and Subsequent Nitric Oxide Release From Endothelial Cells

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Background—Population-based studies suggest a reduced incidence of morbidity and mortality from coronary heart disease caused by moderate and regular consumption of red wine. Endothelial nitric oxide (NO) is a pivotal vasoprotective molecule. This study examines the influence of red wine polyphenols on the regulation of endothelial nitric oxide synthase (eNOS) expression and subsequent NO synthesis, focusing on the putative long-lasting antiatherosclerotic effects of red wine.

Methods and Results—Treatment (20 hours) of human umbilical vein endothelial cells (HUVECs) and of the HUVEC-derived cell line EA.hy926 with an alcohol-free red wine polyphenol extract (RWPE) led to a concentration-dependent (100 to 600 μg/mL), significant increase in NO release (up to 3.0-fold/HUVEC and 2.0-fold/EA.hy926) as shown by use of the fluorescent probe DAF-2. This effect was corroborated by the [14C]-L-arginine/L-citrulline conversion assay in intact EA.hy926 cells. RWPE (20 hours, 100 to 600 μg/mL) also significantly increased eNOS protein levels up to 2.1-fold. Furthermore, we found an increased human eNOS promotor activity (up to 2-fold) in response to red wine polyphenols (18 hours, 100 to 600 μg/mL), as demonstrated by a luciferase reporter gene assay.

Conclusion—We provide conclusive data showing for the first time that a RWPE increases eNOS expression and subsequent endothelial NO release. Increased active eNOS levels may antagonize the development of endothelial dysfunction and atherosclerosis, a hypothesis that supports the view that red wine indeed may have long-term protective cardiovascular properties mediated by its polyphenols. (Circulation. 2002;106:1614-1617.)

Key Words: cardiovascular diseases ■ nitric oxide synthase ■ nutrition ■ pharmacology

A significantly reduced incidence of coronary heart disease despite a high-fat diet, little exercise, and widespread cigarette smoking in certain areas of France has led to the concept of the “French paradox.” This phenomenon was attributed to a higher intake of alcohol and in particular of red wine in France. Although several observations support a protective effect of red wine, there is no clear-cut evidence showing whether red wine is more beneficial than other forms of alcohol. Identification of unique compounds and properties of red wine that are not attributable to alcohol may help to clarify this issue.

Nitric oxide (NO) released from endothelial cells via the endothelial nitric oxide synthase (eNOS) is a pivotal vasoprotective molecule. In addition to its vasodilating feature, endothelial NO has antiatherosclerotic properties, such as inhibition of platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of genes involved in atherogenesis. Thus, eNOS is a significant target in cardiovascular pharmacology. The eNOS enzyme is regulated post-translationally but can also be influenced on the transcriptional level. Some studies suggest a short-term activation of eNOS by red wine. No studies exist that examine the influence of red wine on the regulation of eNOS expression and thus on putative long-lasting antiatherosclerotic effects of red wine, however. Here we show for the first time that an alcohol-free red wine polyphenol extract (RWPE) strongly increases NO release, eNOS activity, and eNOS expression after long-term incubation (20 hours) of human endothelial cells with RWPE.

Methods

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to the method of Sohn et al. The human endothelial cell line EA.hy926 (provided by Dr C.-J.S. Edgell, University of North Carolina, Chapel Hill, NC) were cultivated as described. EA.hy926 stably transfected with a plasmid containing...
3600 bp of the human eNOS promoter controlling a luciferase reporter gene were used for measuring eNOS promoter activity.

Preparation of the Red Wine Polyphenol Extract
RWPE was prepared as described by Caderni et al from a cabernet sauvignon red wine made in southern France. About 3 g RWPE were obtained from 1 L red wine. Its phenolic composition was analyzed as previously described. RWPE was dissolved in PBS/H2O.

Quantification of NO Release by DAF-2
Quantification of NO released from HUVECs or EA.hy926 cells was performed by the 4,5-diaminofluorescein (Alexis Biochemicals, Grünberg, Germany) fluorescence assay as described.

$^{[14C]}\text{L-Arginine}/[14C] \text{L-Citrulline Conversion Assay}$
EA.hy926 cells were stimulated for 20 hours. Cells were washed with PBS and kept in a HEPES buffer (45 minutes) before addition of 0.32 μmol/L $^{[14C]}\text{L-arginine}$ (313 mCi/mmol) and 1 μmol/L A23187. The NO inhibitor NG-amino-L-arginine (NAA, 200 μmol/L) was added 30 minutes after addition of HEPES buffer and incubated for 15 minutes. Cells were then washed and exposed to $^{[14C]}\text{L-arginine}$ and A23187. After incubation for 25 minutes at 37°C, the reaction was stopped by lysing the cells with ice-cold ethanol (96%). Lysed cells were extracted with water (2 mL, agitation) and water-supernatants were dried under vacuum. The extract was resolved in water/methanol (1:1) and spotted on a thin layer chromatography plate. $^{[14C]}\text{L-citrulline}$ was separated from $^{[14C]}\text{L-arginine}$ using the solvent system water/chloroform/methanol/ammonium hydroxide (1/0.5/4.5/v/v/v/v). The thin layer chromatography plates were dried and analyzed by a phosphoimager (FUJIFILM BAS-1500).

Western Blot Analysis
EA.hy926 cells were stimulated with RWPE or phorbol-12-myristate-13-acetate (PMA) for 20 hours as a positive control. Protein isolation and western blotting were performed essentially as described by Li et al eNOS protein was detected by a monoclonal mouse anti-eNOS antibody (1:2500, BD Biosciences, Heidelberg, Germany, clone 3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by a monoclonal anti-GAPDH antibody (1:1200, Chemicon, Holheim, Germany, MAB374) and visualized on a Kodak Image station 440CF using the chemiluminescence reagent Renaissance Plus (both NEN Life Science).

Reporter Gene Assay
EA.hy926 cells stably transfected with a plasmid containing 3600 bp of the human eNOS promoter driving a luciferase gene were stimulated as indicated for 18 hours. PMA (2 nmol/L) served as positive control. Cells were washed and lysed and the assay performed according to the manufacturer’s instructions (Promega) with the use of a luminometer (AutoLumatPlus, Berthold).

Statistical Analysis
One-way ANOVA with Dunnett’s post test was performed with GraphPad Prism version 3.00 (GraphPad Software).

Results
Exposure of EA.hy926 cells and HUVECs to RWPE for 20 hours significantly augmented NO release from endothelial cells in a concentration-dependent manner (100 to 600 μg/mL) up to 2.0- and 3.0-fold, respectively (Figure 1A and 1B). Accordingly, l-citrulline production in EA.hy926 cells was significantly increased (up to 2.6-fold) in response to RWPE (Figure 1C). In contrast, several red wine polyphenols, including resveratrol, delphinidin, and quercetin, applied at 1 and 10 μmol/L had no significant effect on l-citrulline production (Figure 1D).

Figure 1. RWPE increases NO production (A and B) and l-citrulline production in human endothelial cells (C). Selected red wine polyphenols have no effect on l-citrulline production in EA.hy926 cells (D). Cells were either kept untreated (Co) or stimulated with increasing concentrations (100 to 600 μg/mL) of RWPE and several red wine compounds (1 and 10 μmol/L) for 20 hours. Where indicated, cells were treated with the NOS inhibitor NAA (200 μmol/L). A and B, NO was measured with the fluorescence probe DAF-2 in EA.hy926 (A) and in HUVECs (B). C and D, l-citrulline production was determined by the $^{[14C]}\text{L-arginine}/[14C] \text{L-citrulline}$ conversion assay. Graphs show the mean signal intensities obtained from 3 independent experiments by phospho-imaging analysis of $^{[14C]}\text{L-citrulline}$. C, a representative autoradiograph is shown. All data are mean±SD (n=3, in triplicate). **P<0.001 (ANOVA/Dunnett).
Consistent with an increased NO release after long-term stimulation, we detected increased eNOS protein levels. Protein levels rose in a concentration-dependent manner (100 to 600 μg/mL) up to 2.1-fold after treatment with 600 μg/mL RWPE (Figure 2A).

In order to determine whether the increased eNOS protein level is possibly due to an enhanced transcription rate of the eNOS gene we measured the eNOS promotor activity by an eNOS luciferase reporter gene assay. As depicted in Figure 2B, RWPE treatment concentration-dependently led to a significantly enhanced eNOS promotor activity. The promoter activity increased up to 2-fold after treatment with 600 μg/mL RWPE. From the red wine polyphenols, only resveratrol increased eNOS promotor activity significantly (Figure 2C).

Discussion
The presented data show that red wine polyphenols significantly enhance eNOS expression and subsequent NO release from endothelial cells.

In contrast to previous studies that describe acute NO-related effects in aortas, we detected increased NO levels and eNOS protein after long-term exposure (18 to 20 hours) of endothelial cells to RWPE. As reviewed by Stoclet, previously released data suggest that red wine polyphenols induce Ca^{2+} entry in endothelial cells, which leads to an acute eNOS enzyme activation and subsequent vasodilatation. Here we show that RWPE is also able to induce eNOS transcription and thus to increase eNOS protein levels and NO output from endothelial cells. This may lead to a more constant and long-lasting effect compared with short-term Ca^{2+} mobilization. Reduced bioavailability of NO is thought to contribute considerably to the development of vascular diseases like atherosclerosis. Thus, the observed long-term eNOS-inducing effects in response to RWPE indeed support a role of red wine in the prevention of cardiovascular disease. This is in concordance with very recent in vivo data.

Of high interest is the identification of the polyphenolic constituents that may be responsible for the observed RWPE effect. Red wine polyphenols encompass anthocyanins, proanthocyanidins, monomeric flavonols, flavonoids, and phenolic acids, as well as stilbene derivatives. Of these, resveratrol15 (stilbene), delphinidin 16 (anthocyanidin), and quercetin 17 (flavonol) are suggested to mediate red wine effects. With the exception of resveratrol, none of these compounds (1 to 10 μmol/L) showed any influence on eNOS. The resveratrol content in the RWPE we used, however, was extremely low (0.57 mg/g). Thus, a dosis of 400 μg/mL RWPE contained <1 μmol/L resveratrol, a concentration found to be effective on eNOS promotor activity but not on eNOS-L-citrulline production. This indicates that resveratrol is unlikely to be the sole effective constituent in RWPE. Further studies should focus on the identification of polyphenolic compounds or fractions mediating a stimulatory effect on eNOS.

Because of the complex composition of red wine polyphenols, reliable data about the plasma polyphenol levels are scarce. Using the Folin-Ciocalteau method, Nigdikar et al18 found an increase in plasma polyphenols of 6.4±3.0 mg/g protein after subjects had consumed 375 mL red wine/d for 2 weeks. This amount corresponds to a plasma polyphenol concentration of ~450 μg/mL, which was found to be effective in our in vitro study. More work is needed, however, to determine the bioavailability and pharmacokinetics of
polyphenols and to identify metabolites of red wine components that may mediate red wine activity.

In summary, the presented data support the idea that red wine contains unique polyphenolic constituents that may augment eNOS expression and thus endothelial NO output. Increased active eNOS levels may antagonize the development of endothelial dysfunction and subsequent atherosclerosis. The identification of the responsible constituents should help in the design of strategies to prevent atherosclerosis.

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