

## Wine, Nicotine, and Cardiovascular Disease

# Red Wine Increases the Expression of Human Endothelial Nitric Oxide Synthase

## A Mechanism That May Contribute to its Beneficial Cardiovascular Effects

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<b>OBJECTIVES</b>	The study tested the effect of red wine on endothelial-type nitric oxide synthase (eNOS) expression and eNOS activity in human endothelial cells.
<b>BACKGROUND</b>	Endothelial-type nitric oxide (NO) synthase exerts vasoprotective effects. Moderate alcohol consumption has been associated with a reduction of cardiovascular disease, and red wine seems to offer more benefits than any other type of drink. However, the molecular basis of this protective effect is unclear.
<b>METHODS</b>	Human endothelial cells were treated with red wine, and eNOS messenger ribonucleic acid (mRNA) expression was measured by RNase protection assay, eNOS protein expression by Western blotting, and eNOS activity by RFL-6 reporter cell assay. The eNOS promoter activity was analyzed in transfected endothelial cells; binding activities of relevant transcription factors were determined by electrophoretic mobility shift assay.
<b>RESULTS</b>	Incubation of endothelial cells with red wines from France upregulated eNOS mRNA and protein expression. In contrast, red wines from Germany showed little or no effect on eNOS expression. No significant difference in eNOS mRNA expression could be detected between “en barrique” (matured in oak barrels) and “non-barrique” (matured in steel tanks)–produced French red wines. Endothelial cells treated with French red wines produced up to three times more bioactive NO than did control cells. French red wines increased the activity of the eNOS promoter, with the essential trans-stimulated sequence being located in the proximal 326 bp of the promoter sequence. The eNOS mRNA stability was also increased by red wine.
<b>CONCLUSIONS</b>	The increase in eNOS expression and activity brought about by red wines from France (and probably other locations) may contribute to the beneficial effects of this beverage on the cardiovascular system. (J Am Coll Cardiol 2003;41:471–8) © 2003 by the American College of Cardiology Foundation

Epidemiological evidence from various disparate populations has consistently correlated daily moderate wine consumption with lower mortality from cardiovascular and cerebrovascular disease and other causes (1–4). Moderate ethanol intake from any type of beverage improves lipoprotein metabolism and lowers cardiovascular mortality risk (5,6), but wine, particularly red wine with its abundant content of phenolic acids, polyphenols, and flavonoids,

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seems to confer additional health benefits. These include an increase in high-density lipoprotein (HDL) cholesterol levels and decreased oxidation of low-density lipoprotein (LDL) cholesterol, antioxidant activity, decreased platelet aggregation and adhesion, as well as improved endothelium-dependent vasodilation (1,4,7,8). Many of these effects are compatible with the action of endothelium-

derived nitric oxide (NO), implying that NO may be a mediator of the cardiovascular protection provided by red wine. Indeed, some studies have shown that short-term exposure of blood vessels to red wine can promote NO generation (9,10). Although this effect of red wine is caused by an acute activation of endothelial-type nitric oxide synthase (eNOS) through yet unknown mechanisms, we hypothesized that the beneficial effects of long-term wine consumption could involve an upregulation of eNOS expression. This would lead to moderate, but sustained, elevations of vascular NO. The current study demonstrates that French red wines, indeed, upregulate eNOS expression and activity and that this involves transcriptional and posttranscriptional mechanisms.

### METHODS

**Wines.** Wines from different areas were investigated for their effects on eNOS expression. German red wines: “Dornfelder 1997” (area: Mosel, grape: Dornfelder), “Spätburgunder 1999” (Palatine, Spätburgunder), “Kriegsheimer Rosengarten 1997” (Rhine-Hessen, Portugieser/Dornfelder), and French red wines: “Chateau Bonnet 1997”, produced “non-barrique”; “Chateau Bonnet reserve

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Manuscript received May 29, 2002; revised manuscript received August 2, 2002, accepted October 10, 2002.

**Abbreviations and Acronyms**

eNOS	= endothelial-type nitric oxide synthase
GAPDH	= glyceraldehyde-3-phosphate dehydrogenase
HUVEC	= human umbilical vein endothelial cells
mRNA	= messenger ribonucleic acid
NO	= nitric oxide

1997", produced "en barrique"; "Chateau Bellegrave 1997", and "Chateau de la Marechaude 1997" (Bordeaux; Cabernet Sauvignon/Cabernet Franc/Merlot); "Chateau Cabieres 1998" (Rhône; Grenache/Syrah/Mourvedre/Cinsault); "Chateau Rousseau 1998" and "Les Chevaliers de la Reine 1998" (L. Chevaliers; Burgundy; Pinot Noir). All wines used contained 11.5% to 12.5% ethanol, v/v; therefore, an ethanol solution of 12.5%, v/v, in phosphate-buffered saline (PBS) was used as the appropriate control.

**Cell culture.** Both human umbilical vein endothelial cells (HUVECs) and HUVEC-derived EA.hy 926 cells were grown in Dulbecco's modified Eagle's medium as described previously (11,12). Confluent endothelial cells were incubated for the periods of time indicated with the different wines or an ethanol solution as control. Red wines and ethanol solution were diluted in cell culture medium to final concentrations of 1%, 3%, or 10% (v/v), respectively. Then, either RNA or protein was extracted or eNOS activity of intact cells was determined.

**RNase protection assay of eNOS mRNA.** The eNOS messenger ribonucleic acid (mRNA) was measured by RNase protection assay as previously described (11,12). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined in parallel for normalization. The GAPDH probe was generated according to a previously described procedure (11,12) using total RNA from human A673 rhabdomyosarcoma cells and oligonucleotide primers for GAPDH corresponding to positions 501-524 (sense) and 582-605 (antisense) of the human GAPDH cDNA (GenBank accession number: NM\_002046). Resulting cDNA fragments (105 bp) were cloned into the pXcm I-TA-cloning vector (kindly provided by Dr. A. Borovkov [13]). The protected RNA fragments of eNOS and GAPDH were 280 nt and 108 nt, respectively.

**Preparation of eNOS protein and Western blotting.** For the determination of eNOS protein expression, total protein was isolated and Western blots were performed as previously described (11,12).

**Determination of eNOS activity.** Bioactive NO produced by eNOS in EA.hy 926 cells was assayed using the stimulation of soluble guanylyl cyclase in RFL-6 rat lung fibroblasts as previously described (11,12). Results of the RFL-6 assay were verified by measuring  $\text{NO}_2^-/\text{NO}_3^-$  formation as previously described (11). The EA.hy 926 cells were treated as indicated in the Results section, and  $\text{NO}_2^-/\text{NO}_3^-$  levels were measured using a NOA 280 Nitric Oxide Analyzer.

**Reporter gene assay using the 5'-flanking region of the human eNOS gene stably transfected into EA.hy 926 cells.** The EA.hy 926 cells stably transfected with the plasmid p-eNOS-Hu-3500-Luc-neo have been described previously (11,12). Extracts from treated cells were prepared using passive lysis buffer (Promega), and luciferase activities were determined using the luciferase assay system as described (11,12).

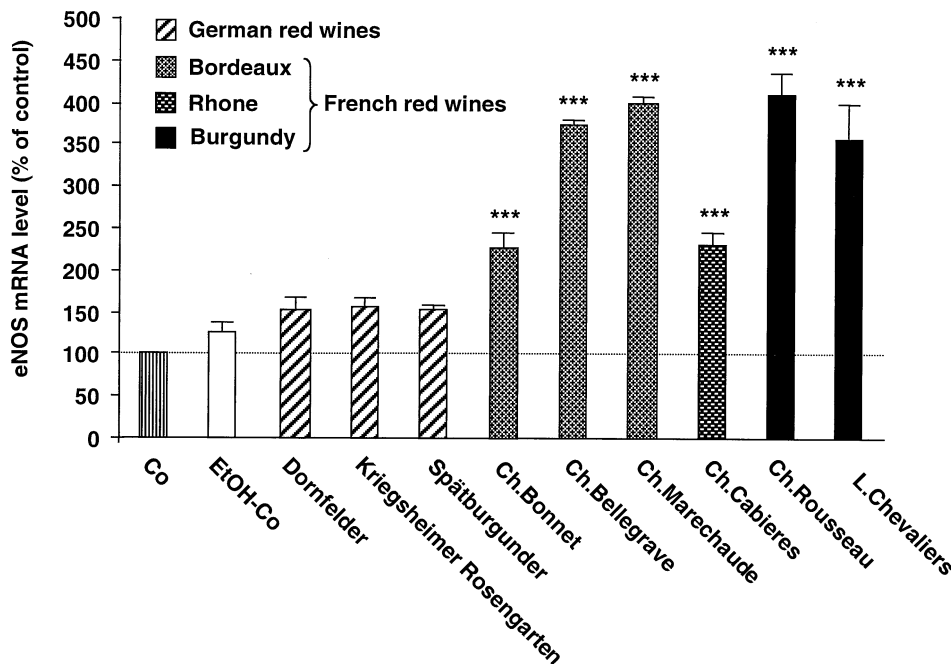
**Transient transfection of EA.hy 926 cells and reporter gene assays.** The EA.hy 926 cells were transfected with luciferase constructs containing human eNOS promoter fragments of various lengths (0.9  $\mu\text{g}$  of p-eNOS-1600-Hu-Luc, p-eNOS-1111-Hu-Luc, p-eNOS-633-Hu-Luc or p-eNOS-326-Hu-Luc) using SuperFect (Qiagen). The plasmid pRL-SV40 (containing the renilla-luciferase gene driven by an SV40 promoter) was co-transfected for normalization. The luciferase- and renilla-luciferase-activities of the extracts were determined using the Dual-Luciferase System (Promega).

**Electrophoretic mobility shift assays.** Binding activities of transcription factors GATA, Sp1/Sp3, PEA3, YY1, and Elf-1 in nuclear extracts from ethanol- or red-wine-treated cells were determined by electrophoretic mobility shift assays as described (11,14). Double-stranded oligonucleotides containing the following transcription factor binding motifs of the human eNOS promoter were used: Sp1-binding motif (positions -95 to -104), Sp1/Sp3-like-binding motif (positions -141 to -146), GATA-binding motif (positions -225 to -230), PEA3-binding motif (positions -24 to -40), YY1-binding motif (positions -117 to -121) or the Elf-1-binding motif (positions -126 to -129). The DNA-protein complexes were analyzed on polyacrylamide gels. The dried gels were autoradiographed on X-ray film.

**Statistics.** Statistical differences between mean values were determined by analysis of variance (ANOVA) followed by the Fisher PLSD test for comparison of means, or by the Student *t* test for unpaired data (as indicated in the figure legends).

## RESULTS

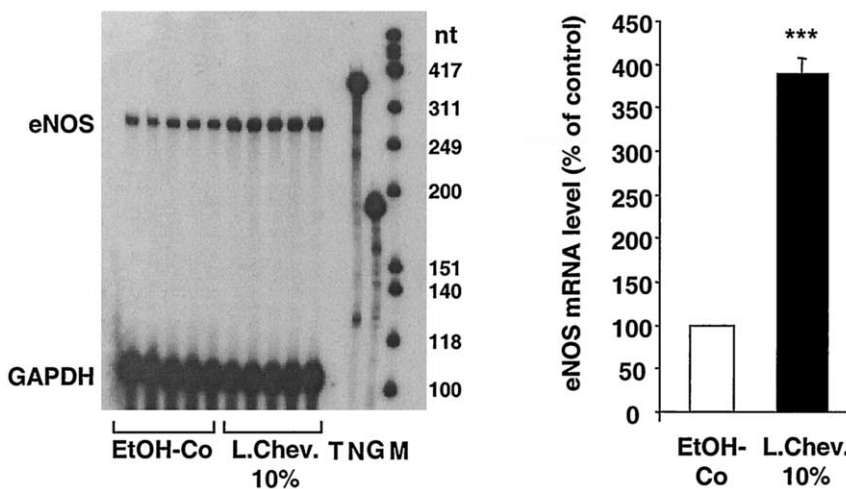
**Upregulation of eNOS mRNA expression by different red wines in human endothelial cells.** Human EA.hy 926 endothelial cells were incubated for 24 h with three different German red wines ("Dornfelder", "Kriegsheimer Rosengarten", and "Spätburgunder"), or six different French red wines ("Ch.Bonnet reserve", "Ch.Bellegrave", "Ch.Marechaude", "Ch.Cabieres", "Ch.Rousseau", and "L.Chevaliers"). The red wines were diluted in the cell culture medium to a final concentration of 10% (v/v). Cells receiving no treatment, or cells exposed to ethanol 1.25% (v/v) in the cell culture medium, served as controls. Cells treated with the different German red wines showed only a moderate increase in eNOS mRNA expression, whereas cells exposed to the French red wines showed a significant



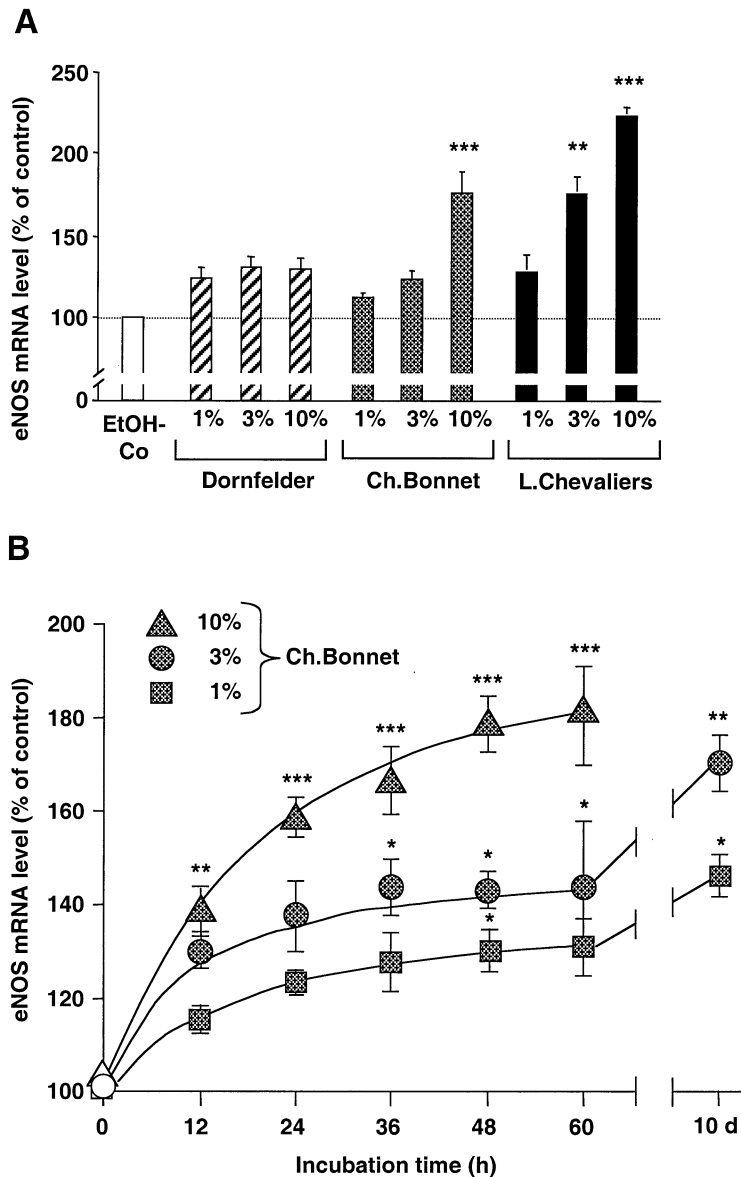
**Figure 1.** Effect of red wines on endothelial-type nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) expression in human endothelial EA.hy 926 cells. Cells were left untreated (control, Co) or exposed for 24 h to ethanol (EtOH-Co, 1.25%, v/v), three different German red wines (“Dornfelder”, “Kriegsheimer Rosengarten”, and “Spätburgunder”) or six different French red wines from Bordeaux (“Ch.Bonnet reserve”, “Ch.Bellegrave”, and “Ch.Marechaude”), Rhone (“Ch.Cabieres”), and Burgundy (“Ch.Rousseau”, “L.Chevaliers”). The red wines were diluted in the cell culture medium to a final concentration of 10% (v/v). RNase protection analyses were performed using antisense RNA probes to human eNOS and GAPDH (for standardization). The **figure** shows densitometric analyses of three different gels. **Bars** represent means  $\pm$  SEM. **Asterisks** indicate significant differences from untreated cells (\*\**p* < 0.001; by ANOVA followed by the Fisher PLSD test).

enhancement of eNOS mRNA (up to fourfold, Fig. 1). The effect of French red wine on eNOS mRNA expression was confirmed with primary HUVECs (Fig. 2). Incubation of these cells with the French red wine “L.Chevaliers” led to a similar increase in eNOS mRNA expression as seen in

EA.hy 926 cells. To test whether the upregulation of eNOS by French red wines was influenced by the way the wine is produced, we tested two different “Ch.Bonnet,” one matured in new oak barrels (“en barrique”) and one matured in steel tanks (“non-barrique”). In EA.hy 926 cells, no signif-



**Figure 2.** Upregulation of endothelial-type nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) by the French red wine “L.Chevaliers” in human umbilical vein endothelial cells (HUVEC). The eNOS mRNA expression was quantified with RNase protection assay. Ribonucleic acids were prepared from HUVEC cells incubated either for 24 h with ethanol (EtOH-Co, 1.25%, v/v) or with the red wine “L.Chevaliers” (L. Chev., 10% v/v). The **left panel** shows an autoradiograph of a representative gel of an RNase protection experiment. T: tRNA control; N: eNOS antisense probe alone; G: GAPDH antisense probe alone; M: molecular weight markers. The **right panel** shows densitometric analyses of three different gels. **Bars** represent means  $\pm$  SEM. **Asterisks** indicate significant differences from ethanol-treated cells (\*\**p* < 0.001; by the Student *t* test for unpaired data).

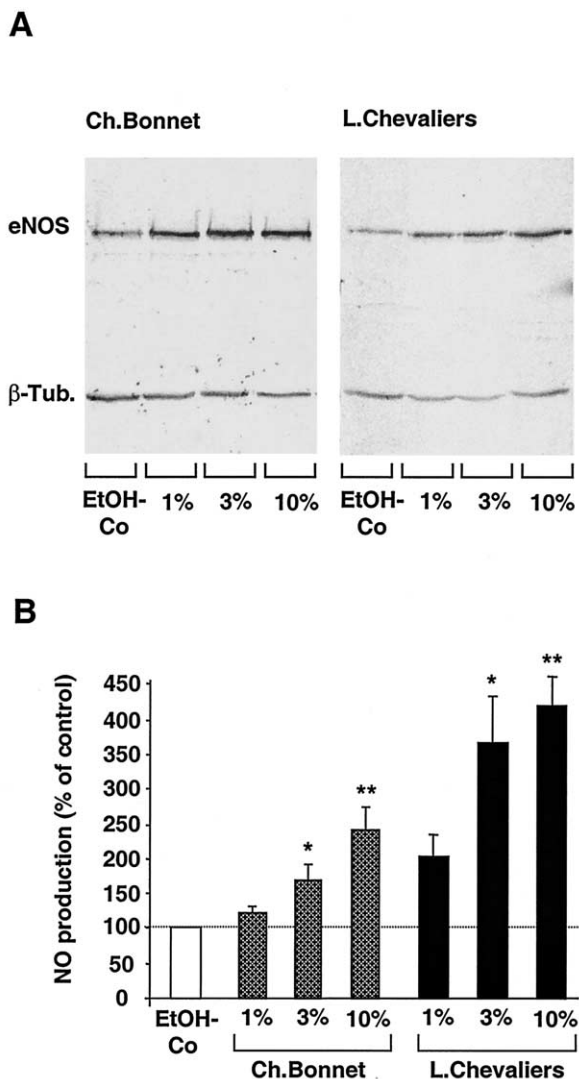


**Figure 3.** Concentration- and time-dependent upregulation of endothelial-type nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) expression by French red wines. The eNOS mRNA expression was quantified with RNase protection assay. **(A)** EA.hy 926 cells were incubated for 24 h with ethanol (EtOH-Co, 1.25%, v/v), a German (“Dornfelder”) and two French red wines (“L.Chevaliers” and “Ch.Bonnet reserve”) diluted to final concentrations of 1%, 3%, and 10% (v/v). **(B)** Cells were exposed to cell culture medium containing 1%, 3%, and 10% (v/v) of “Ch.Bonnet reserve” for different periods of time. Symbols represent means  $\pm$  SEM. Asterisks indicate significant differences from ethanol-treated cells at the same time points (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; by ANOVA followed by the Fisher PLSD test).

icant difference in the upregulation of eNOS mRNA expression could be detected. “Ch.Bonnet reserve” produced “en barrique” (10%, v/v, 24 h) generated an upregulation to  $226\% \pm 17\%$  of the ethanol control and “Ch.Bonnet” produced “non-barrique” (10%, v/v, 24 h) upregulated eNOS expression to  $207\% \pm 13\%$  (means  $\pm$  SEM,  $n = 4$ , each).

**Concentration- and time-dependent upregulation of eNOS mRNA expression by French red wines in human EA.hy 926 endothelial cells.** The EA.hy 926 cells were incubated for 24 h with different concentrations (1%, 3%, and 10%, v/v) of red wine, or with 1.25% (v/v) ethanol as

the control. Then, total RNA was prepared. As shown in Figure 3A, the French red wines (“Ch.Bonnet reserve” and “L.Chevaliers”) increased eNOS mRNA expression in a concentration-dependent manner, whereas the German red wine (“Dornfelder”) showed only a small, nonsignificant enhancement of eNOS mRNA expression. Figure 3B demonstrates that the stimulation of eNOS expression by French red wine (“Ch.Bonnet reserve”) is a function of concentration and time; high concentrations (10%, v/v) produced significant increases already after 12 h; lower concentrations (1%, v/v) required up to 10 days of incubation to reach a similar degree of stimulation.



**Figure 4.** Effect of French red wines on endothelial-type nitric oxide synthase (eNOS) protein expression and eNOS activity in EA.hy 926 cells. (A) Western blot analyses using a polyclonal anti-eNOS- and a monoclonal anti- $\beta$ -tubulin-antibody (for normalization). Cells were incubated for 48 h with ethanol (EtOH-Co, 1.25%, v/v) or two different French red wines (“Ch.Bonnet reserve” and “L.Chevaliers”, 1%, 3%, and 10%, v/v). The blots represent four independent experiments each. (B) This panel shows the effect of a 48-h incubation with “L.Chevaliers” and “Ch.Bonnet reserve” on the nitric oxide (NO) production in EA.hy 926 cells stimulated for 2 min with the calcium ionophore A23187. Conditioned medium from EA.hy 926 cells was transferred to RFL-6 reporter cells and incubated for 2 min. Thereafter, cGMP content in RFL-6 cells was determined by radioimmunoassay. Basal cGMP content of the RFL-6 cells ( $1.7 \pm 0.16$  pmol/ $10^6$  cells) was subtracted from all samples. The NO production of ethanol-treated EA.hy 926 cells (EtOH-Co,  $13.1 \pm 0.19$  pmol cGMP/ $10^6$  RFL-6 cells) was set 100%. Data represent means  $\pm$  SEM of three independent experiments. Asterisks indicate significant differences from ethanol-treated cells (\* $p < 0.05$ ; \*\* $p < 0.01$ ; by ANOVA followed by the Fisher PLSD test).

**Concentration-dependent upregulation of eNOS protein and eNOS activity by French red wines in human EA.hy 926 endothelial cells.** As shown in Figure 4A, French red wines increased eNOS protein expression in EA.hy 926 cells in a concentration-dependent fashion. Densitometric analyses of the eNOS protein bands indicated an increase to

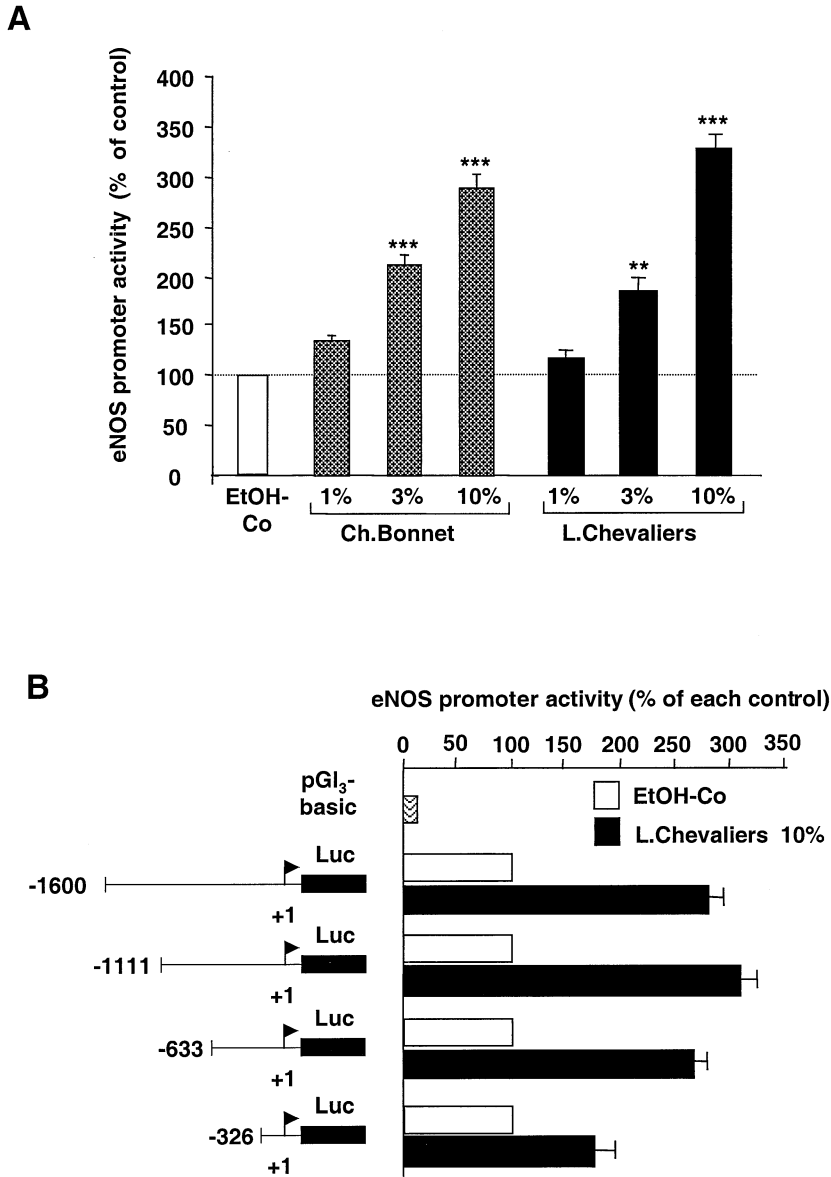
$140 \pm 15$ ,  $182 \pm 11$ , and  $210 \pm 17\%$  for “Ch.Bonnet reserve” and  $130 \pm 13$ ,  $160 \pm 14$ , and  $224 \pm 12\%$  for “L.Chevaliers” compared to ethanol-treated cells.

Also, NO production by EA.hy 926 cells (measured with RFL-6 reporter cell assay) was increased in a concentration-dependent manner after treatment with French red wines for 48 h (Fig. 4B). In additional experiments, eNOS activity was determined by measuring  $\text{NO}_2^-/\text{NO}_3^-$ . Ethanol-treated EA.hy 926 cells (1.25%, v/v, 48 h) produced  $3.7 \pm 1.0$  pmol  $\text{NO}_2^-/\mu\text{g}$  protein after a 10-min stimulation with calcium ionophore A23187 (10  $\mu\text{mol/liter}$ ). Incubation of EA.hy 926 cells with 1%, 3%, or 10% (v/v) “Ch.Bonnet reserve” for 48 h enhanced the calcium ionophore-stimulated  $\text{NO}_2^-$  production to  $4.3 \pm 0.2$ ,  $4.8 \pm 0.3$ , and  $6.3 \pm 0.5$  pmol/ $\mu\text{g}$  protein. Incubation with “L.Chevaliers” (1%, 3%, or 10%, v/v) increased the activity to  $6.6 \pm 1.0$ ,  $7.1 \pm 0.9$ , and  $10.0 \pm 1.3$  pmol/ $\mu\text{g}$  protein ( $n = 3$ , each).

**Concentration-dependent upregulation of eNOS promoter activity by French red wines in human EA.hy 926 endothelial cells.** To analyze the effect of French red wines on eNOS promoter activity, EA.hy 926 cells were stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo. When these cells were incubated for 24 h with the two French red wines “Ch.Bonnet reserve” and “L.Chevaliers” (1%, 3%, and 10%, v/v), the activity of the eNOS promoter was increased in a concentration-dependent manner. The increase reached 2.9-fold for “Ch.Bonnet reserve” and 3.3-fold for “L.Chevaliers” compared with ethanol-treated control cells (Fig. 5A).

In further experiments, EA.hy 926 cells were transiently transfected with different pGL<sub>3</sub>-Basic-derived constructs containing fragments of 1.6-kb to 0.33-kb of the human eNOS promoter cloned before a luciferase reporter gene. The 1.6-kb-eNOS-promoter-luciferase construct showed a significant (>7.5-fold) increase in (basal) promoter activity compared with pGL<sub>3</sub>-Basic. The nonstimulated activities of the shorter promoter fragments did not differ significantly from those of p-eNOS-1600-Hu-Luc: p-eNOS-1111-Hu-Luc had  $118\% \pm 21\%$  of p-eNOS-1600-Hu-Luc, p-eNOS-633-Hu-Luc  $78\% \pm 20\%$ , and p-eNOS-326-Hu-Luc  $143\% \pm 37\%$  ( $n = 3$ , each). The transfected cells were incubated for 24 h with either ethanol (1.25%, v/v) or the French wine “L.Chevaliers” (10%, v/v). The wine increased the activities of the 1.6-kb-, 1.1-kb-, and the 0.6-kb-promoter fragments more than 2.5-fold; the activity of the 0.3-kb-promoter fragment was still increased 1.7-fold (Fig. 5B).

Using electrophoretic mobility shift assay, we tested the effect of the red wines “Ch.Bonnet reserve” and “L.Chevaliers” on the binding activities of transcription factors known to be important for the activity of the human eNOS promoter. We incubated nuclear extracts from ethanol- or red-wine-treated cells with oligonucleotides containing transcription factor binding motifs of the human eNOS promoter for Sp1, Sp1/Sp3-like, GATA, PEA3, YY1, and Elf-1 as previously described (11,14). However, no wine-



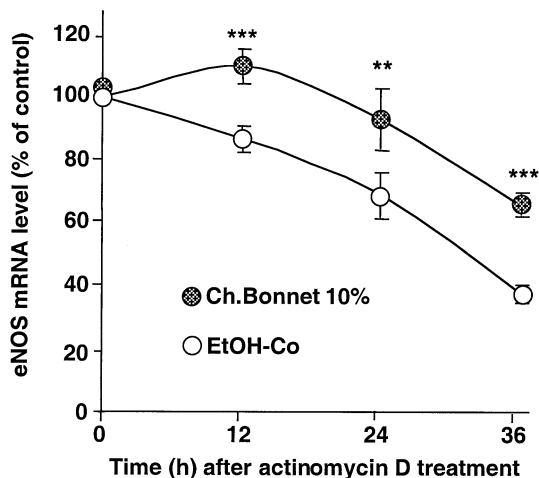
**Figure 5.** Enhancement of endothelial-type nitric oxide synthase (eNOS) promoter activity in EA.hy 926 cells after incubation with French red wines. **(A)** EA.hy 926 cells were stably transfected with p-eNOS-3500-Hu-Luc-neo. Stable cells were incubated for 24 h with ethanol (EtOH-Co, 1.25%, v/v), or various concentrations of two French red wines (“L.Chevaliers” and “Ch.Bonnet reserve”). The luciferase activity (normalized by protein content) was taken as a measure of eNOS promoter activity. **Bars** represent means  $\pm$  SEM of three independent experiments. **Asterisks** indicate significant differences from ethanol-treated cells (\*\*p < 0.01; \*\*\*p < 0.001; by ANOVA followed by the Fisher PLSD test). **(B)** EA.hy 926 cells were transiently transfected with pGl<sub>3</sub>-Basic (containing a promoterless luciferase gene) or different eNOS promoter luciferase constructs (p-eNOS-1600-Hu-Luc, p-eNOS-1111-Hu-Luc, p-eNOS-633-Hu-Luc, and p-eNOS-326-Hu-Luc, containing 1.6-kb to 0.33-kb of the eNOS promoter cloned before a luciferase reporter gene). The different transfected cells were either exposed 24 h to ethanol (EtOH-Co, 1.25%, v/v) or to the red wine “L.Chevaliers” (10%, v/v). The relative luciferase activity (corrected with renilla-luciferase activity; see Methods) was taken as a measure of eNOS promoter activity. Data represent means  $\pm$  SEM of three independent experiments.

induced change in binding activity could be detected for any of these transcription factors (n = 4 each, data not shown).

**Enhancement of eNOS mRNA stability by French red wine.** As shown in Figure 6, eNOS mRNA levels in ethanol (1.25%, v/v)-treated EA.hy 926 cells declined over time after treatment with the transcription inhibitor actinomycin D. The apparent half-life was about 30 h. Treatment of EA.hy 926 cells with “Ch.Bonnet reserve” significantly increased the stability of the eNOS mRNA.

## DISCUSSION

The current study provides evidence that red wines from France (and probably other locations) can upregulate the eNOS gene in human endothelial cells. This upregulation was seen in both EA.hy 926 cells and primary HUVEC. The enhanced mRNA and protein expression resulted in an increased production of bioactive NO, as measured by the RFL-6 reporter cell assay. Recent studies have indicated that an upregulation of eNOS under certain pathophysio-



**Figure 6.** Effect of French red wine on endothelial-type nitric oxide synthase (eNOS) messenger ribonucleic acid (mRNA) stability. The EA.hy 926 cells were preincubated either with ethanol (EtOH-Co, 1.25%, v/v) or with the red wine “Ch. Bonnet reserve” (10%, v/v) for 24 h. To inhibit transcription, actinomycin D (20  $\mu$ g/ml) was added to the culture medium. Ribonucleic acid was prepared 0, 12, 24, and 36 h thereafter. The eNOS mRNA expression was determined by RNase protection assay. The eNOS mRNA levels in both groups at the time of addition of actinomycin D (0 h) were set at 100%. **Circles** represent means  $\pm$  SEM of the densitometric analyses of four different gels. **Asterisks** indicate significant differences from ethanol-treated cells at the same time points (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; by the Student *t* test for unpaired data).

logical conditions can go along with a dysfunctional enzyme (15,16). However, the current study provides no evidence for a dysfunction of eNOS induced by red wine.

**Vasoprotective role of NO.** In addition to its vasodilator properties, NO can convey vasoprotection in several ways. Nitric oxide released toward the vascular lumen is a potent inhibitor of platelet aggregation and adhesion to the vascular wall (17,18). Besides protection from thrombosis, this also prevents the release of platelet-derived growth factors that stimulate smooth muscle proliferation and its production of matrix molecules. Endothelial NO also controls the expression of genes involved in atherogenesis. Nitric oxide decreases the expression of the chemoattractant protein MCP-1 (19), and of surface adhesion molecules such as CD11/CD18 (20), P-selectin (21), vascular cell adhesion molecule-1 (VCAM-1) (22), and intercellular adhesion molecule-1 (ICAM-1) (23), thereby preventing leukocyte adhesion to vascular endothelium and leukocyte migration into the vascular wall. This offers protection against an early phase of atherogenesis. Also, the decreased endothelial permeability, the reduced influx of lipoproteins into the vascular wall (24), and the inhibition of LDL oxidation (25) may contribute to the anti-atherogenic properties of NO. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells (26,27) as well as smooth muscle cell migration (28), thereby protecting against a later phase of atherogenesis. Based on the combination of those effects, NO produced in endothelial cells can be considered an anti-atherosclerotic principle (29).

**Effects of red wine on the endothelial NO system.** Previous evidence in the published data already suggested an interaction of red wine with the endothelial NO system. Red wine or extracts obtained from red wine caused endothelium-dependent, NO-mediated vasorelaxation of rat or rabbit aorta precontracted with norepinephrine (30–32). Also, in human coronary arteries and rat aortic rings in vitro, short-term incubation with red wine induced an endothelium-dependent vasodilation and an increased vascular cGMP content (10). These rapid effects of red wine are likely to involve an acute activation of eNOS (30–32). The molecular mechanisms of this acute enzyme activation are unknown, but they are most probably unrelated to the gene-regulatory effects described in the current study.

**Molecular mechanisms contributing to eNOS upregulation by red wine.** The upregulation of eNOS expression by red wine is brought about by both transcriptional and posttranscriptional (mRNA-stabilizing) events. The predominant transcriptional component seems to involve an activation of rather proximal portions of the eNOS promoter (first 326 bp), because the transcriptional stimulation by red wine was largely preserved with this short promoter fragment (Fig. 5B). This fragment is the binding target of a number of transcription factors, such as Sp1, GATA, PEA3, YY1, and Elf-1, whose functional relevance has been demonstrated previously (for review see Li et al. [33]). In the current study, we performed electromobility shift assays with nuclear extracts from ethanol (1.25%, v/v)-treated, compared with red-wine-treated cells. No wine-induced changes in protein-DNA binding was found with oligonucleotides containing the cognate Sp1-, Sp1/Sp3-like, GATA-, PEA3-, YY1-, or Elf-1-binding motifs of the proximal human eNOS promoter. This suggests that the transcriptional activation caused by red wines is unlikely to be based on the activation of one of the above transcription factors. Rather, it may be a multifactorial process and/or involve yet unidentified transcription factor(s).

**Ingredients of grapes are responsible for eNOS upregulation.** Moderate alcohol intake, also from beverages other than wine, has been shown to lower the risk of cardiovascular disease (4–6). However, the ethanol content of red wine is unlikely to be responsible for the upregulation of eNOS expression observed here, because the equivalent amount of ethanol (1.25%, v/v) alone produced only a marginal upregulation of eNOS (Fig. 1), and all subsequent experiments (Figs. 2–6) were performed with appropriate ethanol controls. In addition, German red wines (which were largely ineffective) and French red wines have the same alcohol content.

Also, no difference was detected between “en barrique” and “non-barrique”-produced red wines made from the same grapes, suggesting that the compounds stimulating eNOS expression derive from the grapes rather than the barrel staves. The numerous phenolic acids, polyphenols, and flavonoids contained in red wine are likely candidates, probably mediating the expressional upregulation of eNOS.

Differences in the content of these compounds between wines (34,35) could explain why French wines were particularly effective in stimulating eNOS expression. French red wines contain high polyphenol levels compared with wines from other regions (34,35).

**Conclusions.** This study provides evidence that red wine can stimulate the expression of the eNOS gene leading to an enhanced production of bioactive NO. In concert with other systemic beneficial effects of red wine, this could contribute to the cardiovascular protection enjoyed by moderate drinkers of red wine.

### Acknowledgments

We appreciate the technical assistance of Isolde Brausch (Department of Pharmacology) and Angelika Karpi (Department of Medicine II), Johannes Gutenberg University, Mainz, Germany.

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