

# Modulation of Expression of Endothelial Nitric Oxide Synthase by Nordihydroguaiaretic Acid, a Phenolic Antioxidant in Cultured Endothelial Cells

SANTHINI RAMASAMY, GRANT R. DRUMMOND, JOON AHN, MICHAL STOREK, JAN POHL, SAMPATH PARTHASARATHY, and DAVID G. HARRISON

*Division of Cardiology (S.R., G.R.D., J.A., D.G.H.), Department of Gynecology and Obstetrics (S.P.), and Microchemical Facility (M.S., J.P.), Emory University, Atlanta, Georgia*

Received March 30, 1999; accepted April 5, 1999

This paper is available online at <http://www.molpharm.org>

## ABSTRACT

Retrospective epidemiological studies have suggested that antioxidant therapy may decrease cardiovascular morbidity and mortality rates, although the mechanisms for this effect remain unclear. In the present study, we demonstrate that selective antioxidants can enhance expression of endothelial nitric oxide synthase (eNOS). We found that the antioxidants nordihydroguaiaretic acid (NDGA), catechol, glutaryl probucol, and *N*-acetylcysteine increased eNOS expression in cultured bovine aortic endothelial cells (BAECs). NDGA seemed to be the most potent of the phenolic antioxidants, producing a 3-fold increase in eNOS mRNA. This effect of NDGA was enhanced by nonphenolic antioxidants such as *N*-acetylcysteine and ascorbic acid. Nuclear run-on studies indicated that NDGA increased eNOS transcription. A similar increase in eNOS protein content

was observed with Western blot analysis after treating BAECs or human aortic endothelial cells with NDGA. Exposure of BAECs to NDGA enhanced NO production, as measured by electron paramagnetic resonance spin trapping and eNOS activity, as measured by [<sup>14</sup>C]arginine-to-[<sup>14</sup>C]citrulline assay. Methylation of the phenolic hydroxyl groups completely inhibited the NDGA effect on eNOS mRNA levels. This effect of NDGA was not due to inhibition of lipoxygenase because *cis*-5,8,11,14-eicosatetraynoic acid did not alter eNOS expression. We conclude that antioxidants may not only increase the bioactivity of nitric oxide but also enhance expression of the eNOS enzyme. Such an effect may prove useful in conditions such as hypertension and atherosclerosis, in which nitric oxide production and/or biological activity is impaired.

Endothelium-derived nitric oxide (NO) plays an important role in the modulation of vascular tone and regulation of blood pressure. Moreover, NO inhibits platelet aggregation, smooth muscle proliferation, and leukocyte adhesion to the endothelium (Moncada and Higgs, 1993; Forstermann et al., 1994). In several pathological conditions, such as hypercholesterolemia, atherosclerosis, diabetes mellitus, and hypertension, endothelium-dependent relaxation is impaired. Reactive oxygen species appear to play an important role in the progression of most of these disease states (Freiman et al., 1986; Giugliano et al., 1995; Keaney and Vita, 1995; Rajagopalan et al., 1996). More specifically, it has been observed

that there is increased production of vascular superoxide, which on reaction with NO results in loss of biological activity of NO.

In patients with non-insulin-dependent diabetes mellitus or hypertension or who are cigarette smokers, acute infusions of ascorbic acid have been shown to improve vasorelaxation (Heitzer et al., 1996; Ting et al., 1996, 1997; Solzbach et al., 1997). In cholesterol-fed rabbits, the impairment in endothelium-dependent relaxation is, in part, reversed by treatment with antioxidants such as vitamin E (Keaney et al., 1993, 1994). Even oral administration of antioxidants has been shown to improve endothelium-dependent vasodilation (Simon et al., 1993; Levine et al., 1996). Although the mechanism by which antioxidant treatment improves vasorelaxation is not clear, it is generally assumed that antioxidants scavenge superoxide and thus increase the availability of functional NO. Kinetic constants, however, suggest that the rate of reactions between common antioxidants and superox-

This work was supported by National Institutes of Health Grants HL390006 and HL48667m as well as American Heart Association Scientist Development Grant Award 9630119N (to S.R.). Portions of this work were presented at the American Heart Association 70th Meeting, November 1997, Orlando, FL; Society of Toxicology 37th Meeting, Seattle, WA, March 1998; and Vascular Biology, 1998, San Francisco, CA, April 1998.

**ABBREVIATIONS:** NO, nitric oxide; NDGA, nordihydroguaiaretic acid; BHT, butylated hydroxy toluene; M199, Medium 199; EPR, electron paramagnetic resonance; MGD, *N*-methyl-D-glucamine dithiocarbamate; eNOS, endothelial nitric oxide synthase; HAEC, human aortic endothelial cell; NAC, *N*-acetylcysteine; L-NAME, *N*<sup>G</sup>-nitro-L-arginine methyl ester; BAEC, bovine aortic endothelial cell; SOD, superoxide dismutase; Sp1, simian virus 40 promoter factor 1; AP-1, activator protein 1; EGM, endothelial cell growth media; ARE, antioxidant response element; ETYA, *cis*-5,8,11,14-eicosatetraynoic acid.

## Materials and Methods

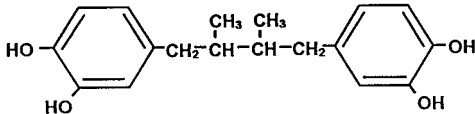
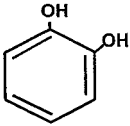
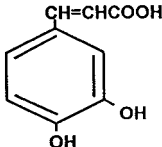
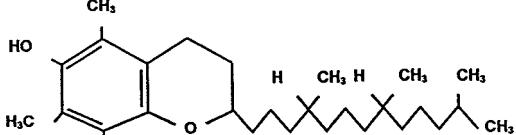
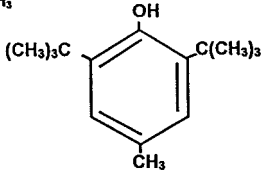
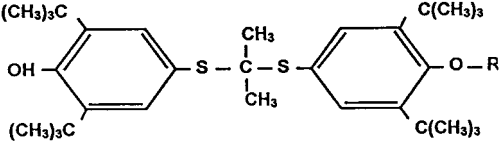
**Cell Culture and Experimental Media Preparation.** Bovine aortic endothelial cells (BAECs) were cultured in Medium 199 (M199; GIBCO, Grand Island, NY) containing 10% FCS (Hyclone Laboratories, Logan, UT) as described previously (Mugge et al., 1991). Postconfluent cells between passages 5 and 10 were used for the experiments. Human aortic endothelial cells (HAECs) were obtained from Clonetics (Walkersville, MD) and cultured in endothelial cell growth media (EGM; Clonetics) containing 2% FCS.

Postconfluent endothelial cells were exposed to control (M199 plus 5% FCS in the case of BAECs or EGM plus 2% FCS in the case of HAECs) or media supplemented with one of several phenolic antioxidants, including nordihydroguaiaretic acid (NDGA), vitamin E, catechol, caffeic acid, butylated hydroxy toluene (BHT), and glutaryl probucol, a water-soluble form of probucol (Table 1). In other experiments, water-soluble antioxidants such as ascorbic acid, cysteine, or *N*-acetylcysteine (NAC) were used. Concentrated stocks of phenolic antioxidants were made in ethanol, and the appropriate aliquots were added to M199 plus 5% FCS in the case of BAECs or EGM in the case of HAECs to prepare antioxidant-enriched media. The concentration of ethanol in the antioxidant-enriched media was never higher than 0.1%. In the case of water-soluble antioxidants, concentrated stocks were made in distilled water and sterile filtered, and appropriate aliquots were added to M199 plus 5% FCS or EGM.

ide is much slower than the rate of the reaction between superoxide and NO (Forman and Fridovich, 1973; Afenas'ev, 1991; Huie and Padmaja, 1993). This raises the possibility that antioxidants may have other effects on the endothelial arginine/NO pathway beyond scavenging superoxide and prolonging the half-life of NO.

Recent evidence suggests that antioxidants can modulate gene expression. For example, antioxidant treatment has been shown to induce expression of chemoprotective enzymes like glutathione *S*-transferase (Rushmore et al., 1991) and NAD(P)H:quinone reductase (Favreau and Pickett, 1993) and thus prevent cells from malignant transformation. In addition, phenolic antioxidants have been shown to inhibit expression of genes involved in inflammatory processes such as vascular cell adhesion molecule and intercellular adhesion molecule (Cominacini et al., 1996). Of interest, the promoter of human endothelial nitric oxide synthase (eNOS) gene contains simian virus 40 promoter factor 1 (Sp-1), activator protein 1 (AP-1), and antioxidant response elements, all of which are regulated by cellular redox state (Sen and Packer, 1996; Palmer and Paulson, 1997). Based on these considerations and the previous findings in experimental animals, we performed the present study to test the hypothesis that antioxidants might modulate eNOS gene expression.

TABLE 1  
Effect of various phenolic antioxidants on eNOS mRNA levels

	Name and Structure of Antioxidant	eNOS/28s	
		$\mu\text{M}$	fold increase above control
NDGA		16	1.8 $\pm$ 0.1
		32	2.9 $\pm$ 0.2
Catechol		50	1.4 $\pm$ 0.04
		100	3.2 $\pm$ 0.2
Caffeic acid		100	0.8 $\pm$ 0.04
		200	0.9 $\pm$ 0.03
Vitamin E		25	1.0 $\pm$ 0.02
		50	1.0 $\pm$ 0.1
BHT		50	0.8 $\pm$ 0.03
		100	Toxic
Glutaryl probucol		10	1.7 $\pm$ 0.1
		25	Toxic

**Synthesis of Methylated NDGA.** NDGA (100 mg; 0.33 mmol) was dissolved in 0.21 ml (3.3 mmol) of iodomethane and 5 ml of dry acetone. Potassium carbonate (0.92 g) was then added to the mixture, and the mixture was refluxed for approximately 10 h. Complete methylation was monitored by thin-layer chromatography on silica plates using ethyl acetate/petrol ether (19:1, v/v) as the mobile phase, and the detection was done using iodine vapors. The reaction mixture was evaporated on a rotary evaporator and extracted with ether. After evaporation of ether, the residue was dissolved in 2.0 ml of methanol/water (17:3, v/v).

Methylated NDGA ( $\text{Me}_4\text{NDGA}$ ) was purified using reversed phase HPLC on a C18 Microsorb-MV silica column ( $5 \times 250$  mm, dp  $\sim 5$   $\mu\text{m}$ , 100-Å pore size; Rainin Instruments) at a flow rate of 0.5 ml/min at room temperature. The column was developed using methanol/water (4:1, v/v) under isocratic conditions, followed by UV detection at 280 nm. The retention time of completely methylated NDGA was 29 min.

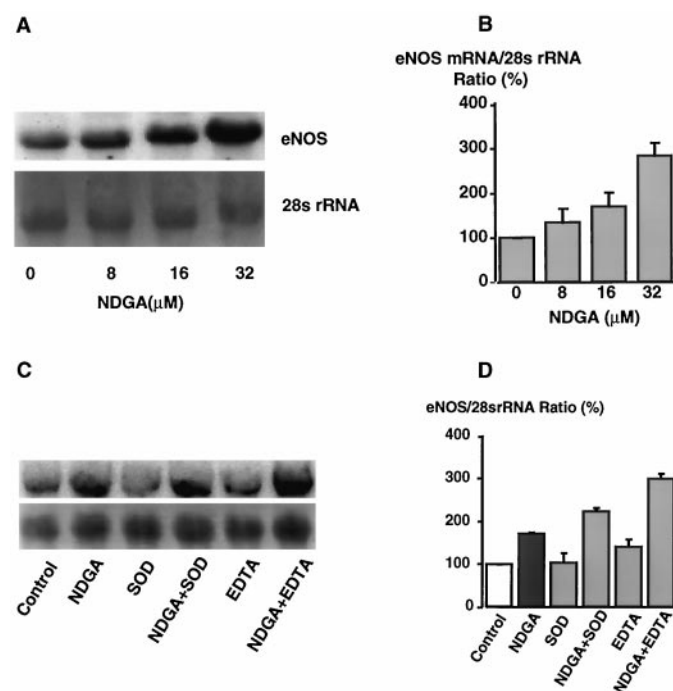
The mass of  $\text{Me}_4\text{NDGA}$  was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, performed using the Bruker ProteinTOF instrument. The sample was prepared according to Storek and Pohl (1997). Briefly, 1.5  $\mu\text{l}$  (1 nmol) of the sample was mixed with 5  $\mu\text{l}$  of the saturated solution of indole-2-carboxylic acid. An aliquot (0.5  $\mu\text{l}$ ) of this mixture was deposited on the target, air dried, and analyzed. Spectra were obtained by averaging 50 single-laser-shot spectra. Parameters were set for laser wavelength of 337 nm, ion acceleration voltage of 17.5 kV, and reflector voltage of 20 kV.

**Northern Blotting.** Total RNA was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA (20  $\mu\text{g}$ ) was loaded on a 1.0% agarose/3% formaldehyde gel, electrophoresed, and transferred to a nitrocellulose membrane overnight. A full-length (4.2 kb) radioactive-labeled eNOS probe was prepared using [ $^{32}\text{P}$ ]dCTP (Dupont NEN, Boston, MA) and oligolabeling kit (Phar-

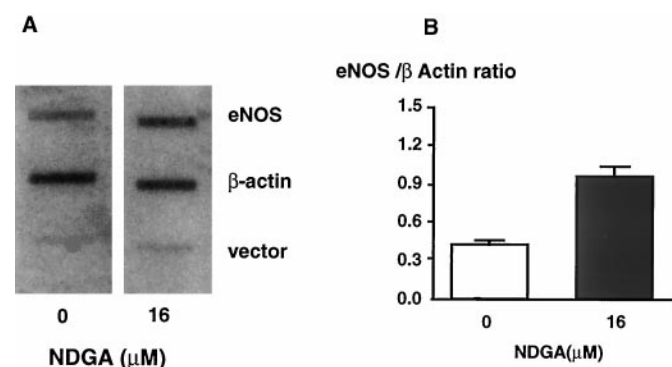
macia Biotech, Piscataway, NJ). Membranes were hybridized for 60 min at 68°C using Quick Hyb (Stratagene) and washed subsequently according to the conditions described previously (Ramasamy et al., 1998). The blots were visualized using a PhosphorImager. Intensity of the eNOS bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The intensities of the eNOS mRNA bands were normalized for the corresponding 28S band or 18S bands.

**NOS Activity Assay.** Postconfluent BAECs were treated with control or antioxidant-containing media for 24 h. Whole-cell homogenates ( $\sim 250$   $\mu\text{g}$  total protein) were then used for NOS activity assay as previously described, and the converted citrulline was separated from labeled L-arginine using a Dowex AG 50WX-8 ( $\text{Na}^+$  form; Bio-Rad, Hercules, CA) (Ramasamy et al., 1998). Radioactivity in the eluate was determined using LS6500 Beckman Liquid Scintillation Counter (Beckman Instruments, Inc., Schaumburg, IL). Protein concentration in the homogenate was determined using Bio-Rad D<sub>50</sub> reagent.

**EPR Spin-trapping for Measurement of NO Production.** Confluent BAECs grown in 6-well plates were treated with M199 with 5% FCS containing 0 or 25  $\mu\text{M}$  NDGA for 24 h. The cells were washed in Krebs/HEPES buffer containing 99.01 mM NaCl, 4.69 mM KCl, 1.87 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 1.03 mM  $\text{K}_2\text{HPO}_4$ , 25.0 mM  $\text{NaHCO}_3$ , 20.0 mM Na-HEPES, and 11.1 mM glucose, pH 7.4, and incubated subsequently with 1.5 ml of the same buffer containing 1.5 mM *N*-methyl-D-glucamine dithiocarbamate (MGD; Oxis International, Inc., Portland, OR), 0.3 mM ferrous sulfate, and 100 U/ml  $\text{Cu}^{2+}/\text{Zn}^{2+}$  superoxide dismutase (SOD) at 37°C. For NO stimulation, cells were incubated in the same buffer mixture containing either 1  $\mu\text{M}$  A23187 or bradykinin. In some experiments, *N*<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 1 mM) was added 30 min before agonist stimulation. After a 30-min incubation, the Krebs/HEPES buffer was removed and immediately frozen in liquid nitrogen. The cell monolayers were dissolved in 0.5% SDS, and total cellular protein content was determined as mentioned above. EPR spectra of the MGD- $\text{Fe}^{2+}$ -NO complex were recorded using a Bruker EMX EPR spectrometer (Bruker Instruments Inc., Billerica, MA) at microwave frequency of 9.785 GHz, microwave power of 20 mW, modulation frequency of 100 kHz, and modulation amplitude of 2.5 G, at room temperature. A standard curve was obtained by incubating the MGD- $\text{Fe}^{2+}$  complex with 0 to 5  $\mu\text{M}$  concentration of the NO donor 2-(*N,N*-diethylamino)diazenolate-2-oxide, sodium salt (Alexis Biochemicals, San Diego, CA) for 30 min at 37°C, and EPR spectra were obtained under the same conditions as mentioned above for samples. Peak intensity of the EPR spectra was proportional to the amount of



**Fig. 1.** Northern blot analysis showing the effect of increasing concentrations of NDGA on eNOS mRNA in BAECs. Postconfluent BAECs were exposed to 0 to 32  $\mu\text{M}$  NDGA in M199 plus 5% FBS for 24 h. A, representative blot. B, combined densitometric ratio of eNOS/28S rRNA. C, effect of concomitant addition of either  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD or  $\text{Ca}^{2+}$ -EDTA with NDGA on eNOS expression. BAECs were exposed to either 16  $\mu\text{M}$  NDGA or SOD (100 U/ml) or 100  $\mu\text{M}$  EDTA individually or in combination for 24 h. D, percent eNOS/28S rRNA ratio from three separate experiments similar to those shown in C. Data are presented as mean  $\pm$  S.E.M.



**Fig. 2.** Nuclear run-off analysis demonstrating the effect of 16  $\mu\text{M}$  NDGA on the transcription rate of the eNOS gene in BAECs. Nuclei were harvested from endothelial cells exposed to control and NDGA-containing media for 4 h. Nascent transcripts were  $^{32}\text{P}$ -labeled, and equal counts from each group were added to the blots containing eNOS,  $\beta$ -actin, and vector DNA. Hybridizations were performed for 48 to 72 h at 65°C. The blots were washed and exposed to PhosphorImager. A, representative blots. B, densitometric ratios of eNOS/ $\beta$ -actin bands. Data are presented as mean  $\pm$  S.E.M. for three separate experiments.

MGD-Fe<sup>2+</sup>-NO complex in the Krebs/HEPES buffer. The total peak intensities of the samples were compared with that obtained for standards allowing quantification of the amount of NO produced by the cells. NO production was normalized to total cellular protein content.

**Western Blotting.** BAECs or HAECs were harvested, and the homogenates were prepared as described in NOS assay. Then, 20  $\mu$ g of protein was electrophoresed using 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Western analysis was performed using a 1:2000 dilution of a monoclonal antibody against human eNOS (Transduction Laboratories, Lexington, KY) and a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham Corporation, Arlington Heights, IL) (Ramasamy et al., 1998). Signals of the immunoreactive bands were measured using the ECL detection system (Amersham).

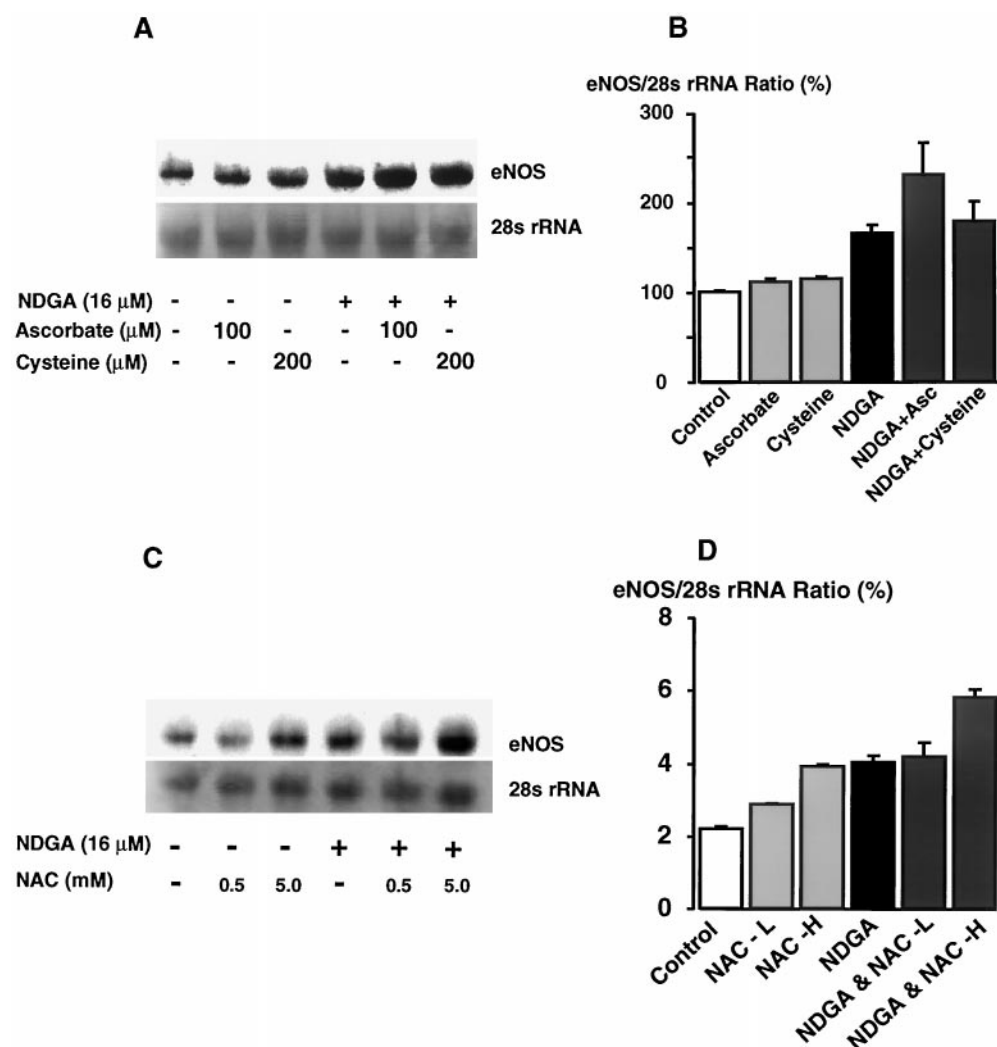
**Nuclear Run-On Assay.** Nuclear run-on assays were performed using a method described by Greenberg (1987) with modifications (Ramasamy et al., 1998). Briefly, BAECs were treated with control or 16  $\mu$ M NDGA-containing media for 4 h. Cells were harvested and lysed in 0.5% Nonidet P-40, and the nuclei were isolated by centrifugation. In vitro transcription was performed using  $5 \times 10^7$  nuclei in a reaction buffer containing a 2 mM concentration each of ATP, GTP, and CTP and 100  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP (DuPont-NEN) for 30 min at 30°C. Total RNA was extracted, and the radiolabeled transcripts (total activity, approximately  $5 \times 10^6$  cpm) were hybridized to nylon membranes previously slot-blotted with eNOS cDNA,  $\beta$ -actin cDNA, and the full-length pBluescript vector. Bands were subsequently

visualized using a PhosphorImager and quantified using ImageQuant software.

## Results

**NDGA Treatment Causes Dose-Dependent Increase in eNOS mRNA Levels.** Exposure of BAECs to increasing concentrations of NDGA (0–32  $\mu$ M) for 24 h caused a dose-dependent increase in eNOS mRNA levels as measured by Northern analysis (Fig. 1A). Densitometric readings of the Northern blots indicated that there was an 80 and a 300% increase in the ratios of eNOS to 28S band after 16 and 32  $\mu$ M NDGA treatments, respectively (Fig. 1B).

Phenolic compounds like NDGA may have both antioxidant and prooxidant effects. In the latter case, the phenolic group is often oxidized to a phenoxyl radical, which then can exert prooxidant effects (Santanam and Parthasarathy, 1995). Common mechanisms involve oxidation catalyzed by transition metals and reactions with superoxide. To examine the possibility that NDGA exerted its effect on eNOS mRNA via such a mechanism, cells were concomitantly treated with NDGA plus either SOD or Ca<sup>2+</sup>-EDTA. Neither Ca<sup>2+</sup>-EDTA nor SOD affected eNOS mRNA levels alone, but both augmented the effect of NDGA (Fig. 1, C and D). These data



**Fig. 3.** Northern blot analysis examining the effect of nonphenolic antioxidants on NDGA-induced eNOS expression. A, Northern blot from BAECs treated for 24 h with 100  $\mu$ M ascorbic acid or 200  $\mu$ M cysteine, either alone or in combination with 16  $\mu$ M NDGA. B, percent densitometric ratio of eNOS/28S rRNA from ascorbic or cysteine groups. C, Northern blot from BAECs treated with 0.5 or 5.0 mM NAC either alone or in combination with 16  $\mu$ M NDGA for 24 h. D, percent densitometric ratio of eNOS/28S rRNA from NAC groups. Data are presented as mean  $\pm$  S.E.M. for three separate experiments.



suggest that the effect of NDGA on eNOS mRNA levels involves an antioxidant rather than a prooxidant effect.

The structures of several antioxidants, including NDGA, and their effects on eNOS mRNA levels are shown in Table 1. In some instances, the use of higher concentrations of some phenolic antioxidants (BHT and probucol) was limited due to cellular toxicity.

**NDGA-Induced Increase in eNOS mRNA Levels Is Transcriptionally Mediated.** NDGA treatment caused an increase in the transcription rate of the eNOS gene but not for  $\beta$ -actin (Fig. 2A). The hybridization of nascent transcripts to eNOS as well as  $\beta$ -actin cDNA in the blots was specific as the hybridization to vector DNA was negligible. Quantitative results from three separate experiments indicated that the ratio of eNOS to  $\beta$ -actin transcription increased 2-fold as a result of NDGA treatment (Fig. 2B).

**Nonphenolic Antioxidants Augment NDGA-Induced Increase in eNOS mRNA Levels.** In BAECs, relatively low concentrations of ascorbic acid (100  $\mu$ M) or cysteine (200  $\mu$ M), when given alone, failed to alter eNOS mRNA levels. In contrast, 100  $\mu$ M ascorbic acid substantially enhanced the effect of 16  $\mu$ M NDGA on eNOS expression. Quantitative densitometry indicated that combined treatment with NDGA

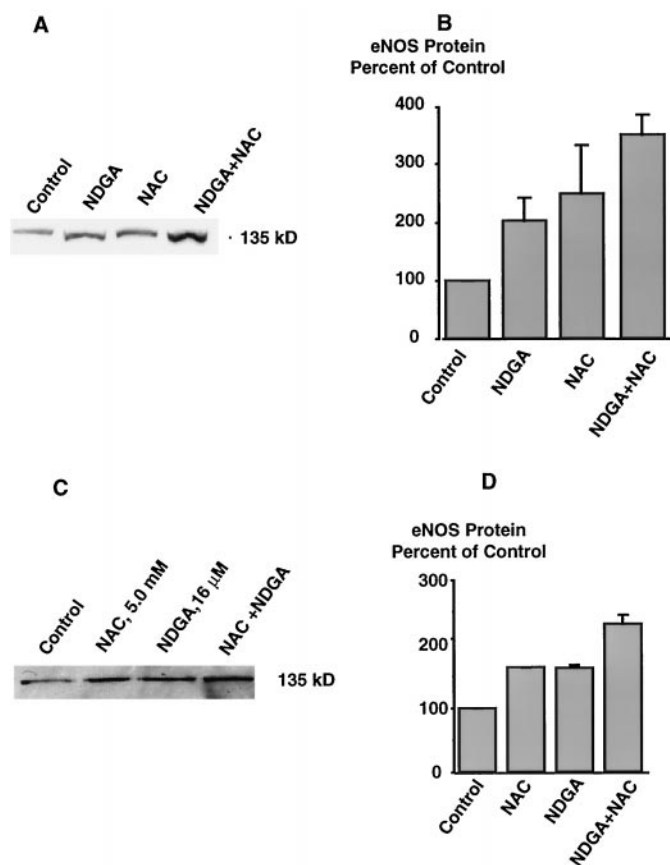
and ascorbic acid or cysteine caused a 2.4- and 1.9-fold increase in eNOS mRNA levels, respectively (Fig. 3B).

Similarly, another nonphenolic antioxidant, NAC, when used alone, had no effect on eNOS mRNA levels when added in low concentrations (0.5 mM) for 24 h. A higher concentration of NAC (5 mM) produced a 90% increase in eNOS mRNA, similar to that observed on exposure of cells to 16  $\mu$ M NDGA. Interestingly, an additive increase in eNOS mRNA levels was found when cells were treated with NDGA and 5.0 mM NAC (Fig. 3, C and D).

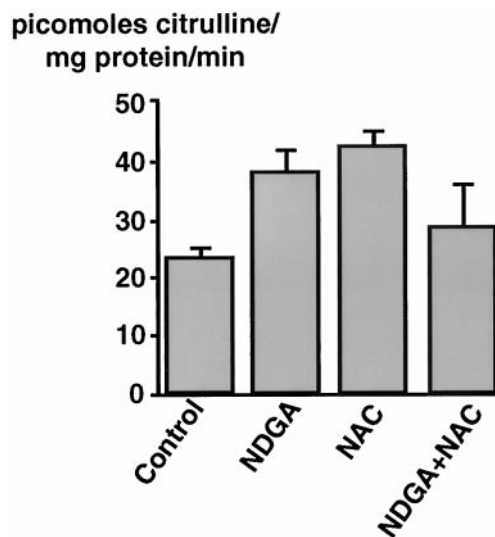
**NDGA and NAC Treatments Increase eNOS Protein and Enzyme Activity.** Because NDGA and NAC exerted the most impressive effects on eNOS mRNA levels, studies examining eNOS protein expression and enzyme activity were focused on the effects of these compounds. When used alone, NDGA (16  $\mu$ M) and NAC (5 mM) increased the eNOS protein content 2- and 2.3-fold, respectively, compared with control BAECs. The combined exposure of NDGA and NAC caused an additive increase (3.5-fold) in eNOS protein content (Fig. 4, A and B). A qualitatively similar effect in eNOS protein expression was observed in HAECs treated with NDGA, NAC, and the combination of the two (Fig. 4, C and D).

In correspondence with the increase in eNOS protein content, both NDGA (16  $\mu$ M) and NAC (5 mM) elevated eNOS enzyme activity by approximately 2-fold (Fig. 5). Interestingly, the combination of NDGA and NAC, which had a dramatic effect on eNOS protein content, did not increase eNOS enzyme activity beyond that observed when either agent was used alone (Fig. 5).

**Effect of NDGA on Endothelial Cell NO Production.** NDGA (25  $\mu$ M) elevated NO production as estimated by EPR (Fig. 6) in unstimulated cells as well as cells treated with either A23187 or bradykinin. Representative spectra of MGD-Fe<sup>2+</sup>-NO complexes obtained from control and NDGA-treated BAECs under basal and agonist-stimulated conditions are depicted in Fig. 6A. Analysis of mean data indicated that NDGA approximately doubled NO production under



**Fig. 4.** Western blot analysis demonstrating the effect of antioxidants on eNOS protein content in BAECs (A and B) and HAECs (C and D). Cells were exposed to control media or media containing either 16  $\mu$ M NDGA, 5 mM NAC, or the combination of NAC with NDGA for 24 h. Each lane was loaded with 15  $\mu$ g of protein. A monoclonal antibody against human eNOS was used to detect the bands. Representative blots are illustrated in A and C from BAECs and HAECs, respectively. The densitometric readings as percent control of Western blots derived from BAECs and HAECs are presented in B and D, respectively. Data are presented as mean  $\pm$  S.E.M. for three separate experiments.



**Fig. 5.** NOS activity as measured by the conversion of [<sup>14</sup>C]arginine to [<sup>14</sup>C]citrulline. Cells were exposed to control media or media containing either 16  $\mu$ M NDGA, 5 mM NAC, or the combination of NAC and NDGA for 24 h. The cells were homogenized, and 250  $\mu$ g of protein was used for the assay. Results represent the mean  $\pm$  S.E.M. from three separate experiments.

basal conditions and in response to A23187 and increased responses to bradykinin by 50%. NO production was virtually eliminated by 1 mM L-NAME treatment (Fig. 6B).

**NDGA-Induced Increase in eNOS Expression Is Not Related to Lipoygenase Inhibition.** Unlike NDGA, the commonly used lipoygenase inhibitor *cis*-5,8,11,14-eicosatetraynoic acid (ETYA) (50  $\mu$ M) had no effect on either eNOS enzyme activity (Fig. 7A) or mRNA levels (7B) on 24 h treatment.

**Phenolic Hydroxyl Groups Are Critical for NDGA-Induced Increase in eNOS Expression.** The MALDI-TOF mass spectrum of NDGA after *O*-methylation confirmed that the molecular weight of the modified molecule, 358.4, amu corresponded to the predicted molecular weight of Me<sub>4</sub>NDGA (Fig. 8A). In addition, fast atom bombardment mass spectrometry analysis (not shown) confirmed the presence of four methyl groups. Unlike native NDGA, Me<sub>4</sub>NDGA had no effect on eNOS mRNA levels (Fig. 8, B and C). These results indicated that the 4-hydroxyl groups were critical in mediating the effect of NDGA on eNOS mRNA expression.

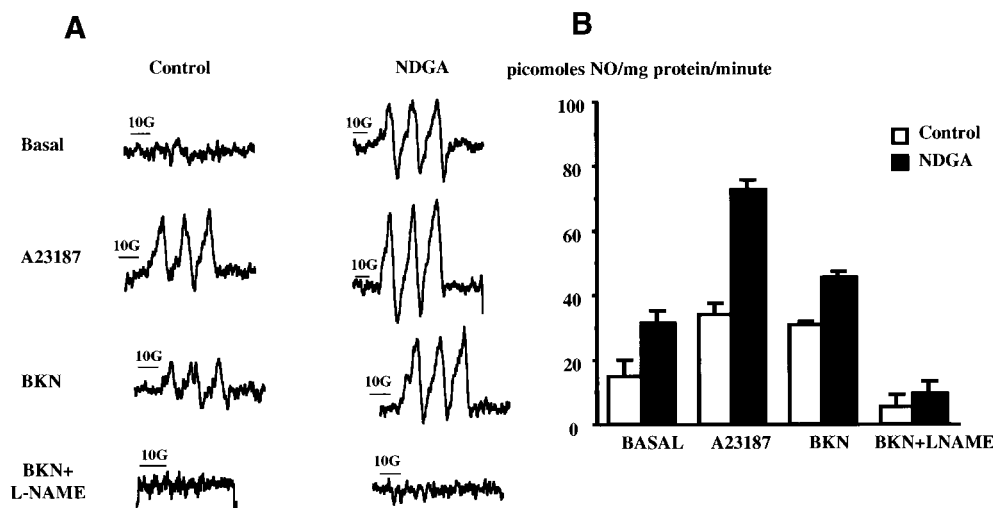
## Discussion

The new finding of this study is that antioxidants, including NDGA, NAC, probucol, and catechol, enhance the expression of eNOS in cultured endothelial cells. The most potent of these, the phenolic antioxidant NDGA, increased eNOS expression by as much as 3-fold. This effect of NDGA is at least in part transcriptionally mediated and is augmented by the presence of nonphenolic small antioxidant molecules such as ascorbic acid and NAC. In addition, increased eNOS gene expression by NDGA in endothelial cells is mediated by its phenolic groups, as the methylation of these groups abolished this effect of the compound. Finally, NDGA-mediated increases in eNOS gene expression are likely due to antioxidant rather than prooxidant effects and are independent of lipoygenase inhibition.

The biological activity of endothelium-derived NO is dimin-

ished in a variety of pathophysiological conditions, resulting in impaired endothelium-dependent vasodilation. In hypercholesterolemia, treatment with SOD, dietary antioxidants including beta carotene or vitamin E (Keaney et al., 1993), or the phenolic antioxidant probucol (Keaney et al., 1993) have been shown to improve endothelium-dependent vasodilation. Recently, antioxidant treatment has also been shown to increase endothelium-dependent vasodilation and reduce blood pressure in angiotensin II-induced hypertension (Laursen et al., 1997). In general, it is thought that antioxidants improve endothelium-dependent vasodilation by scavenging superoxide and thus increasing the availability of functional NO (Harrison and Ohara, 1995). The rate constants for the reaction between superoxide and antioxidant vitamins such as vitamin E and ascorbic acid, however, are approximately 10,000 times slower than the rate constant for the reaction between NO and superoxide (Forman and Fridovich, 1973; Afenasev, 1991; Huie and Padmaja, 1993). Our current study provides an additional mechanism whereby antioxidants may improve endothelial-dependent vasodilation (i.e., by increasing eNOS expression).

Of interest, the effect of the various phenolic antioxidants on eNOS expression varied substantially (Table 1). The antioxidant properties and the propensity of these compounds to undergo redox cycling are affected by their respective *R*-groups attached to the aromatic ring. Among the phenolic antioxidants used in this study, NDGA is the most potent. At a concentration of 32  $\mu$ M, NDGA increased eNOS expression by 3-fold. NDGA has well established antioxidant properties and is known to prevent oxidation of lard stored at room temperature for up to 1 year (Lundberg et al., 1944). NDGA has previously been shown to be more potent than vitamin E, probucol, caffeic acid, or BHT in preventing oxidation of linoleic acid (Sgargali et al., 1993). It is also possible that structural characteristics and hydrophobic properties of the various compounds influence their capacity to interact with redox sensitive transcription factors.



**Fig. 6.** A, EPR spectra of MGD-Fe<sup>2+</sup>-NO complex obtained in control and 25  $\mu$ M NDGA-treated BAECs under basal and agonist-stimulated conditions. EPR spectra were obtained after incubation of the cells in Krebs/HEPES buffer containing 1.5 mM MGD, 0.3 mM ferrous sulfate, and 100 U/ml Cu<sup>2+</sup>/Zn<sup>2+</sup> SOD at 37°C for 30 min. The cells were stimulated either with 1  $\mu$ M A23187 or bradykinin. In some cells, 1 mM L-NAME was added 30 min before agonist stimulation. Spectrometer settings were microwave frequency of 9.785 GHz, microwave power of 20 mW, receiver gain of  $1 \times 10^4$ , modulation frequency of 100 kHz, modulation amplitude of 2.5 G, and scan time of 83.886 s. Images are the averages of five scans subjected to baseline correction. B, quantification of spectra obtained from samples ( $n = 3$ ) after correction for cellular protein. A standard curve generated by exposure of MGD-Fe<sup>2+</sup> to 2-(*N,N*-diethylamino)diazenolate-2-oxide (0–5  $\mu$ M) was used to quantify the actual amount of NO produced by the cells.

Our studies indicate that the phenolic hydroxyl groups of NDGA are necessary for enhancing eNOS expression. When the phenolic hydroxyl groups were methylated, the ability of the modified compound to affect eNOS expression was completely abolished (Fig. 8). Thus, it seems that the presence of the phenolic hydroxyl groups, which are critical for the antioxidant properties of the compound, are also important in enhancing eNOS expression.

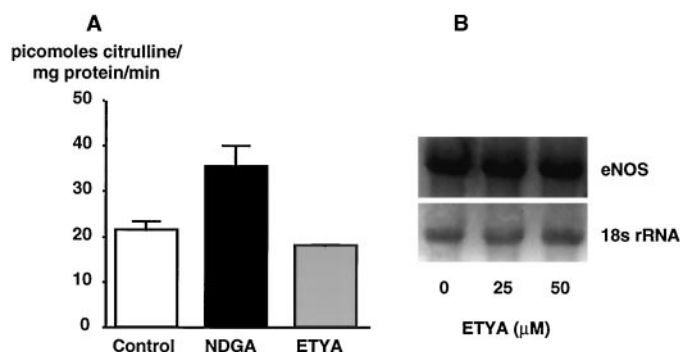
Phenolic hydroxyl-containing compounds may exert both antioxidant and prooxidant properties. When a radical abstracts the hydrogen atom of the phenolic group, the phenolic antioxidant is converted to a phenoxyl radical. Reducing agents such as ascorbic acid or thiol containing compounds promote reduction of the phenoxyl radicals back to the parent compound (Thomas et al., 1995). If the effect of NDGA on eNOS expression were mediated via a prooxidant effect, it would be expected that addition of reducing agents such as ascorbate, cysteine, or NAC would have diminished induction of eNOS by NDGA. This was not found to be the case. Ascorbate and NAC enhanced the effect of NDGA on eNOS expression, whereas cysteine was without effect. Furthermore, the addition of  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD (to scavenge  $\text{O}_2^-$  in the media) or  $\text{Ca}^{2+}$ -EDTA (to chelate transition metals) enhanced the effect of NDGA. Taken together, these lines of evidence suggest that the effect of NDGA on eNOS expression is mediated via antioxidant rather than prooxidant properties (Fig. 1).

NDGA is also known to be nonselective lipoxygenase inhibitor. It is unlikely, however, that its effect on eNOS expression was mediated via lipoxygenase inhibition (Fig. 7). Neither lipoxygenase protein nor enzyme activity has been detected in endothelial cells. Furthermore, another potent inhibitor of lipoxygenase, ETYA (in concentrations exceeding that necessary to inhibit lipoxygenase), had no effect on eNOS expression or enzyme activity (Reddy et al., 1994).

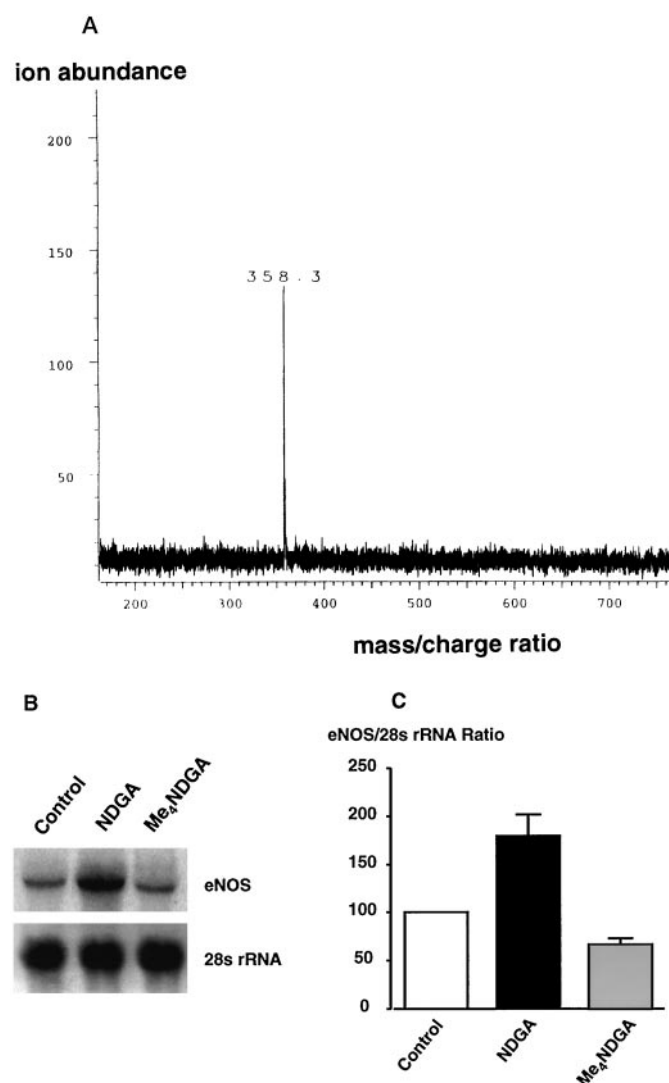
Nuclear run-on analysis suggested that NDGA enhances the rate of eNOS mRNA transcription (Fig. 2). The promoter of eNOS contains *cis*-elements that in the case of other genes, are modulated by cellular redox state. In particular, Sp-1 binding is crucial for basal transcription of eNOS. In other genes and tissues, antioxidant treatment has been shown to increase Sp-1 binding, whereas the addition of hydrogen peroxide or diamide or the depletion of glutathione decreases Sp-1 binding. AP-1-like elements are also present in the

eNOS promoter. Phenolic antioxidants have been shown to induce expression of c-Fos, and c-Jun, resulting in enhanced AP-1 binding activity in hepatocytes (Choi and Moore, 1993). In contrast, phenolic antioxidants may inhibit AP-1 activity by increasing Fra (Yoshioka et al., 1995). It is also possible that NDGA increases eNOS expression through activation of protein or proteins binding to an antioxidant response element (ARE). Both the human and bovine eNOS promoters contain sequences resembling the ARE in the NAD(P)H:quinone reductase and the glutathione *S*-transferase promoters. The presence and significance of ARE-binding proteins in endothelial cells have yet to be examined.

Interestingly, the combination of NDGA with NAC, although increasing eNOS mRNA and protein levels, did not increase eNOS enzyme activity as determined by L-arginine-to-L-citrulline activity (Figs. 3 and 4 versus Fig. 5). The reason for this discrepancy is unclear, but it may be related



**Fig. 7.** Effect of the lipoxygenase inhibitor ETYA on eNOS expression. A, eNOS activity as measured by the conversion of radioactive arginine to citrulline in BAECs treated with either 16  $\mu\text{M}$  NDGA or 50  $\mu\text{M}$  ETYA. Data are presented as mean  $\pm$  S.E.M. of three separate experiments. B, Northern blot indicating the effect of 24-h treatment of ETYA (0–50  $\mu\text{M}$ ) on eNOS steady-state mRNA. The experiments were repeated three times.



**Fig. 8.** Role of the phenolic hydroxyl groups in modulation of eNOS expression by NDGA. After methylation of NDGA, mass spectrometric analysis indicated a single peak at the molecular weight of 358, corresponding to the predicted molecular weight of Me<sub>4</sub>NDGA (A). BAECs were treated with either NDGA or Me<sub>4</sub>NDGA for 24 h, and eNOS mRNA levels were measured. B, representative Northern blot. C, the percent eNOS/28s rRNA ratio from three separate experiments. Data are presented as mean  $\pm$  S.E.M. from three separate experiments.



to either subcellular localization of the enzyme or autoinhibition of enzyme activity as recently described by Griscavage et al. (1995).

In addition to mediating endothelium-dependent vasodilation, endothelium-derived NO has been shown to inhibit platelet aggregation (Alheid et al., 1987), vascular cell adhesion molecule-1 expression (Khan et al., 1996), and smooth muscle growth (Garg and Hassid, 1989), all of which are considered antiatherogenic properties. There is substantial evidence that antioxidants may inhibit the atherosclerotic process, likely via a variety of mechanisms, including prevention of low-density lipoprotein oxidation and inhibition of vascular adhesion molecule expression. Increased expression of NO synthase and production of endothelial NO may be another mechanism whereby antioxidants inhibit the development of atherosclerosis. Modulation of eNOS expression by compounds similar to NDGA may be a useful clinical target for future drug design and clinical studies.

## References

- Afanas'ev I (1991) *Superoxide Ion: Chemistry and Biological Implications*. CRC Press, Boca Raton, FL.
- Alheid U, Frölich JC and Förstermann U (1987) Endothelium-derived relaxing factor from cultured human endothelial cells inhibits aggregation of human platelets. *Thromb Res* 47:561–571.
- Choi H-S and Moore DD (1993) Induction of *c-fos* and *c-jun* gene expression by phenolic antioxidants. *Mol Endocrinol* 7:1596–1602.
- Cominacini L, Garbin U, Pasini AF, Davoll A, Campagnola M, Contessi GB, Pastorino AM and Cascio VL (1996) Antioxidants inhibit the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 induced by oxidized LDL on human umbilical vein endothelial cells. *Free Radic Biol Med* 22:117–127.
- Favreau LV and Pickett CB (1993) Transcriptional regulation of the rat NAD(P)H:quinone reductase gene: Characterization of a DNA-protein interaction at the antioxidant responsive element and induction by 12-*O*-tetradecanoylphorbol 13-acetate. *J Biol Chem* 268:19875–19881.
- Forman HJ and Fridovich I (1973) Superoxide dismutase: A comparison of rate constants. *Arch Biochem Biophys* 158:396–400.
- Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I and Kleinert H (1994) Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* 23:1121–1131.
- Freiman PC, Mitchell GC, Heistad DD, Armstrong ML and Harrison DG (1986) Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ Res* 58:783–789.
- Garg UC and Hassid A (1989) Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest* 83:1774–1777.
- Giugliano D, Ceriello A and Paolisso G (1995) Diabetes mellitus, hypertension, and cardiovascular disease: Which role for oxidative stress? *Metabolism* 44:363–368.
- Greenberg M (1987) Identification of newly transcribed RNA, in *Current Protocols in Molecular Biology* (Ausubel F, Brent R, Kingston R, Moore D, Smith J, Seidman J and Struhl K eds) pp 4.10.1–4.10.11, John Wiley and Sons, New York.
- Griscavage JM, Hobbs AJ and Ignarro LJ (1995) Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. *Adv Pharmacol* 34:215–234.
- Harrison DG and Ohara Y (1995) Physiologic consequences of increased vascular oxidant stresses in hypercholesterolemia and atherosclerosis: Implications for impaired vasomotion. *Am J Cardiol* 75:75B–81B.
- Heitzer T, Just H and Munzel T (1996) Antioxidant vitamin C improves endothelial dysfunction in chronic smokers. *Circulation* 94:6–9.
- Huie RE and Padmaja S (1993) The reaction of NO with superoxide. *Free Radic Res Commun* 18:195–199.
- Keaney JF Jr, Gaziano JM, Xu A, Frei B, Curran-Cellentano J, Shwaery GT, Loscalzo J and Vita JA (1993) Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. *Proc Natl Acad Sci USA* 90:11880–11884.
- Keaney JF Jr, Gaziano JM, Xu A, Frei B, Curran-Cellentano J, Shwaery GT, Loscalzo J and Vita JA (1994) Low-dose  $\alpha$ -tocopherol improves and high-dose  $\alpha$ -tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. *J Clin Invest* 93:844–851.
- Keaney JF Jr and Vita JA (1995) Atherosclerosis, oxidative stress, and antioxidant protection in endothelium-derived relaxing factor action. *Prog Cardiovasc Dis* 38:129–154.
- Khan BV, Harrison DG, Olbrych MT, Alexander RW and Medford RM (1996) Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc Natl Acad Sci USA* 93:9114–9119.
- Laursen JB, Rajagopalan S, Galis Z, Tarpey M, Freeman BA and Harrison DG (1997) Role of superoxide in angiotensin II-induced but not catecholamine-induced hypertension. *Circulation* 95:588–593.
- Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF and Vita JA (1996) Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circulation* 93:1107–1113.
- Lundberg WO, Halvorsen HO and Burr GO (1944) The antioxidant properties of nordihydroguaiaretic acid. *Oil Soap (Chicago)* 21:33–35.
- Moncada S and Higgs A (1993) The L-arginine-nitric oxide pathway. *N Engl J Med* 329:2002–2012.
- Mugge A, Peterson T and Harrison DG (1991) Release of nitrogen oxides from cultured bovine aortic endothelial cells is not impaired by calcium channel antagonists. *Circulation* 83:1404–1409.
- Palmer HJ and Paulson KE (1997) Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr Rev* 55:353–361.
- Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman B, Griendling K and Harrison D (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: Contribution to alterations of vasomotor tone. *J Clin Invest* 97:1916–1923.
- Ramasamy S, Parthasarathy S and Harrison DG (1998) Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J Lipid Res* 39:268–276.
- Reddy RG, Yoshimoto T, Yamamoto S, Funk CD and Marnett LJ (1994) Expression of porcine leukocyte 12-lipoxygenase in a baculovirus/insect cell system and its characterization. *Arch Biochem Biophys* 312:219–226.
- Rushmore TH, Morton MR and Pickett CB (1991) The antioxidant responsive element: Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* 266:11632–11639.
- Santanam N and Parthasarathy S (1995) Paradoxical actions of antioxidants in the oxidation of low density lipoprotein by peroxidases. *J Clin Invest* 95:2594–2600.
- Sen CK and Packer L (1996) Antioxidant and redox regulation of gene transcription. *FASEB J* 10:709–720.
- Sgargali GP, Valoti M, Gorelli B, Fusi F, Palmi M and Mantovani P (1993) Calcium antagonist and antioxidant properties of some hindered phenols. *Br J Pharmacol* 110:369–377.
- Simon BC, Haudenschild CC and Cohen RA (1993) Preservation of endothelium-dependent relaxation in atherosclerotic rabbit aorta by probucol. *J Cardiovasc Pharmacol* 21:893–901.
- Solzbach U, Hornig B, Jeserich M and Just H (1997) Vitamin C improves endothelial dysfunction of epicardial coronary arteries in hypertensive patients. *Circulation* 96:1513–1519.
- Storek M and Pohl J (1997) Indol-2-carboxylic acid: A new matrix for MALDITOF mass spectrometry. Czech Chemical Society Meeting, Zlin. Abstract.
- Thomas SR, Neuzil J, Mohr D and Stocker R (1995) Coantioxidants make  $\alpha$ -tocopherol an efficient antioxidant for low density lipoprotein. *Am J Clin Nutr* 62:1357S–1364S.
- Ting HH, Timimi FK, Boles KS, Creager SJ, Ganz P and Creager MA (1996) Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* 97:22–28.
- Ting HH, Timimi FK, Haley EA, Roddy M-A, Ganz P and Creager MA (1997) Vitamin C improves endothelium-dependent vasodilation in forearm resistance vessels of humans with hypercholesterolemia. *Circulation* 95:2617–2622.
- Yoshioka K, Deng T, Cavigelli M and Karin M (1995) Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra expression. *Proc Natl Acad Sci USA* 92:4972–4976.

**Send reprint requests to:** Santhini Ramasamy, Ph.D., Division of Cardiology, Emory University, 1639 Pierce Drive, Atlanta, GA 30322. E-mail: sramasa@emory.edu