Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid

Santhini Ramasamy,[1] Sampath Parthasarathy,* and David G. Harrison

Division of Cardiology, Department of Medicine, and Department of Obstetrics and Gynecology,*
Emory University School of Medicine, 1639 Pierce Drive, Atlanta, GA 30322

Abstract Hypercholesterolemia is associated with impairments in endothelium-dependent vascular relaxations. Paradoxically, endothelial production of nitrogen oxides is increased in early stages of hypercholesterolemia. Prior work has shown that oxidized low density lipoprotein (LDL) has both stimulatory and inhibitory effects on endothelial nitric oxide synthase expression (eNOS) and has focused on lysophosphatidyl choline (LPC) as a component of oxidized LDL which may modulate this effect. Another biologically active component of oxidized LDL is 13-hydroperoxyoctadecadienoic acid (13-HPODE), an oxidized form of linoleic acid. The purpose of this study was to determine the effect of HPODE on the expression of eNOS in bovine aortic endothelial cells (BAECs). Twenty four hour treatment of endothelial cells with HPODE caused a dose-dependent increase in eNOS mRNA levels as assessed by Northern analysis. The time response studies show that HPODE treatment significantly increased eNOS mRNA levels at 12 and 24 h. Concomitant with the increase in eNOS mRNA levels, 20 μm HPODE treatment significantly increased eNOS protein content and enzyme activity. Nuclear run-on studies indicated that the rate of transcription of eNOS gene was significantly elevated 4 h after HPODE treatment when compared to control cultures. Also, actinomycin D studies demonstrated that the half-life of eNOS mRNA was increased from 6 h to 12 h by HPODE treatment. Thus, HPODE-induced up-regulation of eNOS expression is mediated by both transcriptional and posttranscriptional mechanisms. These observations suggest that endothelial cells may attempt to compensate for oxidative injury by increasing expression of eNOS in early stages of hypercholesterolemia.—**Ramasamy, S., S. Parthasarathy, and D. G. Harrison.** Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J. Lipid Res.* 1998. **39:** 268–276.

Supplementary key words HPODE • endothelial cells • nitric oxide synthase • nuclear run-on assay • eNOS mRNA stability

The endothelial cell nitric oxide synthase, (eNOS) plays an important role in modulating vasomotion, inhibiting platelet aggregation and smooth muscle cell growth (for review see ref. 1). While eNOS is constitutively expressed, there is evidence that its level of expression can be modulated by factors such as shear stress (2), hypoxia (3, 4), tumor necrosis factor alpha (5), and oxidized low density lipoprotein (LDL) (6, 7). Functionally, the vascular release of nitric oxide ($NO^{\bullet}$) is abnormal in the setting of hypercholesterolemia and atherosclerosis (8–10). The mechanisms for this abnormality are likely multifactorial; however, studies of vessels from animals with short-term hypercholesterolemia show that endothelial production of nitrogen oxides is not suppressed, but paradoxically increased (11). In this model, treatment with superoxide dismutase can restore the bioactivity of $NO^{\bullet}$, suggesting that at least a portion of the altered bioactivity of nitric oxide is due to degradation by superoxide anions. The finding that vessels from hypercholesterolemic animals release elevated quantities of nitrogen oxides suggests that eNOS expression is increased at least early after onset of hypercholesterolemia.

In keeping with the concept that hypercholesterolemia can affect expression of eNOS, it has been shown that oxidized LDL can increase eNOS mRNA levels in low concentrations and decrease the eNOS expression at high concentrations (6). It is also reported that the decrease in eNOS expression is positively correlated to TBARS content in the oxidized LDL (7). Lysophosphatidyl choline (LPC) in oxidized LDL can increase eNOS expression in human umbilical vein endothelial cells in a dose-dependent fashion (6, 12).

In addition to LPC, another important component

Abbreviations: eNOS, endothelial nitric oxide synthase; LPC, lysophosphatidyl choline; BAECs, bovine aortic endothelial cells; 13-HPODE, 13-hydroperoxyoctadecadienoate; NO, nitric oxide; FCS, fetal calf serum; PBS, phosphate-buffered saline; TBS-T, Tris-buffered saline containing 0.1% Tween; 12(S) HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NDGA, nordihydroguaretic acid; HODE, 13-hydroxy-octadecadienoate; VCAM-1, vascular cell adhesion molecule 1; NFkB, nuclear factor kappa B; AP-1, activator protein-1.

[1]To whom correspondence should be addressed.

of oxidized LDL is 13-HPODE (HPODE; 13-hydroperoxy-octadecadienoate). Several lines of evidence suggest that the major polyunsaturated fatty acid present in LDL is linoleic acid which is converted to oxidized linoleic acid during the atherosclerotic process (13, 14). HPODE has potent biological effects that include the induction of interleukin-1 (15) and VCAM-1 expression (16). We hypothesized that HPODE is a candidate molecule which might mediate the effect of oxidized LDL on increased NO production. One of the mechanisms by which HPODE might increase nitric oxide production is by up-regulating the eNOS gene expression in atherosclerotic vessels. The present study was performed to test this hypothesis using bovine aortic endothelial cells (BAEC(s)). We examined the effect of HPODE on levels of eNOS mRNA, protein and enzyme activity. Also, the transcriptional activity of eNOS gene and eNOS mRNA stability were measured upon exposure to HPODE treatment.

MATERIALS AND METHODS

Cell culture

Endothelial cells were harvested from bovine aortas and cultured in medium (M199; Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT) as described earlier (17). The post-confluent cells between passages 4 and 9 were used for the experiments.

Experimental media

Post-confluent endothelial cells were exposed to M199 + 1% FCS (control) or M199 + 1% FCS supplemented with HPODE (13-hydroperoxyoctadecadienoic acid). The HPODE-containing media were prepared as described earlier (18). Briefly, 2 mm linoleic acid (Sigma) was incubated with 10 μl of soybean lipoxygenase (10,000 U/μl) (Sigma) in 0.2 m potassium borate buffer (pH 8.0) for 10 min at room temperature. The reaction was monitored by measuring the absorbance of an aliquot of reaction mixture at 234 nm. The reaction was stopped by adding 100 μl of 1.3 N HCl and the HPODE was extracted twice with 2.0 ml of hexane. The hexane was evaporated under nitrogen and HPODE was dissolved in ethanol to yield a 1 mm stock. The concentration of HPODE was calculated using the extinction coefficient of 2.52×10^4 m^{-1}. Lipoxygenase-mediated oxidation of linoleic acid generated predominantly 13-HPODE (19). For the dose response study, the cells were exposed to 0 to 20 μm HPODE-containing media for 24 h. The time point experiments were done using 0 or 20 μm HPODE-containing media up to 24 h.

Northern blotting

Northern analysis was performed as previously described (20). The total RNA from endothelial cells treated with HPODE was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH). The RNA (20 μg) was then size-fractionated on a 1.0% agarose/3% formaldehyde gel and transferred to a nitrocellulose membrane overnight. The transferred RNA was immobilized using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) before hybridization. The full length (4.2 kb) radioactive labeled eNOS probe was prepared using a [^{32}P] dCTP (Dupont NEN, Boston, MA), and oligolabeling kit (Pharmacia Biotech, Piscataway, NJ). The membranes were pre-hybridized for 10 min at 68°C using Quick Hyb (Stratagene) and subsequently hybridized in the same solution containing probe (1×10^7 cpm) for 1 h at 68°C. The membranes were then washed twice with 2 × SSC and 0.1% SDS for 20 min at 55°C, and then once with 0.2 × SSC and 0.1% SDS for 20 min at 55°C. The blots were exposed to phosphor screen, and the screens were scanned in PhosphorImager and the radioactivity of the eNOS bands were quantitated using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA). The negatives of the ethidium bromide-stained blots were scanned and the 28s rRNA bands were quantitated using Bio-Rad Imaging Densitometer. The eNOS mRNA levels were corrected for the corresponding 28s band densities.

Nitric oxide synthase activity assay

NO synthase activity was measured by conversion of l-[^{14}C]arginine to l-[^{14}C]citrulline. The post-confluent cells were treated with 0 or 20 μm HPODE-containing media for 24 h. The cells were washed with ice-cold PBS, and harvested by scraping with a rubber policeman. The cells were centrifuged at 400 *g* for 10 min. The cells were then homogenized using sonication with ultrasonic processor, XL (Misonix, Farmingdale, NY) in 100 μl of homogenization buffer (50 mm Tris-HCl, 0.1 mm EDTA, 0.1 mm EGTA, pH 7.5) containing protease inhibitors (1 μm pepstatin A, 2 μm leupeptin, 1 μm bestatin, and 1 mm phenylmethylsulfonyl fluoride). The homogenates (200–400 μg total protein) were then used for NO-synthase activity assay. Each sample (100 μl) was incubated in 50 mm Tris-HCl/0.1 mm EDTA/0.1 mm EGTA buffer, pH (7.5), containing the cofactors (final concentration: 100 nm calmodulin, 2.5 mm $CaCl_2$, 1 mm reduced nicotinamide adenine dinucleotide phosphate (NADPH), 30 μm tetrahydrobiopterin, 1 mm l-citrulline and the substrate

10 μm cold l-arginine combined with l-[^{14}C]arginine (Amersham; specific activity: 304 mCi/mmol) for 15 min at 37°C. The reaction was quenched by addition of 1 ml of cold stop buffer (20 mm HEPES, pH 5.5, containing 2 mm EDTA and 2 mm EGTA). The reaction mix was loaded onto a 10-ml Bio-Rad column containing 1.0 ml of Dowex AG 50WX-8 (Na+ form, Bio-Rad) resin that had been preequilibrated with 8 ml stop buffer. The eluate containing the product l-[^{14}C]citrulline was collected in a scintillation vial. The column was washed twice with 0.5 ml of stop buffer and the eluates were combined. The radioactivity in the eluate was determined using LS6500 Beckman Liquid Scintillation Counter (Beckman Instruments, Inc., Schaumburg, IL). the protein in the homogenate was determined using Bio-Rad DC reagent (Bio-Rad Laboratories, Hercules, CA).

Western blotting

The homogenates from post-confluent endothelial cells treated with either 0 or 20 μm HPODE-containing media were used for Western analyses. The cells were harvested and the homogenates were prepared as described in NOS assay. Twenty μg of protein was electrophoresed using 7.5% SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane blocked with 5% non-fat dried milk overnight at 4°C in Tris-buffered saline (pH 7.6) containing 0.1% Tween (TBS-T) at room temperature. The membranes were then incubated with a 1:2000 dilution of affinity purified rabbit polyclonal antibody for endothelial NOS, p459. This polyclonal antibody was raised in rabbits against bovine eNOS peptide sequence 628–638 (WRRKRKESSNTC). The membranes were then incubated with a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Amersham Corporation, Arlington Heights, IL). After incubation with each antibody, the membranes were washed 4 times with TBS-T at each step to minimize the background. 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 (21) with modifications. Briefly, endothelial cells were harvested with trypsin and centrifuged at 500 *g* for 10 min. The cell pellets were suspended in buffer A (composition [mmol/L]: 10 Tris-HCl, pH 7.4, 150 KCl, 8 magnesium acetate). After centrifugation at 500 *g* for 10 min, the cells were lysed in buffer A containing 0.5% NP-40. The cell lysates were then layered onto buffer B (composition [mmol/L]: 100 Tris-HCl, 5 $MgCl_2$, 600 sucrose) and the nuclei were isolated by centrifugation at 500 *g* for 10 min. The nuclei were suspended in buffer C (composition: 40% glycerol, 50 mmol/L Tris-HCl, 5 mmol/L $MgCl_2$, and 0.1 mmol/L EDTA) and stored at −80°C until ready for further analysis.

In vitro transcription

Identical numbers of nuclei from cells treated with either 0 or 20 μm of HPODE-containing media for 4 h were used for preparation of nascent transcripts. To perform in vitro transcription, 5×10^7 nuclei were incubated in a reaction buffer [composition: 5 mmol/L Tris-HCl, pH 8.0, 2.5 mmol/L $MgCl_2$, 150 mmol/L KCl, 2 mmol/L each of ATP, GTP, CTP, and 100 μCi of [α-^{32}P]UTP (DuPont NEN)] for 30 min at 30°C. The reaction was stopped by addition of 2000 units of RNase-free DNase (Life Technologies, Inc.) and incubated for 5 min at 30°C. The nuclei were then lysed by the addition of buffer D (composition: 10 mmol/L Tris-HCl, 1% SDS, 5 mmol/L EDTA), and the reaction mixtures were treated with 20 μl of proteinase K (equivalent to 200 μg). RNA was extracted using TRI Reagent (Molecular Research Center Inc., Cincinnati, OH). The 4.2 kb bovine eNOS cDNA was digested from a pBluescript SK+ using EcoR1. The 1.5 kb human β-actin cDNA was restriction digested from a pBluescript SK+ (ATCC) using EcoR1. Equal amounts (5 μg) of linearized, denatured (0.4 mm NaOH and 10 mm EDTA) eNOS, human β-actin cDNA, and 2.9 kb vector, pBluescript+ (Stratagene), were slotblotted onto a Zeta-Probe GT membrane (Bio-Rad) using a Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories). The DNA was immobilized by 0.4 m NaOH treatment. The vector DNA was used to eliminate any nonspecific DNA-nascent transcript interaction. Blots were prehybridized for 3 h at 65°C in a buffer containing 10 mm Tris-HCl, 0.2% SDS, 10 mm EDTA, 2× Denhardts, 0.3 m NaCl and 0.25 mg/ml yeast tRNA. The radiolabeled transcripts (total activity approximately 5×10^6 cpm) were added to the blots and hybridized for 48–72 h at 65°C. Care was taken to assure that identical counts of nascent transcripts for control and HPODE-treated cells were hybridized with the blots. The membranes were washed twice with 2 × SSC and 1%SDS for 15 min at 55°C, and then once with 0.2 × SSC and 0.1% SDS for 30 min at 55°C. The blots were exposed to phosphor screen, and the screens were scanned in PhosphorImager and the radioactivity of the eNOS, β-actin cDNA bands were quantitated using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

eNOS mRNA stability assay

Post-confluent endothelial cells grown in P150 dishes were exposed to 0 or 20 μm HPODE media containing 2.5 μg/ml actinomycin D. The culture media was removed at 0, 6, 12 and 24 h post actinomycin D treat-

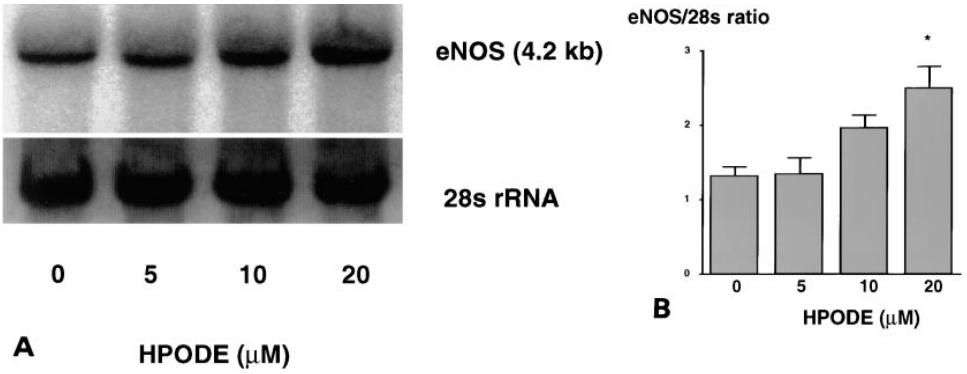

Fig. 1. HPODE treatment causes a dose-dependent increase in eNOS steady state mRNA levels. The endothelial cells were exposed to 0, 5, 10, and 20 μm HPODE for 24 h. The representative blot of Northern analysis of endothelial cells exposed to different doses of HPODE is shown on left side (A). The graphic representation of the average results from four separate experiments after correcting for the RNA loading using ethidium bromide-stained 28s bands is shown in the right side (B). Data are mean ± SEM of four separate experiments. The results are significant at $P < 0.05$ level.

ment. The cell monolayers were lysed in 4.0 ml TRI Reagent and RNA was extracted. Twenty-four μg of total RNA was loaded onto each lane in 1% agarose/3% formaldehyde gel. RNA was transferred overnight into nitrocellulose membrane and hybridized and washed according to the conditions described under Northern blotting.

Statistical analysis

The data in the manuscript are expressed as mean ± SEM. Comparisons of data between control and HPODE treatment were made by paired *t*-tests and where appropriate, by analysis of variance with a Fishers least significant difference post hoc test. Values of $P < 0.05$ were considered significant.

RESULTS

HPODE treatment causes an increase in eNOS steady state mRNA levels

HPODE treatment of bovine aortic endothelial cells caused a dose-dependent increase in eNOS steady state mRNA levels. The endothelial cells were exposed to 0, 5, 10, or 20 μm HPODE for 24 h and eNOS mRNA levels were measured by Northern analysis. **Figure 1A** shows a representative blot of Northern analysis of endothelial cells exposed to HPODE treatment. The eNOS mRNA levels were significantly increased at 20 μm concentrations when compared to 0 μm of HPODE treatment. The radiolabeled eNOS mRNA bands were corrected for loading by the ethidium bromide stained 28 s bands. Figure 1B shows the average results from four separate experiments after correcting for the RNA loading. The eNOS mRNA levels were elevated more than 2-fold by 20 μm HPODE treatment.

Figure 2A shows a representative Northern blot for the time response effect on eNOS gene expression in bovine aortic endothelial cells exposed to 20 μm HPODE-containing media. Both control as well as HPODE-treated cells were harvested at 0, 6, 12, and 24 h. The eNOS mRNA levels were corrected for loading using ethidium bromide-stained 28s rRNA bands. The average results from four separate experiments were combined and plotted in Fig. 2B. HPODE treatment caused a significant increase in eNOS gene expression at 12 and 24 h.

HPODE exposure increases eNOS protein content

Treatment of bovine aortic endothelial cells with 20 μm HPODE also significantly increases eNOS protein content by Western analysis (**Fig. 3A**). Densitometric assessment of three different blots reveal that eNOS protein increased (47%) from 3.80 units in control cultures to 5.59 units in HPODE-treated cells (Fig. 3B).

HPODE treatment elevates eNOS activity

In accordance with eNOS protein content, the activity of the enzyme also increased in bovine aortic endothelial cells treated with 20 μm HPODE for 24 h (**Fig. 4**). The eNOS activity was measured by the conversion of ^{14}C-labeled arginine to ^{14}C-labeled citrulline. The activity of the enzyme significantly increased (57%) from 9.64 to 15.14 picomoles of citrulline per mg protein per min.

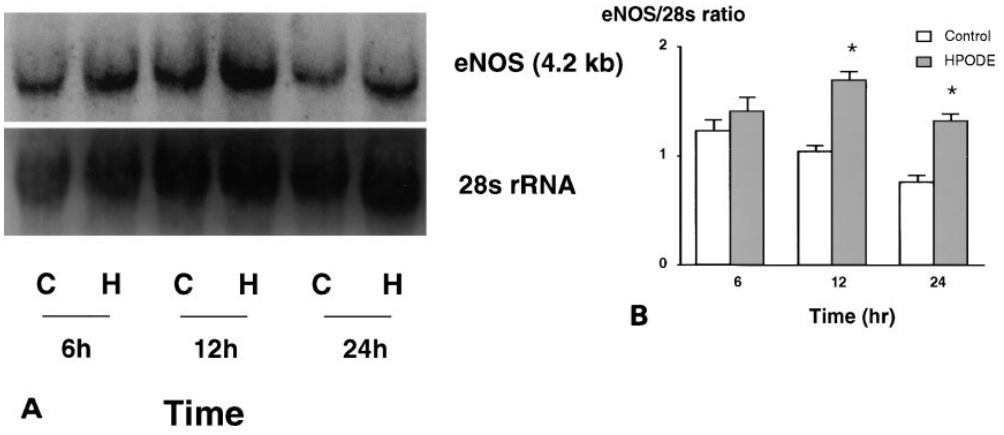

Fig. 2. HPODE treatment causes a time-dependent increase in eNOS steady state mRNA levels. The cells were exposed to 0 or 20 μm HPODE for 6, 12, and 24 h. RNA was harvested and analyzed. The representative blot of Northern analysis is shown in left side panel (A). The graphic representation of the average results from four separate experiments after correcting for the RNA loading using ethidium bromide-stained 28s bands is shown in the right side panel (B). Data are mean ± SEM of four separate experiments. The results are significant at $P < 0.05$ level.

HPODE increases the transcription rate of eNOS gene

The 2-fold increase in steady state eNOS mRNA levels upon HPODE treatment could be due to either an elevated transcription rate or to decreased degradation of eNOS mRNA in the endothelial cultures. The rate of transcription was studied using a nuclear run-on assay. Four-hour exposure of bovine aortic endothelial cells to HPODE caused a significant increase in eNOS transcription rate (**Fig. 5A**). The results also indicate the transcription rate of β-actin remained unchanged in HPODE-treated cells. The blots had negligible background as could be seen from the absence of hybridization of the ^{32}P-labeled nascent transcripts to nonspecific plasmid DNA. Figure 5B shows the average results from three different experiments. The ratio of eNOS/β-actin transcription rate was significantly increased (36%) in cells treated with 20 μm of HPODE.

HPODE exposure increases the eNOS mRNA stability

The degradation rate of eNOS mRNA was studied in bovine aortic endothelial cells after stopping the transcription with actinomycin D. The cells were exposed to 0 or 20 μm HPODE-containing media up to 24 h in the presence of actinomycin D (2.5 μl/ml). The cells

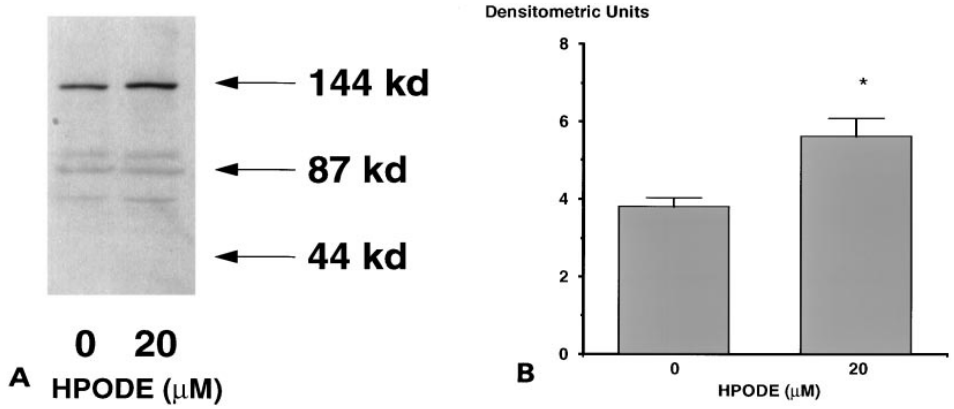

Fig. 3. HPODE treatment elevates eNOS protein content. Western analysis of eNOS protein in endothelial cells exposed to 0 or 20 μm HPODE. eNOS protein (135 kD) bands are shown just below the 144 kD molecular mass marker (A). Densitometric assessment of three different blots are shown in the right side panel (B). Data are mean ± SEM of three separate experiments. The results are significant at $P < 0.05$ level.

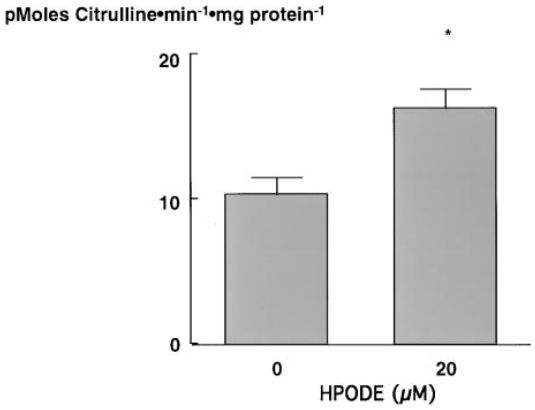

Fig. 4. Effect of HPODE on eNOS enzyme activity. Bar graph shows the eNOS activity measured in cells treated with 0 or 20 μm HPODE-containing media. The specific activity of the enzyme was measured by the conversion of ^{14}C-labeled arginine to ^{14}C-labeled citrulline. The results are expressed in picomoles of citrulline formed per mg protein per minute. Data are mean ± SEM of five separate experiments. The results are significant at $P < 0.05$ level.

were harvested at 0, 6, 12, and 24 h. **Figure 6A** shows a representative Northern blot analysis of three separate experiments. The half-life of eNOS mRNA in control cultures is 6 h and it increased to 12 h after HPODE treatment (Fig. 6B).

DISCUSSION

The results from the present study suggest that HPODE treatment in bovine aortic endothelial cells causes a dose- and time-dependent increase in eNOS mRNA levels. The nuclear run-on and actinomycin D studies reveal that the increase in eNOS mRNA is due to both increased transcription as well as increase in mRNA stability. In accordance with an increase in eNOS mRNA levels, eNOS protein and enzyme activity were increased upon HPODE treatment. Recent studies have shown that hypercholesterolemia increases endothelial cell $^{\bullet}O_2^-$ production, which contributes to inactivation of locally produced nitric oxide (22). Likewise, oxidized LDL has been shown to directly inactivate nitric oxide (23), and LDL has been shown to interfere with signaling events leading to nitric oxide production (24). Thus, an increase in eNOS expression in response to HPODE may be a compensatory mechanism for maintaining the release of nitric oxide in the setting of early hypercholesterolemia. The elevation in HPODE-induced NO production might explain, in part, the increased production of nitric oxide observed in vessels of atherosclerotic animals (11).

Nuclear run-on assays indicated that 4 h of HPODE treatment significantly increased the rate of transcription for eNOS in bovine aortic endothelial cells. Oxidized fatty acids have been shown to alter expression of other genes. For example, oxidized arachidonic acid, hydroxyeicosatetraenoic acid (12(S)-HETE) enhances transcription of integrin αv in lung vascular endothelial cells (25). Transcription rate of the heat-shock protein is increased in HeLa cells by arachidonic acid (26). In addition, 12(R)-HETE increases NFkB binding in rabbit endothelial cells (27). While there are no classical NFkB binding sites in the promoter of eNOS, there are GAGACC sequences that have been shown to bind

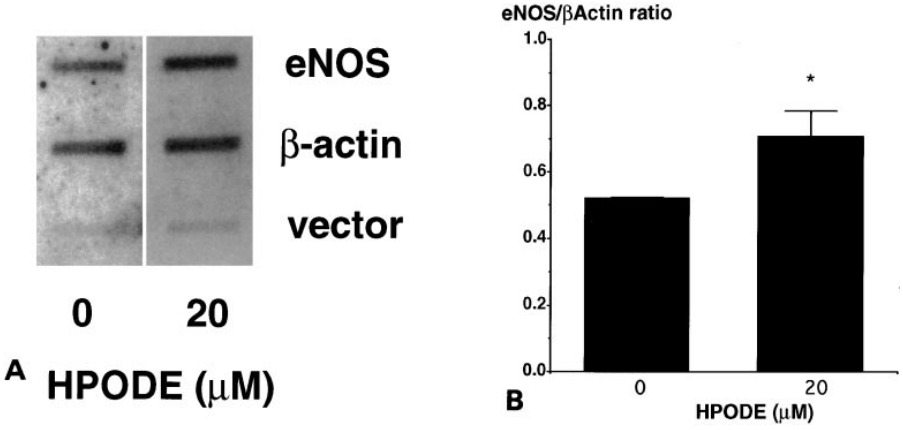

Fig. 5. Effect of HPODE on the transcription rate of eNOS gene by nuclear run-on analysis. Bovine aortic endothelial cells were exposed to HPODE for 4 h and the nascent transcripts were elongated using ^{32}P-labeled UTP. Equal counts of labeled transcripts from 0 to 20 μm HPODE-treated cells were hybridized with blots containing 2 μg each of eNOS, β-actin, and plasmid DNA. A representative blot from nuclear run-on experiment done is shown in panel A. The average ratios of eNOS to β-actin from three different experiments are shown in the bar graph (B).

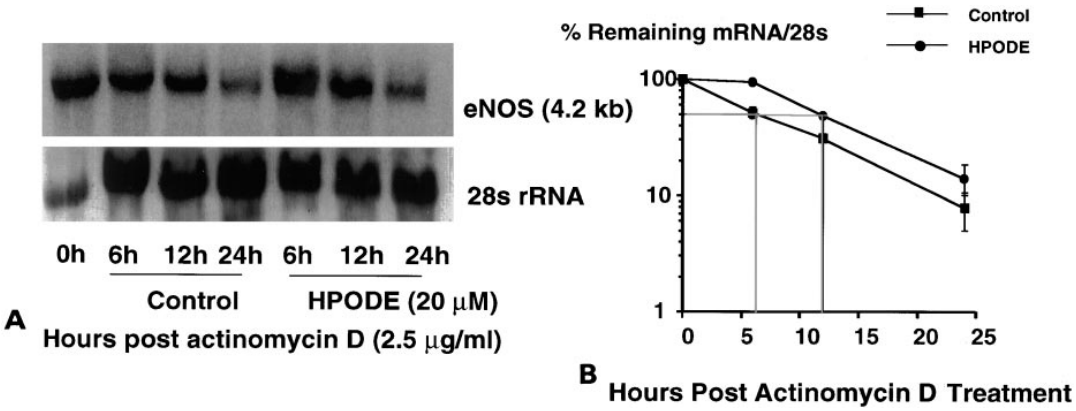

Fig. 6. Effect of HPODE on the stability of eNOS message. Northern blot analysis, measuring the effect of HPODE on the half-life of eNOS mRNA after actinomycin D (2.5 μg/ml) treatment (A). The cells were exposed to 0 or 20 μm HPODE-containing media in the presence of actinomycin D. The cells were harvested at 0, 6, 12, and 24 h for Northern analysis. The average results from three separate experiments are shown in the right side graph (B). Data are mean ± SEM of three separate experiments.

NFkB in response to acute shear in other genes (28). The promoter regions of the bovine and human eNOS contain AP-1 binding sites (29). Recently, Ares et al. (30) reported that when human smooth muscle cells were exposed to either oxidized LDL or lysophosphatidyl choline, AP-1 binding activity increased in a dose-dependent manner. Likewise, the oxidized derivative of arachidonic acid, hydroperoxyeicosatetraenoic acid (HPETE), has been shown to increase AP-1 binding activity in vascular smooth muscle cells (31). Arachidonic acid and its metabolites have been shown to increase expression of c-fos and c-jun in endothelial cells (27), stromal cells (32), and fibroblasts (33, 34). It is therefore conceivable that the increase in eNOS transcription upon exposure to HPODE might be modulated by AP-1. It is important to note that, in preliminary experiments, we found that unoxidized linoleic acid also increased eNOS mRNA levels, but at concentrations four times higher than required for HPODE (data not shown).

In addition to transcriptional regulation, post-transcriptional mechanisms have also been shown to modulate eNOS expression. The basal half-life for eNOS message in our study was found to be 6 h and it increased to 12 h after HPODE treatment. This increase in eNOS mRNA half-life after HPODE treatment appeared to be due to slow degradation of eNOS message in the first 6 h post actinomycin D/HPODE treatment. The eNOS mRNA degradation rate subsequent to 6 h appeared similar between control and HPODE-treated cells. The cause of biphasic pattern of eNOS mRNA decay after HPODE treatment is not clear at this time, although it could be related to the stability of HPODE or the metabolites in the culture media or the cells. Nevertheless, the overall increase in the half-life of eNOS message upon HPODE treatment could contribute to increased steady state mRNA levels. The eNOS mRNA half-life reported in other endothelial cell types varies from 24 to 36 h (3, 5, 7). The reason for the discrepancies in the basal half-life for eNOS between our study and other studies is not clear; however, it might relate to the growth sate of cells studied. Data from our laboratory (unpublished results) have shown that eNOS mRNA in proliferating cells has a substantially longer half-life as compared to confluent cells. In the present studies, we used only confluent cells. There is precedent for fatty acid modulation of mRNA half-life. Metabolites of arachidonic acid have been shown to increase mRNA half-life of the macrophage-specific colony stimulating factor (35).

Interestingly, exposure of endothelial cells to HPODE can have beneficial effects. For example, the hydroxy-derivative of HPODE, HODE inhibits platelet aggregation (36, 37) and production of thromboxane (38). In addition, HPODE has been shown to stimulate prostacyclin synthesis by the endothelium, which may have antiatherogenic effects (39). On the other hand, HPODE treatment could cause pro-atherogenic effects. Exposure of endothelium to HPODE can compromise the endothelial barrier function (18, 40). Similarly, HPODE treatment of human vascular endothelial cells augments TNF-induced expression of VCAM 1 (41), contributing to inflammatory properties in the vascular wall. The present data provide another mechanism whereby HPODE could modulate the atherosclerotic process. Nitric oxide inhibits platelet aggregation, leukocyte adhesion, vascular smooth muscle growth, and the expression of inflammatory molecules (1, 16). It is therefore conceivable that an increase in eNOS expression in response to HPODE could exert antiatheroscle-

rotic effects via these mechanisms, in part offsetting the pro-atherosclerotic processes caused by HPODE.

This work was supported by NIH grants HL390006, HL48667.

Manuscript received 29 July 1997 and in revised form 15 October 1997.

REFERENCES

1. Forstermann, U., E. I. Closs, J. S. Pollock, M. Nakane, P. Schwarz, I. Gath, and H. Kleinert. 1994. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension*. **23:** 1121–1131.
2. Nishida, K., D. G. Harrison, J. P. Navas, A. A. Fisher, S. P. Dockery, M. Uematsu, R. M. Nerem, R. W. Alexander, and T. J. Murphy. 1992. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. *J. Clin. Invest.* **90:** 2092–2096.
3. McQuillan, L. P., G. K. Leung, P. A. Marsden, S. K. Kostyk, and S. Kourembanas. 1994. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. *Am. J. Physiol.* **267:** H1921–H1927.
4. Arnet, U. A., A. McMillan, J. L. Dinerman, B. Ballermann, and C. J. Lowenstein. 1996. Regulation of endothelial nitric-oxide synthase during hypoxia. *J. Biol. Chem.* **271:** 15069–15073.
5. Yoshizumi, M., M. A. Perrella, J. C. Burnett, and M-E. Lee. 1993. Tumor necrosis factor down-regulates an endothelial nitric oxide synthase mRNA by shortening its half-life. *Circ. Res.* **73:** 205–209.
6. Hirata, K., N. Miki, Y. Kuroda, T. Sakoda, S. Kawashima, and M. Yokoyama. 1995. Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine up-regulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *Circ. Res.* **76:** 958–962.
7. Liao, J. K., W. S. Shin, W. Y. Lee, and S. L. Clark. 1995. Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase. *J. Biol. Chem.* **270:** 319–324.
8. Habib, J. B., C. Bossaller, S. Wells, C. Williams, J. D. Morrisett, and P. D. Henry. 1986. Preservation of endothelium-dependent vascular relaxation in cholesterol-fed rabbit by treatment with the calcium blocker PN 200110. *Circ. Res.* **58:** 305–309.
9. Verbeuren, T. J., F. H. Jordaens, L. L. Zonnekeyn, C. E. Van Hove, M. C. Coene, and A. G. Herman. 1986. Effect of hypercholesterolemia on vascular reactivity in the rabbit. *Circ. Res.* **58:** 552–564.
10. Freiman, P. C., G. C. Mitchell, D. D. Heistad, M. L. Armstrong, and D. G. Harrison. 1986. Atherosclerosis impairs endothelium-dependent vascular relaxation to acetylcholine and thrombin in primates. *Circ. Res.* **58:** 783–789.
11. Minor, R. L. J., P. R. Myers, R. J. Guerra, J. N. Bates, and D. G. Harrison. 1990. Diet-induced atherosclerosis increases the release of nitrogen oxides from rabbit aorta. *J. Clin. Invest.* **86:** 2109–2116.
12. Zembowicz, A., J. Tang, and K. K. Wu. 1995. Transcriptional induction of endothelial nitric oxide synthase type III by lysophosphatidylcholine. *J. Biol. Chem.* **270:** 17006–17010.
13. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modification of low-density lipoproteins that increase its atherogenecity. *N. Engl. J. Med.* **320:** 915–924.
14. Thomas, M. J., T. Thornburg, J. Manning, K. Hooper, and L. L. Rudel. 1994. Fatty acid composition of low density lipoprotein influences its susceptibility to autooxidation. *Biochemistry.* **33:** 1828–1834.
15. Thomas, C. E., R. L. Jackson, D. F. Ohlweiler, and G. Ku. 1994. Multiple oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. *J. Lipid Res.* **35:** 417–427.
16. Khan, B. V., D. G. Harrison, M. T. Olbrych, R. W. Alexander, and R. M. Medford. 1996. Nitric oxide regulates VCAM-1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. *Proc. Natl. Acad. Sci. USA.* **93:** 9114–9119.
17. Mügge, A., T. Peterson, and D. Harrison. 1991. Release of nitrogen oxides from cultured bovine aortic endothelial cells is not impaired by calcium channel antagonists. *Circulation*. **83:** 1404–1409.
18. Hennig, B., C. Enoch, and C. K. Chow. 1986. Linoleic acid hydroperoxide increases the transfer of albumin across cultured endothelial monolayers. *Arch. Biochem. Biophys.* **248:** 353–357.
19. Hamberg, M., and B. Samuelsson. 1965. On the specificity of the lipoxidase-catalyzed oxygenation of unsaturated fatty acids. *Biochem. Biophys. Res. Commun*. **21:** 531–536.
20. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
21. Greenberg, M. 1987. Identification of newly transcribed RNA. *In* Current Protocols in Molecular Biology. F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Smith, J. Seidman, and K. Struhl, editors. John Wiley and Sons, New York. 4.10.1–4.10.11.
22. Ohara, Y., T. E. Peterson, and D. G. Harrison. 1993. Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* **91:** 2546–2551.
23. Galle, J., and E. Bassenge. 1991. Effects of native and oxidized low-density lipoproteins on endothelium-dependent and endothelium-independent vasomotion. *Basic Res. Cardiol.* **86:** 127–142.
24. Liao, J. K. 1994. Inhibition of G(i) proteins by low density lipoprotein attenuates bradykinin-stimulated release of endothelial-derived nitric oxide. *J. Biol. Chem.* **269:** 12987–12992.
25. Tang, D. G., C. A. Diglio, R. Bazaz, and K. V. Honn. 1995. Transcriptional activation of endothelial cell integrin αv by protein kinase C activator 12 (S)-HETE. *J. Cell Sci.* **108:** 2629–2644.
26. Jurivich, D. A., L. Sistonen, K. D. Sarge, and R. I. Morimoto. 1994. Arachidonate is a potent modulator of human heat shock gene transcription. *Proc. Natl. Acad. Sci. USA.* **91:** 2280–2284.
27. Laniado-Schwartzman, M., Y. Lavrovsky, R. A. Stoltz, M. S. Conners, J. R. Falck, K. Chauhan, and N. G. Abraham. 1994. Activation of nuclear factor kB and oncogene expression by 12(R)-hydroxyeicosatrienoic acid, an angiogenic factor in microvessel endothelial cells. *J. Biol. Chem.* **269:** 24321–24327.
28. Khachigian, L., N. Resnick, M. Gimbrone, and T. Collins. 1995. Nuclear factor kB interacts functionally with the platelet-derived growth factor B chain shear stress response element in vascular endothelial cells exposed to fluid shear stress. *J. Clin. Invest.* **96:** 1169–1175.

29. Venema, R. C., K. Nishida, R. W. Alexander, D. G. Harrison, and T. J. Murphy. 1994. Organization of the bovine gene encoding the endothelial nitric oxide synthase. *Biochim. Biophys. Acta*. **1218:** 1–8.
30. Ares, M. P., B. Kallin, P. Eriksson, and J. Nilsson. 1995. Oxidized LDL induces transcription factor activator protein-1 but inhibits activation of nuclear factor-kappa B in human vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol*. **15:** 1584–1590.
31. Rao, G. N., W. C. Glasgow, T. E. Eling, and M. S. Runge. 1996. Role of hydroperoxyeicosatetraenoic acids in oxidative stress-induced activating protein (AP-1) activity. *J. Biol. Chem*. **271:** 27760–27764.
32. Rizzo, M. T., H. S. Boswell, L. Mangoni, C. Carlo-Stella, and V. Rizzoli. 1995. Arachidonic acid induces c-jun gene expression in stromal cells stimulated by interleukin-1 and tumor necrosis factor -α: evidence for a tyrosine–kinase-dependent process. *Blood*. **86:** 2967–2975.
33. Danesch, U., P. C. Weber, and A. Sellmayer. 1996. Differential effects of n–6 and n–3 polyunsaturated fatty acids on cell growth and early gene expression in swiss 3T3 fibroblasts. *J. Cell. Physiol*. **168:** 618–624.
34. Sellmayer, A., U. Danesch, and P. C. Weber. 1996. Effects of different polyunsaturated fatty acids on growth-related early gene expression and cell growth. *Lipids*. **31:** S37–S40.
35. Sherman, M. L., B. L. Weber, R. Datta, and D. W. Kufe. 1990. Transcriptional and posttranscriptional regulation of macrophage-specific colony stimulating factor gene expression by tumor necrosis factor. Involvement of arachidonic acid metabolites. *J. Clin. Invest*. **85:** 442–447.
36. Tloti, M. A., D. G. Moon, L. K. Weston, and J. E. Kaplan. 1991. Effect of 13-hydroxyoctadeca-9,11-dienoic acid (13-HODE) on thrombin induced platelet adherence to endothelial cells in vitro. *Thromb. Res*. **62:** 305–317.
37. Buchanan, M. R., M. C. Bertomeu, S. J. Brister, and T. A. Haas. 1991. 13-Hydroxyoctadecadienoic acid (13-HODE) metabolism and endothelial cell adhesion molecule expression: effect on platelet vessel wall adhesion. *Wien Klin. Wochenschr*. **103:** 416–421.
38. Yamaja Setty, B. N., M. Berger, and M. J. Stuart. 1987. 13-Hydroxyoctadeca-9,11-dienoic acid (13-HODE) inhibits thromboxane A2 synthesis, and stimulates 12-HETE production in human platelets. *Biochem. Biophys. Res. Commun*. **148:** 528–533.
39. Yamaja Setty, B. N., M. Berger, and M. J. Stuart. 1987. 13-Hydroxyoctadecadienoic acid (13-HODE) stimulates prostacyclin production by endothelial cells. *Biochem. Biophys. Res. Commun*. **146:** 502–509.
40. Ramasamy, S., D. W. Lipke, G. A. Boissonneault, H. Guo, and B. Hennig. 1996. Oxidized lipid-mediated alterations in proteoglycan metabolism in cultured pulmonary endothelial cells. *Atherosclerosis*. **120:** 199–208.
41. Khan, B. V., R. W. Alexander, and R. M. Medford. 1994. Oxidized low density lipoprotein selectively augments cytokine activated VCAM-1 gene expression in human vascular endothelial cells. *J. Cell. Biochem*. **18A:** 268 (abstract).