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Nitric oxide contributes to 20-HETE-induced relaxation of pulmonary arteries

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Yu, Ming, Ryan P. McAndrew, Rula Al-Saghir, Kristopher G. Maier, Meetha Medhora, Richard J. Roman, and Elizabeth R. Jacobs. Nitric oxide contributes to 20-HETE-induced relaxation of pulmonary arteries. *J Appl Physiol* 93: 1391–1399, 2002. First published June 30, 2002; 10.1152/japplphysiol.00247.2002.—In contrast to its constrictor effects on peripheral arteries, 20-hydroxyeicosatetraenoic acid (20-HETE) is an endothelial-dependent dilator of pulmonary arteries (PAs). The present study examined the hypothesis that the vasodilator effects of 20-HETE in PAs are due to an elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) and the release of nitric oxide (NO) from bovine PA endothelial cells (BPAECs). BPAECs express cytochrome *P*-450 4A (CYP4A) protein and produce 20-HETE. 20-HETE dilated PAs preconstricted with U-46619 or norepinephrine and treated with the cytochrome *P*-450 inhibitor 17-octadecynoic acid and the cyclooxygenase inhibitor indomethacin. The dilator effect of 20-HETE was blocked by the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) or by removal of endothelium. 20-HETE significantly increased $[Ca^{2+}]_i$ and NO production in BPAECs. 20-HETE-induced NO release was blunted by removal of extracellular calcium, as well as NO synthase inhibitors (L-NAME). These results suggest that 20-HETE dilates PAs at least in part by increasing $[Ca^{2+}]_i$ and NO release in BPAECs.

cytochrome *P*-450; calcium transients; pulmonary artery endothelial cells; vasodilation; 20-hydroxyeicosatetraenoic acid

ARACHIDONIC ACID (AA) is metabolized by cytochrome *P*-450 (CYP) enzymes in lung of human, guinea pigs, dog, rabbit, and rat to 20-hydroxyeicosatetraenoic acid (20-HETE) (4, 17, 30–32). A number of CYP isoforms, including CYP4A4, which catalyzes formation of 20-HETE, are dramatically induced in rabbit lungs during pregnancy (22, 23, 28). CYP metabolites of AA modulate a number of important biological functions in other organs, including but not limited to tubuloglomerular feedback, epithelial cell growth, neutrophil adhesion, and regulation of vascular tone in the cerebral, coronary, and renal vascular beds (11, 24). Interestingly, the pulmonary vasoactive effects of 20-HETE appear to be opposite to those of the same metabolite in systemic vessels, decreasing the state of activation of small pulmonary arteries (PAs) (16, 24). Although many explanations might underlie this apparent discrepancy, our observations defining a unique localization of CYP4A to pulmonary vascular endothelial cells (33) suggested that 20-HETE might play a novel role in the lung-regulating release of one or more endothelium-derived vasodilators, such as nitric oxide (NO), prostacyclin, or epoxyeicosatrienoic acids. In addition to strategic localization in endothelial cells, this hypothesis is supported by our recent observations that 20-HETE-induced dilation of human PAs is blocked by removal of the endothelium (4). Thus the purpose of these investigations was to examine the contribution of NO (18) to 20-HETE-evoked relaxation of PAs and to begin to explore the signaling pathways that connect endothelial cell 20-HETE synthesis and relaxation of subjacent vascular smooth muscle cells. In these studies, we report concentration-dependent inhibition of 20-HETE-induced relaxation of bovine PAs by inhibition of NO synthase (NOS) or by removal of endothelium. In addition, we observed 20-HETE-evoked calcium transients and NO release in bovine PA endothelial cells (BPAECs). Together, these data suggest that 20-HETE affects relaxation of PAs through increases in endothelial cell intracellular calcium concentration ($[Ca^{2+}]_i$) and NOS activity, mechanisms of vasoaction that are clearly distinct from those operative in systemic arteries.

METHODS

Cell culture. BPAECs were prepared from PAs harvested from a local abattoir (14). PAs were opened longitudinally under a laminar flow hood, and a sterile blade was used to scrape cells from the luminal surface. Cells were placed into a solution of RPMI-1640 containing 20% serum, 1% penicillin/streptomycin, and 0.5 mg/ml collagenase (Sigma C-8051, 5 ml per vessel). Cells were incubated in the collagenase solution at 25°C for ~10 min after scraping, vortexed vigorously, and then centrifuged at 100 *g* for 10 min. The supernatant was discarded, and the pellet was rinsed twice with 10 ml Hanks' balanced salt solution (Sigma Chemical, H-9269) with 1% penicillin/streptomycin. Finally the cells were resuspended in RPMI-1640 with 20% serum and 1% penicillin/

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streptomycin and plated in appropriate containers. After 48–72 h, the medium was replaced to remove nonadherent cell types and debris, and the cells cultured in media without antibiotics from this point on. Cells were cultured on glass coverslips in 35-mm dishes; all experiments were performed on cells after one to four passages. Representative cells stained with platelet endothelial cell adhesion molecule or acetylated low-density lipoprotein revealed that ~90% of the cells were endothelial in origin. In all of the following experiments with 20-HETE, cells were pretreated with indomethacin (2 μM) and 17-octadecynoic acid (17-ODYA; 10 μM) to block the endogenous synthesis and metabolism of prostanoids and 20-HETE, respectively, unless otherwise stated.

Isolation of PA vascular smooth muscle cells. Vessels of the desired size (~1 mm) were harvested and dissected free of adventitial tissue and were treated with collagenase and trypsin to dissociate individual vascular smooth muscle cells (8). This procedure involved incubation of dissected vessels with Worthington collagenase (0.4 mg/ml), dithiothreitol (0.3 mg/ml), and trypsin inhibitor (0.4 mg/ml) for 30–60 min at 37°C. Supernatant fractions were collected at 5-min intervals and diluted with cold buffered saline solution containing 1.8 mM $CaCl_2$, starting at 30 min. The remaining vessel was incubated with fresh enzyme solution again, and the process was repeated. The first two fractions were discarded because they might be contaminated with endothelial cells. Fractions were inspected under a microscope to ensure morphology of smooth muscle cells and were pooled for Western analysis as well as measurement of endogenous 20-HETE.

Western blot identification of CYP4A protein. Lysates of BPAECs, endothelial cells freshly scraped from medium-size PAs (10–50 mm diameter) or dispersed PA vascular smooth muscle cells were prepared, and the concentration of protein was measured according to the method of Bradford (5). The proteins were separated by electrophoresis on 10% SDS-PAGE and were transferred to nitrocellulose membranes (31, 32). Nonspecific binding was blocked by incubating the membranes for 2 h in Tris-buffered saline containing 0.05% Tween-20 plus 5% nonfat milk. Membranes were incubated with a polyclonal antibody raised against rat liver CYP4A1 purchased from Gentest, which cross-reacts with CYP4A8, -4A2, and -4A3 isoforms (13). Finally, after reaction with horseradish peroxidase-labeled secondary antibodies (1:1,000), membranes were visualized by use of enhanced chemiluminescence.

Radioactive detection of AA metabolites in BPAECs. First passage BPAECs cultured in 100-mm dishes were washed three times, and microsomes from isolated cells were prepared by differential centrifugation in a modification of the method previously reported (4, 31). Cells were washed in isotonic saline and were resuspended in hypotonic buffer before sequential centrifugations at 9,000 *g* (10 min) and 100,000 *g* (1.5 h) to generate a microsomal pellet. Protein was quantified according to the method of Bradford (5). Microsomes were separately resuspended in assay buffer (100 mM KPO_4, 1 mM EDTA, and 10 mM $MgCl_2$, pH 7.7) and incubated at a protein concentration of 1 mg/ml (~200 μl final volume) at 37°C with [1-^{14}C]AA (0.5 μCi/ml; 20 μM), nicotinamide-adenine dinucleotide phosphate (NADPH; 1 mM), and a NADPH-regenerating system containing isocitrate and isocitrate dehydrogenase for 30 min (4, 31). Reactions were terminated by acidification with formic acid, and the products were extracted with ethyl acetate. The organic phase was back-extracted with distilled water, evaporated under nitrogen, and reconstituted in ethanol. Reaction products were separated on a C-18 reverse-phase HPLC column (Supelco, Bellefonte, PA) by using a linear gradient ranging from 100% *solvent A* (acetonitrile-water-acetic acid, 30:70:1) to 100% *solvent B* (acetonitrile-acetic acid, 100:1) over 40 min. ^{14}C-labeled products were detected by using a flow-through liquid scintillation cell (HPLC, Beckman System gold programmable detector module no. 171). Identification of metabolites was based on coelution with authentic standards. The amount of 20-HETE in the sample was calculated by comparing the area of the 20-HETE peak with that of known amount of the substrate AA on the same chromatogram.

Fluorescent measurements of 20-HETE concentrations. These assays were performed according to the methods our laboratory has previously published (20). 20-5(2), 14(2)-hydroxyeicosadienoic acid (WIT002) was added as an internal standard to samples, and the lipids were extracted from cell lysates using chloroform-methanol (2:1) and labeled with 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate. Metabolites of AA were separated on a C18 reverse-phase HPLC column. The 20-HETE peak could be separated from those for dihydroxyeicosatrienoic acids and other HETEs. Levels of epoxyeicosatrienoic acids and 20-HETE could be detected from as little as 0.1 mg of tissue.

Tension measurements. Briefly, cow lungs were obtained from a local abattoir, and PA rings ~1 mm in diameter were dissected free in ice-cold buffered saline solution (31). Indomethacin (2 μM) and 17-ODYA (10 μM) were added to the bathing solutions throughout the experiment to block the endogenous synthesis and metabolism of prostanoids and 20-HETE, respectively, unless otherwise specified. The rings were mounted on tungsten wire, one connected to a fixed holder and other to a force displacement transducer (Model FT03E, Grass Instruments) for continuously measuring isometric tension, and immersed in pH-adjusted, oxygenated physiological salt solution (PSS) at 37°C (15, 31, 32). Tension data were relayed from transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data were acquired and analyzed by using CODAS software (DataQ Instruments). Rings were preloaded with 0.5 g of passive tension and then equilibrated for an additional 30 min before the studies were begun. Viability of the rings was determined by measuring the contractile response to the addition of 25 mM KCl to the bath. We examined 20-HETE-induced relaxation of rings preconstricted with U-46619 (10^{-8} M) or norepinephrine (10^{-6} M) after pretreatment with vehicle or blockers/inhibitors of NOS or removal of endothelium as detailed in the experimental protocol.

Calcium measurements. These experiments examined the effect of 20-HETE on $[Ca^{2+}]_i$ in cultured BPAECs. $[Ca^{2+}]_i$ was measured after loading cells with 5 μM of fura 2-acetoxymethyl ester (fura 2-AM; Molecular Probes, Eugene, OR.) in culture media for 45 min at 37°C in a cell culture incubator. After loading, cells were transferred to a 1-ml perfusion chamber mounted on an inverted microscope and washed with PSS containing (in mM): 119 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4$, 12 $NaHCO_3$, 1.18 NaH_2PO_4, 0.03 EDTA, 10 glucose, and 10 HEPES. The cells were maintained in a dark area for 15 min at 37°C. $[Ca^{2+}]_i$ was measured by use of an InCyto Im2 imaging system (Intracellular Imaging, Cincinnati, OH) mounted on an inverted microscope (IMT-2, Olympus Optical, Tokyo, Japan). The cells were visualized by using a ×40 ultraviolet long working distance fluorescence objective. $[Ca^{2+}]_i$ were calculated on the basis of the fluorescence intensity ratios obtained by using excitation (340 and 380 nm) and emission (510 nm) wavelengths and a standard curve generated by measurements obtained from solutions with known calcium concentrations. After a 2-min control

period, 20-HETE (1 μM final concentration) or vehicle (ethanol) was added to the chamber, and the recording was continued for another 15 min. In a separate group of experiments, U-46619 (10^{-8} M) was added to the bath after a 2-min control period. Ten minutes later, 20-HETE (1 μM) was added to the bath to more closely follow the protocol of the ring tension experiments.

NO measurements. These experiments examined whether 20-HETE alters the production of NO in cultured BPAECs and whether the release of NO is dependent on elevations of $[Ca^{2+}]_i$ and inhibitable by NOS inhibitors. The release of NO was measured by loading the cells with a fluorescent NO indicator, 4-amino-5 methylamino-2′,7′-difluorofluorescein diacetate (10 μM, Molecular Probes), and a NO substrate, L-arginine (1 mM), in PSS for 30 min at 37°C. After loading, cells were transferred to the recording chamber and washed with PSS for 15 min. L-Arginine (1 mM) was added before the recording was started. Fluorescence intensity was recorded in the same imaging system as $[Ca^{2+}]_i$, except for employing single excitation and emission wavelengths of 475 and 510 nm, respectively.

Experiments were performed in four groups of cells. In *group 1*, baseline fluorescence was measured for 15 min, then vehicle was added to the bath, and DAF fluorescence was measured for an additional 15 min. Subsequently, 20-HETE (1 μM) was added to the bath, and DAF fluorescence was measured for another 15 min. In *group 2*, the orders of the treatments (first 20-HETE, then vehicle) were reversed. Cells in *group 3* were pretreated with N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 mM) or N^{ω}-nitro-L-arginine (L-NNA, 1 mM) in the recording chamber. After a 15-min period of measuring basal NO production, 20-HETE (1 μM) was delivered to the bath, and fluorescence was measured for another 15 min. The concentrations of L-NAME and L-NNA were chosen on the basis of preliminary studies, which demonstrated that treatment with lower concentrations of these compounds blunted but did not eliminate the increase in NO signal in response to known agonists such as A-23187. In *group 4*, cells were bathed in calcium-free PSS and pretreated with the intracellular calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM, 10 μM). After establishment of baseline NO production for 15 min, 20-HETE (1 μM) was added to the bath, and DAF fluorescence signals were tracked for an additional 15 min.

Statistical methods. Data are presented as means ± SE. When more than one test condition was being compared with control, e.g., tensions of PAs at baseline and after treatments with agents such as U-46619, U-46619 followed by 20-HETE, or vehicle, differences were assessed by using one-way analysis of variance for repeated measures followed by a Student-Newman-Keuls test when significant differences were identified. Differences between concentrations of calcium or NO at baseline and after addition of 20-HETE or vehicle to the bath were compared by using a two-tailed paired *t*-test (one control and one test condition in the same cell). Differences in relaxation of PAs to 20-HETE pretreated with 17-ODYA and indomethacin or those treated with only 17-ODYA were compared with two-tailed unpaired *t*-test. A P value of <0.05 was considered significant.

RESULTS

BPAECs express CYP4A and synthesize 20-HETE. The results from Western blot experiments are presented in Fig. 1*A*. BPAEC lysates exhibit a band of the expected size (~55 kDa) when probed with a primary antibody raised against rat liver CYP4A. To verify that CYP4A is expressed in noncultured endothelial cells, and to compare relative protein density in endothelial vs. vascular smooth muscle cells, we performed Western blots comparing CYP4A protein in lysates of freshly scraped endothelial cells to that in freshly dissociated bovine PA vascular smooth muscle cells (Fig. 1*B*). Both freshly scraped endothelial cells and early-passage BPAECs express CYP4A. The density of CYP4A protein in native endothelial cells is greater than that of dispersed vascular smooth muscle (see normalized density values in the legend for Fig. 1*B*). BPAECs incubated with [^{14}C]AA in the presence of an NADPH-regenerating system (see METHODS) convert substrate into a product that coelutes with authentic

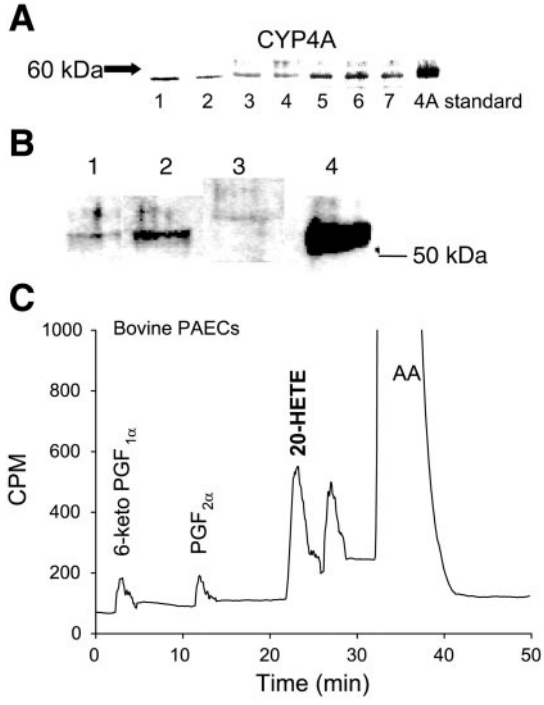

Fig. 1. *A*: expression of cytochrome *P*-450 4A (CYP4A) protein in bovine pulmonary artery endothelial cells (BPAECs; Bovine PAECs). *Lanes 1–7* were loaded with lysates from 7 isolations of BPAECs (*passage 1–4*) containing 50 μg protein/lane. *Lane 8* was loaded with the CYP4A standard. Immunospecific CYP4A protein at ~55 kDa is noted in all test lanes. *B*: comparison of CYP4A protein in lysates of freshly scraped BPAECs to that of lysates of dispersed bovine pulmonary arterial vascular smooth muscle cells from pulmonary arteries ~1–2 mm in diameter. *Lane 1*, BPAEC (5 μg protein); *lane 2*, BPAEC (10 μg protein); *lane 3*, bovine pulmonary arterial smooth muscle cells (7 μg protein); *lane 4*, male liver microsomes (positive control). Densitometric values were quantitated and then normalized for the protein in each sample. Results were *lane 1* = 785 relative density units (rdu)/μg; *lane 2* = 763 rdu/μg, and *lane 3* = 133 rdu/μg, showing that there is more CYP4A protein in BPAECs than bovine pulmonary arterial vascular smooth muscle cells. *C*: representative reverse-phase chromatogram of metabolites of [^{14}C]arachidonic acid (AA) from BPAECs, with a peak at ~23 min, identical to that of authentic 20-hydroxyeicosatetraenoic acid (20-HETE) standard. CPM, counts per minute.

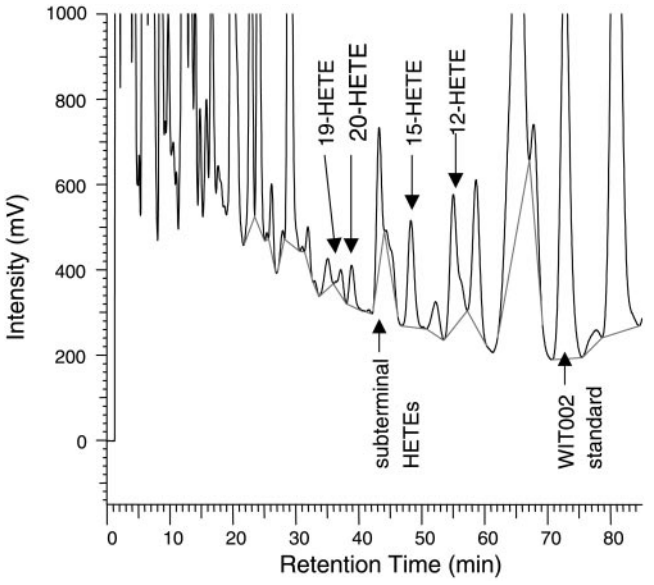

Fig. 2. Representative chromatogram of fluorescently labeled endogenous lipids in BPAECs. A peak around 39 min consistent with elution time of authentic 20-HETE can be clearly seen. Other peaks, including 15- and 19-HETE, and subterminal HETEs, are labeled on the basis of coelution with authentic standards. WIT002, 20-5(2), 14(2)-hydroxyeicosadienoic acid.

20-HETE (Fig. 1*C*). These data demonstrate that BPAECs have the enzymes to synthesize 20-HETE in vitro.

In addition, we investigated the capacity of cultured BPAECs to produce 20-HETE by measuring endogenous 20-HETE in cell cultures. Lipids were extracted from early-passage BPAECs and identified by using a fluorescent assay (see METHODS). A representative chromatogram is presented in Fig. 2. We found 6.1 ± 0.9 ng 20-HETE/mg protein in these cells (n = 4). In addition, we also studied human microvascular PA endothelial cells and found ~3 ng 20-HETE/mg protein (n = 3 isolations). Together, these data provide good evidence that CYP4A is expressed in BPAECs and that 20-HETE is a product of intact bovine and human pulmonary microvascular endothelial cells.

Effects of 20-HETE on bovine PA rings. Bovine PA rings preconstricted with U-46619 relaxed to 20-HETE (see representative tracing in Fig. 3*A*). Relaxation was concentration dependent; 10^{-7} M 20-HETE decreased tension to 94 ± 3% U-46619 baseline (n = 4) compared with 68 ± 4% U-46619 baseline (n = 16) after 10^{-6} M 20-HETE. Furthermore, the 20-HETE-induced relaxation of rings pretreated only with 17-ODYA and not indomethacin (Fig. 3*C*) was modestly increased over that of rings studied in the presence of cyclooxygenase inhibition (58 ± 3 vs. 68 ± 4% precontracted tension,

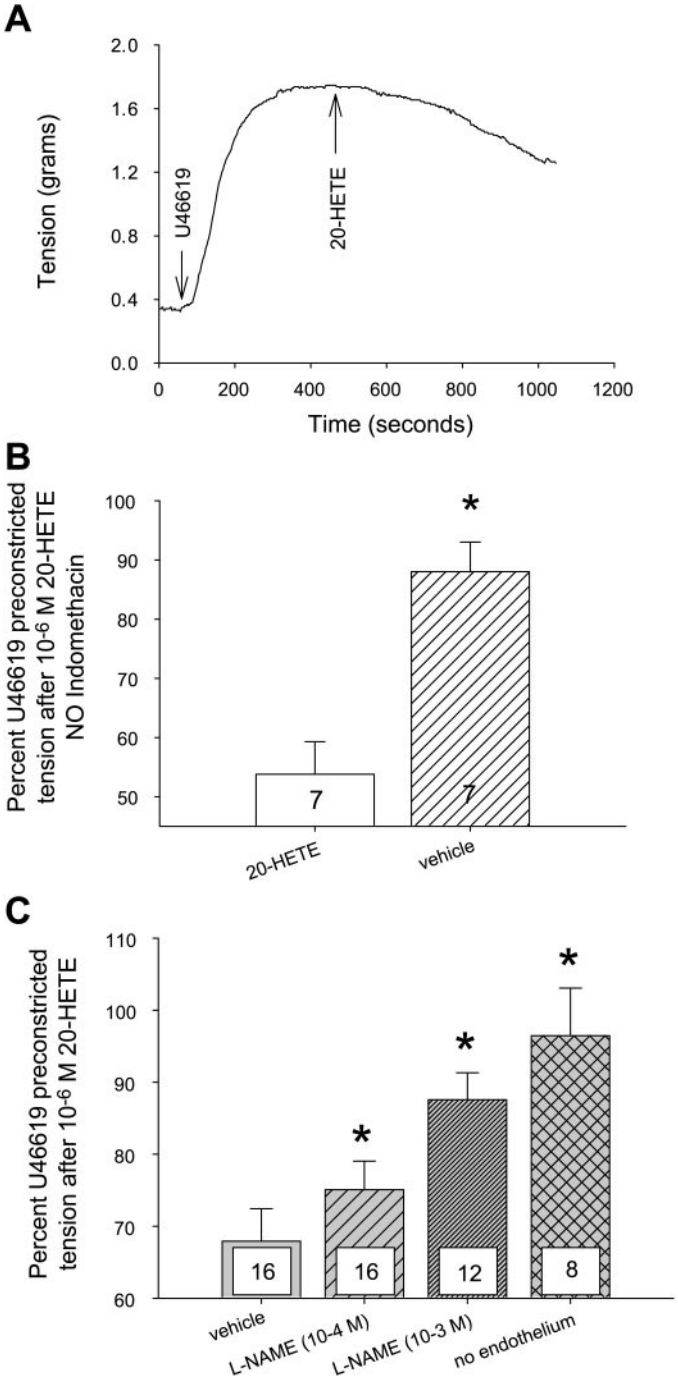

Fig. 3. Effects of 20-HETE on bovine pulmonary artery rings. *A*: a representative experiment demonstrating the responses of a ring pretreated with 17-octadecynoic acid (17-ODYA) and indomethacin to application of U-46619 and then 10^{-6} M 20-HETE. *B*: response of rings pretreated with 17-ODYA and not indomethacin then constricted with U-46619, followed by either 20-HETE or vehicle. Relaxation to 20-HETE is significantly greater than that of time and vehicle controls and is augmented in rings with intact cyclooxygenase compared with those treated with indomethacin. Sample numbers for each experiment are marked inside the bars. *C*: averaged responses and standard error bars, with the number of each experiment appearing in boxes inside of the bars of all rings pretreated with both 17-ODYA and indomethacin. The x-axis labels refer to the pretreatment of the rings; as indicated in the y-axis, all rings in these experiments were exposed to 10^{-6} M 20-HETE in the bath after preconstriction with U-46619. Prorelaxant effects of 20-HETE depend on intact endothelium, are enhanced by cyclooxygenase activity, and can be blocked in a concentration-dependent manner by N^{ω}-nitro-L-arginine methyl ester (L-NAME).

unpaired t-test $P < 0.05$). The response to 20-HETE was significantly greater than time and vehicle control (Fig. 3*B*).

We examined the dependence of HETE-evoked relaxation on several other factors, including intact NOS or endothelium, the agent employed to induce precontraction, and the position of the hydroxyl group on the HETE molecule. Response to 20-HETE could be inhibited in a concentration-dependent manner by pretreatment with L-NAME or removal of the endothelium (Fig. 3*C*). Furthermore, relaxation to 20-HETE was not limited to rings constricted with U-46619. Rings precontracted with norepinephrine (10^{-6} M) relaxed to 20-HETE equally well as those with tension increased by U-46619 (68 ± 3% preconstricted tension; $n = 8$; $P < 0.04$; data not shown). Finally, we tested relaxation of rings precontracted with U-46619 to 10^{-6} M 15-HETE; on an equimolar basis, 20-HETE was more potent than the subterminal 15-HETE (77 ± 2% preconstricted tension $n = 8$, compared with 68 ± 4% $n = 16$ with 20-HETE).

Effect of 20-HETE on $[Ca^{2+}]_i$ in BPAEC. Baseline $[Ca^{2+}]_i$ in cultured BPAEC averaged 68 ± 7 nM ($n = 30$). 20-HETE (10^{-6} M) produced a gradual rise in $[Ca^{2+}]_i$ over a 2- to 3-min period from the control value to 93 ± 8 nM ($n = 70$; Fig. 4*B*). In contrast, addition of vehicle had no effect on $[Ca^{2+}]_i$ (Fig. 4*A*). Removal of calcium from the bathing solution blocked 20-HETE-induced increases in $[Ca^{2+}]_i$ (Fig. 4*C*). These results are summarized in Fig. 4*D*.

The experiment was repeated with pretreatment of each cell with U-46619 followed by application of 20-HETE. The results of these experiments are presented in Fig. 5. The increase in $[Ca^{2+}]_i$ after addition of 20-HETE was significantly augmented by prior stimulation with U-46619 (Fig. 5*B*), which was also different from the action of U-46619 alone (Fig. 5*C*).

Effect of 20-HETE on NO production rate in BPAEC. The effect of 20-HETE on NO production is presented in Fig. 6. In one study, cells were treated with 20-HETE followed by vehicle control (Fig. 6*A*). In another study, treatments were in the reverse order, i.e., vehicle followed by 20-HETE. Value of NO production rate (i.e., fluorescence intensity/min, a slope of a tracing) was taken at 4 min after each treatment. Compared with vehicle controls, 20-HETE significantly increased NO production rate (0.34 ± 0.04 vs. 0.90 ± 0.07 fluorescence intensity unit/min) in BPAEC (Fig. 6*C*). Moreover, vehicle failed to further increase NO production

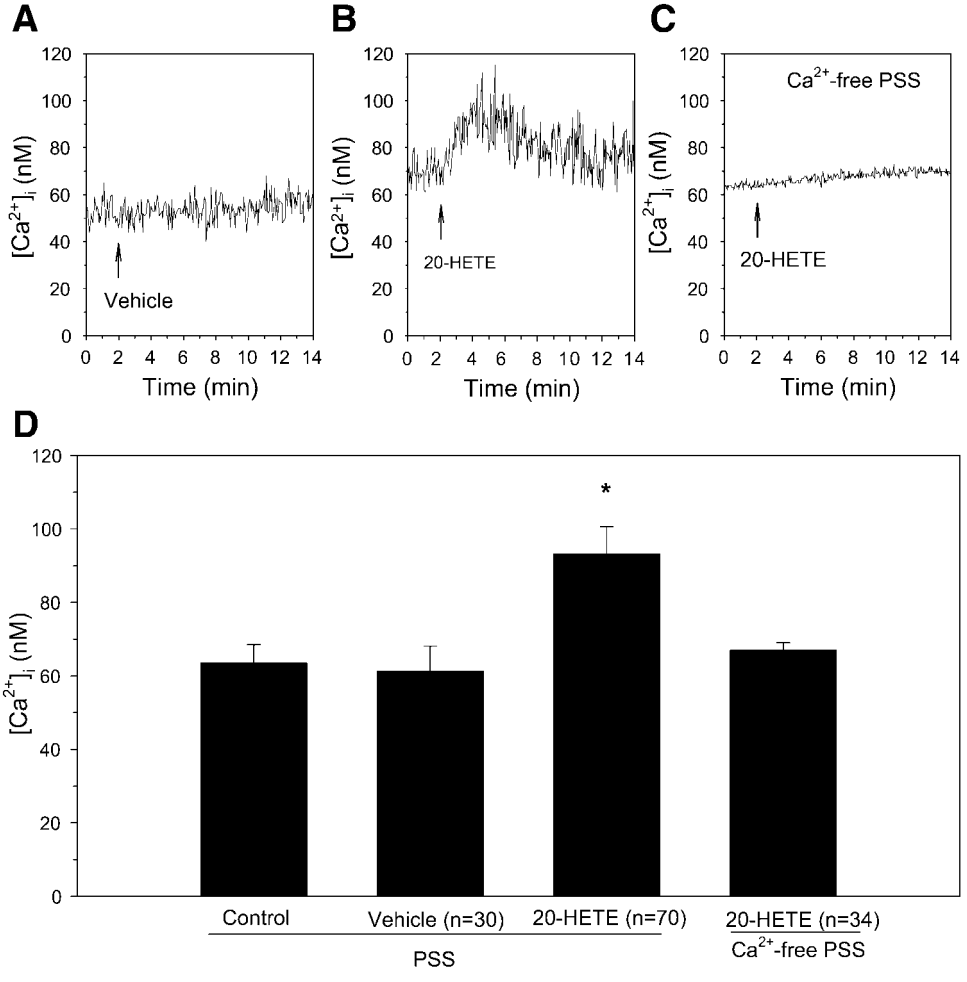

Fig. 4. Effect of 20-HETE on intracellular calcium concentration ($[Ca^{2+}]_i$) in BPAEC. *A–C*: tracings from single cells under baseline conditions after treatment with vehicle (ethanol; *A*), after addition of 20-HETE (*B*), and finally after 20-HETE in a bath with extracellular calcium removed (*C*). *D*: summary of the effect of 20-HETE and vehicle on $[Ca^{2+}]_i$ in all cells studied after pretreatment with 17-ODYA and indomethacin. PSS, physiological salt solution. *$P < 0.05$ compared with the control value. For purposes of graphing, vehicle values for all control experiments were averaged.

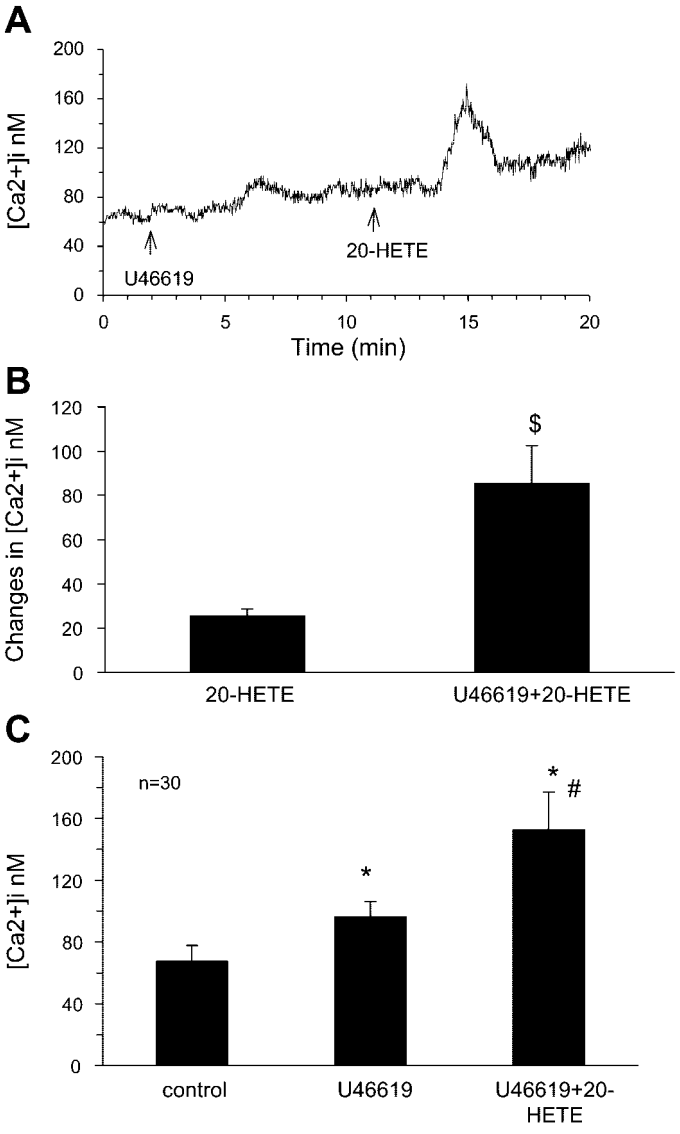

Fig. 5. Effect of pretreatment with U-46619 on increase of $[Ca^{2+}]_i$ by 20-HETE in BPAECs. *A*: tracings from single cells treated with U-46619 followed by stimulation with 20-HETE. Results are graphically represented in *B* and *C*. $\$P < 0.05$ from 20-HETE without treatment with U-46619; * and *#, $P < 0.05$ between vehicle treatment and U-46619 alone and/or U-46619+20-HETE, respectively.

rate after 20-HETE treatment (0.65 ± 0.11 vs. 0.90 ± 0.07 fluorescence intensity unit/min).

Pretreatment with L-NAME or L-NNA significantly attenuated the 20-HETE-induced NO production rate in BPAEC (0.68 ± 0.06 vs. 0.33 ± 0.05, 0.18 ± 0.04 fluorescence unit/min, Fig. 7, *A*, *B*, and *D*). When intracellular calcium was chelated by the calcium chelator BAPTA-AM and extracellular calcium was removed, the change of 20-HETE-induced NO production rate was close to 0 (0.02 ± 0.04 fluorescence unit/min, Fig. 7, *C* and *D*).

DISCUSSION

Recent studies have indicated that 20-HETE plays a critical role in the regulation of vascular tone, autoregulation of renal and cerebral blood flow, and the influence of oxygen on vasculature (24). In contrast to its effects in systemic beds (19), 20-HETE relaxes PAs (4). Previous studies indicated that the vasodilator effect of 20-HETE in human PAs is blunted by pretreatment with indomethacin or by removal of endothelium (4), suggesting that this response is dependent on the release of a dilator from the endothelium. Also germane to the question of pathways that could underlie the vascular effects of 20-HETE on pulmonary and systemic vascular tone are our recent observations identifying a novel CYP4A localization to PA vascular endothelium (33). In the present study, we demonstrate that 20-HETE-dependent relaxation of small PA rings is blocked by pretreatment with the NOS inhibitor L-NAME or removal of the endothelium. These observations suggest that 20-HETE-induced increases in NO release could underlie increases in PA diameter (4) and decreases in PA tone (present work), raising the potential that this eicosanoid could modulate endothelial NOS in vivo.

We next investigated the capacity of 20-HETE to induce increases in intracellular free calcium in BPAECs. A host of endothelial cell functions is modulated by changes in intracellular calcium and/or calcium-calmodulin-regulated processes, including endothelial NOS (eNOS) (10, 21) and a number of phospholipase A_2 enzymes (3). We observed that 20-HETE evoked a relatively slow and modest but sustained increase in intracellular free calcium in early-passage BPAECs. Removal of calcium from the bathing solution effectively blunted this response, indicating that 20-HETE likely increases calcium influx. In addition, 20-HETE triggered an increased NO release from these same cells. 20-HETE-evoked NO release from BPAECs was blocked by NO synthase inhibitors L-NAME or L-NNA, providing evidence that the fluorescent signal indeed represented a NOS-dependent product. Furthermore, we demonstrated that NO release in BPAECs was blocked by removing calcium from the bath and pretreatment with the calcium chelator BAPTA-AM. Thus the effects of 20-HETE on $[Ca^{2+}]_i$ are sufficient to account for increases in NO production. The mechanism(s) by which 20-HETE increases $[Ca^{2+}]_i$ in endothelial cells remains to be determined. However, the present results are consistent with previous findings that 20-HETE (8) increases calcium influx in vascular smooth muscle cells.

The physiological importance of NO in shear/flow-induced pulmonary vasodilation has long been recognized (12). Moreover, a deficiency in the production of NO in the pathogenesis of pulmonary hypertension has been suggested by several investigations. Giaid and Saleh (9) demonstrated previously that PAs of patients with pulmonary hypertension and severe morphological abnormalities exhibit diminished expression of eNOS. Furthermore, eNOS knockout mice exhibit increased pulmonary arterial pressure and impaired relaxation to acetylcholine, suggesting an important role for eNOS in maintaining normal vessel tone (25).

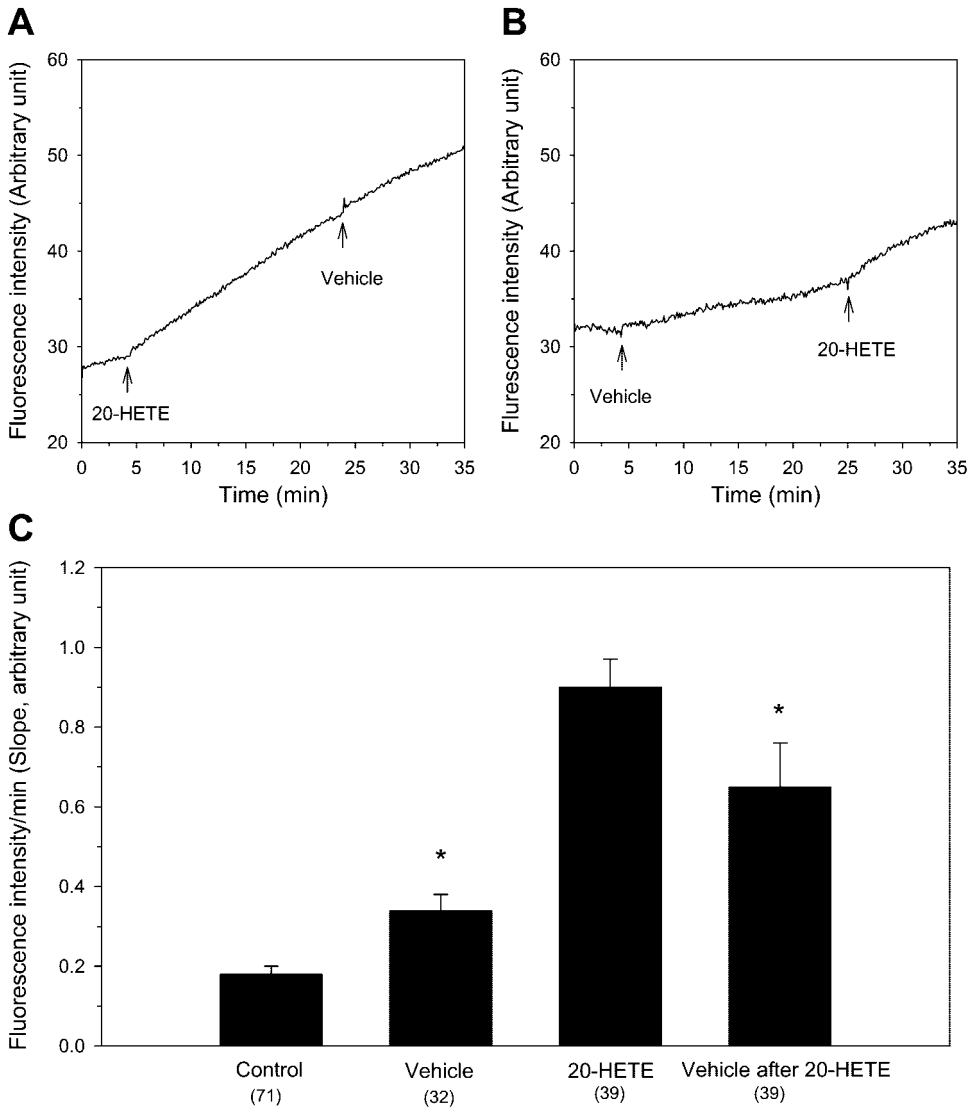

Fig. 6. Effect of 20-HETE on nitric oxide (NO) production rate in BPAEC. *A* and *B*: tracings of a single cell treated with 20-HETE and vehicle in a different order. *C*: summary of the effect of 20-HETE on NO production rate (fluorescence intensity/min, a slope of a tracing) in all cells studied. Numbers in parentheses indicate number of cells studied. *$P < 0.05$ compared with 20-HETE treatment.

Pregnancy is associated with increases in NO biosynthesis, and inhibition of NOS induces some pathological characteristics of preeclampsia (29). However, a good deal less is known about pregnancy-induced changes in maternal PA endothelial NOS or the role of NO in maintaining low pulmonary vascular tone under conditions of increased vascular volume and flow characteristic of pregnancy. Data from this laboratory have likewise supported a functional role for CYP4A/20-HETE in control of pulmonary vascular tone. Inhibition of CYP4A and reduction of 20-HETE levels augment the hypoxic vasoconstrictive response and shift concentration response curves of PA rings to constricting agents to the left, both consistent with loss of a relaxing factor (31). These observations indicate that changes in stimulated release of NO or 20-HETE in a variety of conditions may profoundly impact pulmonary biology and raise the imperative of better understanding the intracellular signaling pathways that contribute to activation of NO or the interaction of these two modulators of pulmonary vascular tone.

The functional implications of NO and CYP4A interactions in cerebral and renal circulations appear to be very different than those that we observe in PAs. Binding of NO to the heme moiety of CYP4A inhibits 20-HETE formation (1, 2), and a fall in 20-HETE levels (attributable to NO binding to CYP4A) contributes to effects of NO in the cerebral circulation (26). Addition of nanomolar concentrations of 20-HETE to the bath completely blocks the ability of NO to activate calcium-activated potassium channels in cerebrovascular smooth muscle cells, and preventing the fall in 20-HETE levels reduces the vasodilator response of isolated pressurized rat middle cerebral arteries to NO by 60% (2, 26). Although the same interaction of NO and the heme moiety of CYP proteins very likely occur in PA smooth muscle cells, the unique localization of CYP4A and 20-HETE in the endothelium of the lung appears to confer to the pulmonary circulation the

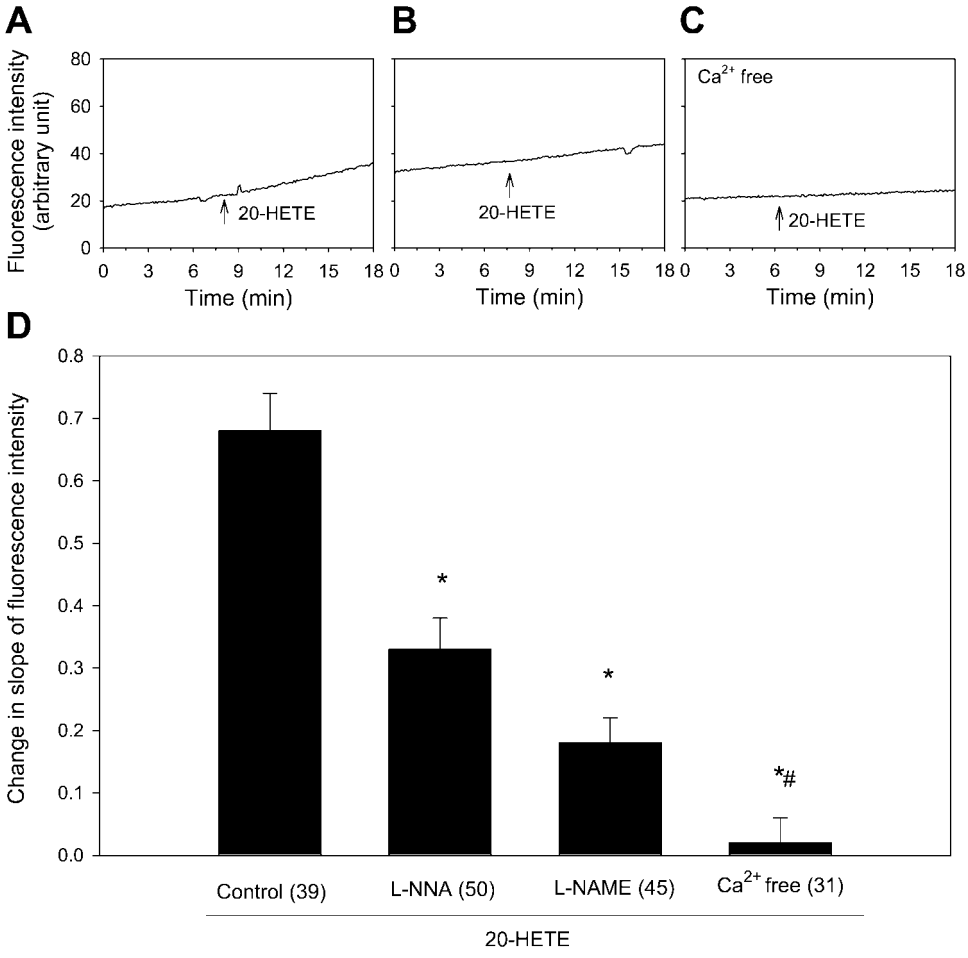

Fig. 7. Effects of reduced $[Ca^{2+}]_i$ and NO synthase inhibitors on 20-HETE-induced NO production rate. *A–C*: tracings of a single cell treated with 20-HETE after pretreatment with N^{ω}-nitro-L-arginine (L-NNA; *A*), L-NAME (*B*), or the intracellular calcium chelator 1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM; *C*). *D*: summary of the changes of 20-HETE-induced NO production rates (change in a slope of a tracing after 20-HETE, fluorescence unit/min) after pretreatment with L-NNA, L-NAME, or BAPTA-AM in a calcium-free PSS bath in all cells studied. Numbers in parentheses indicate number of cells studied. $^*P < 0.05$ compared with 20-HETE treatment alone (Control); $\#P < 0.05$ compared with L-NAME and L-NNA pretreatments.

capacity to induce vasorelaxation due to increases in PA endothelial cell $[Ca^{2+}]_i$.

Experiments performed in the presence or absence of indomethacin confirm a contribution of cyclooxygenase products in the vasoactive effects of 20-HETE in bovine PAs. Indeed, our previously published data suggest a cyclooxygenase-dependent contribution to 20-HETE-induced relaxation of human PAs (4). These observations are well consistent with the present data depicting 20-HETE-associated calcium transients in BPAECs, because many phospholipase A_2 enzymes and diacylglycerols are regulated by calcium (3). These calcium-sensitive enzymes could increase the biosynthesis of cyclooxygenase metabolites via an increase in substrate availability. Therefore, although the data in this study implicate a primary role of NO in 20-HETE-mediated relaxation of bovine PAs, it is probable that the increases in $[Ca^{2+}]_i$ evoked by 20-HETE would increase downstream synthesis of a number of eicosanoids as well.

Our observations raise new questions. Although the calcium dependence of 20-HETE-induced NO release is evident, the source of calcium increases in $[Ca^{2+}]_i$ remains to be determined. Perhaps the most frequent regulatory pathway for calcium in endothelial cells is the store-operated calcium entry (27). Depletion of intracellular calcium stores, membrane hyperpolarization, and shear stress activate capacitative calcium entry through these pores, in which increases in intracellular free calcium result in enhanced activity of eNOS and NO release (6, 27). 20-HETE could gate potassium-selective channels or calcium entry pores in PA endothelial cells, enhance release of calcium from endoplasmic reticulum or mitochondrial sources, or modulate a host of other processes that directly or indirectly change intracellular free calcium. Regardless, the mechanisms of action of 20-HETE in PA endothelial cells are clearly different from those of vascular smooth muscle cells, in which 20-HETE decreases opening of large-conductance potassium channels and increases opening of L-type calcium channels (7, 8, 24). These questions, as well as those pertaining to the functional importance of 20-HETE in triggering NO release in a number of physiological or pathophysiological states, need to be addressed.

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