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Identification of 15-hydroxy-11,12-epoxyeicosatrienoic acid as a vasoactive 15-lipoxygenase metabolite in rabbit aorta

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[1]*Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin;* [2]*Department of Biochemistry, University of Texas Southwestern Medical School, Dallas, Texas; and* [3]*Department of Entomology and Cancer Research Center, University of California, Davis, California*

Chawengsub Y, Aggarwal NT, Nithipatikom K, Gauthier KM, Anjaiah S, Hammock BD, Falck JR, Campbell WB. Identification of 15-hydroxy-11,12-epoxyeicosatrienoic acid as a vasoactive 15-lipoxygenase metabolite in rabbit aorta. *Am J Physiol Heart Circ Physiol* 294: H1348–H1356, 2008. First published January 11, 2008; doi:10.1152/ajpheart.01326.2007.—Arachidonic acid (AA) causes endothelium-dependent smooth muscle hyperpolarizations and relaxations that are mediated by a 15-lipoxygenase-I (15-LO-I) metabolite, 11,12,15-trihydroxyeicosatrienoic acid (11,12,15-THETA). We propose that AA is metabolized sequentially by 15-LO-I and hydroperoxide isomerase to an unidentified hydroxyepoxyeicosatrienoic acid (HEETA), which is hydrolyzed by a soluble epoxide hydrolase (sEH) to 11,12,15-THETA. After incubation of aorta with ^{14}C-labeled AA, metabolites were extracted and the HEETAs were resolved by performing HPLC. Mass spectrometric analyses identified 15-Hydroxy-11,12-epoxyeicosatrienoic acid (15-H-11,12-EETA). Incubation of aortic incubates with methanol and acetic acid trapped the acid-sensitive 15-H-11,12-EETA as methoxydihydroxyeicosatrienoic acids (MDHEs) (367 *m/z*, M-H). Pretreatment of the aortic tissue with the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; 10^{-6} M) increased the formation of 15-H-11,12-EETA, measured as MDHEs. Thus 15-H-11,12-EETA is an acid- and sEH-sensitive precursor of 11,12,15-THETA. Aortic homogenates and endothelial cells contain a 57-kDa protein corresponding to the rabbit sEH. In preconstricted aortic rings, AA (10^{-7}–10^{-4} M) and acetylcholine (10^{-9}–10^{-6} M) caused concentration-related relaxations that were enhanced by pretreatment with AUDA. These enhanced relaxations were inhibited by increasing extracellular $[K^+]$ from 4.8 to 20 mM. AA (3×10^{-6} M) induced cell membrane hyperpolarization (from -31.0 ± 1 to -46.8 ± 2 mV) in aortic strips with an intact endothelium, which was enhanced by AUDA. These results indicate that 15-H-11,12-EETA is produced by the aorta, hydrolyzed by sEH to 11,12,15-THETA, and mediates relaxations by membrane hyperpolarization. 15-H-11,12-EETA represents an endothelium-derived hyperpolarizing factor.

arachidonic acid; endothelial cells; endothelium-derived hyperpolarizing factor; 15-lipoxygenase

UPON STIMULATION WITH ACETYLCHOLINE (ACh), bradykinin, or mechanical stress, vascular endothelial cells (ECs) release mediators that relax smooth muscle (10, 15, 17, 24, 25, 28, 30). The principal mediators are nitric oxide (NO) (26, 35), prostacyclin (PGI_2) (3, 29), and endothelium-derived hyperpolarizing factors (EDHFs) (2, 8, 13). EDHFs cause endothelium-dependent hyperpolarization and relaxation of vascular smooth muscle cells (SMC) that are resistant to inhibition of NO synthase and cyclooxygenase (COX) (6, 10). EDHF activity is blocked by high extracellular K^+ concentration ($[K^+]_o$) and K^+ channel blockers, which prevent cells from being hyperpolarized. The chemical identity of EDHFs has received considerable attention; however, the existence and mechanism of action of EDHFs varies in different vascular beds, sizes, species, aging, and disease (4, 5, 21, 44, 50). Potassium ion, arachidonic acid (AA) metabolites, residual NO, H_2O_2, C-type natriuretic peptide, and myoendothelial cell gap junction are major EDHF candidates (5–7, 9, 12, 14, 21, 27, 44).

In rabbit aorta, ACh and AA induce endothelium-dependent smooth muscle hyperpolarization and vascular relaxation (6). Inhibitors of lipoxygenases (LOs) block non-NO, non-PGI_2 relaxations to ACh and AA (6). Rabbit aorta and aortic ECs predominantly express 15-LO-I isoform (49). We have proposed that AA is metabolized sequentially by 15-LO-I and hydroperoxide isomerase to produce hydroxyepoxyeicosatrienoic acids (HEETAs), which are hydrolyzed by soluble epoxide hydrolase (sEH) to trihydroxyeicosatrienoic acids (THETAs) (40, 42). The 15-LO-I metabolite 11(R),12(S),15(S)-trihydroxyeicosa-5(Z),8(Z),13(E)-trienoic acid (11,12,15-THETA) was isolated and identified from rabbit aorta and mesenteric arteries and shown to function as an EDHF (6, 18, 19, 40, 52). 11,12,15-THETA hyperpolarizes and relaxes the SMCs of rabbit aorta through opening of apamin-sensitive K^+ channels (19). In contrast to the THETA, the chemical identity of HEETA and its role in regulation of vascular tone has not been studied. The present study was designed to identify the HEETA produced by rabbit aorta and to study its effect on vascular tone.

Fractions collected from reversed-phase (RP)-HPLC containing HEETAs relaxed preconstricted rabbit aorta, and these relaxations were blocked by high $[K^+]_o$ (40). However, these fractions contained more than one AA metabolite. Therefore, in this present study, the AA metabolites in the HEETA fractions were further purified and the chemical structure of a purified HEETA was identified with the use of gas chromatography-mass spectrometry (GC-MS) as 15-Hydroxy-11,12-epoxyeicosatrienoic acid (15-H-11,12-EETA). sEH hydrolyzes 15-H-11,12-EETA to 11,12,15-THETA. This finding is consistent with our previous report that 11,12,15-THETA is produced by the rabbit aorta and mesenteric arteries (6, 52). Both rabbit aorta and aortic ECs express sEH. Inhibition of sEH with 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA)

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increased the amount of 15-H-11,12-EETA. Moreover, preincubation of the aorta with AUDA increased AA-induced relaxations of the aorta and hyperpolarizations of SMCs. These findings suggest that 15-H-11,12-EETA is a novel EDHF in rabbit aorta.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care Committee of the Medical College of Wisconsin, and procedures were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (1996). Indomethacin (Indo), nitro-L-arginine (L-NA), AA, and other chemicals were purchased from Sigma Chemical.

Tissue preparation and incubation. Aortas were isolated from 4- to 6-wk-old New Zealand White rabbits (Kuiper Rabbit Ranch, Gary, IN), placed in ice-cold HEPES buffer (in mM: 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, and 6 glucose, pH 7.4), cleaned of adhering connective tissue and fat, and cut into rings (5 mm long) (39, 49). Aortic rings were incubated for 10 min at 37°C in HEPES buffer containing Indo (10^{-5} M). In some experiments, a sEH inhibitor, AUDA or 1-cyclohexyl-3-dodecyl-urea (CDU; 10^{-6} M), was added (31, 32). ^{14}C-labeled AA (1 μCi, 0.25 μM) plus unlabeled AA (10^{-7} M) were added, and the vessels were incubated for an additional 5 min. Calcium ionophore A-23187 (2×10^{-5} M) was added, and the vessels were incubated for another 15 min. The reaction was stopped by the addition of ethanol to a final concentration of 15%. The incubation buffer was removed, acidified (pH <3.5) with glacial acetic acid, and extracted on Bond Elut C-18 extraction columns as previously described (38, 39, 43). The extracts were evaporated to dryness under a stream of N$_2$ and stored at -40°C until analysis with HPLC. In the incubations for mass spectrometric analyses, AA (10^{-4} M) was used. The incubations of the aorta with [^{14}C]AA plus either 10^{-4} or 10^{-7} M AA did not show any difference in the profile of radioactive metabolites when resolved with RP-HPLC.

Chemical trapping of 15-H-11,12-EETA. Aortic rings were incubated with [^{14}C]AA as described above in the presence or absence of a sEH inhibitor, AUDA (10^{-6} M) (31, 32). The incubation buffer was transferred immediately into 25 volumes of methanol acidified with a volume of glacial acetic acid that would acidify the aqueous solution to pH 3.5. The methanolic solution was left at room temperature for 30 min. The excess methanol was removed with a rotary evaporator under vacuum, and the remaining incubation medium was extracted on Bond Elut C-18 extraction columns as described above.

For time-course experiment, aortic rings were incubated with [^{14}C]AA in the presence of AUDA as described above. The incubation medium was removed and glacial acetic acid added. The acidified incubation medium was incubated for various times at room temperature before methanol was added to trap the unhydrolyzed HEETA as monomethoxydihydroxyeicosatrienoic acids (MDHEs). The changes in the formation of MDHEs and THETAs were measured using liquid scintillation spectrometry. 12-Hydroxyeicosatetraenoic acid (Cayman Chemical) was used as an internal standard to quantify the percentage of MDHEs and THETAs produced.

HPLC separation of AA metabolites. Extracted samples were first resolved on RP-HPLC (Nucleosil-C18 column, 5 μm, 4.6 × 250 mm) using solvent system I (40). *Solvent A* was water, and *solvent B* was acetonitrile containing 0.1% glacial acetic acid. The program was a 40-min linear gradient from 50% *solvent B* in *solvent A* to 100% *solvent B*. The flow rate was 1 ml/min. The column eluate was collected in 0.2-ml aliquots, and radioactivity was measured. The fractions containing THETAs (3.5–7.5 min), MDHEs (8–11 min), and HEETAs (12.8–15.8 min) were pooled, acidified, and extracted with cyclohexane-ethyl acetate (50:50). The solvent was dried under a stream of N$_2$. The HEETA extract was derivatized to methyl esters and rechromatographed on normal phase (NP)-HPLC (Nucleosil silica column, 5 μm, 4.6 × 250 mm) using solvent system II. This was a

30-min isocratic system of 0.1% glacial acetic acid and 0.8% isopropanol in hexane with a flow rate of 1 ml/min. HEETA methyl ester fractions (*peak A*, 14–18 min) were collected, dried under a stream of N$_2$, and stored at -40°C until analysis using mass spectrometry.

Functional group derivatizations. Sample was derivatized to methyl ester by being dissolved in 100 μl of acetonitrile and then esterified with 1 ml of ethereal diazomethane for 15 min at room temperature (40). The reaction mixture was evaporated to dryness under a N$_2$ stream. Alternatively, the pentafluorobenzyl (PFB) ester was formed (40, 47). The dried sample was dissolved in acetonitrile containing pentafluorobenzyl bromide and *N,N*-diisopropylethylamine (10:1:5 vol/vol/vol) and incubated for 20 min at room temperature. The solvents were removed under a stream of N$_2$. After the methyl or PFB ester was made, the dried sample was dissolved with 50 μl of acetonitrile and the hydroxyl group was converted to the trimethylsilyl (TMS) ether with 100 μl of bis-TMS-trifluoroacetamide for 2 h at 37°C (40). The reaction mixture was evaporated to dryness under N$_2$ and stored at -40°C for GC-MS analysis.

Mass spectrometry methods. GC-MS analysis was performed with a Hewlett Packard 5989A mass spectrometer coupled to a 5890 series II gas chromatograph. Chemical ionization of the sample was performed using methane as the reagent gas (40). Ions were measured in the positive ion mode for the methyl esters and negative ion mode for the PFB esters. The derivatized sample was resolved using a 30-m capillary DB-5 column. The initial oven temperature of 100°C was maintained for 2 min, increased to 200°C with the rate of 20°C/min, sustained for 0.1 min, then increased to 300°C with the rate of 10°C/min, and maintained for 4.9 min.

The pooled fractions of the MDHEs (8–11 min) were analyzed by electrospray ionization (ESI)-Fourier transform ion cyclotron resonance (FTICR)-MS (IonSpec 7.0-tesla FTICR high-resolution mass spectrometer with a Waters Z spray ESI source). The source temperature was 80°C, and cone voltage was -45 V. The mass-to-charge ratio (*m/z*) of 367 was isolated and fragmented with sustained offresonance irradiation collision-induced dissociation (SORI-CID) by using N$_2$ as the collision gas, and daughter ions were detected with the *m/z* range from 25 to 500. Ions were measured in negative ion mode.

Western immunoblot. Protein lysates of rabbit aortic tissue, rabbit aortic ECs, and rabbit aortic SMCs (25 μg) were loaded in each lane and separated by SDS-PAGE using a 10% resolving gel and 4% stacking gel. Proteins were then transferred to nitrocellulose membranes. Nonspecific binding was blocked by incubation of the membrane with 5% nonfat dry milk in TBST buffer (20 mM Tris base, 50 mM NaCl, and 10% Tween 20, pH 7.8) for 1 h at room temperature. Rabbit serum against human sEH was used (1:5,000 dilution) in 5% milk-TBS buffer (20 mM Tris base and 50 mM NaCl, pH 7.8) and incubated for 1 h at room temperature. The membrane was then rinsed with TBST and TBS buffer. Goat anti-rabbit IgG (1:5,000, horseradish peroxidase conjugated; Zymed) was added as a secondary antibody in 5% milk-TBS buffer and incubated for 1 h at room temperature. Immunoreactive bands were detected using SuperSignal West Pico chemiluminescent substrate (Pierce Chemicals) and Kodak BioMax ML film.

Vascular reactivity. Aortic rings (3 mm long) were suspended in 6-ml tissue baths containing Krebs bicarbonate buffer (in mM: 118 NaCl, 4 KCl, 3.3 CaCl$_2$, 24 NaHCO$_3$, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, and 11 glucose) maintained at 37°C and continuously bubbled with 95% O$_2$-5% CO$_2$ as described previously (40, 49). Aortas were then slowly stretched during an additional 1-h equilibration period to a resting tension of 2 g, which was the optimal preload for active tension development as determined by the length-tension curve method. Contractions were produced by increasing the KCl concentration in the baths to 47 mM. After the vessels reached peak contraction, tissue baths were rinsed and vessels were allowed to return to resting tension. Vessels were pretreated for 30 min with AUDA (10^{-6} M) or vehicle and for 10 min with L-NA (3×10^{-5} M) and Indo (10^{-5} M) or vehicles. Vessels were then preconstricted to 50–60% maximal

KCl constriction with phenylephrine (10^{-7} M). When the constrictions to phenylephrine were stable, cumulative concentrations of AA (10^{-7}–10^{-4} M) or ACh (10^{-9}–10^{-6} M) were added and changes in isometric tension were measured. In some studies, the Krebs buffer was modified to increase the KCl concentration to 20 mM by substituting KCl for NaCl. Experiments were performed on aortic rings from multiple rabbits.

Membrane potential measurements. Rabbit aortic rings were cut open laterally and pinned to a silicone elastomer layer, with the endothelial layer exposed, in a heated (37°C) chamber containing Krebs bicarbonate buffer, equilibrated with a 95% O_2-5% CO_2 gas mixture to maintain a pH of 7.4. Aortic segment was treated with Indo (10^{-5} M) and phenylephrine (10^{-7} M) for 30 min before initiation of experiment protocols. Impalements of SMCs were performed in a section of the artery where a small patch of endothelium had been removed by gentle rubbing with a needle tip. Intracellular membrane potential was recorded using published methods (6, 20). In brief, glass microelectrodes were filled with 3 M KCl and had estimated tip sizes of 0.1–0.2 μm and tip resistances of 30–80 MΩ. Electrodes were attached to a high-impedance biological amplifier (Dagan Cell Explorer; Dagan Instruments, Minneapolis, MN) and digitized for analysis with a MacLab system (AD Instruments). Electrode polarization was eliminated by an Ag-AgCl half-cell. Criteria for successful cell impalement included an abrupt return to the original baseline when the electrode was retracted from the tissue. Impalement of less than −20 mV was excluded. AUDA (10^{-6} M) and/or AA (3×10^{-5} M) were added to the aortic segments, and after 10 min, membrane potential was measured. Experiments were performed on aortic rings from multiple rabbits.

Statistical analysis. Statistical evaluations of the vascular activity data and membrane potential were preformed using one-way analysis of variance followed by Student-Newman-Keuls post hoc analysis when significant differences were present. All statistical comparisons are made at the 95% confidence level, $P \leq 0.05$.

RESULTS

Identification of aortic HEETA. After incubation of the aortic segments with [^{14}C]AA, the metabolites were extracted and resolved using RP-HPLC with solvent system I. ^{14}C metabolites comigrating with the THETAs, HEETAs, 15-HETE, 12-HETE, and AA standards were observed (Fig. 1*A*). The HEETA fractions were collected, derivatized to methyl esters, and further purified using a NP-HPLC with solvent system II. NP-HPLC resolved the methyl esters of the HEETA fractions into two radioactive peaks, *peak A* and *peak B* (Fig. 1*C*).

The HEETA fractions (12.8–15.8 min from Fig. 1*A*) were collected, derivatized to the PFB esters, TMS ethers, and analyzed using GC-negative ion chemical ionization MS. The mass spectrum of the major product eluting at 16.96 min is shown in Fig. 2*A*. The major ions (*m/z*) were 407 (M-PFB), 335 [M-PFB-(CH$_2$)$_4$CH$_3$], 317 [M-PFB-HO-Si-(CH$_3$)$_3$], 307 (M-PFB-100), 235 {M-PFB-CH[O-Si-(CH$_3$)$_3$]-(CH$_2$)$_4$CH$_3$}, and 195 {M-PFB-CH-CH=CH-CH[O-Si-(CH$_3$)$_3$]-(CH$_2$)$_4$CH$_3$}. This structure is consistent with the PFB ester, TMS ether of 15-H-11,12-EETA.

Peak A of Fig. 1*C* was collected, derivatized to the TMS ether, and analyzed using GC-positive ion chemical ionization-MS. *Peak A* showed a peak on GC eluting at 14 min. The mass spectrum indicated the presence of major ions at 451 (M+CH$_2$CH$_3$), 423 (M+H), 333 [M+H-90; M+H-HO-Si-(CH$_3$)$_3$], 301, {M-[HO-Si-(CH$_3$)$_3$]-(OCH$_3$)}, 241 [M-181; loss of (CH$_2$-CH=CH)$_2$-(CH$_2$)$_3$COOCH$_3$], 223 {M-199; loss of CH=CH-CH[O-Si-(CH$_3$)$_3$]-(CH$_2$)$_4$CH$_3$}, 199 [M-223; loss of
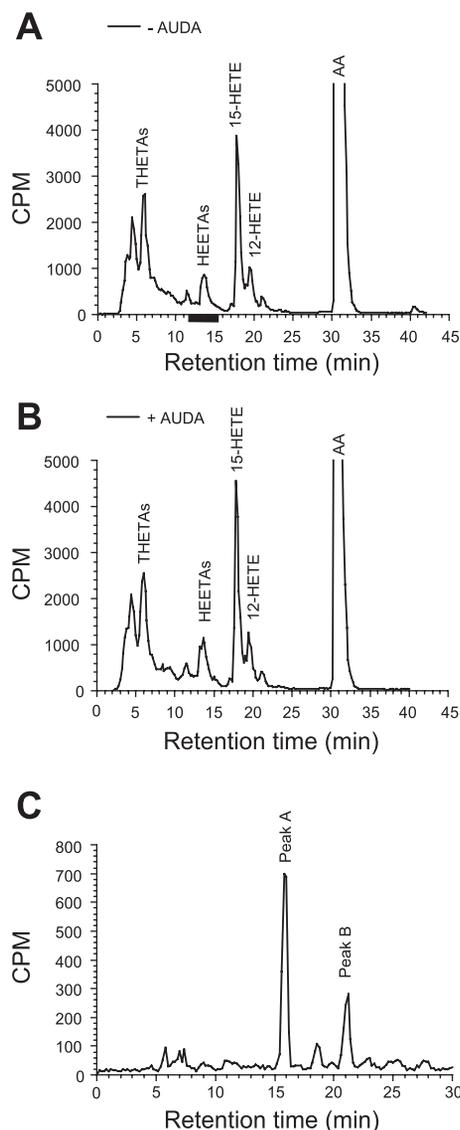

Fig. 1. Metabolism of ^{14}C-labeled arachidonic acid (AA) by rabbit aorta in the absence (*A*) and presence (*B*) of the epoxide hydrolase inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA; 10^{-6} M). Rabbit aortic rings were incubated with [^{14}C]AA (10^{-7} M) in the presence of indomethacin (Indo; 10^{-5} M), and the metabolites were separated using reversed-phase (RP)-HPLC solvent system I (*A* and *B*). The migration times of known standards are shown above the chromatogram; CPM, counts per minute. The HEETA fractions (12.8–15.8 min) from *A* were collected, derivatized to the methyl esters, and rechromatographed on normal phase (NP)-HPLC (*C*). Two major peaks were observed (*peak A* and *peak B*). Results are representative of 5 experiments. THETAs, trihydroxyeicosatrienoic acids; HEETAs, hydroxyepoxyeicosatrienoic acids; 15-HETE, 15-hydroxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid.

CH-O-CH-(CH$_2$-CH=CH)$_2$-(CH$_2$)$_3$COOCH$_3$], and 173 [M-249; loss of CH=CH-CH-O-CH-(CH$_2$-CH=CH)$_2$-(CH$_2$)$_3$COOCH$_3$] (Fig. 2*B*). This mass spectrum is consistent with the methyl ester, TMS ether of 15-H-11,12-EETA.

HEETA metabolism by sEH. sEH hydrolysis of 15-H-11,12-EETA to 11,12,15-THETA was investigated. First, the expression of sEH in rabbit aorta was shown. Using a rabbit anti-serum against human sEH, we detected a single 57-kDa protein by performing immunoblotting in rabbit aorta and RA-EC protein lysates (Fig. 3). This molecular mass of the protein was
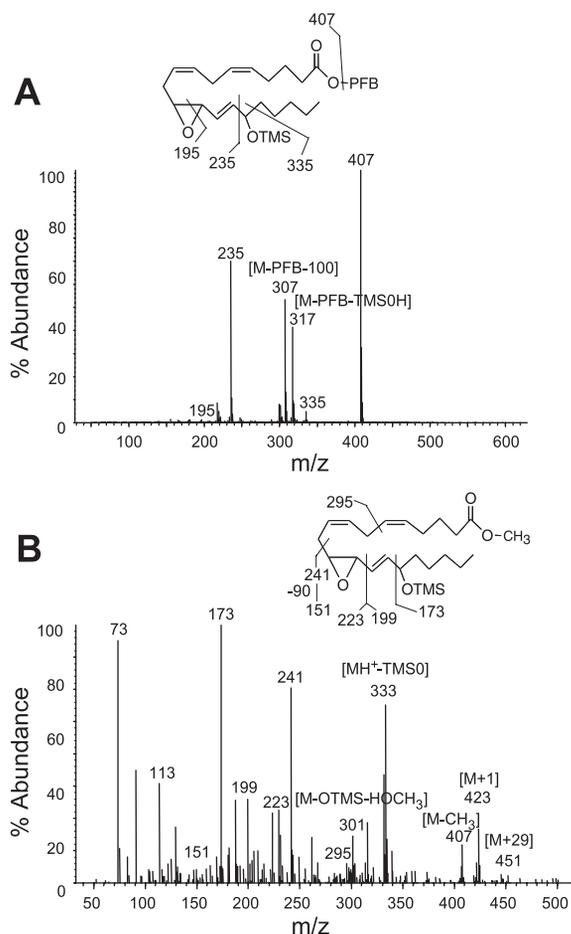
Fig. 2. Gas chromatography-mass spectrometry (GC-MS) analyses of HEETA indicating mass spectra of HEETA derivatives. *A*: HEETA fractions from Fig. 1*A* were derivatized to the pentafluorobenzyl (PFB) esters, trimethylsilyl (TMS) ethers and were analyzed using negative ion chemical ionization MS. *B*: *peak A* from Fig. 1*C* was derivatized to the methyl ester, TMS ether and analyzed using positive ion chemical ionization MS. *m/z*, mass-to-charge ratio.

consistent with the rabbit isoform of sEH (51). Recombinant human sEH, human microvascular EC, human liver cytosol, and human atrial protein lysates were included as positive controls. A 62-kDa protein corresponding to a sEH human isoform (1) was detected in each human sample.

The possibility that inhibition of sEH with AUDA would alter the production of HEETAs and THETAs was tested with sEH inhibitors AUDA or CDU (31, 32). As shown in Fig. 1*A*, rabbit aorta metabolized [^{14}C]AA to ^{14}C metabolites comigrating with THETAs, HEETAs, and 15-HETE. There was a small increase in HEETA production with AUDA (10^{-6} M) pretreatment for 30 min (Fig. 1*B*). CDU (10^{-6} M) did not change the pattern of [^{14}C]AA metabolism to a great extent (data not shown). The proximity of the olefin to the epoxy group activates the epoxide of 15-H-11,12-EETA to hydrolysis in acid to 11,12,15- and 11,14,15-THETAs (22). For example, at pH 3.0, 15-H-11,12-EETA has a half-life of ~50 s. For efficient extraction of the HEETAs and THETAs, the samples must be acidified to pH 3.5. Thus the extraction process may hydrolyze the 15-H-11,12-EETA to the THETAs independently of sEH. However, metabolites comigrating with the HEETAs on RP-HPLC were observed following acidification and extraction,

indicating that the 12.8- to 15.8-min fractions may contain acid-stable metabolites in addition to the acid-sensitive 15-H-11,12-EETA. These acid-stable metabolites have not been identified and may represent the metabolites in *peak B* of Fig. 1*C*.

Since thromboxane A$_2$ rapidly degrades in aqueous solution and aqueous acid to thromboxane B$_2$, Hamburg et al. (23) treated incubates of platelets with an excessive amount of methanol to trap thromboxane A$_2$ as the methoxy derivative of thromboxane B$_2$. This was used to access the formation of the unstable thromboxane A$_2$ in platelets. Similarly, to determine the synthesis of acid-labile HEETAs and to study the effect of sEH inhibition on HEETA formation in aorta, an excessive amount of methanol was used to trap the acid-labile HEETA as MDHEs. Aortic rings were incubated with [^{14}C]AA, and 25 volumes of methanol were added before acidification and extraction. Without the sEH inhibitor AUDA, radioactive peaks comigrating with the THETAs, HEETAs, and 15-HETE were observed (Fig. 4*A*). In addition, a small radioactive peak eluting at 8–11 min comigrating with MDHEs was observed. In the presence of AUDA, a similar profile of metabolites was observed; however, the 8- to 11-min peak comigrating with MDHEs was increased and the THETA peak was reduced (Fig. 4*B*). The differences between MDHEs and THETAs productions were quantified by integrating the radioactivity counts under individual peaks and normalized by total radioactive counts. Without AUDA treatment, the synthesis of THETAs and MDHEs was 17.7 ± 2 (*n* = 3) and 6.0 ± 1% (*n* = 3), respectively. With AUDA, THETA production decreased to 10.7 ± 2% (*n* = 3), whereas MDHE production increased to 13.6 ± 1% (*n* = 3). These data indicate that an acid-labile HEETA can also be hydrolyzed by sEH, and inhibition of this enzyme with AUDA increases the production of the HEETA, measured indirectly as MDHEs.

The 8- to 11-min peak was collected, and its structure was analyzed using high-resolution FTICR-MS. With the negative ionization mode, the major ion (M-H) was *m/z* 367.24900, indicating a chemical formula of $C_{21}H_{35}O_5^-$. This formula and molecular mass are consistent with the MDHE structure. The *m/z* 367 ion was then selected for fragmentation by SORI-CID, and the daughter ions were detected. The MS/MS spectra revealed the presence of ions *m/z* 367 (M-H), 317 [M-H-(H$_2$O)-(CH$_3$OH)], 299 [M-H-2(H$_2$O)-(CH$_3$OH)], 273 [M-H-(H$_2$O)-(CH$_3$OH)-(CO$_2$)], 255 [M-H-2(H$_2$O)-(CH$_3$OH)-(CO$_2$)], 235 [M-H-132; loss of CH(OH)-(CH$_2$)$_4$CH$_3$ and CH$_3$OH], and 167 [M-H-200; loss of CH=CH-CH$_2$-CH=CH-(CH$_2$)$_3$COO] (Fig. 5*A*). Other fragments identified the metabolites as 12-methoxy-11,15-dihydroxy- and 14-methoxy-11,15-dihydroxy-eicosatrienoic acids.
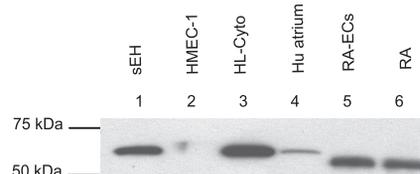

Fig. 3. Western immunoblot for soluble epoxide hydrolase (sEH) in rabbit aortic endothelial cells (RA-ECs) and rabbit aorta (RA). Recombinant sEH, human microvascular endothelial cells (HMEC-1), human liver cytosol (HL-Cyto), and human atrial tissue lysates (Hu atrium) were included as positive controls. The blot is representative of 3 experiments.
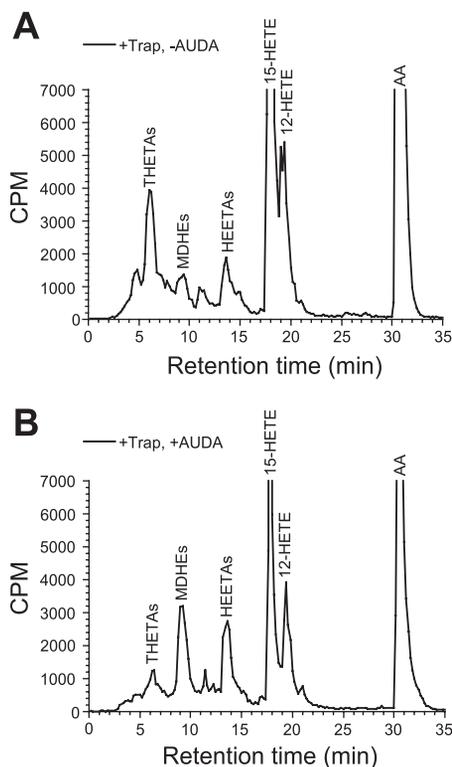
Fig. 4. Effect of methanol trapping and epoxide hydrolase inhibition on the metabolism of [^{14}C]AA (10^{-7} M) by rabbit aorta. Aortic rings were incubated as described in Fig. 1 in the absence (A) and presence (B) of AUDA (10^{-6} M). The incubation buffer was treated with methanol (25 volumes) and acetic acid before extraction. Metabolites were resolved with RP-HPLC using system I. The methoxydihydroxyeicosatrienoic acids (MDHEs) eluted with a retention time of 8–11 min. Results are representative of 3 experiments.

GC-MS analysis of the methyl ester, TMS ether derivatives of MDHEs was also performed. With positive chemical ionization mode, two major products were observed at 13.325 and 13.398 min, respectively. Mass spectra of the 13.325-min product revealed the presence of major ions of m/z 511 (M-CH$_3$), 405 {M-[HO-Si-(CH$_3$)$_3$]-(OCH$_3$)}, 315 {M-2[HO-Si-(CH$_3$)$_3$]-(OCH$_3$)}, 283 {M-243; loss of CH(OCH$_3$)-CH=CH-CH[O-Si-(CH$_3$)$_3$]-(CH$_2$)$_4$CH$_3$}, 243 {M-283; loss of CH[O-Si-(CH$_3$)$_3$]-(CH$_2$-CH=CH)$_2$-(CH$_2$)$_3$COOCH$_3$}, and 173 {M-353; loss of CH=CH-CH(OCH$_3$)-CH[O-Si-(CH$_3$)$_3$]-(CH$_2$-CH=CH)$_2$-(CH$_2$)$_3$COOCH$_3$} (Fig. 5B). Mass spectra of the 13.398-min product showed prominent ions of m/z 511 (M-CH$_3$), 283, and 173 (Fig. 5C). These spectra indicate a molecular mass of 526 for methyl esters, TMS ethers of MDHEs. These two compounds differed in the intensity of the m/z 173 and 283. Based on these mass spectra, the MDHE peak (8–11 min) collected from RP-HPLC contained two regioisomers of 12-methoxy-11,15-di-hydroxy- and 14-methoxy-11,15-dihydroxy-eicosatrienoic acids. Thus, when the epoxide is hydrolyzed with acidified methanol, the methoxy group can be added at either the epoxide group or the allylic position. This property also holds true for the addition of water to 15-H-11,12-EETA in aqueous buffer with acidification and accounts for the presence of both 11,12,15- and 11,14,15-THETAs in the THETA fractions (40).

To evaluate HEETA hydrolysis to THETAs under acidic conditions, we incubated aortas with [^{14}C]AA in the presence of a sEH inhibitor, AUDA. These conditions promoted the accumulation of the HEETA in the incubation buffer. The incubation buffer containing the HEETA was treated with acetic acid with different time periods. After 0, 10, 30, 45, 60, and 300 s, 25 volumes of methanol were added to the reaction. As the time of incubation with acid increased, the hydrolysis of HEETA to THETAs increased and less MDHE was detected
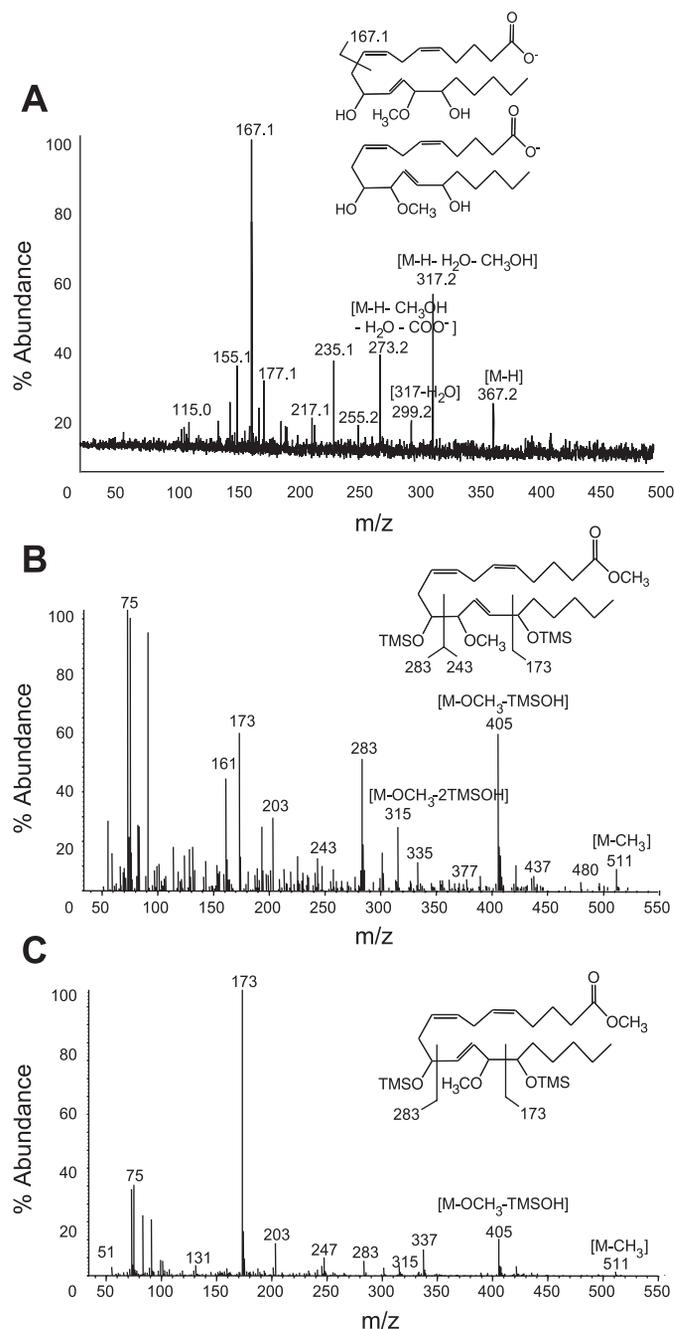

Fig. 5. Mass spectrometric analyses of MDHEs. A: the MDHE fractions (8–11 min) from Fig. 4B were collected and analyzed using electrospray ionization-Fourier transform ion cyclotron resonance-MS. The mass spectrum indicates a M-H ion of m/z 367.24900 (data not shown). The m/z 367 was selected for further analysis using sustained off-resonance irradiation collision-induced dissociation. The MS/MS spectra are shown in A. B and C show the positive ion chemical ionization GC-MS of methyl ester, TMS ether derivatives of MDHE fractions. The structures of compounds eluted from GC at 13.325 (B) and 13.398 min (C) are illustrated and their prominent ions are indicated.
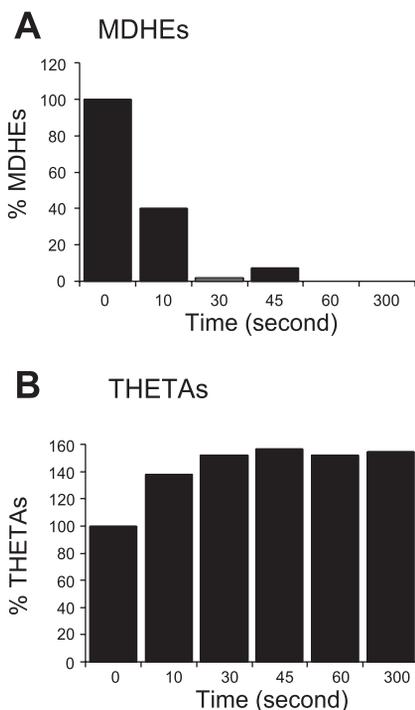
Fig. 6. Effect of acid treatment on the conversion of HEETA to THETAs and MDHEs. Rabbit aortas were incubated with $[^{14}C]AA$ (10^{-7} M) in the presence of Indo (10^{-5} M) and AUDA (10^{-6} M). The incubation buffer was treated with acetic acid for 0, 10, 30, 45, 60, and 300 s before 25 volumes of methanol were added. The AA metabolites were extracted and resolved with RP-HPLC. The percent decrease in MDHEs (*A*) and increase in THETAs (*B*) were measured with liquid scintillation counting. Standard 12-HETE was used as an internal standard.

(Fig. 6, *A* and *B*). At 30 s, both the decrease of MDHE and the increase of THETA formation reached a plateau. This indicates that the HEETA, identified as 15-H-11,12-EETA, is an unstable precursor of THETAs and is easily hydrolyzed by acidic conditions.

Role of sEH inhibitor in relaxations to AA and ACh. We (40) previously demonstrated that the biological HEETAs isolated from RP-HPLC relaxed preconstricted rabbit aortas. However, it is not known whether the HEETAs are direct vasodilators or whether the HEETAs are metabolized to vasodilatory THE-TAs. We used the sEH inhibitor AUDA to prevent the hydrolysis of HEETAs to THETAs. AA- and ACh-induced relaxations of preconstricted rabbit aorta were compared in the absence and presence of the sEH inhibitor AUDA (10^{-6} M). In Indo-treated vessels to inhibit the COX pathway, AA caused concentration-related relaxations, which were enhanced by AUDA pretreatment (Fig. 7*A*). The maximum relaxations were 49.9 $\pm$ 4 ($n = 14$) and 68.9 $\pm$ 3% ($n = 22$) in the absence and presence of AUDA, respectively. AUDA alone did not relax the preconstricted vessels (data not shown). In the presence of Indo (10^{-5} M) and L-NA (3×10^{-5} M) to inhibit COX and NO synthase, respectively, ACh also induced concentration-related relaxations. These relaxations were also enhanced in the presence of AUDA. The maximum relaxations were 43.0 $\pm$ 3 ($n = 8$) and 51.6 $\pm$ 3% ($n = 16$) in the absence and presence of AUDA, respectively (Fig. 7*B*). These data indirectly indicate that the HEETA relaxes the rabbit aorta and does not require conversion to a THETA for activity. The enhanced relaxations

by AUDA to both AA and ACh were inhibited with high $[K^+]_o$ (20 mM) [maximum relaxations were 16.0 $\pm$ 3 ($n = 8$) and 4.6 $\pm$ 1% ($n = 7$), respectively]. In the presence of AUDA, the relaxations to AA were inhibited by the LO inhibitor nordihydroguaiaretic acid [NDGA; maximum relaxation was 17.2 $\pm$ 4% ($n = 8$)]. Therefore, HEETA relaxed preconstricted vessels by causing K^+ channel activation and cell hyperpolarization.

Role of sEH inhibitor in AA-induced hyperpolarization of cell membrane. AA-induced hyperpolarization of smooth muscle was investigated in Indo- and phenylephrine-treated aortic segments with intact endothelium (Fig. 8). Under control conditions without AUDA, the membrane potential averaged -31.0 ± 1 mV ($n = 14$) and did not change with AUDA treatment (-32.6 ± 1 mV, $n = 27$). AA (3×10^{-5} M) induced a 15.8-mV ($n = 12$) hyperpolarization of aortic smooth muscle. AUDA enhanced this effect of AA, causing a 26.2-mV hyperpolarization ($n = 8$). In the presence of LO inhibitor NDGA, AA did not cause hyperpolarizations in the absence or the presence of AUDA (-31.8 ± 2 mV, $n = 13$, and -33.6 ± 3 mV, $n = 14$, respectively).

DISCUSSION

Furchgott and Zawadzki (17) first demonstrated that relaxations to ACh in rabbit aorta were dependent on an intact endothelium. These endothelium-dependent relaxations were
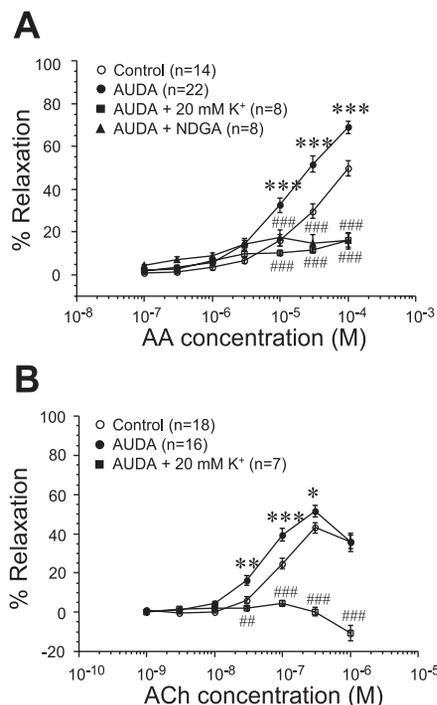

Fig. 7. Effect of the sEH inhibitor AUDA on AA- and ACh-induced relaxations of rabbit aorta. AUDA (1 μM) or its vehicle was added 30 min before testing of the agonists. Aortic rings were precontracted with phenylephrine. Relaxation responses to AA (*A*) were performed in the presence of Indo (10^{-5} M), and relaxation responses to ACh (*B*) were performed in the presence of both Indo (10^{-5} M) and nitro-L-arginine (L-NA; 3×10^{-5} M). In the AUDA-treated rings, the lipoxygenase (LO) inhibitor nordihydroguaiaretic acid (NDGA; 5×10^{-5} M) was added 10 min before testing of AA. In some experiments, the AUDA-enhanced relaxations to AA and ACh were determined in Krebs solution containing 20 mM K^+. Values are means $\pm$ SE. *$P <$ 0.05; **$P <$ 0.01; ***$P <$ 0.001 compared with controls. ##$P <$ 0.01; ###$P <$ 0.001 compared with the presence of AUDA.
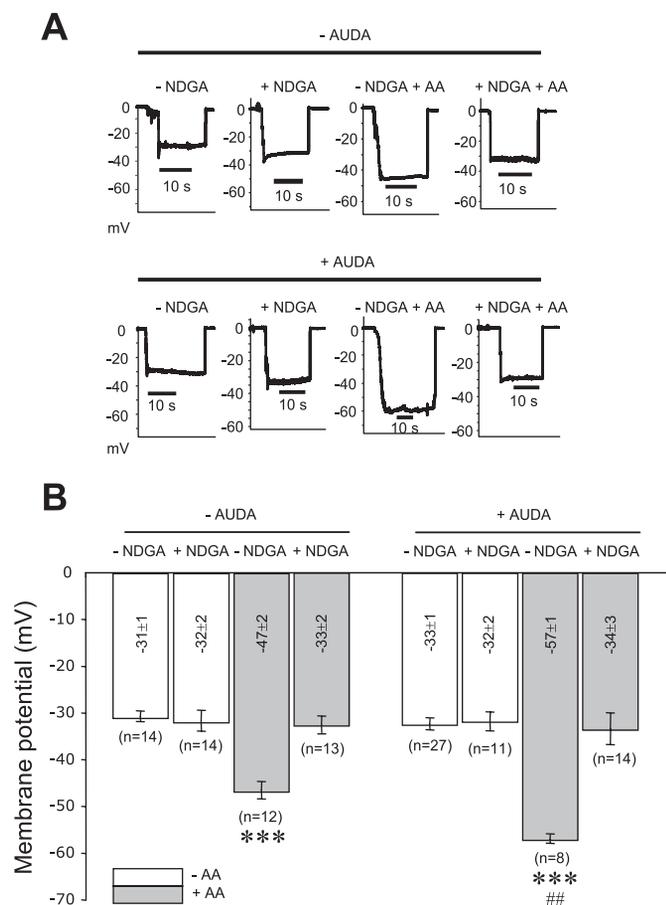
A

B

Fig. 8. Effect of AA on membrane potential of rabbit aortic smooth muscle cells. Studies were performed in the presence and absence of AUDA (10^{-6} M) and/or NDGA (5×10^{-5} M). Indo (10^{-5} M) and phenylephrine (10^{-7} M) were present in all experiments. A: typical traces of impalements under various conditions. B: summarized data. Value are means $\pm$ SE. ***$P < 0.001$ compared with control. ##$P < 0.01$ compared with the absence of AUDA.

acid (6, 18) and showed that this THETA caused hyperpolarization of rabbit aorta smooth muscle by opening apamin-sensitive K$^+$ channels (19). Thus 11,12,15-THETA functions as an EDHF in the rabbit aorta. Presently, we propose that 15-H-11,12-EETA is a biosynthetic intermediate in the synthesis of 11,12,15-THETA (6, 40, 42).

We demonstrated previously that the HEETA fractions collected from RP-HPLC relaxed the rabbit aorta (40). These fractions, however, contained a mixture of compounds, so the definitive structure(s) could not be determined (40). In this present study, the HEETA fraction from RP-HPLC was further purified by NP-HPLC and resolved into two peaks, called *peaks A* and *B. Peak A* was identified by GC-MS analyses as 15-H-11,12-EETA. The chemical structure of *peak B* needs further characterization. Nevertheless, this study is the first to provide direct evidence of the production of 15-H-11,12-EETA in rabbit aorta.

15-H-11,12-EETA has an epoxide group in conjugation with a double bond, so it is easily hydrolyzed in acid. Hamberg et al. (22) reported that the half-life of 15-H-11,12-EETA in aqueous solution at pH 3.0 and pH 7.4 is about 50 s and 33 h, respectively. The acid-labile nature of 15-H-11,12-EETA became a problem for evaluating the effect of sEH inhibition on the production of HEETA, since aortic incubates must be acidified for efficient extraction of the AA metabolites. Therefore, even with the sEH inhibitor present, a large portion of HEETA was converted to THETAs by acid hydrolysis and
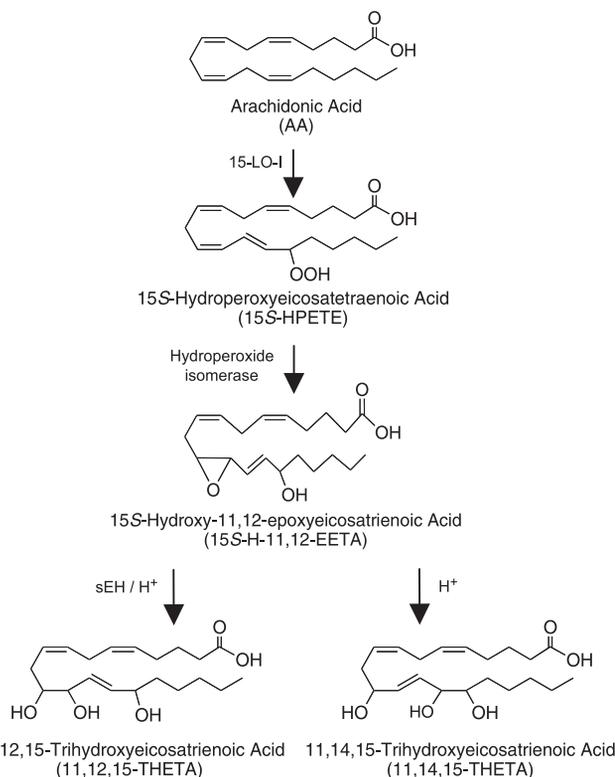
inhibited by LO inhibitors and suggested a role for LO metabolites of AA as relaxing factors. Subsequent studies showed that NO was the endothelium-derived relaxing factor (26, 35). However, there is evidence for other endothelium-derived factors that are LO metabolites. Singer and Peach (45, 46) showed that AA relaxed the rabbit aorta, and these relaxations were inhibited by LO inhibitors. Our laboratory (6, 48) also demonstrated that ACh- and AA-induced relaxations of rabbit aorta were blocked by LO inhibitors or antisense oligonucleotides against 15-LO-I.

Pace-Asciak et al. (34) first reported that the rat lung produced novel 12-LO metabolites of AA. The 12-LO product, 12-hydroperoxyeicosatetraenoic acid (12-HPETE), was converted to both the 8-H-11,12-EETA and 10-H-11,12-EETA (hepoxilin A$_3$ and B$_3$, respectively). Hepoxilins were converted by an epoxide hydrolase into the corresponding 8,11,12-THETA and 10,11,12-THETA (trioxilin A$_3$ and B$_3$, respectively) (34). It is unclear whether this epoxide hydrolase is sEH or a specific hepoxilin epoxide hydrolase (33). Since 15-LO-I is the major LO expressed in rabbit aorta (49), it does not produce hepoxilins or trioxilins (41). We identified a 15-LO-I metabolite of AA that is produced by the aortic endothelium as 11(R),12(S),15(S)-trihydroxyeicosa-5(Z),8(Z),13(E)-trienoic

Fig. 9. Proposed biosynthesis pathway of HEETA and THETAs. AA is converted to 15S-HPETE by 15-LO-I. 15S-HPETE is converted by a hydroperoxide isomerase to the HEETA. 15-H-11,12-EETA can be hydrolyzed by either sEH or acid to the corresponding THETAs. However, in acid hydrolysis, 15-H-11,12-EETA can be hydrolyzed to both 11,12,15-THETA and 11,14,15-THETA. The exact stereochemistry of the HEETA is not known but is deduced from published reports (see DISCUSSION).

there was no significant change in HEETA production. We overcame this problem by using an excess of acidified methanol to trap the acid-labile HEETA into the form of MDHEs. Thus the accumulation of HEETA after inhibition of sEH can be measured as MDHE production. Using this method, we showed that inhibition of sEH with AUDA increased MDHE formation (Fig. 4, *A* and *B*), suggesting that HEETA is a substrate for sEH. The presence of sEH in rabbit aorta and rabbit aortic ECs further supports this conclusion.

With acidified methanol treatment, the methoxy group can add at either the epoxide group or allylic to the epoxide. Therefore, 15-H-11,12-EETA will produce two regioisomers of 12-methoxy-11,15-dihydroxy- and 14-methoxy-11,15-dihydroxyeicosatrienoic acids when trapped with acidified methanol. In addition to the acid-labile 15-H-11,12-EETA, the HEETA fractions contain another metabolite or metabolites that are not hydrolyzed to THETAs or other polar metabolites in acid. These metabolites may be the components of *peak B* of Fig. 1*C* and may be HEETAs or metabolites of similar polarity. The identity and biological activity of these metabolites are not known but are important questions for future studies.

In agreement with our current and previous data demonstrating that 11,12,15-THETA is a vasodilator produced in rabbit aorta and that 11,12,15-THETA is the hydrolysis product of 15-H-11,12-EETA (6, 40), we propose the biosynthetic pathway of HEETA and THETA synthesis shown in Fig. 9. In rabbit aortic endothelium, AA is first converted to 15-HPETE by 15-LO-I. 15-HPETE is then metabolized to 15-H-11,12-EETA by the hydroperoxide isomerase. The epoxide group of HEETA is hydrolyzed by sEH and/or acid to 11,12,15-THETA. The nonspecific hydrolysis of 15-H-11,12-EETA by acidic conditions will produce both 11,12,15-THETA and 11,14,15-THETA.

15-LO catalyzes the insertion of molecular oxygen into the carbon 15-position of AA in the S configuration to produce 15*S*-HPETE (11, 16). The principal LO of the rabbit aorta is 15-LO-I, and aorta produces 96% 15*S*-HETE and 4% 15*R*-HETE (37, 49). Based on this evidence, we propose that the stereochemistry of the hydroxyl group at the carbon-15 position is S stereoisomer. Because of the small amount of HEETA produced by the rabbit aorta and its sensitivity of acid hydrolysis, we were unable to purify an adequate mass of the HEETA for directly determination of the stereochemical configuration.

The biological role(s) of the HEETAs in regulating vascular tone has not been studied. We showed that the HEETA fractions collected from RP-HPLC relaxed preconstricted rabbit aorta in a concentration-dependent manner (40). These relaxations were blocked with high $[K^+]_o$, suggesting that HEETA activity is dependent on K^+ channel activation, K^+ efflux, and membrane hyperpolarization. Since HEETAs are hydrolyzed to THETAs, it is not known whether the HEETAs have direct vasoactivity or if they require hydrolysis to the THETAs. We have previously shown that AA and ACh cause endothelium-dependent, concentration-related relaxations in Indo- and L-NA-treated aortas (6, 36). These relaxations are inhibited by LO inhibitors and antisense oligonucleotides against rabbit 15-LO (6, 36, 48, 49). Thus these relaxations are mediated by a 15-LO metabolite of AA. In this study, we demonstrated that ACh- and AA-induced relaxations were enhanced by AUDA. This finding indirectly suggests that HEETAs relax rabbit

aorta. The mechanism of the HEETA relaxation involves K^+ efflux and membrane hyperpolarization, since increasing $[K^+]_o$ from 4.8 to 20 mM inhibited these enhanced relaxations to both AA and ACh. Results from electrophysiological studies support this conclusion by demonstrating that AUDA also increases AA-induced hyperpolarization of aortic smooth muscle cells. Increased vascular relaxation and hyperpolarization of smooth muscle with AUDA treatment would also suggest HEETA is more potent than THETA. Nevertheless, caution must be used in the interpretation of these data, since it is not known whether AUDA completely inhibits sEH under these experimental conditions. The presence of small amounts of THETA could complicate the interpretation. A stable analog of the HEETA is needed to evaluate its potency with confidence. In other studies, we observed the synthesis of THETAs and HEETAs in arteries from mice, guinea pigs, and humans (unpublished observation). Therefore, these LO metabolites may have a general role in the vasculature of many species.

In summary, results from this study demonstrate that the 15-LO-I metabolite 15-H-11,12-EETA is produced by the endothelium of the rabbit aorta and is metabolized by sEH to 11,12,15-THETA. Along with 11,12,15-THETA, 15-H-11,12-EETA represents an endothelium-derived hyperpolarizing factor.

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