Identification of 13-Hydroxy-14,15-epoxyeicosatrienoic Acid as an Acid-stable Endothelium-derived Hyperpolarizing Factor in Rabbit Arteries*

Yuttana Chawengsub1,1, Kathryn M. Gauthier1,2, Kasem Nithipatikom3, Bruce D. Hammock1, John R. Falck3, Dubasi Narshmhaswamy4, and William B. Campbell1,3

From the 1Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, the 2Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, and the 3Department of Entomology and Cancer Research Center, University of California, Davis, California 95616

Arachidonic acid (AA) is metabolized by endothelial 15-lipoxygenase (15-LO) to several vasodilatory eicosanoids such as 11,12,15-trihydroxyicosatetraenoic acid (11,12,15-THETA) and its proposed unstable precursor 15-hydroxy-11,12-epoxyeicosatrienoic acid (15-H-11,12-EETA). In the present study, the acid-stable 13-hydroxy-trans-14,15-epoxy-eicosatrienoic acid (13-H-14,15-EETA) was identified and its vascular activities characterized. Rabbit aorta, mesenteric arteries, and the combination of 15-LO and cytochrome P450 2J2 converted AA to two distinct HEETA metabolites. The HEETA metabolites were resistant to acidic hydrolysis but were hydrolyzed by recombinant sEH to a more polar metabolite identified by mass spectrometry as 13,14,15-THETA. Mass spectrometric analyses and HPLC comigration identified the HEETA identified by mass spectrometry as 13,14,15-THETA. Mass but were hydrolyzed by recombinant sEH to a more polar metabo-

The on-line version of this article (available at http://www.jbc.org) contains additional data.
Experimental protocols were 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, AA, and other chemicals were purchased from Sigma. The erythro- and threo-HETA standards were prepared from methyl 15(S)-HPETE according to Corey et al. (21, 22) and had spectral properties consistent with literature values. Additional threo- and erythro-HETA standards were provided by Dr. Alan Brash, Vanderbilt University Medical Center, Nashville, TN.

Tissue Preparation and Incubation—Thoracic and abdominal aortas were isolated from 4–6-week-old New Zealand White rabbits (Kuiper Rabbit Ranch, IN), placed in ice-cold HEPES buffer (in mM; 10 HEPES, 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 6 glucose, pH 7.4), cleaned of adhering connective tissue and fat and cut into rings (5-mm long) (23, 24). Aortic rings were incubated for 10 min at 37 °C in HEPES buffer containing indomethacin (10⁻⁵ M). [¹⁴C]AA (10⁻⁶ Ci, 2.5 × 10⁻⁷ m) plus unlabeled AA (10⁻⁷ m) was added, and the vessels were incubated for an additional 5 min. A23187 (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 to pH 3.5 with glacial acetic acid, incubated for 3 min at room temperature and extracted on Bond Elut C-18 extraction columns (23, 25, 26). The extracts were evaporated to dryness under a stream of N₂ and stored at −40 °C until HPLC analysis.

In the incubations for mass spectrometric analyses, AA (10⁻⁴ m) was used. Aortic incubations with [¹⁴C]AA plus either 10⁻⁴ m or 10⁻⁷ m AA did not show differences in the profile of radiolabeled metabolites when resolved with reverse phase (RP)-HPLC. In some experiments, AA was incubated with soybean 15-LO (20 μg/ml) and/or recombinant human CYP2J2 (Genetest, 50 pmol/ml) for 10 min, and the metabolites were extracted as described above.

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. 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. Column eluate was collected in 0.2-ml aliquots, and radioactivity was measured with a liquid scintillation counter. Fractions containing THETA (6.8–8.8 min) and HEETA (12.8–15.8 min) were pooled, acidified, and extracted with cyclohexane/ethyl acetate (50:50). The solvent was dried under a stream of N₂. HEETA extracts were further resolved on normal phase (NP)-HPLC (Nucleosil silica column, 5 μm, 4.6 × 250 mm) using solvent system II. This was an isocratic system of 0.1% glacial acetic acid and 1% isopropanol in hexane for 40 min at a flow rate of 1 ml/min. Radioactive peaks were collected, dried under a stream of N₂, and stored at −40 °C for further analyses with mass spectrometry. All solvents were HPLC grade.

Chemical Modifications—To aid in the identification of the HEETAs, a number of chemical modifications were performed on column fractions containing the HEETAs. The HEETA fractions were divided into two parts with one-half being treated with various conditions as described below and the other half retained for comparison. The resulting products were resolved by RP-HPLC using solvent system I. The column eluate was collected in 0.2-ml aliquots, and radioactivity was measured by liquid scintillation spectrometry. A change in migration time suggested a modification of the molecule. In each case, authentic compounds with known functional groups were treated with the same reagents and analyzed in a similar manner by RP-HPLC. The chemical modification procedures of the AA metabolites included the following.

Acid Treatment—The sample was dissolved in 1 ml of 1 N acetic acid. After incubation at room temperature for 18 h, the sample was extracted with one volume of ethyl acetate five times. The organic layer was separated and evaporated to dryness under N₂ stream and analyzed by HPLC.

Recombinant sEH Incubation—The sample was dissolved in 1 ml of HEPES buffer. Recombinant human sEH (5 μg) was added, and the sample was incubated for 30 min at 37 °C. After the incubation, the sample was extracted with 1 volume of ethyl acetate five times. The organic layer was separated and evaporated to dryness under N₂ stream and analyzed by HPLC.

Mass Spectrometry Methods—The analyses were performed by an electrospray ionization (ESI)-Fourier transform ion cyclotron resonance mass spectrometer (FTICR-MS) (IonSpec 7.0 Tesla FTICR high resolution mass spectrometer with a Waters Z spray ESI source) (27). The source temperature was 80 °C and cone voltage −45 V. The m/z of interest was isolated and fragmented with sustained off-resonance irradiation collision-induced dissociated (SORI-CID) using N₂ 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.

Vascular Reactivity—Thoracic aortic rings (3-mm long) were suspended in 6-ml tissue baths containing Krebs bicarbonate buffer (in mM; 118 NaCl, 4 KCl, 3.3 CaCl₂, 24 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, and 11 glucose) maintained at 37 °C and continuously bubbled with 95% O₂, 5% CO₂ as described previously (14, 24). Isometric tension was measured with force-displacement transducers (FT-03C, Grass) and amplifiers (ETH-400, AD Instruments) and recorded on a Macintosh computer using MacLab Chart software. Aortas were 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. Contraction 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. At this point, the vessels were ready for the experiment.
Vessels were pretreated for 10 min with indomethacin (10^{-5} M) and 30 min with AUDA (10^{-6} M). Vessels were then preconstricted to 50–60% of maximal KCl constriction with phenylephrine (10^{-7} M). When the constrictions to phenylephrine were stable, cumulative concentrations of 13-H-trans-14,15-EETA isomers were added, and changes in isometric tension were measured.

Wire Myograph—Small mesenteric arteries corresponding to a second- or third-order branch from superior mesentery arteries (diameter 200–300 μm) were isolated from 3–6-week-old, male New Zealand White rabbits and placed in ice-cold HEPES buffer. The tissue was carefully cleaned of adhering fat and connective tissue and cut into rings (1.5 mm long). Arterial segments were threaded on two stainless steel wires (40-μm diameter) and mounted on a four-chamber wire myograph (model 610 M, Danish Myo Technology A/S) (28) in physiological saline containing indomethacin (10^{-6} M) and 30 min with AUDA (10^{-6} M) for 3–5 min at 10 min intervals. Arteries were then allowed to equilibrate for another 30 min before the initiation of experimental protocols.

To examine relaxation responses, a submaximal concentration of phenylephrine (10^{-6} M) was added to the bath to precontract the arteries to 50–75% of maximal constriction to 50 mM KCl. After the contraction reached steady state, cumulative concentration-response curves to synthetic 13-H-trans-14,15-EETA isomers were determined. Relaxations were examined as concentration-response curves to synthetic 13-H-trans-14,15-EETA (1–3 × 10^{-6} M, 3 ml each concentration). Results are graphed as mean open state probability (NPo).

Cell Migration Assay—Rabbit aortic smooth muscle cells (RA-SMCs) were grown to confluence in T175 flasks (31). The cells were seeded into 35 mm × 10 mm cell culture dishes and maintained in culture for 3 days or until cell confluency was achieved. The cell culture dish was denuded of cells along a single line across the dish using a small sterile flat head screwdriver. The cell culture dishes were washed with phosphate-buffered saline and then twice with serum-free medium after wounding to remove the debris as well as the feeding medium. Angiotensin II (10^{-7} M) was added to stimulate cell migration. Threeo- and erythro-13-H-trans-14,15-EETA (10^{-9} to 10^{-5} M) were added to the experimental wells. Control wells were exposed to serum-free medium and vehicle alone. Images of the wound region were recorded after scraping (0 h) and 24 h later in the same region of the cell culture dish. Migration was reported as the percentage of control cell migration. In some experiments, mitomycin C (10^{-7} M) was added to inhibit SMC proliferation.

Statistical Analysis—Statistical evaluation of the vascular activity and patch-clamp data were performed by using one-way analysis of variance followed by Student-Newman-Keuls post hoc analysis when significant differences were present. All statistical comparisons were made at the 95% confidence level, \( p \leq 0.05 \).

RESULTS

Purification of HEETA Stereoisomers—After incubation of rabbit aorta with [^{14}C]AA, the metabolites were extracted and resolved by RP-HPLC with solvent system I. Metabolites comigrating with THETA, HEETA, and HETE standards, and non-metabolized AA were observed (Fig. 1A). HEETA fractions (12.8–15.8 min) were collected, extracted, and further purified with NP-HPLC (solvent system II). NP-HPLC resolved HEETA into two distinct radioactive peaks named peak A and peak B (Fig. 1B). During the extraction process, the incubation buffer was acidified to pH 3 and incubated for 3 min to hydrolyze the acid-sensitive allylic epoxyalcohols (15-H-11,12-EETA or 11-H-14,15-EETHA) to THETAs. This was necessary since an unstable HEETA comigrates with peak A on NP-HPLC (solvent system II). NP-HPLC resolved HEETA into two distinct radioactive peaks named peak A and peak B. When tested for vascular activity, peak A and peak B relaxed phenylephrine preconstricted rabbit aortas in a concentration-dependent manner in the presence of indomethacin (10^{-5} M) and AUDA (10^{-6} M) (supplemental Fig. S1). The relaxations produced by peak A exceeded peak B. Increasing [K^+]_o from 4.8 mM to 20 mM reduced the relaxations to both peak A and B.

Characteristic of HEETA Metabolites—Because metabolites comigrating with the HEETAs were observed following acidification and extraction, the 12.8–15.8-min fractions on RP-HPLC may contain acid stable metabolites in addition to the acid sensitive HEETAs (15). To test this possibility, peak A and B resolved and collected from NP-HPLC were incubated with 1 N acetic acid for 18 h at room temperature. After incubation, the samples were extracted and analyzed with RP-HPLC (Fig. 2). Peaks A and B eluted as single peaks comi-
incubated with $^{14}$C-AA in the presence of indomethacin (10 nM) when both peaks were treated with acid (Fig. 2, A and B). Biological peak A and peak B co-eluted on NP-HPLC with synthetic erythro- and threo-13-H-trans-14,15-EETA (C). Elution times of the biological HEETAs were determined by measurement of radioactivity in the column eluate, whereas elution times of the synthetic HEETAs were determined by UV absorbance at 205 nm.

Integrating with HEETA (Fig. 2, A and D), A hydrolysis product with a retention time consistent with THETA was observed when both peaks were treated with acid (Fig. 2, B and E). The hydrolysis product was more prominent with the acid incubation of peak B than peak A. Nevertheless, unlike the acid-labile HEETA, which is completely hydrolyzed to THETAs within 30 s at pH 3 (15), the majority of peaks A and B were intact despite acid treatment for 18 h. Under this same acid treatment conditions, ~70% of 14,15-epoxyicosatrienoic acid was hydrolyzed to a product comigrating with 14,15-dihydroxyeicosatrienoic acid (data not shown).

When peaks A and B were incubated with recombinant human sEH and analyzed with RP-HPLC, each peak was hydrolyzed to a more polar compound with a retention time consistent with a THETA (Fig. 2, C and F). Since the compounds are hydrolyzed by sEH, they must contain an epoxide group. However, the possibilities that the functional group being a keto or hydrofuran were also considered. To test for the presence of a keto group, HEETA was derivatized by methoxylamine to produce a methoxime derivative, which is less polar than a keto group. When analyzed with RP-HPLC, there was no change in migration times of the HEETAs with or without methoxime treatment (data not shown). In contrast, the migration time of 6-keto PGF$_{1\alpha}$, shifted from 4 to 5 min with methoxime treatment. Therefore, the possibility that the HEETAs contain a keto group was excluded. 11,14-hydrofuran-15-hydroxy-eicosatrienoic acid was resistant to both sEH and acid treatment (data not shown); hence, the possibility of HEETA containing a hydrofuran group was also excluded.

Structural Characterization of Peak A and Peak B HEETAs—Chemical structures of peak A and peak B were analyzed separately with FTICR-MS using the negative ionization mode. The MS/MS spectra of both peaks were practically identical. Mass spectra of peak A (Fig. 3B) and B (Fig. 3C) showed prominent ions at $m/z$ 335 (M-H), 317 (M-H-(H$_2$O)), 299 (M-H-2(H$_2$O)), 273 (M-H-(H$_2$O)-(CO$_2$)), 255 (M-H-2(H$_2$O)-(CO$_2$)), 235 (loss of O-CH-(CH$_2$)$_4$CH$_3$), 217 (235-H$_2$O), 205 (loss of CH-O-CH-(CH$_2$)$_4$CH$_3$-(H$_2$O)), 189 (loss of CH(CH$_2$)$_4$CH$_3$-(H$_2$O)-(CO$_2$)), 173 (235-(H$_2$O)-(CO$_2$)), and 161 (205-CO$_2$). These data indicate that both peak A and peak B are 13-H-14,15-EETAs. Intensity of $m/z$ 235 was lower in peak A than in peak B, while that of $m/z$ 189 was higher suggesting that the chemical structure of peak A favored the cleavage of the epoxide group on the allylic side while peak B favored the cleavage on the carboxyl side. Note that ion $m/z$ 191 in peak B was a contaminant. When peak B was analyzed by LC-FTICR-MS, ion $m/z$ 191 was not present. The difference in stereochemistry of the adjacent hydroxyl group may contribute to the fragmentation pattern of the epoxide group. This implies that peak A and peak B are diastereomers.

To gain additional structural information, double-bond hydrogenation was performed on each peak and the products were analyzed by FTICR-MS. The mass spectra of saturated peak A and B revealed major ions at $m/z$ 341 (M-H), 323 (M-H-(H$_2$O)), 305 (M-H-2(H$_2$O)), 279 (M-H-(H$_2$O)-(CO$_2$)), 257 (loss of CH-(CH$_2$)$_4$CH$_3$), 241 (loss of O-CH-(CH$_2$)$_4$CH$_3$), 227 (271-CO$_2$), or (M-H-114; loss of CH$_2$-O-CH-(CH$_2$)$_4$CH$_3$, and 197 (241-CO$_2$). These mass spectra are consistent with the structure of 13-hydroxy-14,15-epoxy-eicosanoic acid (supplemental Fig. S2).

It can be predicted that hydrolysis of 13-H-14,15-EETAs by sEH will yield 13,14,15-THETA. The chemical structures of the sEH-hydrolysis products of peaks A (Fig. 4B) and B (Fig. 4C) were characterized with FTICR-MS and compared with authentic 13,14,15-THETA (27). sEH-hydrolysis product major ions ($m/z$) were 353 (M-H), 335 (M-H-(H$_2$O)), 317 (M-H-2(H$_2$O)), 299 (M-H-3(H$_2$O)), 253 (loss of CH-OH-(CH$_2$)$_4$CH$_3$), 235 (253-H$_2$O), 217 (235-H$_2$O), 205 (223-H$_2$O), 193 (loss of (CHOH)$_2$-(CH$_2$)$_4$CH$_3$, 173 (217-CO$_2$), and 161 (223-CO$_2$). These mass spectra are consistent with 13,14,15-THETA, especially the characteristic peak of $m/z$ 193 (27).
Characterization of Synthetic 13-H-14,15-EETA Isomers—
Based on structural data of the biological HEETAs, threo- and erythro-isomers of 13-H-trans-14,15-EETAs were synthesized. The physical and chemical properties of these synthetic HEETAs were compared with biological peak A and B. Standard HEETA with a similar structure, 15-H-13,14-EETA, was also compared. Migration times of the synthetic HEETAs were determined by coinjection of the synthetic HEETAs with biological HEETAs on NP-HPLC. Migration times of the biological 14C-peak A and B were identical to those of the erythro- and threo-13-H-trans-14,15-EETAs, respectively (supplemental Fig. S3, B and C). Mass spectra of 15-H-13,14-EETA, though similar with those of 13-H-14,15-EET, did not contain ion m/z 189, which can be used to differentiate between these two regioisomers (supplemental Fig. S4).

To confirm these structural assignments, MS analyses of peak A and B (Fig. 3) were compared with those of the synthetic counterparts. The MS/MS of peak A and peak B were identical to those of the erythro- and threo-13-H-trans-14,15-EETAs, respectively (supplemental Fig. S3, B and C). Mass spectra of 15-H-13,14-EETA, though similar with those of 13-H-14,15-EET, did not contain ion m/z 189, which can be used to differentiate between these two regioisomers (supplemental Fig. S4).

The acid stability of threo- and erythro-13-H-trans-14,15-EETAs was also tested (supplemental Fig. S5). Similar to biological peak A and B, erythro- and threo-13-H-trans-14,15-EETAs were resistant to acid hydrolysis with the more notable hydrolysis of the threo-isomer. sEH also hydrolyzed synthetic threo- and erythro-13-H-trans-14,15-EETAs to 13,14,15-THETA and mass spectra of the hydrolytic product from erythro- and threo-isomers were identical to those from peak A and peak B, respectively (supplemental Fig. S3, F and G). These data confirmed the structural assignments of peak A and peak B as erythro- and threo-isomers of 13-H-trans-14,15-EET, respectively.

Biosynthetic Pathway of 13-H-14,15-EETAs—To gain an insight into the mechanisms of the HEETA biosynthetic pathway, the synthesis of 13-H-14,15-EETAs was reconstituted with individual enzyme components. We proposed that the pathway of 13-H-14,15-EET synthesis involves sequential actions of 15-LO and cytochrome P450 2J2 (CYP2J2) (32, 33). 15-LO converts AA to 15-hydroperoxyeicosatetraenoic acid (15-HPETE) and CYP2J2 rearranges the hydroperoxide group to form 13-H-14,15-EETAs. Incubation of [14C]AA with a combination of soybean 15-LO and recombinant human CYP2J2 produced metabolites that comigrated with standard for THE-TAs, HEETAs, and 15-HETE on RP-HPLC (solvent system I) (Fig. 5C). Another metabolite eluting at 19.8 min was also detected and comigrated with 12-HETE and 15-keto-eicosatetraenoic acid (15-KETE) standards. FTICR-MS analysis indicated prominent ions of m/z 317.2, 158.6, and 105.7. This mass
spectrum was identical to the mass spectrum of the 15-KETE standard. In the absence of 15-LO or CYP2J2, the production of HEETA, THETA, and 15-KETE were not observed (Fig. 5, A and B). 15-LO alone metabolized AA to products comigrating with 15-HETE. These data indicate that HEETA and THETA production is an enzymatic rearrangement of 15-HPETE by CYP2J2. There was no evidence for the non-enzymatic formation of these products. From RP-HPLC, the HEETA fractions were collected and the chemical structure was characterized with FTICR-MS. The mass spectra of the HEETAs (Fig. 5D) is consistent with 13-H-14,15-EETA and its major daughter ions are shown in panel A.

Biological Activities of Peak A and Peak B—The threo- and erythro-13-H-trans-14,15-EETA were tested for their ability to relax rabbit aorta and small mesenteric arteries. Both isomers relaxed phenylephrine preconstricted rabbit aortas in a concentration-dependent manner in the presence of indomethacin (CDC) inhibited the synthesis of THETAs, HEETAs, and HETEs in both rabbit aorta and mesenteric artery (34, 35). Interestingly, the imidazole containing CYP inhibitor ketoconazole (10^{-4} M) (supplemental Fig. S6) and ebastine (5 × 10^{-5} M) (data not shown), which inhibit epoxygenase activity of CYP, did not alter the production of HEETAs from either rabbit aorta, mesenteric arteries, or in vitro system of 15-LO and CYP2J2.
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FIGURE 5. Synthesis of 13-H-14,15-EETAs by a combination of soybean 15-LO and CYP2J2. [14C]AA was incubated with soybean 15-LO (A) or CYP2J2 (B) or a combination of 15-LO and CYP2J2 (C), and the metabolites were resolved with RP-HPLC solvent system I. HETE fractions (12.8–15.8 min) were collected and analyzed by ESI-FTICR-MS using a negative ionization mode. Ion m/z 335 indicating the HETE molecular weight was isolated and fragmented with SORI-CID (D). The HETE fractions (19–21 min) were collected and analyzed by ESI-FTICR-MS producing ions of m/z 317.2, 158.6, and 105.7 consistent with 15-KETE.

(10−5 m) and AUDA (10−6 m). Maximum relaxations produced by the erythro- and threo-isomers were 28.7 ± 3.1% and 19.5 ± 6.1%, respectively (Fig. 6A). Similarly, they relaxed the endothelium-denuded mesenteric arteries in the presence of indo- methacin (10−5 m) and N-ω-nitro-L-arginine (L-NNa) (3 × 10−5 m) (Fig. 6B). Maximum relaxation of erythro- and threo-isomers were 22.6 ± 6.0% and 8.6 ± 4.3%, respectively. Apamin (10−7 m) inhibited the mesenteric artery relaxations to the erythro-isomer (maximum relaxation = 1.2 ± 5.6%) (Fig. 6C). When the mesenteric arteries were preconstricted with K+ (30 mm), the erythro-isomer caused constrictions (maximum constriction = −21.4 ± 2.8%) (Fig. 6D). Therefore, the erythro-13-H-trans-14,15-EETA (equivalent to the biological peak A) was more active than the threo counterpart and relaxed the arteries in an endothelium-independent fashion, which suggests a direct action on smooth muscle. The mechanism of action of the erythro-isomer involves apamin-sensitive SK channel activation.

To demonstrate the effect of erythro-13-H-trans-14,15-EETAs on K+ channels, cell-attached K+ currents of isolated rabbit mesenteric artery SMCs were measured at holding potential of −80 mV (Fig. 7). Small K+ currents were observed with a conductance of 13–14 pS. Perfusion with increasing concentrations of erythro-13-H-trans-14,15-EETAs (1 × 10−6 to 3 × 10−4 m) increased the open state probability (NPo) of these channels from 0.0007 ± 0.0007 to 0.0053 ± 0.0042 at 3 × 10−6 m. This activation was inhibited by the presence of apamin (10−7 m). There was no indication that the HEETA affected K+ channels with a larger conductance. These results confirm the role of smooth muscle apamin-sensitive SK channel activation in vascular relaxations induced by erythro-13-H-trans-14,15-EETA.

13-H-14,15-EETAs were also tested on migration of rabbit aortic SMCs. Angiotensin II (10−7 m) stimulated SMC migration (129 ± 5.0% compared with control) (Fig. 8). Erythro-13-H-trans-14,15-EETA inhibited angiotensin II-stimulated SMC migration in a concentration-dependent manner (10−9 to 10−5 m) with complete inhibition at 10−6 m (Fig. 8B). With 10−5 m HEETA, the migration was reduced further to 81 ± 6% of the control. A similar inhibition by erythro-13-H-trans-14,15-EETA was observed in cells treated with mitomycin C (10−7 m) to inhibit SMC proliferation (data not shown). Thus, the wound closure by angiotensin II and its inhibition by HEETA were due to SMC migration and not proliferation. In contrast, threo-13-H-trans-14,15-EETA was without effect (maximum inhibition of 106 ± 6% at 10−5 m) (Fig. 8C). These results suggest that like most

DISCUSSION

Our understanding of the mechanisms underlying cardiovascular diseases has improved since the discovery that endothelial cells, besides being the interface between blood and vascular wall, also regulate vascular tone by releasing several soluble mediators. These vasodilatory mediators are NO, PGI2, and EDHF. Unlike NO and PGI2, EDHF is not a single molecule, but instead a group of chemically diverse compounds that hyperpolarize and relax the smooth muscle cells (6, 36). EDHF activity is described as the vasodilator response, which remains after administration of NO synthase and cyclooxygenase inhibitors, and this response is inhibited by high [K+]o (6, 36). These criteria were used in this study to define an EDHF.

AA causes endothelium-dependent relaxations and endothelium-dependent hyperpolarization of SMCs (16, 37–39). These relaxations are blocked by LO inhibitors, 15-LO-I anti-sense oligonucleotides, high [K+]o, and an inhibitor of SK channels, apamin (11, 12, 16). 15-LO-I was characterized as the prominent LO expressed in rabbit aorta (12). Therefore, rabbit aortic endothelial cells release EDHF’s that are AA metabolites of the 15-LO-I pathway. These 15-LO-I metabolites act on SMCs to open K+ channels, hyperpolarize the cell membrane, and induce relaxation. The proposed mediators of this EDHF activity are 11(R),12(S),15(S)-THETA (17) and its possible precursor 15-H-11,12-EETA (15). In this study, acid treatment of
Aortic incubates eliminated the acid labile allylic epoxyalcohols such as 15-H-11,12-EETA and 11-H-14,15-EETA (15) and allowed characterization of the structure of the acid stable 13-H-14,15-EETA and determination of its actions on SMCs.

Other investigators have previously demonstrated the synthesis of 13-H-14,15-EETA from 15-HPETE by liver microsomes, polymorphonuclear leukocytes (PMN) cytosol and recombinant prostacyclin synthase and thromboxane synthase (33, 40, 41). These reports disagree on whether the synthesis of 13-H-14,15-EETA is enzymatic or non-enzymatic. Boiling liver microsomes to inactivate enzymatic activity reduces 13-H-14,15-EETA synthesis by 70% (33) whereas boiling PMN cytosol increases HEETA production (40). As with liver microsomes, boiled prostacyclin or thromboxane synthases fail to convert 15-HPETE to 13-H-14,15-EETA (41). Our studies also support enzymatic synthesis since no 13-H-14,15-EETA formation was observed when AA and 15-LO were incubated alone. Addition of CYP2J2 to the mixture of AA and 15-LO was required for HEETA formation.

This study is the first to demonstrate the production of 13-H-14,15-EETA by vascular endothelial cells, to establish the identities of the specific 13-H-14,15-EETA stereoisomers, to suggest potential enzymes involved in the vascular synthetic pathway and to describe direct vascular actions for a 13-H-

FIGURE 6. Effect of three- and erythro-13-H-trans-14,15-EETA on preconstricted rabbit aortas and mesenteric arteries. A, relaxations of endothelium-intact rabbit aortas in the presence of indomethacin (10^{-5} M) and AUDA (10^{-6} M). B, relaxations of endothelium-denuded rabbit mesenteric arteries in the presence of indomethacin (10^{-5} M) and L-NNA (3 \times 10^{-5} M). C, effect of apamin (10^{-7} M) on erythro-13-H-trans-14,15-EETA relaxations of mesenteric arteries pretreated with indomethacin (10^{-5} M) and L-NNA (3 \times 10^{-5} M). D, effect of high K+ (3 \times 10^{-5} M) on erythro-13-H-trans-14,15-EETA relaxations of mesenteric arteries. Arteries were contracted with phenylephrine (10^{-6} M; A–D). Values represent the mean \pm S.E. **, p < 0.01; ***, p < 0.001 compared with control.

FIGURE 7. Effect of apamin (10^{-7} M) on erythro-13-H-trans-14,15-EETA activation of cell-attached K+ currents of isolated mesenteric SMCs. Channel activity is expressed as NPo; \Em = -80 \text{mV}. Channel conductance = 13–14 pS. n = 7 each. *, p < 0.05 control, 3 \times 10^{-6} M versus Control. 0, **, p < 0.05 apamin; 3 \times 10^{-6} M versus Control, 3 \times 10^{-6} M.
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FIGURE 8. Effect of threeo- and erythro-13-H-trans-14,15-EETA on rabbit aortic SMC migration. A, representative photographs of rabbit aortic SMC migrations in the wound healing assay taken at 24 h with angiotensin II (Ang II, 10−7 M) and Ang II plus erythro-13-H-14,15-EETA (Ang II + Erythro [10−5 M]). B, effect of increasing concentrations of erythro-13-H-trans-14,15-EETA on Ang II-stimulated SMC migration. C, effect of increasing concentrations of threeo-13-H-trans-14,15-EETA on Ang II-stimulated SMC migration, n = 4–16. Values are expressed as percentage of migration ± S.E. *, p < 0.05; **, p < 0.001 compared with Ang II alone. #, p < 0.05, compared with no Ang II.

14,15-EETAs, in the endothelium.

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radioactive peaks called peak A and peak B. Peak A and unstable HEETAs elute on NP-HPLC but can be resolved by gas chromatography (15). To ensure that the unstable allylic HEETAs did not interfere with the identification of the acid stable HEETAs, the incubation buffer was acidified to hydrolyze allylic HEETAs to THETAs leaving peak A as the single chemical species. Peak A and B were not hydrolyzed to THETAs when incubated with acetic acid for 18 h. Mass spectra of both peaks were consistent with the structure of 13-H-14,15-EETAs. Along with these data, sEH hydrolysis products of peak A and B were identified as 13,14,15-THETAs.

Because the quantities of biological peak A and B were not sufficient for the stereochemical determination, synthetic 13-H-14,15-EETA isomers were used for comparison with the biological HEETAs. Peak A comigrated with erythro-13-H-trans-14,15-EETA while peak B comigrated with threeo-13-H-trans-14,15-EETA on NP-HPLC. Comparison of mass spectra of synthetic 13-H-trans-14,15-EETA isomers with peak A and peak B support the conclusion that peak A and peak B are erythro- and threeo-13-H-trans-14,15-EETAs, respectively.

An epoxide group in conjugation with a double bond is highly reactive and easily hydrolyzed in acid (42–44). Because 13-H-14,15-EETAs has a hydroxyl group separating the double bond and the epoxide group, it can be postulated that the hydroxyl group protects the epoxide from acid hydrolysis by a combination of steric shielding and inductive effects. Additionally, 5,6-trans-EET is more stable and resistant to hydrolysis than its cis-isomer (45). Thus, the trans-epoxide of 13-H-14,15-EETAs could also contribute to the acid stability. Hydrolysis of epoxides by sEH may vary with substituents. The trans isomers of epoxysterate and the EETAs were better substrates for sEH than the cis isomers (46, 47). The hydration of epoxides alpha to carbons with a hydroxyl substituent (glycidols) such as 13-H-14,15-EETAs occurs so slowly that they may function as inhibitors of the sEH (48). This may be a possible action of 13-H-14,15-EETAs in the endothelium.

The physiological cardiovascular importance of the acid stable 13-H-14,15-EETAs versus the acid labile 15-H-11,12-EETAs is unknown. Hepoxilin A3 has a role to recruit PMNs across intestinal epithelia (49). The labile nature of hepoxilin A3 is required to prevent excessive PMN migration. Similarly, the acid labile and acid stable properties of the HEETAs could also be important for the regulation of other biological systems besides the cardiovascular system.

The 12-LO pathway of AA metabolism was characterized by Pace-Asciak et al. (50). 12(S)-HPETE, formed by oxygenation of AA by 12-LO, undergoes reduction to 12(S)-HETE or isomerization that is catalyzed by hydroperoxide isomerase (hepoxilin synthase) to hepoxilins. Hydroperoxide isomerase activity in this pathway is an intrinsic activity of 12-LO (51). In rabbit arteries, there was no indication of hepoxilin production; however, AA metabolism by the 15-LO pathway does occur (14). AA is first converted to 15-HPTE by 15-LO. 15-HPTE is reduced to 15-HETE or metabolized to HEETAs by hydroperoxide isomerases. Unlike 12-LO, rabbit reticulocyte 15-LO is devoid of intrinsic hydroperoxide isomerase activity (14, 51). In our current study, CYP2J2 shows hydroperoxide isomerase activity resulting in 13-H-14,15-EETAs production. The combi-
nation of 15-LO and CYP2J2 metabolized AA to products that comigrated on RP-HPLC with THETAs, threo- and erythro-13-H-trans-14,15-EETA, 15-KETE and 15-HETE. The THETAs are assumed to arise from acid hydrolysis of an unstable HEETA. CYP2J2 expression was observed in rabbit aorta (32). Other CYP isozymes are present in endothelial cells and may convert 15-HPETE to HEETAs (32, 41). The ability of CYPs to reduced 15-HPETE to 15-HETE and convert 15-HPETE to 15-KETE was previously reported (41, 52), which may explain the 15-HETE and 15-KETE observed in this in vitro incubation. When the CYP inhibitor ketoconazole was added to the incubations of 15-LO plus CYP2J2 or vessel incubations, decrease in THETA production occurred but no change in HEETA or HETE formation was observed. CYP hydroperoxide isomerase activity does not require NADPH or dioxygen for the isomerization reaction (33). The roles of CYPs as hydroperoxide isomerases in LO pathways of AA metabolism have been demonstrated previously (32, 33, 41, 52–54). The typical CYP inhibitors block EET production (miconazole (2 × 10⁻⁵ m), clotrimazole (5 × 10⁻⁵ m), ketoconazole (10⁻⁴ m), and ebastine (5 × 10⁻⁵ m)); however, none of these drugs inhibited the production of 13-H-14,15-EETAs without interfering with 15-LO activity. Thus, 15-HPETE must bind to a different site on the CYP than the inhibitors. Further study of the hydroperoxide isomerase is required.

Interestingly, the erythro-13-H-trans-14,15-EETA relaxed arteries to a greater extent than the threeo-13-H-14,15-EETA indicating a structural specificity and potentially a HEETA specific receptor. In mesenteric arteries, relaxations by erythro-13-H-trans-14,15-EETA were independent of the endothelium, cyclooxygenase and NO synthase and were blocked by high [K⁺]o, or apamin. These findings suggest that its action is on the smooth muscle and involves SK channel activation. Using the cell-attached patch clamp configuration, the erythro-13-H-trans-14,15-EETA opened apamin-sensitive, small-conductance K⁺ channels in vascular SMCs. Hence, erythro-13-H-trans-14,15-EETA functions as an EDHF. Erythro-13-H-trans-14,15-EETA produced a maximum relaxation of about 29% in both rabbit aorta and mesenteric arteries.

In contrast, 11(R),12(S),15(S)-THETA produced a maximum relaxation of about 70% in rabbit aorta (17). Based on these results, we speculate that the contribution of the 11,12,15-THETA pathway to the regulation of vascular tone seems to be more prominent than the contribution of the 13-H-14,15-EETAs pathway. In this regard, PGL₂ is synthesized by the rabbit aorta but does not cause relaxation (26, 55). Like many other eicosanoids, biological actions of 13-H-14,15-EETA, other than the EDHF activity, is very likely. Supporting this hypothesis, the erythro, but not the threeo, isomer of 13-H-trans-14,15-EETA concentration-dependently inhibited rabbit aortic SMC migration. The mechanism of this inhibition is unknown.

EDHF activity plays an important role in cardiovascular physiology and pathology in various animal models as well as in humans (56). For example, in patients with essential hypertension, the EDHF system becomes active when the endothelium-derived NO pathway is diminished (57–59) suggesting its role as an important compensatory mechanism to maintain endothelium-dependent vasodilation. In isolated aorta, mesenteric arteries, and renal arteries of hypercholesterolemic rabbits, an enhanced contribution of EDHF compensates for the decrease in NO-mediated relaxation (60, 61). Given the biological importance of EDHF, characterization of a new EDHF will widen the field, set new ground for EDHF research, and lead to a new therapeutic target for the treatment of cardiovascular diseases.

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