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11,12,15-Trihydroxyeicosatrienoic acid mediates ACh-induced relaxations in rabbit aorta

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Campbell, William B., Nancy Spitzbarth, Kathryn M. Gauthier, and Sandra L. Pfister. 11,12,15-Trihydroxyeicosatrienoic acid mediates ACh-induced relaxations in rabbit aorta. *Am J Physiol Heart Circ Physiol* 285: H2648–H2656, 2003. First published August 7, 2003; 10.1152/ajpheart.00412.2003.—Rabbit aortic endothelium metabolizes arachidonic acid (AA) by the 15-lipoxygenase pathway to vasodilatory eicosanoids, hydroxyepoxyeicosatrienoic acids (HEETAs), and trihydroxyeicosatrienoic acids (THETAs). The present study determined the chemical identity of the vasoactive THETA and investigated its role in ACh-induced relaxation in the rabbit aorta. AA caused endothelium-dependent, concentration-related relaxations of the rabbit aorta. Increasing the extracellular KCl concentration from 4.8 to 20 mM inhibited the relaxations to AA by ~60%, thereby implicating K^+-channel activation in the relaxations. In addition, AA caused an endothelium-dependent hyperpolarization of aortic smooth muscle from -39.6 ± 2.7 to -56.1 ± 3.4 mV. In rabbit aortic rings, [^{14}C]AA was metabolized to prostaglandins, HEETAs, THETAs, and 15-hydroxyeicosatetraenoic acid. Additional purification of the THETAs by HPLC resolved the mixture into its ^{14}C-labeled products. Gas chromatography/mass spectrometry identified the metabolites as isomers of 11,12,15-THETA and 11,14,15-THETA. The 11,12,15-THETA relaxed and hyperpolarized the rabbit aorta, whereas 11,14,15-THETA had no vasoactive effect. The relaxations to 11,12,15-THETA were blocked by 20 mM KCl. In aortic rings pretreated with inhibitors of nitric oxide and prostaglandin synthesis, ACh caused a concentration-related relaxation that was completely blocked by 20 mM KCl. Pretreatment with the phospholipase A_2 inhibitors mepacrine and 7,7-dimethyl-5,8-eicosadienoic acid, the lipoxygenase inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate, nordihydroguaiaretic acid, and ebselen, or the hydroperoxide isomerase inhibitors miconazole and clotrimazole also blocked ACh-induced relaxations. ACh caused a threefold increase in THETA release. These studies indicate that AA is metabolized by endothelial cells to 11,12,15-THETA, which activates K^+ channels to hyperpolarize the aortic smooth muscle membrane and induce relaxation. Additionally, this lipoxygenase pathway mediates the nonnitric oxide, nonprostaglandin relaxations to ACh in the rabbit aorta by acting as a source of an endothelium-derived hyperpolarizing factor.

trihydroxyeicosatrienoic acid; arachidonic acid; endothelium-derived hyperpolarizing factor; potassium channels; membrane potential; lipoxygenase

ACETYLCHOLINE AND BRADYKININ STIMULATE the release of soluble mediators from the vascular endothelium that act on the adjacent vascular smooth muscle to cause vasodilation (3, 11, 15, 19, 20). These mediators include prostacyclin and nitric oxide (NO). However, when the synthesis of NO and prostacyclin are inhibited by the combination of nitro-L-arginine (L-NNA) and indomethacin, a portion of the relaxation response to ACh persists (2–4, 6, 9, 21). These endothelium-dependent, L-NNA- and indomethacin-resistant relaxations are associated with smooth muscle hyperpolarization and are blocked by inhibitors of K^+ channels such as tetraethylammonium, charybdotoxin, and apamin and therefore are attributed to endothelium-dependent hyperpolarizing factors (EDHFs). The chemical identity of EDHFs is controversial and may be species and vessel specific. In coronary arteries, epoxyeicosatrienoic acids (EETs), which are cytochrome *P*-450 metabolites of arachidonic acid (AA), act as EDHFs (1, 7, 12). In rat hepatic arteries, this function is provided by K^+ ions (5). Rabbit aortas and carotid arteries do not synthesize EETs from AA (25). Regardless, these arteries demonstrate L-NNA- and indomethacin-resistant relaxations to ACh that are inhibited by K^+-channel blockers and high extracellular K^+ concentrations (4). The mediator(s) of these relaxations has not been defined and is the subject of the present study.

In the initial description of endothelium-derived relaxing factor (EDRFs), Furchgott and Zawadzki (11) reported that ACh relaxed rabbit aortas in an endothelium-dependent manner. These relaxations were inhibited by blockers of the lipoxygenase pathway of AA metabolism but not by inhibitors of cyclooxygenase. These authors concluded that a lipoxygenase metabolite of AA mediated the endothelium-dependent relaxations. This conclusion was confirmed by others (8). Subsequently, we and others reported that AA relaxed rabbit aortas, and these relaxations were endothelium dependent, inhibited by lipoxygenase inhibitors, and enhanced by cyclooxygenase inhibitors (22, 26, 27, 30, 31). Although EDRF was identified as NO (16, 23), the identity of the lipoxygenase-derived mediator of AA relaxations was not identified until recently. We reported that AA was metabolized to a polar metabolite that relaxed rabbit aortas (27). The production of this metabolite was endothelium dependent, inhibited by lipoxygenase inhibitors, and increased by cyclooxygenase

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inhibition. Using a variety of chemical methods including gas chromatography-mass spectrometry (GC/MS) and HPLC, we found that AA is metabolized by 15-lipoxygenase to hydroxyepoxyeicosatrienoic acids (HEETAs), which are hydrolyzed to 11,14,15- and 11,12,15-trihydroxyeicosatrienoic acids (THETAs). The THETAs relax the rabbit aorta.

The present study was designed to determine *1*) whether a THETA metabolite mediates the L-NNA- and indomethacin-resistant relaxations to ACh in rabbit aortas, and *2*) which THETA regioisomer(s) mediates the relaxation response to AA. We found that 11,12,15-THETA was the regioisomer that relaxed rabbit aortas and that the relaxations to THETA, AA, and ACh were reduced by inhibition of K^+-channel activity with high extracellular K^+ concentration. Furthermore, inhibitors of THETA synthesis blocked the L-NNA- and indomethacin-resistant relaxations to ACh in rabbit aortas.

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 *Guide for the Care and Use of Laboratory Animals* (21a) published by the National Institutes of Health.

Tissue preparation and incubation. Aortas were isolated from 1- to 2-mo-old New Zealand White rabbits and were cleaned of adhering connective tissue and fat (26, 27). The vessels were rinsed in Tris buffer (0.05 M, pH 7.5) and then cut into 3-mm-long rings. Vessels were incubated for 15 min at 37°C in HEPES buffer (in mM: 10 HEPES, 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 6 glucose; pH 7.4) that contained indomethacin (10^{-5} M) and [^{14}C]AA (0.05 μCi, 5×10^{-5} M). A23187 (2×10^{-5} M) was added, and the vessels were incubated for an additional 5 min. The reaction was stopped by adding ethanol to a final concentration of 15%, and the HEPES buffer was removed, acidified (pH < 3.5) with glacial acetic acid, and extracted on ODS solid-phase extraction columns as previously described (26, 27). The extracted metabolites were evaporated to dryness under a stream of nitrogen and stored at −40°C until analysis via HPLC. Parallel incubations were conducted without vessels and analyzed in a similar manner to determine the extent of autooxidation.

Perfused rabbit aorta. Rabbit aorta was isolated as described and was cannulated on both ends. Major branches were tied off and placed in a tissue bath that contained Krebs buffer at 37°C. The vessel was then perfused with HEPES buffer that contained indomethacin (10^{-5} M) at 3 ml/min in a nonrecirculating system. Perfusion pressure was maintained at 60 mmHg by an outflow restrictor and was measured continuously by a pressure transducer. The vessel was treated with KCl (20 mM) every 5 min over a 30-min period. After 45 min, [^{3}H]AA (5 μCi, 10^{-4} M) was added to the perfusate, and the perfusate was recirculated through the vessel for 45 min. To remove unesterified [^{3}H]AA, the vessel was then perfused in a nonrecirculating system with fresh HEPES buffer that contained fatty acid-free bovine serum albumin (2 mg/ml) and indomethacin (10^{-5} M). Once the amount of radioactivity in the perfusate stabilized, the perfusate was collected for 6 min (control). The vessel was then perfused with the same buffer that contained ACh (10^{-6} M), and the perfusate was collected for 6 min (ACh). The perfusate was extracted and analyzed by HPLC as described below. Column fractions that contained the THETAs were collected, derivatized to their pentafluorobenzyl (PFB) ester-trimethylsilyl (TMS) ethers, and analyzed by negative ion chemical ionization GC/MS (see below).

Purification of AA metabolites by HPLC. The extracted samples were first resolved into their components by reverse-phase HPLC (Nucleosil-C18 column, 5 μm, 4.6 × 250 mm) using *solvent system I* (27). *Solvent A* was water and *solvent B* was acetonitrile that contained 0.1% glacial acetic acid. The program was a 40-min linear gradient from 50% *solvent B* in *solvent A* to 100% *solvent B*. Flow rate was 1 ml/min. The column eluate was collected in 0.2-ml fractions by a fraction collector. An aliquot of each fraction was removed, and radioactivity was determined by liquid scintillation spectrometry. The fractions corresponding to the THETAs (*fractions 27–35*; 5–7.5 min) were collected, acidified, and extracted with a 50:50 cyclohexane-ethylacetate mixture. The solvent was removed under a stream of nitrogen, and the extract was redissolved in the HPLC mobile phase. The THETA fraction was rechromatographed on reverse-phase HPLC using *solvent system II* (27). In *solvent system II*, *solvent A* was water that contained 0.1% glacial acetic acid, and *solvent B* was acetonitrile. The program consisted of a 5-min isocratic phase with 35% *solvent B* in *solvent A*, followed by a 35-min linear gradient to 85% *solvent B*. Flow rate was 1 ml/min. The column eluate was collected in 0.2-ml aliquots, and radioactivity was determined as described. The fractions that contained the THETAs (*fractions 87–93;* 17.5–18.5 min) were collected, acidified, and extracted with a 50:50 cyclohexane-ethylacetate mixture. The THETA fraction was further purified by normal-phase HPLC using a Nucleosil silica column (5 μm, 4.6 × 250 mm). *Solvent system III* consisted of an isocratic separation using a 995:4:1 ratio of hexane, isopropranol, and glacial acetic acid at a flow rate of 1 ml/min. The column eluate was collected in 0.2-ml fractions, and the radioactivity was determined as described. The radioactive peaks from the THETAs (*solvent system III*) were collected, dried under a stream of nitrogen, and tested for biological activity on the phenylephrine-pretreated rabbit aorta or were derivatized and analyzed by GC/MS as described below.

Gas chromatography-mass spectrometry. The pooled THETA fractions isolated from *solvent systems II* or *III* were evaporated to dryness under nitrogen and derivatized for GC/MS as previously described (25, 27). The samples were dissolved in 120 μl of acetonitrile and then treated with ethereal diazomethane for 6 min at 0°C to form the methyl ester. Alternatively, they were dissolved in acetonitrile that contained PFB bromide and *N,N*-diisopropylethanolamine (10:1:5 vol/vol/vol ratio) and incubated for 20 min at room temperature to form the PFB esters. The reacted sample was evaporated to dryness under nitrogen, and the hydroxyl groups were then converted to the TMS ethers by 60 min of incubation at 37°C with 15 μl of bis-trimethylsilyl-trifluoroacetamide. GC/MS was performed with a Hewlett-Packard 5989A mass spectrometer coupled to a 5890 series 2 gas chromatograph. Chemical ionization of the samples was performed at 65–70 eV using methane as the reagent gas. Ions were measured in the positive (methyl esters) or negative (PFB esters) ion mode. The derivatized metabolites were resolved using a 14-m capillary DB-5 column with a linear gradient from 100 to 300°C. Standards were derivatized and analyzed by GC/MS using the identical methods described for the biological samples. For measuring THETA release by ACh, negative ion chemical ionization GC/MS was used in the selective-ion monitoring mode using the 569 *m/z* (M-1) ion.

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Vascular reactivity. Thoracic aorta was obtained from 1- to 2-mo-old New Zealand White rabbits and placed in Krebs bicarbonate buffer (in mM: 118 NaCl, 4 KCl, 3.3 $CaCl_2$, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 11 glucose) as previously described (24, 26). The tissue was carefully cleaned of adhering fat and connective tissue and was cut into rings (3-mm long) while care was taken not to damage the endothelium. Aortic rings were suspended in 6-ml tissue baths that contained Krebs bicarbonate buffer maintained at 37°C and were continuously bubbled with 95% O_2-5% CO_2. 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 8e software. Resting tension was adjusted to its length tension maximum of 2 g, and vessels were allowed to equilibrate for 1 h. Contractions were produced by increasing the KCl concentration of the baths to 40 mM. After the vessels reached peak contraction, tissue baths were rinsed and vessels were returned to resting tension. Once the aortic rings had reproducible, stable responses to KCl, the tissue was contracted with phenylephrine (10^{-8} to 10^{-7} M) to 50–75% maximal KCl contraction. The column fractions were dissolved in ethanol and tested for activity. In some studies, the Krebs buffer was modified to increase the KCl concentration to 20 mM by substituting KCl for NaCl.

In other studies, vessels were treated with L-NNA (3×10^{-5} M) and indomethacin (10^{-5} M) for 10 min before precontraction with phenylephrine (10^{-8} to 10^{-7} M). ACh (10^{-9} to 10^{-6} M) was then added in cumulative concentrations, and the relaxation responses were followed. Similar studies were performed in vessels pretreated for 10 min with the phospholipase A_2 inhibitors mepacrine (10^{-5} M) and 7,7-dimethyl-5,8-eicosadienoic acid (DEDA, 1.5×10^{-5} M), the lipoxygenase inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC, 10^{-6} M), nordihydroguaiaretic acid (NDGA, 5×10^{-5} M), and ebselen (2.5×10^{-5} M), or the hydroperoxide isomerase inhibitors miconazole (2×10^{-5} M) and clotrimazole (5×10^{-5} M) (26, 27, 32). Another series of studies involved treating the vessels with indomethacin for 10 min before precontacting the vessels with phenylephrine (10^{-7} M). AA (10^{-7} to 10^{-4} M) was then added in cumulative concentrations, and relaxation responses were followed. These studies were repeated in Krebs solution with 20 mM KCl substituted for NaCl. In an additional series of experiments, AA-induced relaxations were performed in vessels pretreated for 10 min with mepacrine, DEDA, CDC, NDGA, ebselen, clotrimazole, or miconazole.

Membrane potential measurements. Rabbit aortic rings were cut open laterally, pinned to a Silastic layer with the endothelial layer exposed in a heated perfusion chamber (37°C), and perfused with a physiological salt solution (PSS) of the following composition (in mM): 119 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.17 $MgSO_4$, 5.5 glucose, 24 $NaHCO_3$, 1.18 NaH_2PO_4, and 0.0026 EDTA. Perfusate solutions were equilibrated with a 21% O_2-5% CO_2-74% N_2 gas mixture to maintain a pH of 7.4 and a Po_2 of 140 mmHg as previously described (13). Indomethacin (10^{-5} M) and phenylephrine (10^{-7} M) were present in all perfusate solutions. Aortic segments were continually superfused for 30 min before initiation of experimental protocols. Impalements of smooth muscle cells were performed only in a small section of the artery where the endothelium was removed by gentle rubbing with a small cotton swab. In a subset of experiments, the entire endothelial layer was removed from the aortic strip. Intracellular membrane potential (E_m) values were recorded using published methods (1, 14). Briefly, glass microelectrodes were filled with 3 M KCl and had estimated tip sizes of 0.1–0.2 μm, tip resistances of 30–80 MΩ, and tip potentials of ≤3 mV. Electrodes were attached to a high-impedance biological amplifier (Dagan Cell Explorer, Dagan Instruments; Minneapolis, MN). Electrode polarization was eliminated by a Ag/AgCl half cell. Criteria for a successful impalement included an abrupt drop in potential to a new steady-state value, which was maintained for a minimum of 5 s, an E_m value greater than −20 mV, and an abrupt return to the original baseline when the electrode was retracted from the tissue. AA (10^{-5} M) was added to the aortic segments, and after 10 min E_m was measured. In other studies in vessels without endothelium, column fractions that contained 11,12,15-THETA were added during an impalement and E_m was continuously monitored.

Statistical analysis. The vascular reactivity and E_m data are expressed as means ± SE. Statistical evaluation of the data was performed by a one-way ANOVA, followed by the Student-Newman-Keuls multiple-comparison test when significant differences were present, or data were analyzed by the Student's *t*-test for paired observations. $P < 0.05$ was considered statistically significant.

Materials. Phenylephrine, AA (sodium salt), indomethacin, miconazole, mepacrine, and ebselen were obtained from Sigma Chemical, CDC was obtained from Biomol Research Laboratories, and DEDA was from Cayman Chemicals. Unless otherwise indicated, drugs and column fractions were dissolved in ethanol and added in a final ethanol concentration of <0.07%. Arachidonate sodium was dissolved in distilled water previously sparged with nitrogen gas. Stock solutions and dilutions were made fresh daily for each experiment and stored on ice under nitrogen. All solvents were HPLC grade and purchased from Burdick and Jackson, and [^{14}C-U]AA (920 mCi/mmol) was obtained from New England Nuclear.

RESULTS

In aortic rings contracted with phenylephrine and pretreated with indomethacin, AA caused a concentration-related relaxation (Fig. 1*A*). The relaxations to AA were almost completely blocked by removal of the endothelium. Inhibition of lipoxygenase with CDC, NDGA, or ebselen inhibited AA-induced relaxations to a similar extent as removal of the endothelium (Fig. 1*B*; Refs. 27, 32). A similar inhibition was observed with the hydroperoxide isomerase inhibitors miconazole and clotrimazole (Fig. 1*C*; Ref. 27). Mepacrine, a nonspecific inhibitor of phospholipase A_2, had no effect (Fig. 1*A*). Similarly, another phospholipase A_2 inhibitor, DEDA, had no effect. Increasing the KCl concentration in the Krebs solution from 4.8 to 20 mM completely blocked the relaxations to 10^{-6} and 10^{-5} M AA, and the relaxation to 10^{-4} M was inhibited by ~60% (Fig. 1*D*). These studies suggest that the endothelium-dependent relaxations to AA are mediated by lipoxygenase metabolite(s) and K^+-channel activation.

The E_m value of the aortic smooth muscle cells was measured in indomethacin- and phenylephrine-treated aortic segments with and without an intact endothelium (Fig. 2). Under these conditions, the E_m value of the endothelium-intact aorta averaged −39.6 ± 2.7 mV. Similar values were obtained in vessels without an intact endothelium (−32.4 ± 1.2 mV). AA (10^{-5} M) induced a pronounced 16.5-mV hyperpolarization of the aortic smooth muscle cells, whereas in vessels

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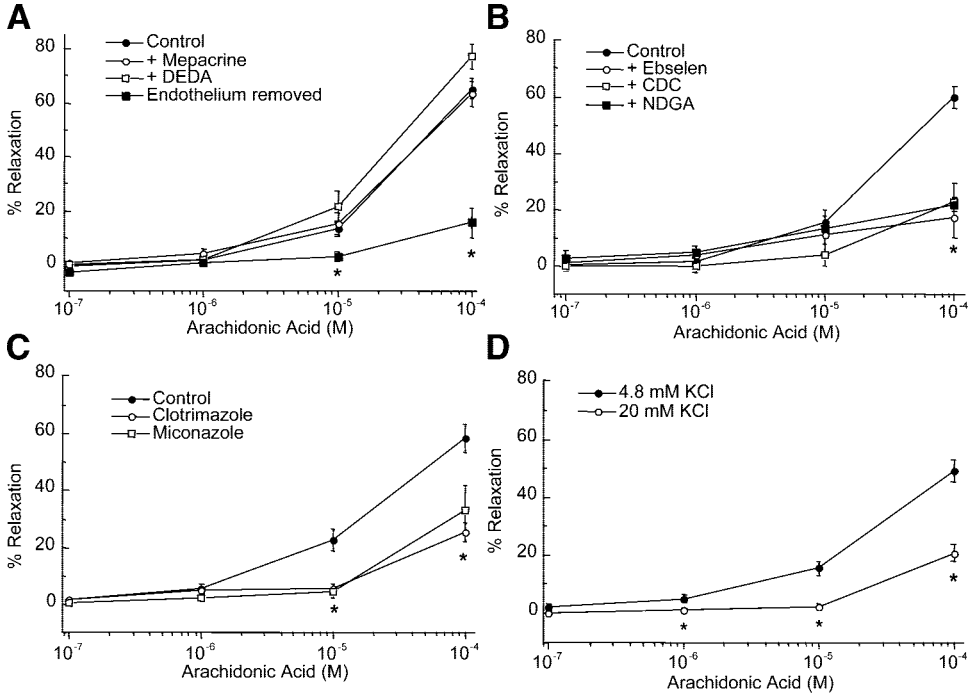

Fig. 1. Effects of inhibitors on arachidonic acid (AA)-induced relaxation of rabbit aortic rings. Aortic rings with an intact endothelium were pretreated with indomethacin (10^{-5} M) and contracted with phenylephrine (10^{-7} M). Isometric tension was measured. Vessels were pretreated as follows. *A*: phospholipase A_2 inhibitors mepacrine (10^{-5} M) and 7,7-dimethyl-5,8-eicosadienoic acid (DEDA, 1.5×10^{-5} M). *B*: lipoxygenase inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC, 10^{-5} M), nordihydroguaiaretic acid (NDGA, 5×10^{-5} M), and ebselen (2.5×10^{-5} M). *C*: hydroperoxide isomerase inhibitors miconazole (2×10^{-5} M) and clotrimazole (5×10^{-5} M). In *A*, the endothelium was removed by gentle rubbing. *D*: K^+ concentration of the Krebs solution was increased from 4.8 to 20 mM. Values are means ± SE; $n = 12$ vessels; $^*P \leq 0.05$ compared with controls.

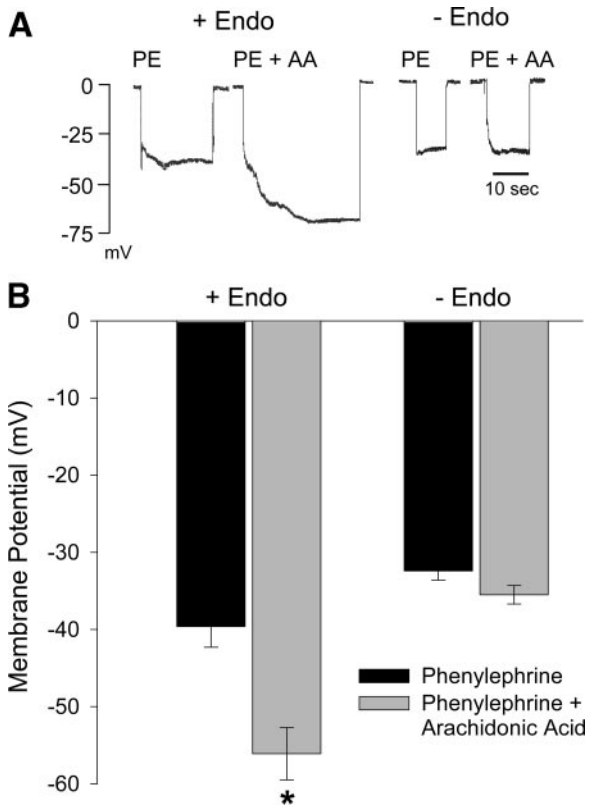

Fig. 2. Effects of AA (10^{-5} M) on membrane potential (E_m) of vascular smooth muscle cells in aortas with (+Endo) and without (−Endo) an intact endothelium. Vessels were pretreated with indomethacin (10^{-5} M) and phenylephrine (PE, 10^{-7} M). *A*: typical impalements under the various conditions. *B*: summarized data. Values are means ± SE for $n = 7$–14 impalements; $^*P < 0.05$ compared with controls.

without an intact endothelium, AA (10^{-5} M) did not significantly change the E_m value. Thus AA causes an endothelium-dependent hyperpolarization of the vascular smooth muscle.

Pfister et al. (27) previously reported that 11,12,15- and/or 11,14,15-THETA mediate AA-induced relaxations in rabbit aorta. We developed an HPLC procedure that resolved the two THETA regioisomers to determine the identity of the active vasodilator eicosanoid. Aortic rings were incubated with indomethacin and [^{14}C]AA, and the metabolites were extracted and resolved by reverse-phase HPLC using *solvent system I*. The radioactive metabolites comigrated with the THETAs, HEETAs, and 15-hydroxyeicosatetraenoic acid (Fig. 3*A*). No metabolites were observed in incubations without aortic rings or rings without an intact endothelium. The column fractions that contained the THETAs (*fractions 27–35*) were collected and further purified by reverse-phase HPLC using *solvent system II*. A single major radioactive peak (*peak 2*) was observed eluting in *fractions 87–93* (Fig. 3*B*). Previous studies indicated that these fractions contained a mixture of the THETA regioisomers (27). This was confirmed by derivatization to the PFB ester and TMS ether and analysis by negative ion chemical ionization GC/MS. Figure 4*A* illustrates a typical mass spectrum with a major ion of 569 *m/z* (loss of PFB, M−1). This indicates a mol wt of 570, which is consistent with a THETA-TMS ether. *Fractions 87–93* containing the THETAs were further purified by normal-phase HPLC and resolved into four radioactive products (*peaks A–D*; Fig. 3*C*). These products were derivatized and analyzed by positive ion chemical ionization GC/MS. The four products had similar mass spectra with major ions (*m/z*) of the following (Fig. 4): 585 (M^++1), 569 (M−15,

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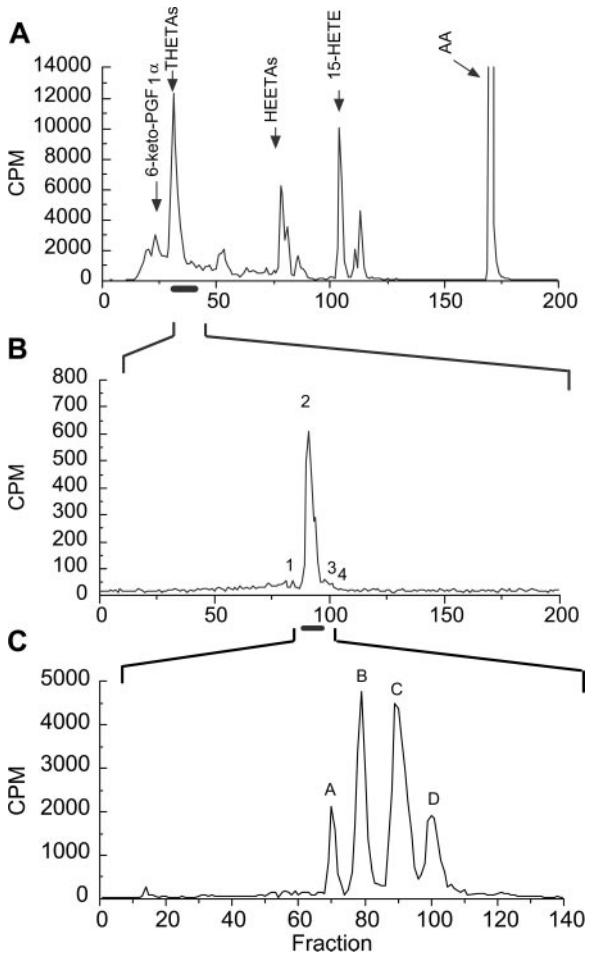

Fig. 3. Metabolism of [^{14}C]AA by rabbit aorta. Aortic rings were incubated with indomethacin (10^{-5} M) and [^{14}C]AA. Metabolites were extracted and resolved by sequential reverse-phase and normal-phase HPLC. *A*: separation of AA metabolites by reverse-phase HPLC using *solvent system I* (see METHODS). Migration times of known standards are shown above the chromatogram. *B*: separation of the trihydroxyeicosatrienoic acid (THETA) fraction (*fractions 27–35*, dark bar) from *A* by reverse-phase HPLC using *solvent system II*. *C*: separation of THETA fraction *peak 2* (*fractions 87–93*, dark bar) from *B* by normal-phase HPLC using *solvent system III*. CPM, counts/min; 15-HETE, 15-hydroxyeicosatetraenoic acid; HEETA, hydroxyepoxyeicosatrienoic acid.

loss of CH_3); 405 [M−179, loss of $(CH_3)_3SiOH$ and $(CH_3)_3SiO^+$], 301 (M−283), 283 [M−301, $(CH_3)_3SiO$-CH-$(CH_2$-CH=CH$)_2$-$(CH_2)_3$-$COOCH_3$], and 173 [M−411, $(CH_3)_3SiO$-$(CH_2)_4$-CH_3]. However, they differed in the intensity of the 173- and 283-*m/z* ions indicating the favored cleavage between the 14,15- and 11,12-vicinal diols, respectively (27). The derivatized metabolite in *peak B* eluted from the GC at 13.75 min. The mass spectrum for *peak B* is shown in Fig. 4*B* and is consistent with *peak B* being 11,12,15-THETA. The metabolite in *peak C* eluted from the GC at 13.87 min. The mass spectrum for *peak C* is illustrated in Fig. 4*C* and indicates that *peak C* is 11,14,15-THETA. *Peak D* was a mixture of both regioisomers eluting from the GC at 13.75 and 13.87 min. *Peak D* contained stereoisomers of the two regioisomers. These findings indicate that AA is metabolized to a mixture of regioisomers and stereoisomers of 11,12,15- and 11,14,15-THETA that can be resolved by normal-phase HPLC.

The four radioactive products described in Fig. 3*C* were also tested for their ability to relax the phenylephrine-precontracted rabbit aorta. The fractions that contained *peaks A, C*, and *D* were without effect. However, the fractions that contained *peak B* relaxed aortic rings in a concentration-related manner (Fig. 5*A*). When the concentration of KCl was increased from 4.8 to 20 mM, the fractions of *peak B* no longer caused relaxations, which implicates activation of K^+ channels in their action. When the same fractions from

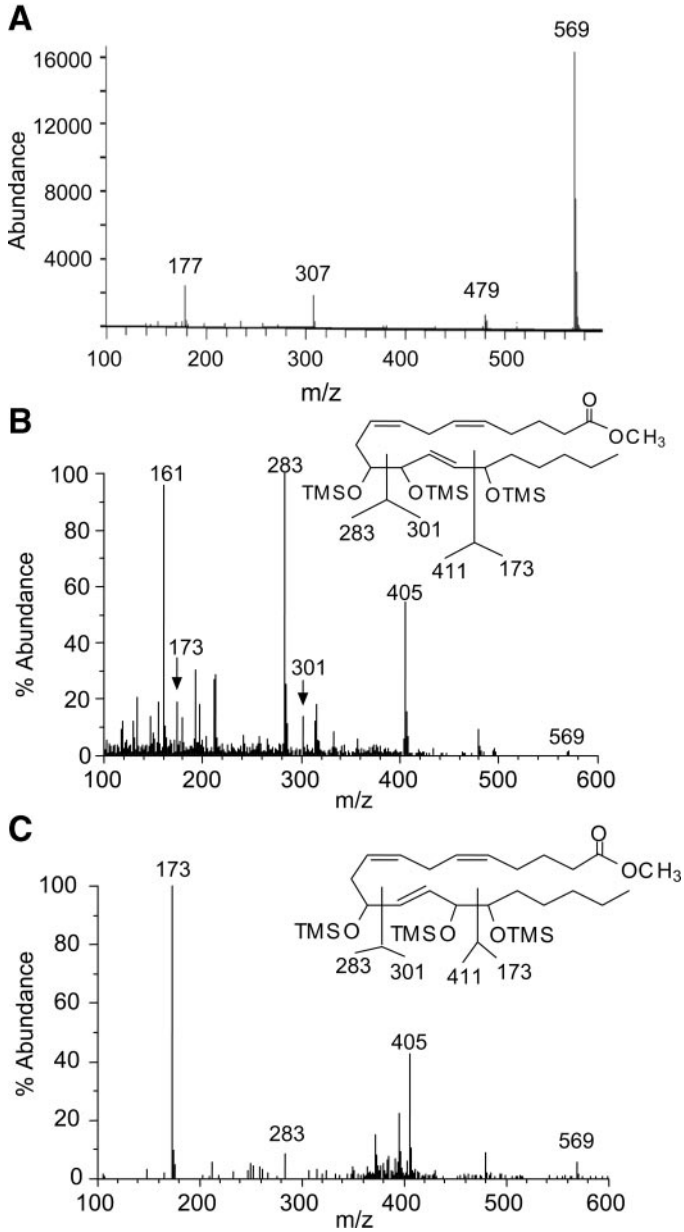

Fig. 4. Mass spectra of THETAs isolated from rabbit aorta. *A*: negative ion chemical ionization mass spectrum of the pentafluorobenzyl (PFB) ester, trimethylsilyl (TMS) ether of *peak 2* of Fig. 3*B*. *B*: positive ion chemical ionization mass spectrum of the methyl ester, TMS ester of *peak B* of Fig. 3*C*. *C*: positive ion chemical ionization mass spectrum of the methyl ester, TMS ester of *peak C* of Fig. 3*C*.

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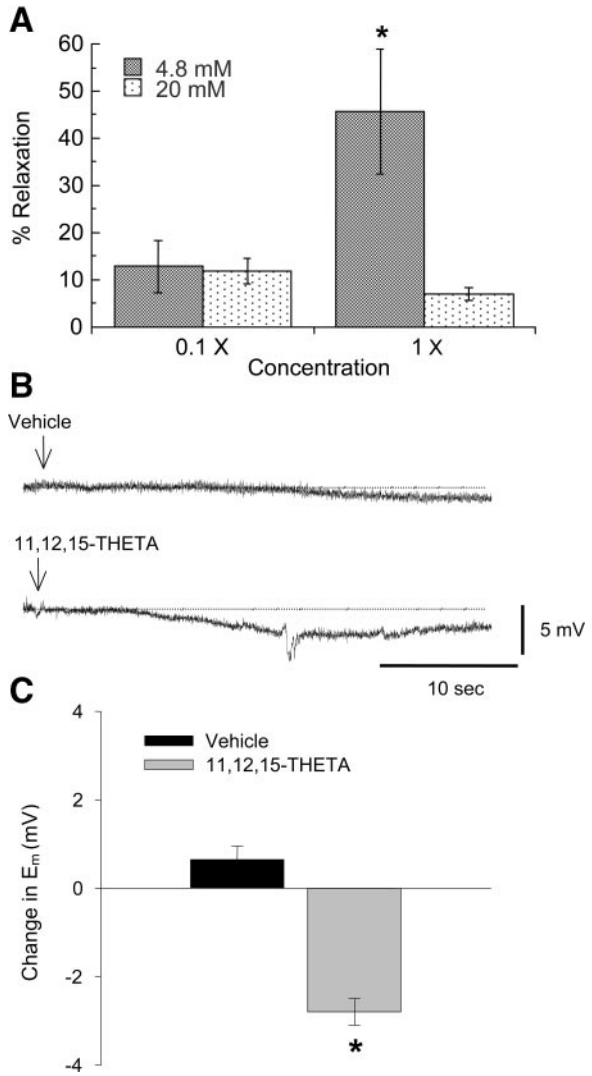

Fig. 5. Effects of 11,12,15-THETA (*peak B* of Fig. 3*C*) on isometric tension and membrane potential (E_m) in rings of rabbit aorta. *A*: rabbit aortic rings were incubated in Krebs solution that contained 4.8 or 20 mM KCl. Vessels were precontracted with phenylephrine (10^{-7} M). Dilutions of *peak B* of Fig. 3*C* were added to the precontracted vessel and isometric tension was measured. Each value represents the mean ± SE for $n = 6$ vessels; $^*P \leq 0.05$ for 1× concentration compared with 0.1×concentration at 4.8 mM KCl or 1× concentration at 4.8 mM KCl compared with 1×concentration at 20 mM KCl. *B* and *C*: effects of 11,12,15-THETA (*peak B* of Fig. 3*C*) on E_m of vascular smooth muscle cells in aortas without an endothelium. Vessels were pretreated with indomethacin (10^{-5} M) and phenylephrine (10^{-7} M). Resting E_m was −32.4 ± 3.0 mV. Typical tracings of changes in E_m with time (*B, top*) and summarized data from four vessels (*B, bottom*) are shown. Each value represents mean ± SE for $n = 4$; $^*P < 0.05$ compared with controls.

incubations without vessels were tested, they were without effect. The fractions from *peak B* were also tested on the smooth muscle E_m value. Application of *peak B* caused a consistent increase in the E_m value with time (Fig. 5*B*). The E_m value increased by 2.8 ± 0.3 mV with *peak B* and decreased by 0.6 ± 0.3 mV with vehicle ($P < 0.05$; Fig. 5*C*). The resting E_m value was −32.4 ± 3 mV. These findings identify 11,12,15-THETA as the AA metabolite that causes vasorelaxation and hyperpolarization.

The lipoxygenase inhibitors CDC and NDGA and hydroperoxide isomerase inhibitor miconazole inhibited the metabolism of AA to THETAs (26, 27). To determine whether THETAs contribute to ACh-induced relaxations, we tested the effects of mepacrine, DEDA, CDC, NDGA, ebselen, clotrimazole, and miconazole on ACh-induced relaxations in aortic rings in which the synthesis of NO and prostaglandins were inhibited with L-NNA and indomethacin (Fig. 6). ACh caused concentration-related relaxations of phenylephrine-contracted aortic rings. In the presence of L-NNA and indomethacin, the relaxations to ACh were reduced by ~50% (Fig. 6*A*). Thus ~50% of the relaxation responses to ACh are independent of NO and prostaglandins. When the KCl concentration of the Krebs solution was increased to 20 mM, the L-NNA- and indomethacin-resistant relaxations to ACh were completely blocked (Fig. 6*A*). These findings implicate the activation of K^+ channels in the L-NNA- and indomethacin-resistant relaxations. Mepacrine, DEDA, CDC, NDGA, ebselen, clotrimazole, and miconazole also blocked the L-NNA- and indomethacin-resistant relaxations to ACh (Fig. 6, *B–D*). Thus blockade of any of these steps in THETA synthesis inhibits ACh-induced relaxations. In the mepacrine-, ebselen-, and clotrimazole-treated vessels, the relaxation responses to ACh were converted to a contraction. These data suggest that 11,12,15-THETA contributes to ACh-induced relaxations. Furthermore, blockade of EDRFs with mepacrine, clotrimazole, or ebselen in combination with L-NNA and indomethacin unmasks ACh-induced constriction.

The release of THETAs by ACh was determined in perfused aorta in which the endothelial phospholipids were prelabeled with [^{3}H]AA. Samples were collected under control conditions and after ACh (10^{-6} M) stimulation. THETAs were analyzed by sequential HPLC using *solvent system I*, followed by *system II* (Fig. 7, *A* and *C*). A radioactive peak comigrating with the THETAs was observed in the control and ACh-treated samples. The radioactivity in the peak was greater in the ACh-treated sample. *Fractions 87–93* (17.5–18.5 min) were collected, derivatized, and analyzed by GC/MS using selective ion monitoring of the 569-*m/z* (M-1) ion for the THETAs (Fig. 7, *B* and *D*). Two peaks were observed on the ion chromatogram eluting at 12.5 and 12.8 min. The peak heights were approximately threefold greater in the ACh-treated sample compared with the control sample. These studies indicate that ACh stimulates THETA release from rabbit aorta.

DISCUSSION

Endothelial cells metabolize AA by cyclooxygenase, lipoxygenase, and cytochrome *P*-450 pathways (28, 29). Each of these pathways produces metabolites with vasoactive properties. Consistent with these biochemical studies, AA causes endothelium-dependent relaxation of rabbit aorta (22, 24, 26, 30). These AA-induced relaxations are blocked by inhibitors of the lipoxygenase pathway and enhanced by inhibitors of the cyclo-

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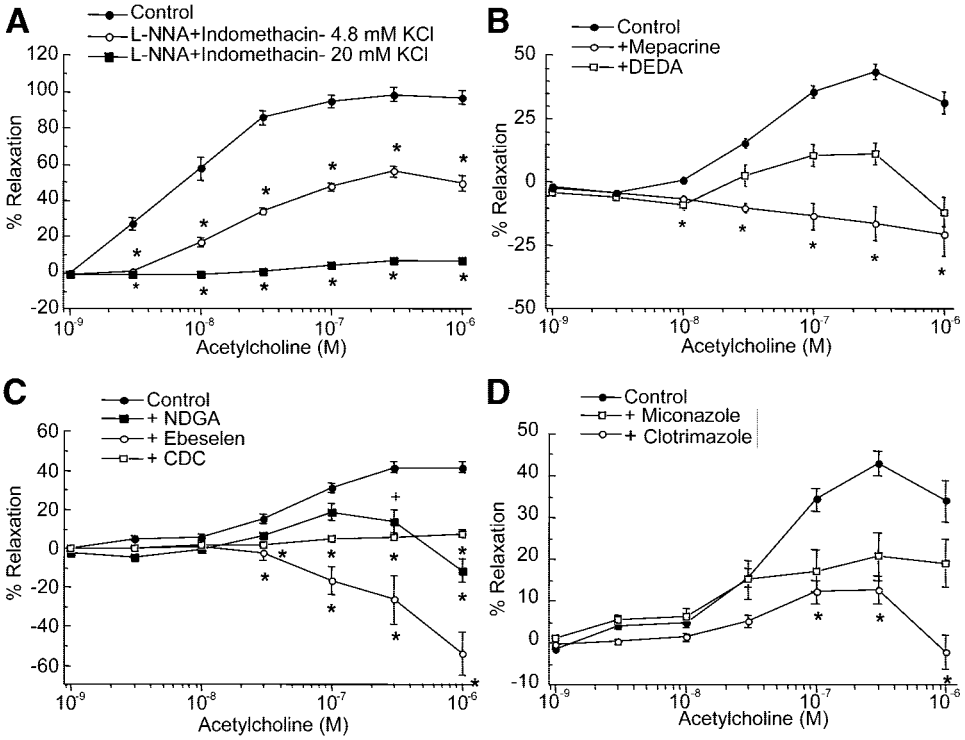

Fig. 6. Effects of inhibitors on nitric oxide (NO) and prostaglandin-independent relaxations of the rabbit aortic rings to ACh. Aortic rings were precontracted with phenylephrine, and cumulative concentration-response curves were obtained to ACh. Vessels were pretreated with nitro-L-arginine (L-NNA, 3×10^{-5} M) and indomethacin (10^{-5} M) in 4.8 or 20 mM KCl, and the relaxation responses to ACh were determined (*A*). In L-NNA- and indomethacin-pretreated aortic rings, relaxation responses to ACh were determined in the presence and absence of the phospholipase inhibitors mepacrine or DEDA (*B*); lipoxygenase inhibitors NGDA, CDC, or ebselen (*C*); or hydroperoxide isomerase inhibitors clotrimazole or miconazole (*D*). Value represent means ± SE for $n = 12$; $^*P \leq 0.05$ for treatment vs. control.

oxygenase pathway, which suggests that the mediator of these relaxations is a lipoxygenase metabolite. We isolated and identified the major metabolites of AA produced by the endothelium of rabbit aorta (25–27). These studies revealed a polar lipoxygenase metabolite that relaxed the rabbit aorta (27). There was no evidence of vasodilatory cytochrome *P*-450 metabolites such as EETs. The production of this polar metabolite was also inhibited by lipoxygenase inhibitors and enhanced by cyclooxygenase inhibition. The vasodilator

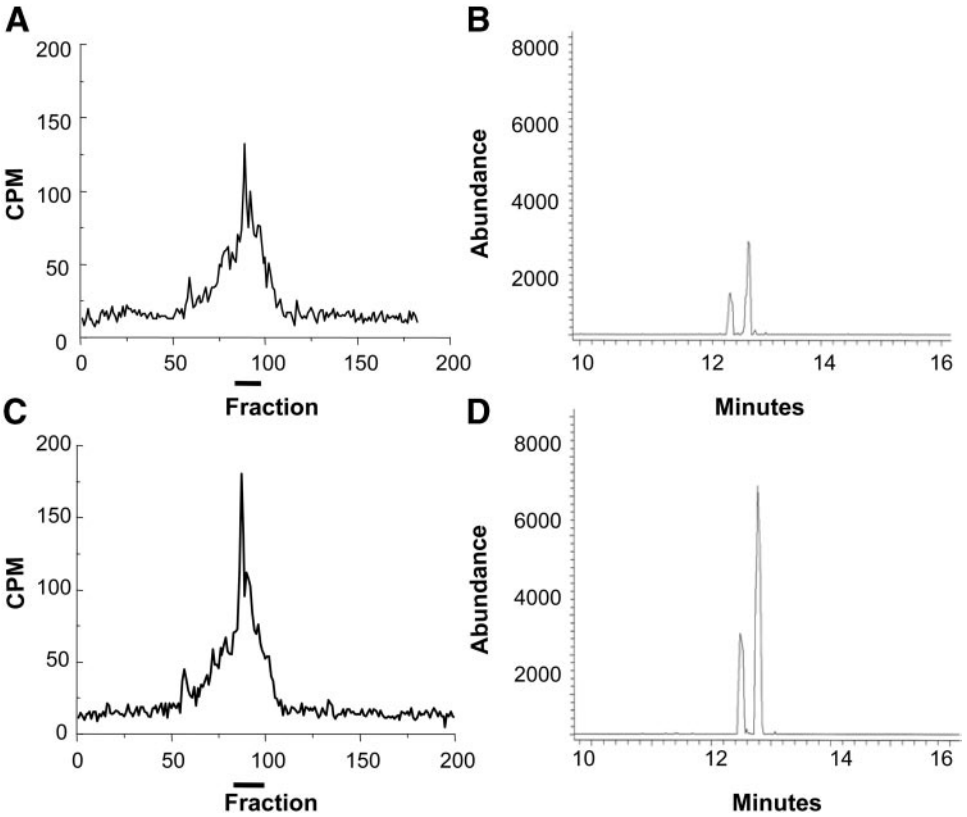

Fig. 7. ACh stimulation of THETA release from perfused rabbit aorta. Rabbit aorta was perfused with [^{3}H]AA for 45 min to label the endothelial phospholipids. Nonesterified [^{3}H]AA was removed by perfusing the vessel with buffer that contained fatty acid-free bovine serum albumin. Samples were collected under control conditions (*A* and *B*) and after ACh (10^{-6} M) stimulation (*C* and *D*) and were subjected to sequential analysis by HPLC (*solvent systems I* then *II*). *A* and *C*: the release of [^{3}H]THETAs from the control and ACh-treated samples, respectively, is shown. Black bars under the chromatograms indicate fractions that were collected, derivatized, and analyzed by gas chromatography-mass spectrometry by selective ion monitoring of the 569 *m/z* (M-1) ion for THETAs. *B* and *C*: ion chromatogram for control and ACh-treated samples, respectively.

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metabolite was purified and identified by GC/MS as a mixture of 11,12,15- and 11,14,15-THETA. In the present study, we developed a normal-phase HPLC method that resolved the THETA mixture into four isomers. Only one of the four isomers relaxed rabbit aorta, namely, 11,12,15-THETA. These studies establish 11,12,15-THETA as a mediator of AA-induced relaxation of rabbit aorta. Eight stereoisomers of 11,12,15-THETA are possible. We previously documented the synthesis of 15(*S*)-hydroxyeicosatetraenoic acid by rabbit aorta (25). This would indicate that the 15-hydroxyl of 11,12,15-THETA is in the *S* configuration. However, the stereochemistry of the other hydroxyl groups is not known. The identity of the active stereoisomer of 11,12,15(*S*)-THETA will require the synthesis and evaluation of the dilator activity of the four possible isomers.

The synthesis of the THETAs involves the metabolism of AA by 15-lipoxygenase to 15-hydroperoxyeicosatetraenoic acid (27). This intermediate is converted to HEETAs by a heme-containing hydroperoxide isomerase (27). Hydrolysis of the epoxy group of the HEETA by an epoxide hydrolase results in THETA formation. In previous studies, we described inhibitors of each of these biosynthetic steps. The 15-lipoxygenase is inhibited by CDC, NDGA, or ebselen, whereas miconazole and clotrimazole inhibit the hydroperoxide isomerase (26, 27, 32).

The mechanism of AA-induced relaxation of rabbit aorta has not been described previously. AA failed to increase the cAMP or cGMP contents of aorta (24). In contrast, AA increased both cAMP and cGMP accumulation in bovine pulmonary arteries (17, 18). It was concluded that a cyclooxygenase metabolite stimulated cAMP accumulation, whereas NO promoted cGMP accumulation. In the current studies, the relaxations to AA were inhibited by increasing the extracellular KCl concentration from 4.8 to 20 mM. In addition, AA hyperpolarized the smooth muscle cells of endothelium-intact vessels. Similarly, increasing the extracellular KCl concentration inhibited the relaxations to 11,12,15-THETA. The 11,12,15-THETA also hyperpolarized the aortic smooth muscle cells. These findings implicate a K^+-channel-mediated hyperpolarization in the action of AA and its active metabolites on the vascular muscle. The identity of the K^+ channel(s) involved in regulating vascular tone and membrane potential in the arterial smooth muscle cells of rabbit is not clearly defined. In rabbit mesenteric arteries, Murphy and Brayden (21) reported that endothelium-dependent hyperpolarization to ACh was blocked by apamin but not tetraethylammonium, iberiotoxin, glibenclamide, or 4-aminopyridine. These findings suggest that EDHF activated an apamin-sensitive K^+ channel in rabbit mesenteric artery. In contrast to these findings, Fujimoto and coworkers (9) found that tetraethylammonium, 4-aminopyridine, charybdotoxin, or the combination of apamin and charybdotoxin inhibited L-NNA- and indomethacin-resistant relaxations to ACh in rabbit mesenteric artery. Apamin was without effect. In rabbit aorta, Cowan et al. (4) reported L-NNA- and indomethacin-resistant relaxations to ACh that were blocked by increasing the extracellular KCl concentration to 25 mM. In addition, these relaxations were inhibited by tetraethylammonium, charybdotoxin and glibenclamide. The reasons for these differences in the effects of K^+-channel blockers are unclear. The K^+-channel-mediating THETA-induced relaxation and hyperpolarization in the rabbit aorta remains to be characterized.

Using rabbit aorta, Furchgott and Zawadzki (11) first described that the relaxations to ACh were dependent on an intact endothelium. They demonstrated that the endothelium released an EDRF. Because the relaxations to ACh were inhibited by lipoxygenase inhibitors, the authors concluded that EDRF was a lipoxygenase metabolite of AA. Subsequent studies revealed that EDRF was NO (16, 23). However, NO is not the only mediator of ACh-induced relaxations in the rabbit aorta. When the syntheses of NO and prostaglandins were inhibited by L-NNA and indomethacin, the relaxations to ACh were reduced but not blocked by this drug combination (4). However, the relaxations resistant to L-NNA and indomethacin were inhibited by K^+-channel blockers. These studies led to the conclusion that NO and EDHF mediate the relaxations to ACh. In the coronary and renal circulation, EDHF has been identified as an EET, a cytochrome *P*-450 metabolite of AA (1, 7, 10, 12). However, rabbit aorta does not synthesize EETs under normal circumstances, so some other mediator must be involved (25). The current studies indicate that ACh stimulates the release of THETAs, and the L-NNA- and indomethacin-resistant relaxations are blocked by the phospholipase A_2 inhibitors mepacrine and DEDA, the lipoxygenase inhibitors CDC, NDGA, and ebselen, and the hydroperoxide isomerase inhibitors miconazole and clotrimazole. Because several of these inhibitors have actions other than the ones ascribed, experiments with an individual inhibitor may not be definitive. However, these findings with inhibitors in the aggregate and the increased release of THETAs by ACh support the original conclusions of Furchgott and Zawadzki (11) that a lipoxygenase metabolite of AA mediates the relaxations to ACh. Futhermore, it suggests that EDHF is a lipoxygenase metabolite in rabbit aorta. As indicated above, 11,12,15-THETA is the vasoactive lipoxygenase metabolite of AA that is produced by the aortic endothelium (26, 27). This metabolite relaxes and hyperpolarizes the precontracted rabbit aorta, and the relaxations to 11,12,15-THETA are blocked by high extracellular K^+ concentration. These findings support the conclusion that 11,12,15-THETA represents an EDHF in rabbit aorta.

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DISCLOSURES

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