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14,15-Dihydroxyeicosatrienoic acid relaxes bovine coronary arteries by activation of K_{Ca} channels

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Campbell, William B., Christine Deeter, Kathryn M. Gauthier, Richard H. Ingraham, J. R. Falck, and Pin-Lan Li. 14,15-Dihydroxyeicosatrienoic acid relaxes bovine coronary arteries by activation of K_{Ca} channels. *Am J Physiol Heart Circ Physiol* 282: H1656–H1664, 2002. First published January 17, 2002; 10.1152/ajpheart.00597.2001.—Epoxyeicosatrienoic acids (EETs) cause vascular relaxation by activating smooth muscle large conductance Ca^{2+}-activated K^+ (K_{Ca}) channels. EETs are metabolized to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase. We examined the contribution of 14,15-DHET to 14,15-EET-induced relaxations and characterized its mechanism of action. 14,15-DHET relaxed U-46619-precontracted bovine coronary artery rings but was approximately fivefold less potent than 14,15-EET. The relaxations were inhibited by charybdotoxin, iberiotoxin, and increasing extracellular K^+ to 20 mM. In isolated smooth muscle cells, 14,15-DHET increased an iberiotoxin-sensitive, outward K^+ current and increased K_{Ca} channel activity in cell-attached patches and inside-out patches only when GTP was present. 14,15-[^{14}C]EET methyl ester (Me) was converted to 14,15-[^{14}C]DHET-Me, 14,15-[^{14}C]DHET, and 14,15-[^{14}C]EET by coronary arterial rings and endothelial cells but not by smooth muscle cells. The metabolism to 14,15-DHET was inhibited by the epoxide hydrolase inhibitors 4-phenylchalcone oxide (4-PCO) and BIRD-0826. Neither inhibitor altered relaxations to acetylcholine, whereas relaxations to 14,15-EET-Me were increased slightly by BIRD-0826 but not by 4-PCO. 14,15-DHET relaxes coronary arteries through activation of K_{Ca} channels. Endothelial cells, but not smooth muscle cells, convert EETs to DHETs, and this conversion results in a loss of vasodilator activity.

endothelium-derived hyperpolarizing factor; epoxyeicosatrienoic acids; potassium channels; epoxide hydrolase

ENDOTHELIAL CELLS relax vascular smooth muscle through the release of a number of soluble mediators such as nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) (4, 13, 22). Studies have implicated metabolites of arachidonic acid, K^+, and hydrogen peroxide as mediators of the EDHF response (2, 7, 10, 16, 20, 26, 28). The mediator may vary with the vascular bed. In the coronary artery, evidence from a number of laboratories indicates that EDHF is an epoxyeicosatrienoic acid (EET), a cytochrome *P*-450 metabolite of arachidonic acid (2, 10, 12, 15, 16, 29). The coronary endothelium synthesizes four regioisomeric EETs, and agonists such as acetylcholine and bradykinin stimulate their release (2, 24, 29, 30). The endothelium-dependent relaxation and hyperpolarization of coronary smooth muscle to acetylcholine and bradykinin is blocked by inhibitors of cytochrome *P*-450 and by inhibitors of Ca^{2+}-activated K^+ (K_{Ca}) channels (2, 16). Like EDHF, EETs relax and hyperpolarize coronary smooth muscle. The relaxations to the EETs are independent of the endothelium (27, 29). The EETs open a large conductance K_{Ca} channel of the smooth muscle cell membrane, resulting in membrane hyperpolarization (2). The activation of the K_{Ca} channel by EETs involves a guanine nucleotide-binding protein, most likely G_s (11, 15, 18).

While most of these studies have been conducted in vitro in isolated coronary arteries, these conclusions have been confirmed in vivo in anesthetized dogs (21, 23, 35). In coronary microvessels, acetylcholine causes vasodilation that is not blocked by inhibitors of nitric oxide synthase or cyclooxygenase but eliminated by inhibitors of K^+ channels. These findings implicated EDHF as a mediator of vasodilation in coronary microvessels. The nonnitric oxide, nonprostaglandin relaxation to acetylcholine was also blocked by inhibitors of cytochrome *P*-450. These experiments support the conclusion that a cytochrome *P*-450 metabolite mediates a portion of the coronary vasodilation in vivo.

EETs are hydrolyzed to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase (34, 36). The effects of DHETs on vascular tone vary. 5,6-EET relaxes the rat tail artery; however, 5,6-DHET is without effect (3). In contrast, 11,12-DHET relaxes porcine coronary arteries and is equipotent to 11,12-EET (33). Canine coronary microvessels are much more sensitive to the relaxing effects of 11,12-EET and 11,12-DHET, and

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11,12-DHET is more potent than 11,12-EET (25). Inhibition of epoxide hydrolase to prevent DHET formation from EETs did not alter the EET-induced relaxation or the relaxations to bradykinin (34). Thus the conversion of EETs to DHETs is not required for relaxation. It is not known whether DHETs act by the same mechanism as EETs.

In the present study, we compared the vascular effects of 14,15-EET and 14,15-DHET and examined the role of 14,15-DHET in the action of 14,15-EET. 14,15-DHET relaxed the bovine coronary artery; however, 14,15-DHET was approximately fivefold less potent than 14,15-EET. Additional studies indicate that 14,15-DHET, like 14,15-EET, opens a large conductance K_{Ca} channel by a G protein-dependent mechanism (11, 15, 18). Endothelial cells, but not smooth muscle cells, converted 14,15-EET to 14,15-DHET. In bovine coronary arteries, the conversion of EET to DHET represents an inactivation pathway and causes a loss of activity.

METHODS

Vascular reactivity of bovine coronary arteries. Bovine hearts were purchased from a local slaughterhouse, and the left anterior descending coronary artery was dissected and cleaned of connective tissue. Vessels were cut into 2-mm-diameter rings (3 mm width), and isometric tension measured as previously described (2, 18, 29). Vessels were stored in Krebs buffer consisting of (in mM) 119 NaCl, 4.8 KCl, 24 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 11 glucose, 0.02 EDTA, and 3.2 $CaCl_2$. In some studies, the K^+ concentration was increased from 4.8 to 20 mM by substituting KCl for NaCl.

Basal tension was set at the length-tension maximum of 3.5 g and equilibrated for 1.5 h. KCl (40 mM) was added to the chamber until reproducible maximal contractions were maintained. U-46619 (10–20 nM), a thromboxane agonist, was used to precontract the vessels from basal tension to between 50% and 90% of the maximal KCl contraction. Cumulative additions of acetylcholine, 14,15-EET, 14,15-DHET, or 14,15-EET methyl ester (Me) were added to the chamber. Between concentration-response curves, the chambers were rinsed with fresh Krebs buffer, 40 mM KCl was administered to determine the maximum contraction, and the vessels were rinsed. Pretreatment with charybdotoxin (100 nM), iberiotoxin (50 nM), BIRD-0826 (10^{-5} M), 4-phenylchalcone oxide (4-PCO; 2.2×10^{-5} M), or vehicle occurred 10 min before precontraction with U-46619. Tension is presented as a percentage of relaxation, where 100% relaxation is basal pre-U-46619 tension.

Isolation of coronary smooth muscle cells. Pieces of bovine heart ($2 \times 3 \times 1$ cm) were obtained from where the left anterior descending coronary artery branches. These pieces of heart were sliced into 300-μm sections, the microvessels were dissected, and smooth muscle cells were isolated as previously described (18, 19). The dissociated cells were stored at 4°C until they were placed in the recording chamber.

Patch-clamp methods. Single channel K^+ currents were recorded using the patch-clamp technique (14) as previously described (18, 19, 27). Whole cell, cell-attached, and inside-out configurations were used to determine the effect of 14,15-DHET on K^+ currents in vascular smooth muscle cells. Patch pipettes were made from borosilicate glass capillaries and had a tip resistance of 8–10 MΩ for single channel recording when filled with 145 mM KCl solution. Smooth muscle cells were placed in a 1-ml perfusion chamber that was mounted on the stage of a Nikon inverted microscope. After the tip of the pipette was positioned on a cell, a high-resistance seal (5–15 GΩ) was formed between the pipette tip and the cell membrane by light suction. This configuration was used for the cell-attached mode. For the whole cell mode, an additional acute suction was applied to rupture the membrane under the pipette. Inside-out membrane patches were excised by lifting the pipette membrane complex to the air-solution interface.

Whole cell patch-clamp recordings were obtained using standard pulse protocols. Macroscopic K^+ currents were generated by progressive stepwise 10-mV depolarizing pulses (400-ms duration, 100-ms intervals) from a constant holding potential of −60 mV. Cells were dialyzed with a pipette solution that contained (in mM) 145 potassium glutamate, 1 $MgCl_2$, 10 HEPES, 1 EGTA, 1 Na_2ATP, and 0.05 Na_2GTP and 100 nM ionized calcium (pH 7.2) and perfused with a bath solution composed of (in mM) 145 NaCl, 4 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 2 $CaCl_2$ (pH 7.4). Control trials were performed using a bath solution with 0.095% ethanol. Trials were performed in triplicate and averaged to estimate current amplitude. Subsequently, cells were perfused with a bath solution containing 14,15-DHET (10 μM) and/or iberiotoxin (100 nM). The membrane capacitance of each cell was estimated by integrating the capacitative current generated by a 20-mV hyperpolarizing pulse after electronic cancellation of pipette-patch capacitance. Currents are expressed in picoamperes per picofarad to normalize for differences in cell membrane area between individual cells.

For single channel recordings in the cell-attached mode, the bath solution contained (in mM) 145 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, 10 glucose, and 5 HEPES (pH 7.4), and the pipette solution contained (in mM) 145 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, and 5 HEPES (pH 7.4). For single channel recordings using the inside-out excised membrane patch, the bath solution contained (in mM) 145 KCl, 1.1 $MgCl_2$, 10 HEPES, and 2 EGTA and 300 nM ionized calcium (pH 7.2). The pipette solution contained (in mM) 145 KCl, 1.8 $CaCl_2$, 1.1 $MgCl_2$, and 10 HEPES (pH 7.4). All patch-clamp experiments were performed at room temperature, ~20°C. Channel open probability (NP_o) was used to represent the activity of the K_{Ca} channels.

The current-voltage relationship was established by recording the currents at patch membrane potentials of −60 to +60 mV. A K^+ channel with a conductance of 256 ± 5 pS was identified (2, 18, 19, 27). This large conductance K_{Ca} channel increases NP_o with depolarization and increases in calcium concentration. The channel was blocked by tetraethylammonium chloride, iberiotoxin, and charybdotoxin.

To determine the effects of 14,15-DHET on the activity of K_{Ca} channels, cell-attached patches were studied first. A 3-min control recording at a membrane potential of +40 mV was obtained after a tight seal was established. The bath solution was then rapidly changed by flushing the perfusion chamber with 10 ml of the same solution containing 14,15-DHET (0.01, 0.1, or 1 μM, $n = 10$), and a series of 3-min recordings was obtained. To determine whether a G protein was involved in the effect of 14,15-DHET on the activity of the K_{Ca} channels, the inside-out patch modes were used. After inside-out patches were established, a 3-min control recording was obtained at a membrane potential of +40 mV. The bath solution was then rapidly changed by flushing the perfusion chamber with 5–10 ml of the same solution containing 0.01, 0.1, or 1 μM 14,15-DHET ($n = 4$–5) with or

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without 0.5 mM GTP and 1 mM ATP, and a second successive 3-min recording was obtained.

Metabolism of 14,15-[^{14}C]EET-Me. Bovine coronary endothelial cells and smooth muscle cells were cultured in 75-cm^2 flasks as previously described (30). The culture medium was removed, and the cells washed twice with HEPES buffer. 14,15-[^{14}C]EET-Me (8,000 counts/min, 10^{-6} M) was incubated for 10 min at 37°C in HEPES buffer (see above) in the presence and absence of rings of the coronary artery, endothelial cells, or smooth muscle cells (29, 30). In some studies, the incubation time was varied, and in others, the cells or tissue were pretreated with the epoxide hydrolase inhibitors BIRD-0826 (10^{-5} M) or 4-PCO (2.2×10^{-5} M). After incubation, the media were removed, and radioactive metabolites were extracted by solid phase extraction using C-18 extraction columns (Varian) as previously described (2, 30). The extract was resolved into its components by reverse-phase HPLC using a linear gradient from 50% acetonitrile in water to 100% acetonitrile as previously described (2, 30). In parallel analyses, the migration times of known standards (14,15-EET, 14,15-EET-Me, 14,15-DHET, and 14,15-DHET-Me) were determined. Epoxide hydrolase activity was expressed as the conversion of 14,15-EET-Me to 14,15-DHET-Me and 14,15-DHET.

Statistical analysis was performed using an analysis of variance to determine the significant differences within groups and Student-Newman-Keuls tests to determine the significance between groups. Data are expressed as means ± SE.

Chemicals. Charybdotoxin and iberiotoxin were purchased from Research Biochemicals International (Natick, MA). U-46619 was purchased from Cayman Chemical (Ann Arbor, MI). BIRD-0826 was provided by Boehringer-Ingelheim Pharmaceutical. It inhibits soluble epoxide hydrolase (6) with an IC_{50} of 0.46 μM but did not affect microsomal epoxide hydrolase in concentrations up to 10 mM. Its solubility in 20 mM sodium phosphate buffer (pH 7.4) was 16.3 mg/ml. Collagenase and elastase were purchased from Worthington (Lakewood, NJ). 14,15-EET was prepared as previously described by the method of Corey et al. (2, 5). 14,15-EET-Me was prepared by treating 14,15-EET with ethereal diazomethane for 15 min at room temperature (30). The solvent was removed under a stream of nitrogen. The purity was determined by HPLC as described above. 14,15-DHET was prepared by acid hydrolysis of 14,15-EET. The 14,15-EET was treated with 0.1 N hydrochloric acid for 2 h at room temperature. The eicosanoids were extracted and purified by HPLC as described above. All other chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

Vascular relaxations to 14,15-EET, 14,15-DHET, and 14,15-EET-Me. 14,15-EET relaxed the U-46619-precontracted bovine coronary artery in a concentration-related manner as previously described (Fig. 1). Similar relaxation responses were obtained with 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET (2, 29). 14,15-EET-Me also relaxed the coronary artery and was less potent than 14,15-EET. The activity of the methyl ester may represent conversion of 14,15-EET-Me to 14,15-EET by vascular cells (31). 14,15-DHET also relaxed the bovine coronary artery, as was previously indicated in the canine coronary artery (25, 33). However, in our studies, the concentration-response curve for 14,15-DHET was shifted approximately fivefold to the right of the curve for 14,15-EET with no change in maximal effect. Thus the hydrolysis of the epoxide to a vicinal diol results in loss of potency but retention of full agonist activity. The relaxations to 14,15-DHET were inhibited by increasing the extracellular K^+ concentration from 4.8 to 20 mM (Fig. 2). Similarly, pretreatment with the K_{Ca} channel blocker iberiotoxin (50

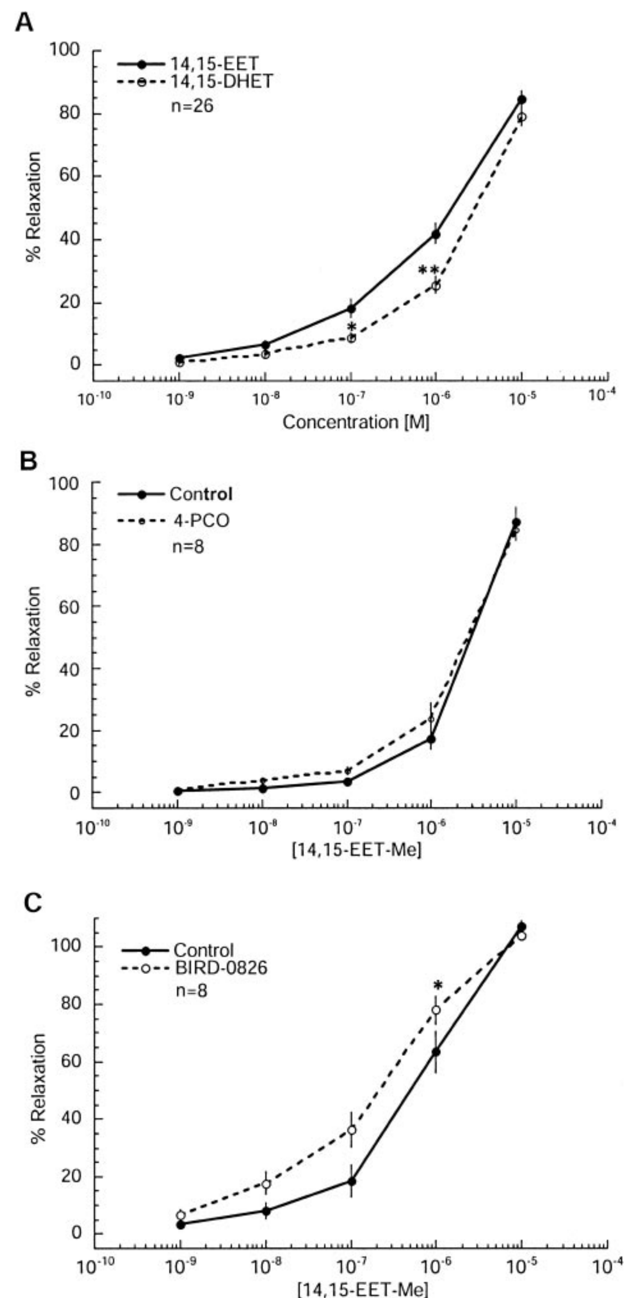

Fig. 1. Effect of 14,15-epoxyeicosatrienoic acid (EET), 14,15-dihydroxyeicosatrienoic acid (DHET), and 14,15-EET methyl ester (Me) on vascular tone in U-46619-precontracted bovine coronary arteries. *A*: 14,15-EET and 14,15-DHET were added in cummulative concentrations, and changes in isometric tension were measured. *B* and *C*: effect of inhibitors of epoxide hydrolase on 14,15-EET-Me-induced relaxations. Vessels were pretreated with 4-phenylchalcone oxide (4-PCO; 2.2×10^{-5} M; *B*), BIRD-0826 (10^{-5} M; *C*), or their vehicle. Cumulative concentration-responses to 14,15-EET-Me were then determined. Each value represents the mean ± SE for the number of arteries (*n*) indicated. *$P < 0.05$ and **$P < 0.01$, DHET compared with EET.

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nM) blocked 14,15-DHET-induced relaxations. Another K_{Ca} channel inhibitor, charybdotoxin (100 nM), shifted the 14,15-DHET concentration-response curve to the right by ~10-fold. Similar effects of these inhibitors have been described for EETs (2). These studies indicate a role for K^+ channels in the relaxations to 14,15-DHET.

Activation of K_{Ca} channels in coronary smooth muscle by 14,15-DHET. Figure 3*A* shows tracings of macroscopic, outward K^+ currents that were generated by 10-mV depolarizing steps from −60 to +60 mV in an isolated smooth muscle cell in the whole cell mode. The application of 14,15-DHET (10 μM) activated outward K^+ currents by ~50%, and the further application of iberiotoxin (100 nM) eliminated most of the outward K^+ current of this cell. The averaged data show that 14,15-DHET significantly activated the current density (Fig. 3*B*). At +60 mV, 14,15-DHET increased current density by 43%. The subsequent addition of iberiotoxin blocked current density by 42% below control levels. Membrane capacitance, an indicator of cell membrane area, averaged 20.5 ± 3.4 pF.

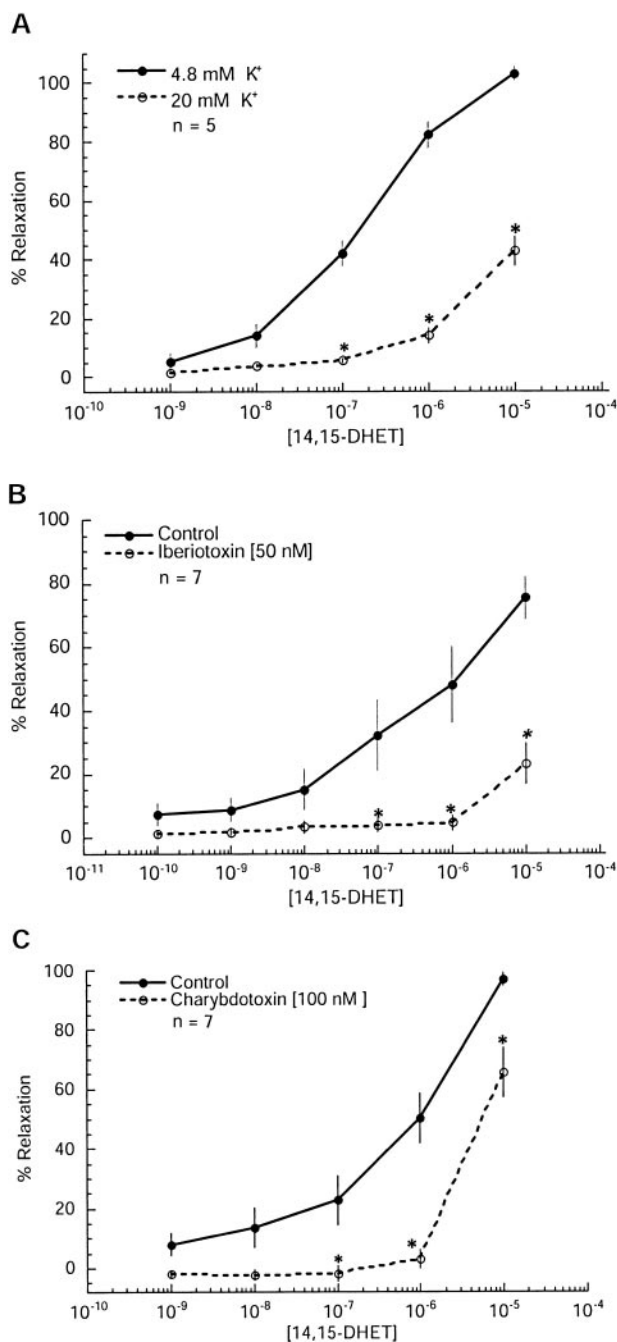

Fig. 2. Effect of inhibitors of K^+ channels on the relaxations to 14,15-DHET. *A*: effect of increasing the extracellular K^+ from 4.8 to 20 mM. *B* and *C*: effects of iberiotoxin (IbTX; *B*) and charybdotoxin (*C*), inhibitors of Ca^{2+}-activated K^+ (K_{Ca}) channels, respectively. Each value represents the mean ± SE for the *n* indicated. $^*P <$ 0.001, control compared with inhibitor.

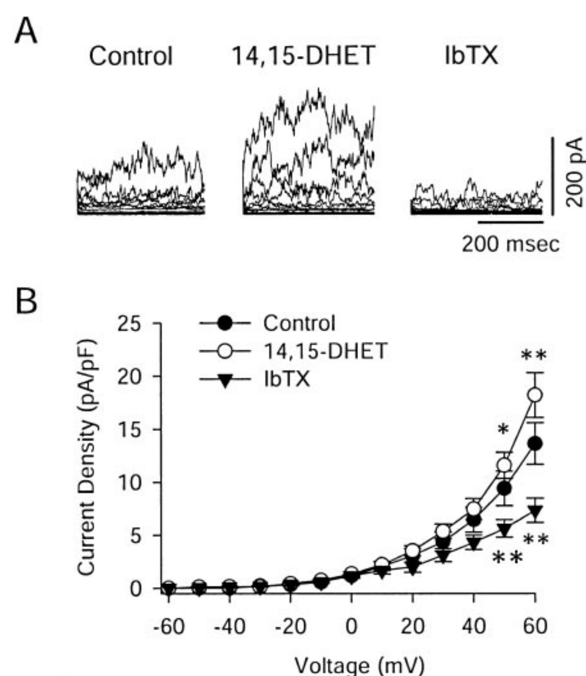

Fig. 3. Effect of 14,15-DHET on whole cell, outward K^+ currents of bovine coronary arterial smooth muscle cells. *A*: tracings of outward K^+ currents from a single smooth muscle cell (capacitance = 19.4 pF) in the presence and absence of 14,15-DHET (10 μM) and IbTX (100 nM). *B*: averaged whole cell current density stimulated by increasing voltage of isolated smooth muscle cells. 14,15-DHET (10 μM) significantly activated current density, and IbTX (100 nM) blocked the current density below control levels. Each value represents the mean ± SE for n = 6. $^*P < 0.05$ and $^{**}P < 0.01$ compared with control.

Isolated coronary smooth muscle cells contain a large conductance (256 ± 5 pS) K_{Ca} channel that is activated by 14,15-EET (2, 18, 19). The activity of this K_{Ca} channel was inhibited by tetraethylammonium chloride, charybdotoxin, and iberiotoxin. In the cell-attached mode, 14,15-DHET increased openings of the K_{Ca} channel in a concentration-related manner (Fig. 4*A*). DHET increased the NP_o without changing the current amplitude (Fig. 4, *B* and *C*). The threshold for activity with 14,15-DHET was observed at 10 nM. In contrast, 14,15-EET was ~10-fold more potent, increasing channel activity at 1 nM (2, 18). In the inside-out configuration, 14,15-DHET failed to change channel opening in concentrations from 10^{-8} to 10^{-5}M (NP_o = 0.030 ± 0.011 for control and 0.026 ± 0.010

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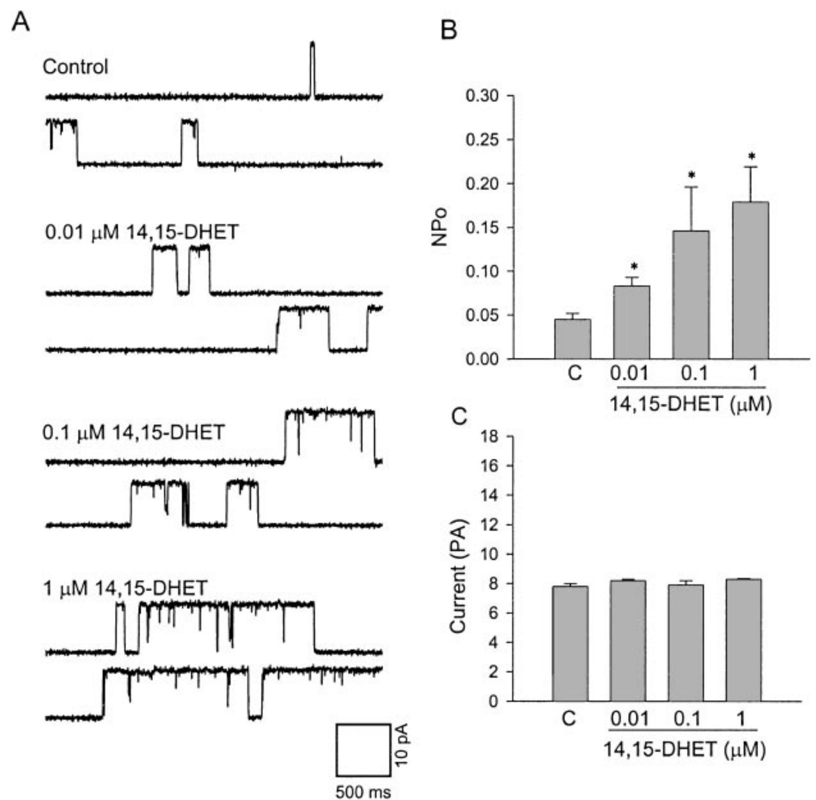

Fig. 4. Effect of 14,15-DHET on the activity of K_{Ca} channels of smooth muscle cells from bovine coronary arteries using the cell-attached patch-clamp mode. *A*: typical tracings of channel activity in the presence and absence of various concentrations of 14,15-DHET. Upward deflections indicate channel openings. *B*: open state probability (NP_o) of the K^+ channel. *C*: effect on current amplitude. Each value represents the mean ± SE for $n = 10$.

with 10 μM 14,15-DHET, $n = 5$). If GTP (0.5 mM) and ATP (1 mM) were added to the bathing solution of inside-out patches, 14,15-DHET increased K_{Ca} channel opening (Fig. 5*A*). As with cell-attached patches, 14,15-DHET caused a concentration-related increase in NP_o of inside-out patches incubated with GTP without changing the current amplitude (Fig. 5, *B* and *C*). The concentrations of 14,15-DHET that increased K_{Ca} channel activity were similar in cell-attached patches and inside-out patches exposed to GTP. A similar dependence on GTP was reported for 11,12-EET in inside-out patches (11, 15, 18).

Metabolism of 14,15-[^{14}C]EET-Me by coronary arteries and vascular cells. Incubation of coronary arterial rings or endothelial cells with 14,15-[^{14}C]EET showed the same conversion of 14,15-DHET as cell-free incubations (data not shown). Because fatty acid methyl esters are taken up by cells and metabolized to free acids (17, 31), we incubated 14,15-[^{14}C]EET-Me (10^{-6} M) with coronary arteries and resolved the radioactive metabolites by HPLC (Fig. 6*B*). Four radioactive peaks were detected. The major product comigrated with 14,15-DHET. The minor products comigrated with 14,15-EET and the methyl esters of 14,15-EET and 14,15-DHET. When 14,15-[^{14}C]EET-Me was incubated under identical conditions in the absence of the coronary artery, the major radioactive peak comigrated with 14,15-EET-Me and a minor peak with 14,15-EET (Fig. 6*A*). Pretreatment with BIRD-0826 (10^{-5} M) or 4-PCO (2.2×10^{-5} M) inhibited the conversion of 14,15-EET to 14,15-DHET by 86% and 93%, respectively (Fig. 6*C*). These studies indicate that coronary arteries contain esterases that convert fatty acid methyl esters to fatty acids and epoxide hydrolase to convert the epoxide to a vicinal diol.

In a similar manner, endothelial cells converted 14,15-EET-Me to 14,15-EET and 14,15-DHET, and this conversion increased with the time of incubation (Fig. 7, *A* and *C*). In contrast, smooth muscle cells only converted 14,15-EET-Me to 14,15-EET (Fig. 7*B*). These findings suggest that endothelial cells contain epoxide hydrolase and an esterase(s), whereas smooth muscle cells only contain the esterase(s).

The relaxations to 14,15-EET-Me and acetylcholine were measured in the presence and absence of epoxide hydrolase inhibitors to determine whether hydrolysis of EET to DHET contributes to the relaxations. 14,15-EET-Me caused a concentration-related relaxation of the coronary artery (Fig. 1, *B* and *C*). Pretreatment with 4-PCO or BIRD-0826 failed to affect the constrictor response to U-46619. However, BIRD-0826 increased the relaxations to 14,15-EET-Me (Fig. 1*C*). It caused a shift to the left in the 14,15-EET-Me concentration-response curve. The response to 14,15-EET-Me in 4-PCO-treated vessels was not significantly different from 14,15-EET-Me alone (Fig. 1*B*). Acetylcholine relaxed indomethacin-treated coronary arteries (Fig. 8). Pretreatment with BIRD-0826 or 4-PCO failed to alter the relaxations to acetylcholine.

DISCUSSION

EETs are endothelium-derived eicosanoids that mediate a portion of the vasodilator effect of agonists such

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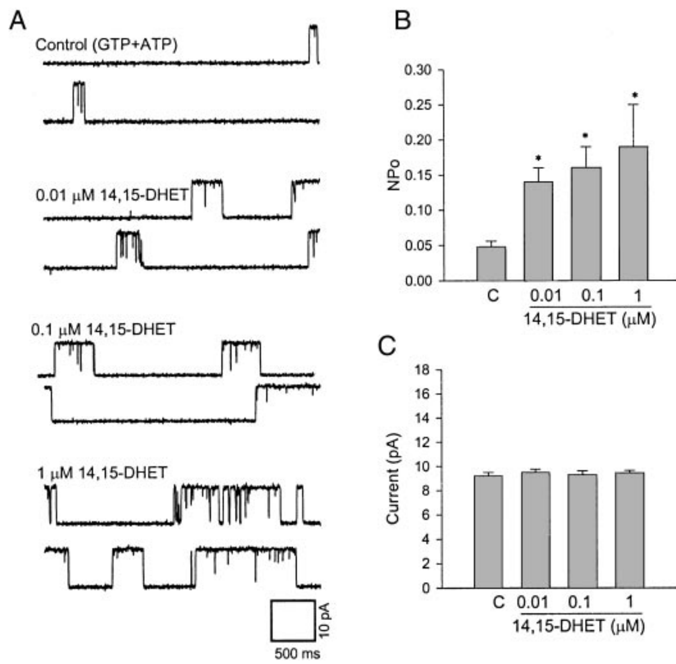

Fig. 5. Effect of 14,15-DHET on K_{Ca} channel activity in coronary smooth muscle cells in the inside-out patch-clamp mode. GTP (0.5 mM) and ATP (1 mM) were added to the bathing solution. *A*: typical tracings of channel activity in the presence and absence of various concentrations of 14,15-DHET. Upward deflections indicate channel openings. *B*: NP_o; *C*: current amplitude. Each value represents the mean ± SE for $n = 5$.

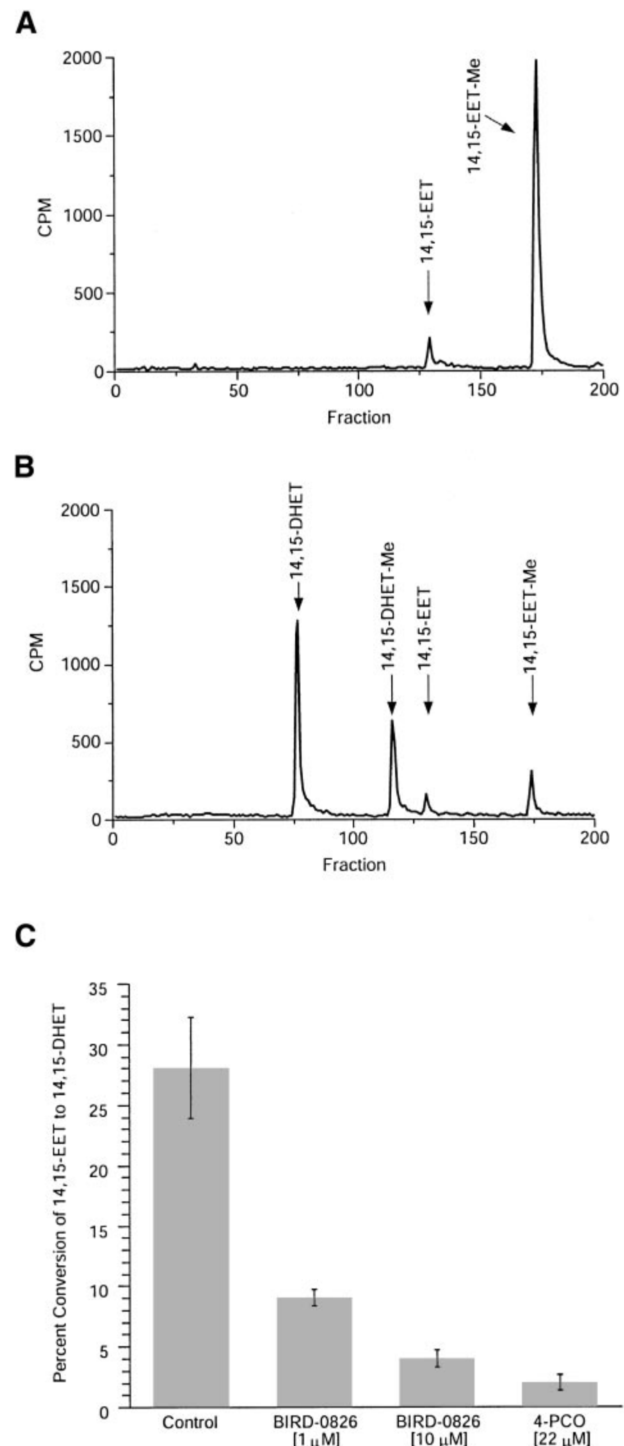

Fig. 6. Metabolism of 14,15-EET-Me by bovine coronary arteries. 14,15-[^{14}C]EET-Me was incubated for 10 min in the presence (*B*) or absence (*A*) of bovine coronary artery. Metabolites were extracted and resolved by HPLC. Migration times of known standards are indicated by the arrows above the chromatograms. *C*: effect of epoxide hydrolase inhibitors on the percent conversion of 14,15-EET-Me to 14,15-DHET/DHET-Me by bovine coronary arteries. Each value represents the mean of 3 measurements. CPM, counts per minute.

as bradykinin and acetylcholine (2, 10, 12, 16, 29). They act on vascular smooth muscle to open K_{Ca} channels, which results in membrane hyperpolarization and vasodilation. The activation of K_{Ca} channels by EETs requires a G protein, G_s (11, 15, 18). These findings may indicate that EETs function as EDHFs. EETs may be metabolized in the vasculature to DHETs by epoxide hydrolase, to 16- or 18-carbon metabolites by β-oxidation, or to 22-carbon metabolites by chain elongation (8, 9, 34). The EETs may also be esterified into phospholipids and neutral lipids (32, 34).

Interestingly, 14,15-DHET also relaxed the bovine coronary artery; however, DHET was five times less potent than 14,15-EET. This finding indicates that the vicinal diol can partially substitute for the epoxide group. This finding confirms published studies that 14,15-DHET is a potent vasorelaxant in porcine coronary arteries and canine coronary microvessels (9, 25). In these studies, 11,12-EET and 11,12-DHET were equipotent in relaxing porcine coronary arteries (9). In contrast, 11,12-DHET relaxed coronary microvessels in concentrations as low as 10^{-18} M and was 1,000 times more potent than 11,12-EET (25). This study suggests that the coronary microcirculation is more sensitive to the relaxant effect of DHET. Like 14,15-EET, the relaxations to 14,15-DHET were blocked by increasing the extracellular K^+ concentration from 4.8 to 20 mM and by inhibitors of K_{Ca} channels (2, 16, 25). These findings implicate K^+ channels in the action of DHET. This conclusion was confirmed by directly measuring whole cell K^+ currents in isolated arteriolar

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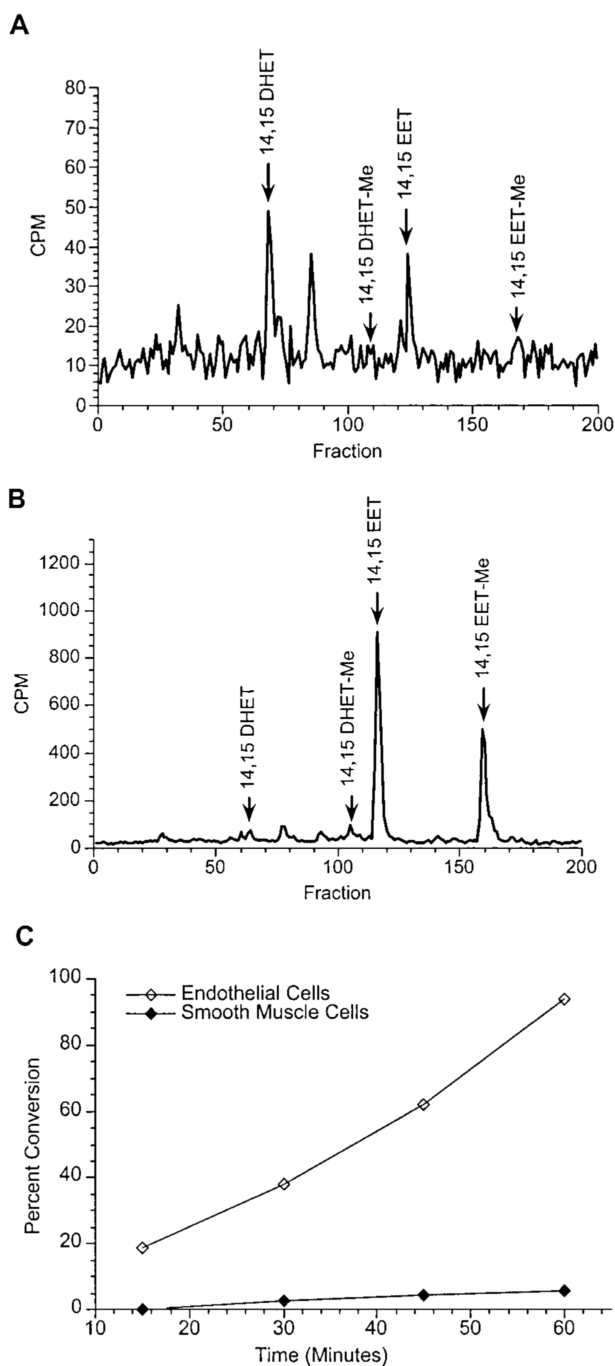

Fig. 7. Metabolism of 14,15-EET-Me by bovine coronary arterial endothelial cells (*A*) or smooth muscle cells (*B*). Cells were incubated for 10 min with 14,15-[^{14}C]EET-Me. The metabolites were extracted and resolved by HPLC. Migration times of known standards are indicated by the arrows above the chromatogram. *C*: percent conversion of 14,15-EET-Me to 14,15-DHET/DHET-Me with time of incubation. Each value represents the mean of 2 determinations.

smooth muscle cells. 14,15-DHET increased outward K^+ currents, which was blocked by iberiotoxin. Similar results were reported for 14,15-EET (1). Additionally, 14,15-DHET activated K_{Ca} channels in a concentration-related manner in cell-attached patches. When compared with our previous studies, 14,15-DHET was ~10-fold less potent than 14,15-EET in opening K_{Ca} channels (2). Also, like 14,15-EET, 14,15-DHET failed to open K_{Ca} channels in inside-out patches unless GTP was added to the bathing solution (11, 15, 18). Thus 14,15-DHET appears to have the same mechanism of action as 14,15-EET, with both eicosanoids requiring a G protein for K_{Ca} channel activation.

EETs are hydrolyzed to DHETs by epoxide hydrolase (8, 34, 36). When 14,15-EET was incubated with coronary arterial rings, there was little metabolism to 14,15-DHET (29). It was not clear whether this lack of metabolism was due to a failure of 14,15-EET uptake into the vascular cells or the absence of epoxide hydrolase. Because previous studies indicate that long-chain fatty acid methyl esters are taken up by cells and hydrolyzed to their fatty acids (17, 31), we examined the metabolism of 14,15-EET-Me by coronary arteries and coronary vascular cells. 14,15-[^{14}C]EET-Me was taken up by vascular cells and metabolized mainly to 14,15-DHET and 14,15-DHET-Me and to a lesser extent to 14,15-EET. These biochemical studies indicate that the coronary artery contains an epoxide hydrolase to convert EET to DHET and esterases to convert methyl esters to free acids. The hydrolysis of EET-Me

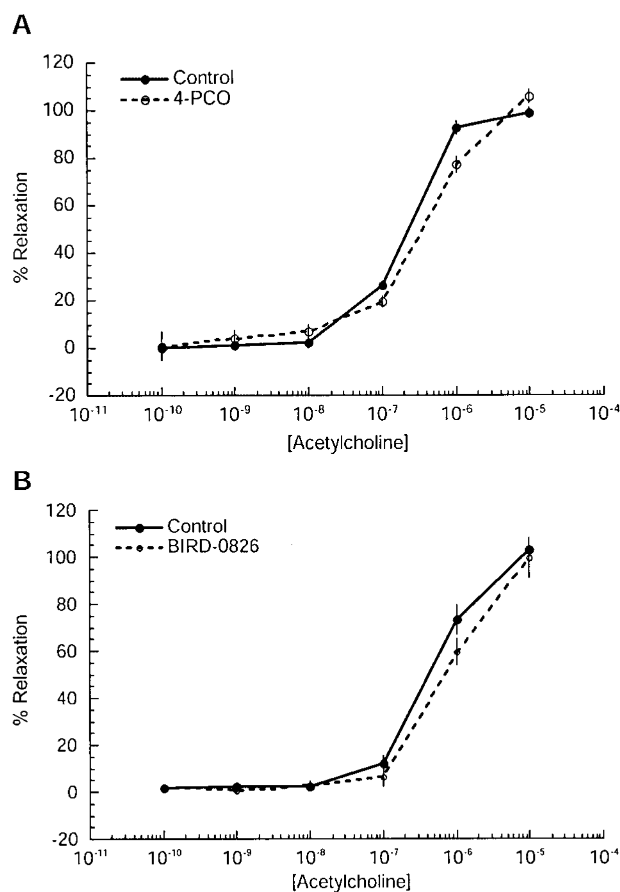

Fig. 8. Effect of epoxide hydrolase inhibition on acetylcholine-induced relaxation of bovine coronary arterial rings. All vessels were pretreated with indomethacin (10^{-5} M) and precontracted with U-46619. Vessels were also pretreated with 4-PCO (2.2×10^{-5} M), BIRD-0826 (10^{-5} M), or vehicle. Cumulative concentrations of acetylcholine were added, and changes in isometric tension were measured. Each value represents the mean ± SE for n = 16.

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to EET occurred in both the endothelial cell and smooth muscle cell. However, only endothelial cells converted 14,15-EET to 14,15-DHET. This finding is consistent with the presence of epoxide hydrolase in coronary endothelial cells (8, 34). The hydrolysis of 14,15-EET to 14,15-DHET was inhibited by two epoxide hydrolase inhibitors, 4-PCO and BIRD-0826. Thus endothelial cells synthesize EETs and contain the epoxide hydrolase necessary for DHET formation (30).

14,15-EET-Me relaxed the coronary artery and was less active than 14,15-EET. The relaxations to 14,15-EET-Me were enhanced slightly by pretreatment with the epoxide hydrolase inhibitor BIRD-0826 but not by 4-PCO. The lack of effect of 4-PCO may be because it is a substrate for epoxide hydrolase and not active for the duration of the tension experiments. These data are consistent with 14,15-EET and to a lesser extent 14,15-DHET mediating a portion of the action of 14,15-EET-Me. Because 14,15-DHET was fivefold less potent than 14,15-EET in causing relaxation, it is not surprising that 14,15-EET-Me was less active than 14,15-EET if 14,15-DHET mediates a portion of its effect.

In bovine coronary arteries, the relaxations to acetylcholine are mediated in part by an EDHF (2). EETs appear to represent EDHF in these arteries. In the current study, the relaxations to acetylcholine were not affected by inhibition of epoxide hydrolase by BIRD-0826 or 4-PCO. Similarly, in porcine coronary arteries, 4-PCO failed to alter the relaxations to bradykinin (34). These studies would suggest that metabolism of endogenous EETs by epoxide hydrolase is not an important determinant of EET action.

In summary, 14,15-DHET, like 14,15-EET, relaxes the bovine coronary artery. However, 14,15-DHET was less active than 14,15-EET. DHET shares the same mechanism of action as EET. It acts by opening K_{Ca} channels through a G protein-dependent mechanism. This opening of K^+ channels allows the efflux of K^+ that hyperpolarizes the smooth muscle cell membrane and causes relaxation. The endothelial cell is the vascular source of EETs and the vascular source of epoxide hydrolase that converts EETs to DHETs (30). Because DHET is less active than EET, the metabolism of EETs to DHETs represents an inactivation pathway.

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REFERENCES

1. **Campbell WB, Falck JR, and Gauthier K.** Role of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factor in bovine coronary arteries. *Med Sci Monitor* 7: 578–584, 2001.
2. **Campbell WB, Gebremedhin D, Pratt PF, and Harder DR.** Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* 78: 415–423, 1996.
3. **Carroll MA, Schwartzman M, Capdevila J, Falck JR, and McGiff JC.** Vasoactivity of arachidonic acid epoxides. *Eur J Pharmacol* 138: 281–283, 1987.
4. **Cohen RA and Vanhoutte PM.** Endothelium-dependent hyperpolarization: beyond nitric oxide and cyclic GMP. *Circulation* 92: 3337–3349, 1995.
5. **Corey EJ, Niwa H, and Falck JR.** Selective epoxidation of eicosa-*cis*-5,8,11,14-tetraenoic (arachidonic) acid and eicosa-*cis*-8,11,14-trienoic acid. *J Am Chem Soc* 101: 586–587, 1979.
6. **Dietze EC, Kuwano E, and Hammock BD.** Spectrophotometric substrates for cytosolic epoxide hydrolase. *Anal Biochem* 216: 176–187, 1994.
7. **Edwards G, Dora KA, Gardener MJ, Garland CJ, and Weston AH.** K is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* 396: 269–272, 1998.
8. **Fang X, Kaduce TL, Weintraub NL, Harmon S, Teesch LM, Morisseau C, Thompson DA, Hammock BD, and Spector AA.** Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells: implications for the vascular effects of soluble epoxide hydrolase inhibition. *J Biol Chem* 276: 14867–14874, 2001.
9. **Fang X, Kaduce TL, Weintraub NL, VanRollins M, and Spector AA.** Functional implications of a newly characterized pathway of 11,12-epoxyeicosatrienoic acid metabolism in arterial smooth muscle. *Circ Res* 79: 784–793, 1996.
10. **Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, and Busse R.** Cytochrome *P*450 2C is an EDHF synthase in coronary arteries. *Nature* 401: 493–497, 1999.
11. **Fukao M, Mason HS, Kenyon JL, Horowitz B, and Keef KD.** Regulation of BK_{Ca} channels expressed in human embryonic kidney 293 cells by epoxyeicosatrienoic acid. *Mol Pharmacol* 59: 16–23, 2001.
12. **Fulton D, Mahboubi K, McGiff JC, and Quilley J.** Cytochrome *P*450-dependent effects of bradykinin in the rat heart. *Br J Pharmacol* 114: 99–102, 1995.
13. **Furchgott RF and Zawadzki JW.** The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373–376, 1980.
14. **Hamill OP, Marty A, Neher E, Sackmann B, and Sigworth FJ.** Improved patch-clamp technique for high resolution current recording from cells and cell free membrane patches. *Pflügers Arch* 391: 85–100, 1981.
15. **Hayabuchi Y, Nakaya Y, Matsuoka S, and Kuroda Y.** Endothelium-derived hyperpolarizing factor activates Ca^{2+}-activated K^+ channels in porcine coronary artery smooth muscle cells. *J Cardiovasc Pharmacol* 32: 642–649, 1998.
16. **Hecker M, Bara AT, Bauersachs J, and Busse R.** Characterization of endothelium-derived hyperpolarizing factor as a cytochrome *P*450-derived arachidonic acid metabolite in mammals. *J Physiol (Lond)* 481: 407–414, 1994.
17. **Kuhl WE and Spector AA.** Uptake of long-chain fatty acid-methyl esters by mammalian cells. *J Lipid Res* 11: 458–465, 1970.
18. **Li P-L and Campbell WB.** Epoxyeicosatrienoic acids activate potassium channels in coronary smooth muscle through guanine nucleotide binding protein. *Circ Res* 80: 877–884, 1997.
19. **Li P-L, Zou A-P, and Campbell WB.** Regulation of potassium channels in coronary arterial smooth muscle by endothelium-derived vasodilators. *Hypertension* 29: 262–267, 1997.
20. **Matoba T, Shimokawa H, Nakashima M, Hirakawa Y, Mukai Y, Hirano K, Kandaide H, and Takeshita A.** Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* 106: 1521–1530, 2000.
21. **Ming Z, Parent R, and Lavallee M.** Nitric oxide-independent dilation of conductance coronary arteries to acetylcholine in conscious dogs. *Circ Res* 81: 977–987, 1997.
22. **Moncada S and Vane JR.** Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. *Pharmacol Rev* 30: 293–331, 1979.
23. **Nishikawa Y, Stepp DW, and Chilian WM.** In vivo location and mechanism of EDHF-mediated vasodilation in canine coronary microcirculation. *Am J Physiol Heart Circ Physiol* 277: H1252–H1259, 1999.
24. **Nithipatikom K, Pratt PF, and Campbell WB.** Determination of EETs using microbore liquid chromatography with fluorescence detection. *Am J Physiol Heart Circ Physiol* 279: H857–H862, 2000.
25. **Oltman CL, Weintraub NL, VanRollins M, and Dellsperger KC.** Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. *Circ Res* 83: 932–939, 1998.

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26. **Pfister SL, Spitzbarth N, Nithipatikom K, Edgemond WS, Falck JR, and Campbell WB.** Identification of 11,14,15- and 11,12,15-trihydroxyeicosatrienoic acids as endothelium-derived relaxing factors of rabbit aorta. *J Biol Chem* 273: 30879–30887, 1998.
27. **Pratt PF, Li P, Hillard CJ, Kurian J, and Campbell WB.** Endothelium-independent, ouabain-sensitive relaxation of bovine coronary arteries by EETs. *Am J Physiol Heart Circ Physiol* 280: H1113–H1121, 2001.
28. **Randall MD, Alexander SPH, Bennett T, Boyd EA, Fry JR, Gardiner SM, Kemp PA, McCulloch AI, and Kendall DA.** An endogenous cannabinoid as an endothelium-derived vasorelaxant. *Biochem Biophys Res Commun* 229: 114–120, 1996.
29. **Rosolowsky M and Campbell WB.** Role of PGI_2 and EETs in the relaxation of bovine coronary arteries to arachidonic acid. *Am J Physiol Heart Circ Physiol* 264: H327–H335, 1993.
30. **Rosolowsky M and Campbell WB.** Synthesis of hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) by cultured bovine coronary artery endothelial cells. *Biochim Biophys Acta* 1299: 267–277, 1996.
31. **Spector AA and Soboroff JM.** Long chain fatty acid methyl ester hydrolase activity in mammalian cells. *Lipids* 7: 186–190, 1972.
32. **VanRollins M, Kaduce TL, Knapp HR, and Spector AA.** 14,15-Epoxyeicosatrienoic acid metabolism in endothelial cells. *J Lipid Res* 34: 1931–1942, 1993.
33. **Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, and Spector AA.** Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circ Res* 81: 258–267, 1997.
34. **Weintraub NL, Fang X, Kaduce TL, VanRollins M, Chatterjee P, and Spector AA.** Epoxide hydrolase regulates epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. *Am J Physiol Heart Circ Physiol* 277: H2108–H2108, 1999.
35. **Widmann MD, Weintraub NL, Fudge JL, Brooks LA, and Dellsperger KC.** Cytochrome *P*-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo. *Am J Physiol Heart Circ Physiol* 274: H283–H289, 1998.
36. **Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, and Capdevila JH.** Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J Biol Chem* 268: 6402–6407, 1993.

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