Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids

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Weintraub, Neal L., Xiang Fang, Terry L. Kaduce, Mike VanRollins, Papri Chatterjee, and Arthur A. Spector. Epoxide hydrolases regulate epoxyeicosatrienoic acid incorporation into coronary endothelial phospholipids. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H2098–H2108, 1999.—Cytochrome P-450-deriv ed epoxyeicosatrienoic acids (EETs) are avidly incorporated into and released from endothelial phospholipids, a process that results in potentiation of endothelium-dependent relaxation. EETs are also rapidly converted by epoxide hydrolases to dihydroxyeicosatrienoic acid (DHETs), which are incorporated into phospholipids to a lesser extent than EETs. We hypothesized that epoxide hydrolases functionally regulate EET incorporation into endothelial phospholipids. Porcine coronary artery endothelial cells were treated with an epoxye hydrolyase inhibitor, 4-phenylchalcone oxide (4-PCO, 20 μmol/l), before being incubated with [3H]-labeled 14,15-EET (14,15-[3H]EET). 4-PCO blocked conversion of 14,15-[3H]EET to 14,15-[3H]DHET and doubled the amount of radiolabeled products incorporated into cell lipids, with >80% contained in phospholipids. Moreover, pretreatment with 4-PCO before incubation with 14,15-[3H]EET enhanced A-23187-induced release of radiolabeled products into the medium. In contrast, 4-PCO did not alter uptake, distribution, or release of [3H]arachidonic acid. In porcine coronary arteries, 4-PCO augmented 14,15-EET-induced potentiation of endothelium-dependent relaxation to bradykinin. These data suggest that epoxide hydrolases may play a role in regulating EET incorporation into phospholipids, thereby modulating endothelial function in the coronary vasculature.

dihydroxyeicosatrienoic acids; arachidonic acid; cytochrome P-450; porcine coronary artery

Cytochrome P-450 epoxygenases convert arachidonic acid to four epoxyeicosatrienoic acid (EET) regioisomers, 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET, corresponding to the double-bond position at which the epoxide group is inserted (4, 8, 16, 17). EETs possess a variety of biological actions, including the capacity to potently relax blood vessels by activating Ca2+-activated K+ (KCa) channels (2, 9, 33). Recent studies have established that EETs are produced by the vascular endothelium, and EETs have been identified by Campbell et al. (2, 19–21) as endothelium-derived hyperpolarizing factors in coronary blood vessels. EETs are avidly incorporated into cell lipids (7, 25), a property that is not shared by other previously characterized endothelium-derived relaxing factors. The biological importance of EET incorporation into cell lipids is suggested by recent reports that these eicosanoids are present esterified to phospholipids in platelets and heart, liver, and kidney tissues (3, 13, 30, 32). The most abundant EET regioisomer present in cell lipids in these tissues was 14,15-EET. Endothelial cells in culture also actively incorporate EETs into phospholipids, from which they are released after agonist-induced phospholipase activation (26). Furthermore, incubation of porcine coronary arteries with 14,15-EET or 11,12-EET resulted in potentiation of endothelium-dependent relaxation to bradykinin, an effect that was prevented when EET incorporation into phospholipids was blocked by inhibition of acyl-CoA synthases (26). These data suggest that EET incorporation into the lipids of cells present in the blood vessel wall can importantly influence vascular function. However, the mechanisms that regulate EET incorporation into vascular cell lipids are unknown.

Besides incorporating EETs into cell lipids, vascular endothelial and smooth muscle cells rapidly convert EETs to the corresponding dihydroxyeicosatrienoic acid (DHET) metabolites by the action of epoxide hydrolases (5, 7, 25, 31). Smaller amounts of DHETs are incorporated into endothelial cell lipids than the corresponding EETs (24, 25). Thus, in contrast to EETs, DHETs are primarily released from endothelial cells. Therefore, one potential mechanism to regulate the amount of EET incorporated into vascular cell lipids is the epoxide hydrolase-mediated conversion to DHETs. In support of this possibility, inhibition of epoxide hydrolases by 4-phenylchalcone oxide (4-PCO) enhanced the incorporation of 14,15-EET into the phospholipids of rat astroglial cells (22).

In the present study, we investigated the effects of inhibition of epoxide hydrolases on endothelial cell incorporation and release of 14,15-EET. We also addressed the functional effects of epoxide hydrolase inhibition on 14,15-EET-induced potentiation of relaxation to bradykinin in porcine coronary arteries.

MATERIALS AND METHODS

14,15-EET and 8,9-EET were purchased from Cayman Chemical (Ann Arbor, MI), U-46619 and bradykinin from Sigma (St. Louis, MO), and triacsin C and 4-PCO from Biomol (Plymouth Meeting, PA). The vehicle used in all experiments in which 4-PCO was added was dimethyl sulfoxide. MEM nonessential amino acids, MEM vitamin solution, HEPES, and trypsin were obtained from GIBCO (Grand Island, NY), fetal bovine serum (FBS) from HyClone Laboratories (Logan, UT), fatty acid-free bovine serum albumin from Miles Laboratories (Elkhardt, IN), and gentamicin from Schering (Kenilworth, NJ). Radiolabeled arachidonic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO) or Amer-

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sham (Arlington Heights, IL), and phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL). Radiolabeled EETs were synthesized, purified, and quantitated as described previously (7, 25, 26). The specific activity of the [3H]-labeled 14,15-EET (14,15-[3H]EET) used for most experiments was 333 Ci/mmol, that of 8,9-[3H]EET was also 333 Ci/mmol, and that of 14,15-[1-14C]DHET was 14.2 Ci/mmol. Some experiments were performed with 14,15-[3H]EET (232 Ci/mmol) obtained from DuPont-NEN (Boston, MA). Whatman Linear K thin-layer chromatography (TLC) plates were purchased from Alltech (Deerfield, IL). All other reagents were purchased from Sigma.

Cell culture and incubations. Porcine coronary endothelial cells (PCAEC) were isolated as reported previously (26, 28) and grown in medium 199 (M199) supplemented with 10% FBS, MEM nonessential amino acids, MEM vitamin solution, 2 mmol/l L-glutamate, 50 µmol/l gentamicin, and 15 mmol/l HEPES. The cultures were maintained until confluent in a humidified atmosphere containing 5% CO2 at 37°C.

Analyses of cell lipids. Cell lipids were extracted and analyzed as described previously (7, 25, 26). The amount of radioactivity in the medium was determined by scintillation counting. The final concentration of the tritiated substrate with which the cells were incubated was measured by combining the column effluent with Budget-Solve scintillation solution, assayed for radioactivity.

The cell lipid extracts were separated by TLC, using Whatman Linear K plates. Neutral lipids were separated with heptane-ethyl ether-glacial acetic acid (75:60:1.5 vol/vol), and phospholipids were separated with chloroform-methanol-glacial acetic acid-Milliapore water (50:50:2:1 vol/vol/vol/vol). The distribution of radioactivity on the TLC plate was determined with a gas flow proportional scanner. The values were corrected to picomoles, calculated from the specific activity of the tritiated substrate with which the cells were incubated.

Some of the cell lipid extracts were saponified for 1 h at 50°C with 0.5 ml methanolic 0.2 N NaOH containing 10% H2O. After the pH was brought to 8.0 with 0.1 mol/l phosphate buffer, the lipids were extracted twice with 5 ml of ice-cold ethyl acetate saturated with water. The solvent was removed under N2, and the lipids were dissolved in acetonitrile and assayed by reverse-phase HPLC.

Effects of 4-PCO on conversion of 14,15-[3H]EET to 14,15-[3H]DHET. Porcine coronary arteries. Coronary arteries were dissected from pig hearts immediately after removal at a local slaughterhouse. The arteries were placed in ice-cold Krebs-Ringer bicarbonate solution (KRB (in mmol/l): NaCl, 118.3, KCl, 4.7, CaCl2, 2.5, MgSO4, 1.2, KH2PO4, 2, 6, 40–80% of the maximal tension obtained with 60 mmol/l KCl. The arteries were incubated in KRB containing 5 ml of KRB continuously aerated with 95% O2-5% CO2. Basal ring tension was gradually adjusted to 10 g, a value previously reported to yield maximal contractions to KCl (60 mmol/l) in porcine coronary artery rings (27). The rings were suspended in water-jacketed (37°C) organ baths containing 5 ml of KRB continuously aerated with 95% O2-5% CO2. Basal ring tension was gradually adjusted to 10 g.

Measurement of porcine coronary arterial ring vasoactivity. Porcine coronary arteries were cut into 35-mm-wide rings and mounted onto stainless steel triangles, which in turn were attached by thread to isometric force transducers coupled to a polygraph for continuous recording of ring tension. The rings were suspended in water-jacketed (37°C) organ baths containing 5 ml of KRB continuously aerated with 95% O2-5% CO2. Basal ring tension was gradually adjusted to 10 g, a value previously reported to yield maximal contractions to KCl (60 mmol/l) in porcine coronary artery rings (27). The rings were contracted several times with 60 mmol/l KCl until the ring was maximally contracted. After 1 µmol/l 14,15-EET and 1 µCi of 14,15-[3H]EET were added and the incubation continued for 30 min. Dimethyl sulfoxide was used as the vehicle at a final concentration of 0.1%, and corresponding amounts of dimethyl sulfoxide were added to the control incubations. The incubation solution was removed, and an aliquot was subjected to scintillation counting. Lipids were extracted from the KRB and analyzed by reverse-phase HPLC as described in Assay of incubation medium. The values were corrected to picomoles, calculated from the specific activity of the 14,15-[3H]EET with which the rings were incubated, and normalized to tissue wet weight.

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relaxation in all experiments. The organ chambers were then rinsed, and ring tension was allowed to return to baseline.

In protocols to examine the effects of 4-PCO on bradykinin-induced relaxation, rings were treated with 4-PCO or vehicle. After 1 h, the rings were contracted again with U-46619 to a level of tension similar to the previous contraction, and when a stable level of tension was achieved, bradykinin was administered in a cumulative fashion. Although 4-PCO concentrations between 0.1 and 20 µmol/l were tested, 20 µmol/l was added in most of the experiments.

In protocols to examine the effects of 4-PCO on relaxation to 14,15-EET and on 14,15-EET-induced potentiation of relaxation to bradykinin, rings were treated with 4-PCO (20 µmol/l) or vehicle. After 1 h, the rings were contracted again with U-46619 to a level of tension similar to the previous contractions; when a stable level of tension was achieved, 14,15-EET (0.05–5 µmol/l) was administered. Thirty minutes after the last 14,15-EET dose (5 µmol/l) was added, the rings were rinsed six times with fresh KRB. 4-PCO or vehicle was immediately reapplied for 30 min, and the rings were recontracted with U-46619 to a level of tension similar to the previous contractions. When a stable level of tension was achieved, bradykinin was administered in a cumulative fashion. The final concentrations of all vehicles were <0.1%.

Relaxation responses were expressed as the percent decrease from the agonist-induced tension, as described previously (6, 26–28).

Statistical analysis. All data are expressed as means ± SE. The EC50 of bradykinin was calculated for each concentration–response curve before and after treatment with 14,15-EET (26). The EC50 values (expressed as −log[M]) before and after treatment were compared by paired Student’s t-tests or repeated-measures ANOVA followed by a Newman-Keuls post hoc analysis (26).

The posttreatment effects of 4-PCO on 14,15-EET-induced potentiation of relaxation to bradykinin were analyzed by repeated-measures ANOVA. Also, differences between the pre- and posttreatment percent changes in tension to each dose of bradykinin were calculated for both treatment groups (4-PCO and vehicle). The values were analyzed by repeated-measures ANOVA with two repeated factors, treatment group and dose of bradykinin. The treatment effects were individually compared post hoc at each level of stimulus. All P values were adjusted for multiple comparisons using the Bonferroni method.

All other data were analyzed by Student’s t-tests or ANOVA followed by a Newman-Keuls post hoc analysis. Probability values of ≤0.05 were considered to be statistically significant.

RESULTS

Effects of 4-PCO on conversion of 14,15-[3H]EET to 14,15-[3H]DHET by PCAEC. To investigate whether 4-PCO blocked the conversion of 14,15-[3H]EET to 14,15-[3H]DHET, PCAEC were pretreated with vehicle or 4-PCO (20 µmol/l) for 1 h and incubated with 1 µmol/l 14,15-[3H]EET for an additional 1 h. The incubation medium was removed, and the products were separated by HPLC. At this time, the medium of cultures treated with vehicle contained twice as much 14,15-[3H]DHET as 14,15-[3H]EET (Fig. 1A). Thus, like endothelial cells from other species of animals and from porcine aorta, PCAEC rapidly convert EETs to DHETs (24–27). Treatment with 4-PCO almost completely blocked the formation of 14,15-[3H]DHET (Fig. 1B). Consequently, considerably more of the medium-associated radioactivity remained as 14,15-[3H]EET after treatment with 4-PCO as compared with vehicle. When cells were incubated with 1 µmol/l 8,9-[3H]EET instead of 14,15-[3H]EET under identical conditions,
most of the EET was converted to 8,9-[3H]DHET by cells treated with vehicle (Fig. 1C), but appreciable amounts of DHET were not formed when 20 µmol/l 4-PCO was added (Fig. 1D).

In some experiments, cells were pretreated with vehicle or 20 µmol/l 4-PCO and then incubated with 1 µmol/l 14,15-[3H]EET for 1 h, after which the incubation medium was removed and the cells were washed and incubated with 2 µmol/l A-23187 for 30 min. The medium was then collected and analyzed by HPLC. In the case of vehicle-pretreated cells, most of the medium-associated radioactivity was in the form of 14,15-[3H]DHET, whereas no detectable 14,15-[3H]DHET was formed by 4-PCO-pretreated cells (not shown).

To investigate the specificity of 20 µmol/l 4-PCO to inhibit EET metabolism, we determined its effects on the metabolism of [3H]arachidonic acid. The cells were pretreated with 4-PCO or vehicle and then incubated with 1 µmol/l [3H]arachidonic acid under the conditions described above, followed by washing and incubation with A-23187 (2 µmol/l) for 30 min. The incubation medium was then removed and analyzed by HPLC. In both vehicle- and 4-PCO-treated cells, prominent peaks were observed which comigrated with arachidonic acid and 6-keto-PGF1, as well as two other unidentified PGs with slightly longer retention times than 6-keto-PGF1. No differences in the profiles of these peaks were observed among the vehicle- and 4-PCO-treated cells (n = 3, data not shown).

Effects of 4-PCO on EET incorporation into cell lipids. Depending on the conditions of incubation, from 10 to 50 times more EETs than DHETs are incorporated into endothelial cell lipids (24, 25). We therefore investigated whether inhibition of epoxide hydrolases would enhance the amount of radioactivity incorporated into PCAEC lipids during incubation with 14,15-[3H]EET or 8,9-[3H]EET. The cells were pretreated with 4-PCO (20 µmol/l) or vehicle, and 1 h later, 14,15-[3H]EET or 8,9-[3H]EET (1 µmol/l) was added. After 1 h the cultures were washed to remove EET that had not been taken up into the cells, and fresh medium containing 4-PCO or vehicle was added. After 30 min the cells were washed, and cell lipids were extracted and assayed for radioactivity. Treatment with 4-PCO enhanced the amount of radioactivity retained in cells during incubations with 14,15-[3H]EET or 8,9-[3H]EET (Fig. 2A). TLC analysis of vehicle- and 4-PCO-treated cells indicated that >80% of the radioactivity was present in phospholipids. In cells incubated with 14,15-[3H]EET, the order of distribution of radioactivity was phosphatidylinositol (PI) > phosphatidylethanolamine (PE) > phosphatidylcholine (PC) (Fig. 2B), with smaller amounts present in neutral lipids and diglycerides. Each fraction contained more incorporated radioactivity when the cultures were treated with 4-PCO, indicating that the increase in retention was not caused by selective incorporation into specific lipids.

In cells incubated with 8,9-[3H]EET, the incorporated radioactivity was distributed equally among the PI, PC, and PE fractions (not shown). As was observed after incubation with 14,15-[3H]EET, 4-PCO did not alter the distribution of radioactivity incorporated into cells incubated with 8,9-[3H]EET.

The effect of 4-PCO concentration on the incorporation of 1 µmol/l 14,15-[3H]EET into PCAEC and its conversion to 14,15-[3H]DHET was also investigated. As in the previous experiment, the cells were exposed initially for 1 h to 4-PCO and then incubated for 1 h with 14,15-[3H]EET. Most of the increase in radioabeled EET uptake into the cells occurred at 4-PCO concentrations between 0.3 and 1 µmol/l (Fig. 3A). The uptakes obtained at 1 and 3 µmol/l were in the same range as those observed in the previous experiment with 14,15-[3H]EET when the 4-PCO concentration was 20 µmol/l (Fig. 2A). Likewise, the amount of 14,15-[3H]DHET formed in the medium at the end of the 1-h incubation was markedly suppressed at 4-PCO concentrations as low as 0.1 µmol/l (Fig. 3B), and the amount formed at 3 µmol/l 4-PCO was as low as that observed at 20 µmol/l (Fig. 1B).
The presence of 14,15-DHET did not affect the uptake of 1 µmol/l 14,15-[3H]EET by the PCAEC. Concentrations of 1 and 5 µmol/l 14,15-DHET were tested, and 2 µmol/l 4-PCO was added to prevent the formation of additional DHET from the radiolabeled EET during the 1-h incubation. The uptakes were 162\(\pm\)2.4 (no added DHET), 146\(\pm\)26 (1 µmol/l DHET), and 153\(\pm\)14 (5 µmol/l DHET) pmol (n = 3, P > 0.1).

To determine whether treatment with 4-PCO would also enhance 14,15-[3H]EET labeling of vascular smooth muscle cell lipids, PASMC were preincubated with vehicle or 4-PCO and then exposed to 1 µmol/l 14,15-[3H]EET, after which the medium and cell lipids were extracted and analyzed as described in MATERIALS AND METHODS. Similar to the results observed with PCAEC, treatment with 4-PCO increased the amount of 14,15-[3H]EET retained by the PASMC without altering the distribution of incorporation, with most of the uptake present in phospholipids (n = 3, data not shown).

After incubation of PCAEC with 14,15-[3H]EET, some of the extracted cell lipids were hydrolyzed by saponification and analyzed by HPLC to determine whether the incorporated radioactivity remained primarily as 14,15-EET. In vehicle-treated cells, ~90% of the cell-associated radioactivity was present as 14,15-[3H]EET, whereas <3% was present as 14,15-[3H]DHET (Fig. 4A). In addition, several unidentified radiolabeled peaks were observed, including a prominent peak that eluted after 14,15-[3H]EET, with a retention time of 47 min. In contrast, in 4-PCO-treated cells virtually all of the cell-associated radioactivity was present as 14,15-[3H]EET, and no 14,15-[3H]DHET peak was detected (Fig. 4B).

An additional experiment indicated that the PCAEC were able to take up a small amount of 14,15-DHET, but the amount was not affected by exposure to 4-PCO. Cultures were incubated for 1 h with 20 µmol/l 4-PCO and subsequently for 1 h with 4 µmol/l 14,15-[1-14C]DHET. The uptake of radiolabeled DHET was 20.7 ± 1.2 pmol (dimethyl sulfoxide added) as compared with 16.7 ± 0.9 pmol (4-PCO in dimethyl sulfoxide added) (n = 3, P > 0.05).

Effects of 4-PCO on release of incorporated 14,15-[3H]EET from PCAEC lipids. To determine whether the enhanced incorporation of 14,15-[3H]EET into PCAEC lipids consequent to treatment with 4-PCO would result in an increase in the amount of 14,15-[3H]EET subsequently released from the cells, PCAEC were pretreated with 4-PCO or vehicle and then incubated with 1 µmol/l 14,15-[3H]EET, as described in Effects of 4-PCO on EET incorporation into cell lipids. After 1 h the incubation medium was removed, and the cells

![Fig. 3. Effect of 4-PCO concentration on uptake of 14,15-EET and its conversion to 14,15-DHET by PCAEC. Cultures were pretreated with vehicle or indicated concentrations of 4-PCO for 1 h and then incubated with 1 µmol/l 14,15-[3H]EET for 1 h. Dimethyl sulfoxide was used as vehicle for 4-PCO. Radioactivity taken up by PCAEC (A) was assayed as described in Fig. 2, and distribution of radioactivity remaining in medium (B) was determined by HPLC as described in Fig. 1. Each point is average of 2 separate cultures, and the 2 values were within 10% agreement.](http://ajpheart.physiology.org/)

![Fig. 4. Separation of radiolabeled products incorporated into PCAEC lipids during incubation with 14,15-[3H]EET in presence of vehicle (A) or 4-PCO (B). After incubation of cells as described in Fig. 2, cell lipid extracts were hydrolyzed with 0.5 ml of methanolic 0.2 N NaOH containing 10% H2O. After extraction twice with 5 ml of ice-cold ethyl acetate saturated with water, lipids were dissolved in acetonitrile and assayed by reverse-phase HPLC.](http://ajpheart.physiology.org/)
were washed and incubated in the absence (control) or presence of 2 µmol/l A-23187 for 30 min. The medium was then removed and analyzed as described previously. During the 30-min control incubation (in the absence of A-23187), little radioactivity was released from cells treated with either 4-PCO or vehicle and no differences were detected between the two groups (Fig. 5A). Exposure to A-23187 resulted in marked increases in the amount of radioactivity released from both 4-PCO- and vehicle-treated cells; however, only slightly more radioactivity was released from cells treated with 4-PCO than from vehicle-treated cells.

We investigated whether the distribution of incorporated 14,15-[3H]EET that remained in the cells after exposure to A-23187 might have been altered by pretreatment with 4-PCO. In some experiments after exposure to A-23187 and removal of the incubation medium, the cells were washed and cell lipids were extracted, separated by TLC, and assayed for radioactivity. Although twice as much radioactivity was recovered from the 4-PCO-pretreated cells as from the vehicle-pretreated cells (120 ± 7 (4-PCO) vs. 57 ± 7 (vehicle) pmol/well; n = 3, P < 0.01), the distribution of the incorporated radioactivity did not differ between the two groups. Over 70% was contained in phospholipids, predominately in PI and PC, with lesser amounts in PE. When cells were incubated with 1 µmol/l 8,9- [3H]EET instead of 14,15-[3H]EET under conditions identical to those described above, most of the radioactivity was also incorporated into phospholipids, and the distribution was not changed by the presence of 20 µmol/l 4-PCO (not shown).

In summary, inhibition of epoxide hydrolases enhanced the uptake of 14,15-[3H]EET and 8,9-[3H]EET but did not alter distribution into cell lipids, with most of the radioactivity still being incorporated into phospholipids. However, despite a greater than twofold increase in the amount of 14,15-[3H]EET incorporated into phospholipids, the amount of 14,15-[3H]EET released into the medium after exposure to A-23187 was only modestly enhanced.

[3H]arachidonic acid incorporation into and release from PCAEC lipids. We next examined [3H]arachidonic acid uptake and release in the absence and presence of treatment with 4-PCO. Experiments were performed exactly as described for EETs, except that after pretreatment with vehicle or 4-PCO the cells were incubated with 1 µmol/l [3H]arachidonic acid instead of 14,15-[3H]EET or 8,9-[3H]EET. Treatment with 4-PCO did not alter the amount of [3H]arachidonic acid incorporated into cell lipids (Fig. 6A) or the amount released into the medium during control incubations or in response to A-23187 (Fig. 6B). In vehicle-pretreated cells, 263 ± 27 pmol/well of arachidonic acid was incorporated into cell lipids, more than twice as much the amount of 14,15-[3H]EET incorporated under the same conditions (Fig. 2A). However, in response to treatment with A-23187, the cells released 24 ± 1 pmol of [3H]arachidonic acid into the medium, only one-third as much as the amount of 14,15-[3H]EET that was released from the cells (Fig. 5A). As was observed after incubation with 14,15-[3H]EET, most of the incorporated [3H]arachidonic acid was contained in phospholipids in both vehicle- and 4-PCO treated cells (not shown).

Effects of inhibition of acyl-CoA synthase activity on release of incorporated 14,15-[3H]EET and on formation of 14,15-[3H]DHET by PCAEC. Previous work from our laboratories (25, 26) indicates that EETs are rapidly incorporated into endothelial lipids via an acyl-CoA synthase-dependent pathway, with the maximal rate of uptake occurring at 30 min. We therefore investigated whether, in the presence of 4-PCO, some of the incorporated 14,15-[3H]EET might have been released from the cells in response to A-23187 but then reincorporated during the 30-min incubation period. To test this possibility, cells were pretreated with 4-PCO and then incubated with 1 µmol/l 14,15-[3H]EET for 1 h as described previously. The medium was removed, and the cells were washed and incubated for 20 min in medium containing 4-PCO and 2 µmol/l triacsin C or vehicle. This concentration of triacsin C, an inhibitor of acyl-CoA synthase (20), selectively blocks 14,15-EET incorporation into PCAEC lipids (26). Incubations were...
then continued for 30 min in the absence (control) or presence of A-23187 (2 µmol/l). Under these conditions, treatment with triacsin C did not alter the amount of 14,15-[3H]EET recovered in the medium after control incubations [30.0 ± 1.6 (triacsin C) vs. 31.6 ± 0.7 (vehicle) pmol; n = 3, P > 0.05] or after incubation with A-23187 [68.2 ± 2.8 (triacsin C) vs. 71.1 ± 0.2 (vehicle) pmol; n = 3, P > 0.05]. Furthermore, the amount of incorporated radioactivity present in cell lipids after the 30-min incubation with A-23187 did not differ between the two groups [112.6 ± 6.7 (triacsin C) vs. 119.5 ± 6.0 (vehicle) pmol/well; n = 3, P > 0.05]. These results suggest that in cells treated with 4-PCO, there was little reincorporation of released, unesterified 14,15-[3H]EET during the 30-min incubation with A-23187.

We proceeded to investigate whether inhibition of EET incorporation into cell lipids with triacsin C would alter the amount of 14,15-[3H]DHET formed from 14,15-[3H]EET by PCEC. To test this possibility, cells were treated with triacsin C (2 µmol/l) or its vehicle for 30 min and then incubated with 0.5 µmol/l 14,15-[3H]EET for 1 h. After incubation, the medium- and cell-associated lipids were extracted and analyzed as described previously. As expected, triacsin C markedly reduced the amount of radioactivity incorporated into cell lipids (n = 3, data not shown), whereas the amount of radioactivity present in the medium was increased [411 ± 4 (triacsin C) versus 356 ± 3 (vehicle) pmol/ml, n = 3, P < 0.001]. Analysis of the incubation medium by HPLC indicated that slightly more 14,15-[3H]DHET was present in the medium of cells treated with triacsin C compared with vehicle (255 ± 4 (triacsin C) vs. 230 ± 4 (vehicle) pmol/ml; n = 3, P < 0.03). These results suggest that inhibition of 14,15-EET incorporation into cell lipids increased the amount of 14,15-DHET formed during the incubation, possibly by increasing the amount of unesterified 14,15-EET available for conversion by epoxide hydrolases.

Effects of 4-PCO on 14,15-EET-induced vasorelaxation and potentiation of bradykinin-induced relaxation in porcine coronary arteries. We next investigated the functional consequences of inhibition of epoxide hydrolases on 14,15-EET-induced relaxation and potentiation of relaxation to bradykinin in porcine coronary arteries. To demonstrate the efficacy of 4-PCO to block conversion of 14,15-[3H]EET to 14,15-[3H]DHET by porcine coronary arteries, arterial rings were treated with vehicle or 4-PCO (20 µmol/l) for 1 h and then incubated with 14,15-[3H]EET for an additional 30 min, after which the incubation buffer was removed and analyzed by HPLC. Rings treated with vehicle formed 1.4 ± 0.4 pmol/g of 14,15-[3H]DHET (Fig. 7A) during the 30-min incubation, which represented a conversion of ~10% of the added 14,15-EET. No detectable 14,15-[3H]DHET was produced by rings treated with 4-PCO (Fig. 7B).

In endothelium-intact rings contracted with U-46619, 5 µmol/l 14,15-EET produced ~55% relaxation (Fig. 8A). Neither the duration (not shown) nor the magnitude of the 14,15-EET-induced relaxation was altered by treatment with 4-PCO. These results confirmed that the vasorelaxant activity of 14,15-EET in porcine coronary arteries is not dependent on its conversion to 14,15-DHET.

Before examining the effects of 4-PCO on 14,15-EET-induced potentiation of relaxation to bradykinin, we determined whether the compound directly affected U-46619-induced tension or relaxation to bradykinin (in the absence of incubation with 14,15-EET). Treatment with 20 µmol/l 4-PCO alone for 1 h did not alter basal or U-46619-induced tension (not shown) or relaxation to bradykinin [EC50 8.22 ± 0.13 (pre-4-PCO) vs. 8.23 ± 0.06 (post-4-PCO)-log[M]; n = 4, P > 0.05].

Pretreatment responses to bradykinin (before application of dimethyl sulfoxide or 4-PCO in dimethyl sulfoxide followed by incubation with 14,15-EET) did not differ between the two groups of rings (EC50 8.37 ± 0.11 vs. 8.32 ± 0.11-log[M]). After treatment with either 14,15-EET (Fig. 8B) or 4-PCO plus 14,15-EET (Fig. 8C), bradykinin-induced relaxation was potentiated [EC50 8.61 ± 0.08 (no 4-PCO) and 8.74 ± 0.03-log[M]
Comparison of posttreatment bradykinin-induced responses [14,15-EET (Fig. 8B) vs. 14,15-EET + 4-PCO (Fig. 8C)] by repeated-measures ANOVA showed that treatment with 4-PCO significantly enhanced the relaxation response (P < 0.05), but an interaction between treatment and dose of bradykinin could not be detected by post hoc Bonferroni t-testing. Analysis of the differences between pre- and posttreatment relaxation responses, however, indicated a significant effect of treatment with 4-PCO that depended on the dose of bradykinin. Post hoc comparisons of the treatment effects demonstrated unadjusted P values of <0.05 at bradykinin doses of 3, 10, and 30 µmol/l. When corrected for multiple comparisons using the Bonferroni method, an effect of treatment with 4-PCO was detected only at 30 µmol/l bradykinin. As was observed in the cultured cell experiments, maximal effects of 4-PCO were observed at concentrations as low as 1 µmol/l (Fig. 8D).
DISCUSSION

In the present study, we found that treatment of coronary endothelial cells with an inhibitor of epoxide hydrolases blocked the conversion of 14,15-EET and 8,9-EET to their corresponding DHETs and enhanced the amount of EET incorporated into cell lipids. Moreover, in coronary arteries, the inhibitor 4-PCO enhanced the 14,15-EET-induced potentiation of relaxation to bradykinin, a phenomenon that we recently showed is related to EET incorporation into cell lipids (26). Taken together, these observations suggest that endothelial epoxide hydrolases may play an important role in regulating EET incorporation into complex lipids, which in turn may modulate endothelial function.

Although 4-PCO increased the amount of 14,15-[^3H]EET and 8,9-[^3H]EET incorporated into PCAEC lipids, it did not alter the distribution of incorporated radioactivity in phospholipids and neutral lipids. Furthermore, the compound did not alter the amount of structurally similar arachidonic acid incorporated into, or released from, PCAEC. These observations suggest that 4-PCO did not directly affect EET uptake or release. Accordingly, our findings suggest that the 4-PCO-induced enhancement in EET incorporation most likely resulted from a reduced conversion of EET to DHET, resulting in an increased availability EET for incorporation into cell lipids.

The largest incorporation of 14,15-EET radioactivity in the PCAEC occurred in PI, followed by PC and PE. This differs from what was observed in murine mast cells, in which PE contained the largest amount of the incorporated radioactivity (1). Most likely, this is caused by either tissue or species differences, because substantial incorporation of 14,15-EET into PI also has been observed in porcine aortic endothelial cells (25).

Substantial effects on 14,15-EET uptake by PCAEC and conversion to DHET were observed at 4-PCO concentrations as low as 0.1–1 µmol/l. Also, the effects of 4-PCO on 14,15-EET-induced potentiation of relaxation to bradykinin appeared maximal at a concentration of 1 µmol/l. No toxicity was observed in either the PCAEC or coronary rings when we added up to 30 µmol/l 4-PCO. Many of the coronary ring studies required prolonged incubations with changes of the medium and repeated washing of the tissue. To ensure that adequate amounts of the inhibitor were present throughout these experiments, a concentration of 20 µmol/l 4-PCO was used routinely in the coronary ring studies and, to permit comparisons, in the PCAEC studies.

Compared with the other EET regioisomers, 14,15-EET was reported to have the highest rate of hydration by cytosol preparations from rat and rabbit liver (5, 22, 31), suggesting that it may be a preferred substrate for cytosolic epoxide hydrolases. Furthermore, Shivachar et al. (22) recently reported that substantially more DHET was formed when rat brain astrocytes were incubated with 14,15-[^3H]EET than with 8,9-[^3H]EET, whereas far more 8,9-[^3H]EET than 14,15-[^3H]EET was incorporated into cell lipids. However, we previously reported (25) that after incubation of porcine aortic endothelial cells with 14,15-[^3H]EET, [^3H]11,12-EET or 8,9-[^3H]EET for 2 h, most of the radioactivity present in the medium was in the form of DHETs, suggesting that the epoxide hydrolases of endothelial cells may not be regioisomeric specific. In the present study, similar results were obtained when PCAEC were incubated with 8,9-[^3H]EET. Perhaps more importantly, inhibition of epoxide hydrolases clearly enhanced the amount of 8,9-[^3H]EET incorporated into PCAEC lipids. Thus epoxide hydrolase-mediated conversion of EETs to DHETs may broadly limit the incorporation of EET regioisomers within the endothelium. Because 4-PCO can inhibit membrane-bound and cytosolic epoxide hydrolases (11, 16), the present results do not necessarily prove where this conversion occurs.

14,15-EET and 14,15-DHET were found previously to produce comparable amounts of relaxation of porcine coronary arteries (26). This observation raised the possibility that 14,15-DHET could mediate 14,15-EET-induced vasorelaxation. However, treatment with 4-PCO, which blocked 14,15-DHET production by PCAEC and porcine coronary arteries, did not reduce the magnitude or duration of 14,15-EET-induced relaxation, suggesting that the vasorelaxation does not require the formation of 14,15-DHET.

In confirmation of our previous observations (26), preincubation of porcine coronary arteries with 14,15-EET potentiated bradykinin-induced relaxation. In the former studies, the potentiation was obviated by triacsin C, suggesting a dependence on incorporation of 14,15-EET into cell lipids (26). In the present study, 4-PCO markedly increased the amount of 14,15-EET incorporated into endothelial cell lipids and modestly enhanced the amount subsequently released from the cells in response to A-23187. Moreover, treatment with 4-PCO augmented the 14,15-EET-induced potentiation of relaxation to bradykinin. Increased incorporation and/or release of EET could potentiate vasorelaxation by acting on either endothelial or smooth muscle cells. For example, EETs have been shown to enhance Ca^{2+} influx into endothelial cells, thereby stimulating production of autacoids that could produce vasorelaxation (10). Once released from endothelial cells, EETs could also interact with smooth muscle cell receptors or G proteins to elicit vasorelaxation (15, 29). Taken together, these findings provide additional support for the contention that incorporated EETs potentiate endothelium-dependent relaxation. Whether potentiation is directly related to the amount of EET esterified to a specific membrane phospholipid or to the amount of EET released from the cells in response to agonist stimulation remains to be determined.

Consistent with our previous reports (25), we found that after a 1-h incubation of PCAEC with 14,15-[^3H]EET in the absence of treatment with 4-PCO, most of the medium-associated radioactivity was in the form of 14,15-[^3H]DHET, whereas ~90% of the cell-associated radioactivity remained as 14,15-[^3H]EET. A very small amount of 14,15-[^3H]DHET was detected in cell...
lipids, along with a larger amount of an unidentified metabolite that eluted from the reverse-phase HPLC column after 14,15-[3H]EET. When the cells were treated with triacsin C to inhibit 14,15-EET incorporation into PCAEC lipids, the conversion of 14,15-[3H]EET to 14,15-[3H]DHET was enhanced. These observations demonstrate that unesterified 14,15-EET probably is the main substrate for the epoxide hydrolases present in endothelial cells. Because the amount of 14,15-[3H]EET incorporated into cell lipids was enhanced when the epoxide hydrolases were inhibited by 4-PCO, it appears that epoxide hydrolases and acyl-CoA synthases compete for the same 14,15-EET substrate so that inhibition of one enzymatic pathway enhances the amount of 14,15-EET available for the other pathway.

Although PCAEC incorporated more than twice as much [3H]arachidonic acid as 14,15-[3H]EET into cell lipids, the amount of [3H]arachidonic acid recovered in the medium after exposure to A-23187 was far less. The reason for this difference is not clear. We considered the possibility that, unlike 14,15-EET, arachidonic acid might be rapidly reincorporated after its release from cell lipids. However, pretreatment with triacsin C did not affect the amount of arachidonic acid present in medium or cell lipids after a 30-min incubation with A-23187 (n = 3, data not shown), suggesting that rapid reincorporation of arachidonic acid did not occur. We also considered whether the arachidonic acid might have been distributed to different classes of intracellular lipids than 14,15-EET. However, analyses of cell lipids after incubations with either 14,15-[3H]EET or [3H]arachidonic acid demonstrated that most of the radioactivity was contained in phospholipids, particularly PI and PC. Although 14,15-EET and arachidonic acid were similarly distributed, the phospholipid pools in which the two fatty acids were incorporated could be located within different intracellular sites, with different accessibility to activated phospholipases. Alternatively, phospholipids to which oxidized fatty acids are esterified may be preferred substrates for phospholipase A2, phospholipase C, or platelet-activating factor acetylhydrolase (18). Further studies are required to investigate these possibilities.

The biological importance of EET incorporation into cell lipids is suggested by recent reports that these eicosanoids are present esterified to phospholipids in platelets and heart, liver, and kidney tissues (3, 13, 30, 32). It is possible that such endogenous EETs may have contributed to the effects produced by 4-PCO in the coronary rings. Furthermore, Karara et al. (14) reported that most endogenous EETs in rat plasma were esterified to phospholipids in circulating lipoproteins; in fact, only 3% was present as the free fatty acid. These observations suggest that, normally, the amount of EET incorporated into cell lipids greatly exceeds the concentration of unesterified EET, as is the case with arachidonic acid (12). In contrast, much less DHETs are incorporated into phospholipids. Our results therefore suggest that by converting EETs to DHETs, endothelial epoxide hydrolases may play an important role in regulating EET incorporation into complex lipids, a function that could modulate the biological effects of EETs within the vasculature.

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