Effect of soluble epoxide hydrolase inhibition on epoxyeicosatrienoic acid metabolism in human blood vessels

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Fang, Xiang, Neal L. Weintraub, Ryan B. McCaw, Shanming Hu, Shawn D. Harmon, James B. Rice, Bruce D. Hammock, and Arthur A. Spector. Effect of soluble epoxide hydrolase inhibition on epoxyeicosatrienoic acid metabolism in human blood vessels. *Am J Physiol Heart Circ Physiol* 287: H2412–H2420, 2004. First published July 29, 2004; doi:10.1152/ajpheart.00527.2004.—We investigated the effects of soluble epoxide hydrolase (sEH) inhibition on epoxyeicosatrienoic acid (EET) metabolism in intact human blood vessels, including the human saphenous vein (HSV), coronary artery (HCA), and aorta (HA). When HSV segments were perfused with 2 μmol/l 14,15-[³H]EET for 4 h, >60% of radioactivity in the perfusion medium was converted to 14,15-dihydroxyeicosatrienoic acid (DHET). Similar results were obtained with endothelium-denuded vessels. 14,15-DHET was released from both the luminal and adventitial surfaces of the HSV. When HSVs were incubated with 14,15-[³H]EET under static (no flow) conditions, formation of 14,15-DHET was detected within 15 min and was inhibited by the selective sEH inhibitors *N,N'*-dicyclohexyl urea and *N*-cyclohexyl-*N'*-dodecanoic acid urea (CUDA). Similarly, CUDA inhibited the conversion of 11,12-[³H]EET to 11,12-DHET by the HSV. sEH inhibition enhanced the uptake of 14,15-[³H]EET and facilitated the formation of 10,11-epoxy-16:2, a β-oxidation product. The HCA and HA converted 14,15-[³H]EET to DHET, and this also was inhibited by CUDA. These findings in intact human blood vessels indicate that conversion to DHET is the predominant pathway for 11,12- and 14,15-EET metabolism and that sEH inhibition can modulate EET metabolism in vascular tissue.

dihydroxyeicosatrienoic acids; cytochrome *P*-450; saphenous vein; β-oxidation

EPOXYEICOSATRIENOIC ACIDS (EETs) are endogenous bioactive lipid mediators synthesized from arachidonic acid by cytochrome *P*-450 epoxygenase (2, 29, 33, 40). EETs potently dilate coronary, renal, internal mammary, intestinal, and cerebral arteries, in part by activating maxi-Ca^{2+}-activated K^+ channels (2, 29, 33, 40), and they have been proposed to act as endothelium-derived hyperpolarizing factors in some vascular beds (1, 15). EETs also modulate smooth muscle, endothelial, and epithelial cell proliferation and have an anti-inflammatory effect in vascular tissue (5, 11, 24). Putative EET receptors (32), epidermal growth factor receptors (4, 19), $G_s\alpha$ proteins (25), and intracellular signaling molecules such as Ca^{2+} (14, 16), protein kinase C, tyrosine kinase (17, 26), and p27^{Kip1} (27) may mediate these cellular processes.

EETs are rapidly taken up by vascular cells and converted either to the corresponding dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH) or to chain-shortened fatty acid epoxides by β-oxidation (7, 8, 10, 13, 33). These metabolic processes likely play an important role in modulating the bioactivity of EETs. Increased excretion of 11,12-DHET and 14,15-DHET has been observed in patients with pregnancy-induced hypertension (3), suggesting a role for epoxide hydrolase in the regulation of blood pressure. Selective inhibition of sEH by *N,N'*-dicyclohexyl urea (DCU) or *N*-cyclohexyl-*N'*-dodecyl urea (CUDA) decreased blood pressure in rodent models of hypertension (18, 39), and sEH gene knockout reduced blood pressure in male mice (31). These observations suggest that sEH inhibition could represent a novel approach for the treatment of hypertension.

Although sEH has been detected in various human cells and tissues (33), recent studies indicate that β-oxidation, rather than sEH, is the primary pathway for EET metabolism in cultured human vascular cells and skin fibroblasts (7, 13). Thus it is necessary to delineate the pathways of EET metabolism in intact human blood vessels to evaluate the potential utility of sEH inhibition in human vascular tissues. In the present study, we examined the metabolism of EETs and tested the effects of a novel sEH inhibitor with enhanced potency and solubility in intact human blood vessels (21, 22). Our findings demonstrate that the main EET metabolic pathway in human blood vessels is conversion to DHET, that selective sEH inhibitors are effective in inhibiting this process, and that EET conversion to β-oxidation products only occurs in intact human vascular tissue when sEH is inhibited.

METHODS

Human Intact Vessel Collection and Cell Culture

Unused human saphenous veins (HSVs) harvested for coronary artery bypass surgery and human aortas (HAs) and coronary arteries (HCAs) removed at the time of heart transplantation surgery were obtained from the operating room at the University of Iowa Hospitals and Clinics according to a protocol approved by the University of Iowa Human Subjects Office (28). Tissues were maintained overnight 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 in a humidified atmosphere containing 5% CO_2 at 37°C. HSV endothelial cells (HSVECs) and smooth muscle cells (HSVSMCs) were isolated from HSVs using the

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method described previously (28). The cells were grown in modified M199 with 10% FBS and maintained until confluent at 37°C in a humidified atmosphere containing 5% CO_2.

Metabolism of 14,15-EET by HSV

Perfusion studies. Freshly collected intact HSVs were placed in M199 supplemented with 10% FBS, examined for leaks, and cut into segments of ≈2 cm in length. Teflon tubes were connected to each end of the vein, and the tubing was passed through a Rabbit Peristaltic Pump (Rainin Instruments, flow rate 0.5 ml/min). After being rinsed thoroughly in PBS, the HSV segments were perfused with M199 containing 0.1 μmol/l BSA. Perfusion medium containing 2 μmol/l 14,15-[³H]EET was pumped from a reservoir through the tubing and vein segments and recycled over the duration of incubation. The vein segment was suspended in an organ bath containing 11 ml M199 supplemented with 0.1 μmol/l BSA. The entire apparatus was placed into a humidified 37°C incubator with 5% CO_2.

In some experiments, the endothelium was denuded by placing a 2-0 silk suture through the lumen of the HSV, followed by rolling the vessel back and forth over the suture. A cotton swab was then inserted into the HSV to remove remaining endothelial cells. The removal of endothelium was confirmed by staining and microscopic examination of the tissue. After incubation, the perfusion and organ bath media were collected, and the tissues were weighed and stored at −20°C for analysis.

Organ bath studies. Vessels were cut into segments of ≈1 cm in length and suspended into individual wells of a 12-well tissue culture plate containing M199 supplemented with 10% FBS. The vessels were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. After the medium was removed and vessels were washed with PBS, the vessels were incubated with either 2 μmol/l 14,15-[³H]EET or 11,12-[³H]EET in M199 supplemented with 0.1 μmol/l BSA for various times in the presence or absence of sEH inhibitors. After incubation, the medium was collected, and the tissues were weighed and stored at −20°C for analysis (8, 20).

Metabolism of 14,15-EET by HSVECs and HSVSMCs

HSVECs and HSVSMCs in individual six-well plates were incubated with 2 μmol/l 14,15-[³H]EET in 1 ml M199 containing 0.1 μmol/l BSA. After incubation, the medium was collected, and the cells were harvested by being scraping into methanol. Lipids in the medium and cells were extracted and analyzed (8, 13).

Assay of Incubation Medium

After the incubation with 14,15-[³H]EET or 11,12-[³H]EET, the medium was extracted twice with 3 volumes of water-saturated ethyl acetate. After the solvent was evaporated under N_2, we dissolved the lipid residue in acetonitrile for separation by reverse-phase HPLC using a Gilson 302 pump plus a 2050 ultraviolet detector and a 4.6 × 250-mm column containing 5-μm spherical particles of EQC C_{18} (Whatman). The elution profile, developed with a Gilson 715 gradient controller, consisted of water adjusted to pH 3.4 with phosphoric acid and an acetonitrile gradient increasing from 25% to 100% over 60 min at a flow rate of 0.7 ml/min. Radioactivity was measured by combining the column effluent with Budget-Solve scintillation solution and passing the mixture through a Radiomatic Flo-One Beta isotope detector or CR Flo-One, as described previously (8, 13).

Analyses of Cell Lipids

After each experiment, human vessel segments were placed on filter paper to absorb liquid and then weighed. The tissues were minced using a scalpel and dissecting scissors. Tissue lipids were extracted and analyzed as described previously (8, 12). Briefly, 6 ml of chloroform-methanol [2:1 (vol/vol)] followed by 2 ml of acidified saline (4 mmol/; HCl in 0.09% NaCl) solution were added to the minced tissue,

and the organic phase was removed after centrifugation. The aqueous phase was washed with chloroform-methanol-acidified saline [86:14:1 (vol/vol/vol)], and the resulting organic layer was combined with the original chloroform extract. The solvent was evaporated under N_2, and the lipids were suspended in chloroform-methanol [2:1 (vol/vol)]. An aliquot of this solution was dried and, after the addition of 5 ml of Budget-Solve scintillation solution, assayed for radioactivity. Some of the tissue lipid extracts were saponified for 1 h at 50°C with 0.5 ml methanolic 0.2 N NaOH containing 10% H_2O. 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 N_2, and the lipids were dissolved in acetonitrile and assayed by reverse-phase HPLC (8).

Detection of sEH Protein in HSVs and Cultured Cells

The sEH protein in HSVs and cultured HSV vascular cells was detected by Western blot analysis using a specific antibody against human sEH protein. Briefly, segments of HSV tissues were washed with cold PBS to remove red blood cells, and homogenates were prepared in a lysis buffer containing 150 mmol/l NaCl, 50 mmol/l Tris (pH 7.5), 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mmol/l diethyldithiocarbamic acid, 1% Nonidet P-40, and 1% sodium deoxycholate using a Tempest homogenizer (Vertis) and a Tekmar sonic disruptor. The cultured cells were sonicated in cold PBS with three 30-s bursts of a Vibra Cell sonicator (Sonics and Materials) at 10% output and 80% duty cycle. The homogenates were centrifuged to remove particulate material, the protein content was measured colorimetrically, and the sEH protein content of the supernatant solution was determined by Western blot analysis (11, 20). Proteins (50 μg) of each sample extracted from the HSV and cultured cells along with purified sEH protein (0.2 μg) were separated by electrophoresis on a 10% polyacrylamide gel at 180 V for 45 min. After proteins were transferred to a nitrocellulose membrane, the membrane was blocked and then incubated with rabbit polyclonal anti-sEH antibody (1:2,500) (37). The blot was then incubated with a horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:4,000). Antibody labeling was detected by enhanced chemiluminescence. The blots were then stripped and reprobed for β-actin with a mouse monoclonal IgG (1:2,500) and anti-mouse IgG-horseradish peroxidase (1:4,000). β-Actin was used as the control for protein loading and transfer.

RESULTS

Metabolism of 14,15-EET by Perfused HSV

HSVs were perfused with 14,15-[³H]EET to investigate its metabolism in intact human blood vessels (Fig. 1). When HSVs were perfused with 2 μmol/l 14,15-[³H]EET for 4 h, 63% of the radioactivity present in the perfusion medium was converted to a radiolabeled metabolite that coeluted with 14,15-DHET, with a 28-min retention time (Fig. 1*A*). An additional unidentified radiolabeled product with a retention time of 42 min accounted for 9% of radioactivity. The formation of 14,15-[³H]DHET was time dependent and was detected as early as 15 min after the HSVs were exposed to 14,15-[³H]EET. In control experiments (i.e., perfusion of 14,15-[³H]EET through tubing without HSVs), all of the radioactivity in the perfusion medium remained as 14,15-EET.

Tissue lipids contained 3.5% of added radioactivity; 54% was present as 14,15-EET and 19% was converted to 14,15-DHET. After a 4-h incubation, about 5% of total added radioactivity was recovered in the organ bath medium. Analysis of the organ bath medium by HPLC demonstrated the presence of a single metabolite, 14,15-[³H]DHET (Fig. 1*B*).
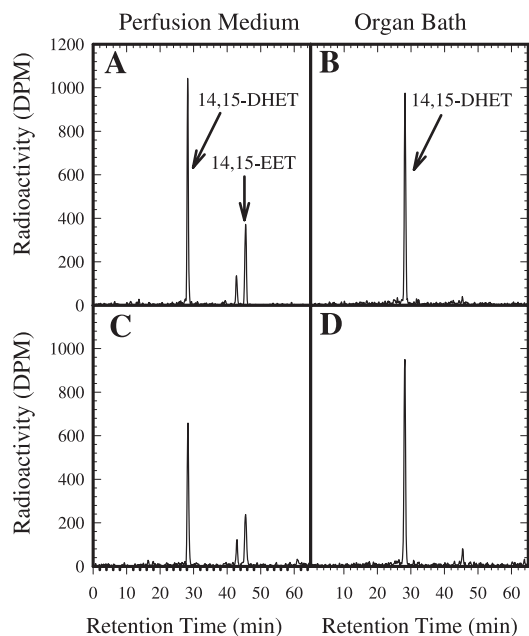
Fig. 1. Metabolism of 14,15-epoxyeicosatrienoic acid (14,15-EET) by perfused human saphrenous veins (HSVs). HSV segments (~2 cm in length) with (*A* and *B*) or without (*C* and *D*) endothelium were perfused with 2 μmol/l 14,15-[³H]EET in medium 199 (M199) containing 0.1 μmol/l BSA. After 4 h of incubation, lipids in the perfusion medium and organ bath were extracted and analyzed by reverse-phase HPLC with an in-line flow scintillation counter. *A* and *C*: samples of the perfusion medium; *B* and *D*: samples of the organ bath. Radiochromatograms obtained with a HSV from a single patient are shown; similar results were obtained using HSVs from two additional patients. 14,15-DHET, 14,15-dihydroxyeicosatrienoic acid; DPM, disintegrations per minute.

Perfusion of 14,15-[³H]EET through HSVs in which the endothelium was denuded resulted in a similar profile of metabolites as was observed with nondenuded vessels. After a 4-h perfusion, 61% of the radioactivity present in the perfusion medium was converted to 14,15-DHET, whereas the unidentified product accounted for ~10% of the total radioactivity (Fig. 1*C*). About 5% of total radioactivity was present in the organ bath medium, almost entirely as 14,15-DHET (Fig. 1*D*).

Effect of sEH Inhibitors on EET Metabolism

We next examined 14,15-[³H]EET metabolism by HSV segments under static (no flow) conditions. The major metabolite, 14,15-[³H]DHET, accounted for 52% of the radioactivity in the medium after a 3-h incubation (Fig. 2, *top left*). The formation of 14,15-[³H]DHET was substantially reduced by CUDA, a selective sEH inhibitor (22). In the presence of 10 μmol/l CUDA, only 14% of the radioactivity in the medium was present as 14,15-DHET (Fig. 2, *middle left*). However, the formation of 14,15-[³H]DHET was not reduced by 10 μmol/l dodecylamine, a microsomal epoxide hydrolase inhibitor (23) (Fig. 2, *bottom left*). Moreover, coincubation with CUDA and dodecylamine did not further reduce 14,15-[³H]DHET formation compared with CUDA alone (not shown). Similarly, when HSV segments were incubated with 11,12-[³H]EET, 11,12-DHET was the major radiolabeled metabolite detected in the medium, and 11,12-DHET formation was inhibited by CUDA but not by dodecylamine (Fig. 2, *right*).

Under static conditions, the formation of 14,15-[³H]DHET was time dependent (Fig. 3*A*). The amount of 14,15-DHET formed normalized to tissue weight varied considerably between HSVs obtained from individual patients as indicated by the large SEs at each time point. CUDA or DCU inhibited 14,15-DHET production over at least a 6-h period (Fig. 3*B*). Taken together, these findings indicate that sEH is the primary enzyme involved in metabolism of 11,12- and 14,15-EET in intact HSVs.

Formation of 10,11-Epoxy-16:2 by HSV in the Presence of sEH Inhibition

When the HSV segments were incubated with 2 μmol/l 14,15-[³H]EET for 6 h, 85% of the total radioactivity in the medium was present as 14,15-DHET (Fig. 4*A*). However, when 10 μmol/l CUDA was added to the incubation with 14,15-[³H]EET, a radiolabeled metabolite with a HPLC retention time of 32 min was detected (Fig. 4*B*). This product coeluted with 10,11-epoxy-16:2, a chain-shortened fatty acid epoxide metabolite of 14,15-EET formed through β-oxidation (8, 13). 10,11-Epoxy-16:2 was detected as early as 4 h when CUDA was added and accounted for 5–6% of total radioactivity remaining in the medium after 6 h of incubation (Fig. 4*C*). Epoxy-16:2 also was detected when DCU was added (Fig.
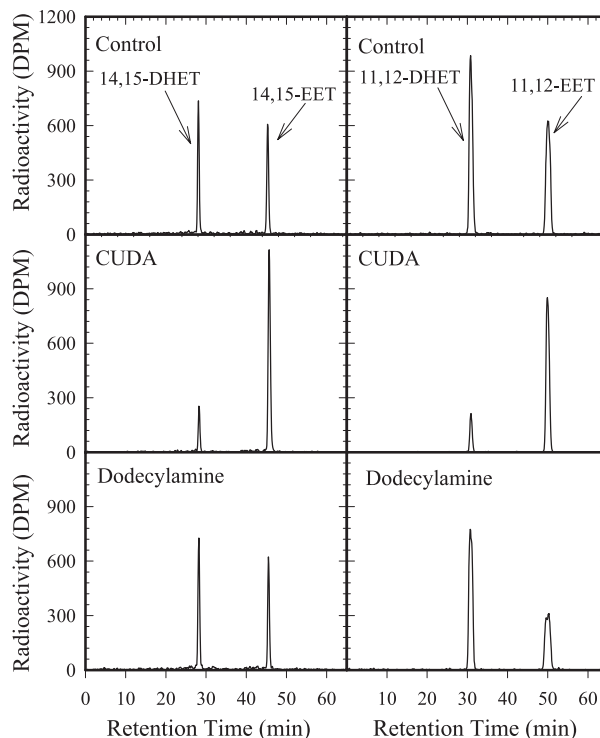

Fig. 2. Effects of *N*-cyclohexyl-*N'*-dodecanoic acid urea (CUDA) and dodecylamine on 11,12- and 14,15-EET metabolism in HSVs. HSV segments were incubated with vehicle (DMSO), 10 μmol/l CUDA, or 10 μmol/l dodecylamine for 45 min. This medium was removed and replaced with medium containing DMSO, CUDA, or dodecylamine, and either 2 μmol/l 14,15-[³H]EET (*left*) or 11,12-[³H]EET (*right*) was added. After incubation for 3 h, the medium was removed, and the lipids were extracted and analyzed for radioactivity by HPLC. Results shown are from segments of a HSV obtained from a single patient; similar results were obtained with a HSV from an additional patient.
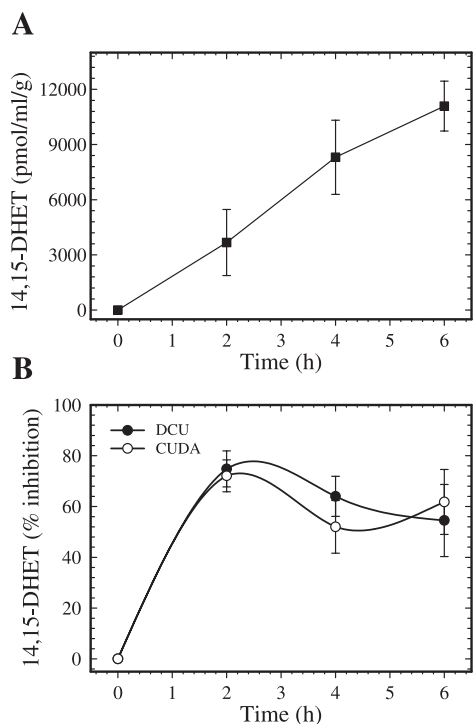
Fig. 3. Time-dependent formation of 14,15-DHET: effect of CUDA and N,N'-dicyclohexyl urea (DCU). HSV segments were incubated with 2 μmol/l 14,15-[^{3}H]EET for various times with or without CUDA or DCU. After incubation, the medium was assayed by HPLC. The picomole values of 14,15-DHET were calculated from the specific activity of 14,15-[^{3}H]EET added to the cultures and are normalized to tissue wet weight. A: time-dependent formation of 14,15-DHET. B: percent inhibition of 14,15-[^{3}H]D-HET production by CUDA and DCU. Results are expressed as means ± SE; $n = 4$ for A and 5 for B.

4C), but no 10,11-[^{3}H]epoxy-16:2 was detected in the absence of sEH inhibitors. Therefore, when sEH activity is inhibited, β-oxidation emerges as an alternative pathway of 14,15-EET metabolism in HSVs.

Tissue Lipid Analysis

Under control conditions, 8.3% of the radioactivity initially added to the incubation was present in HSV lipids after a 6-h control incubation. The addition of 10 μmol/l CUDA or DCU produced a 52% and 49% increase, respectively, in the uptake of radioactivity into the tissue (Fig. 5A). HPLC analysis of the hydrolyzed tissue lipid extract indicated that 73% of the radioactivity was present as 14,15-DHET in the control incubation (Fig. 5B). When CUDA was added, the amount of 14,15-[^{3}H]DHET in the tissue decreased by 50%, and 14,15-EET accounted for the remainder of the tissue-associated radioactivity (Fig. 5C). A similar HPLC result was obtained with DCU (not shown).

Stability of sEH Activity in HSVs

We investigated whether sEH activity was retained when HSV segments were incubated under culture conditions for an extended period of time. HSVs were maintained in M199 containing 10% or 20% FBS in a humidified 5% CO_2 atmosphere at 37°C. The HSVs were then incubated with 14,15-[^{3}H]EET to determine the conversion to 14,15-DHET as an index of sEH activity. Compared with results obtained in freshly collected HSV, conversion of 14,15-[^{3}H]EET to 14,15-DHET was not decreased even when HSVs were maintained in vitro for 4 days, the longest time tested.

Metabolism of 14,15-EET by HCAs and HAs

We next examined 14,15-[^{3}H]EET metabolism in HCAs and HAs. Because of limited supplies of arterial tissues, these
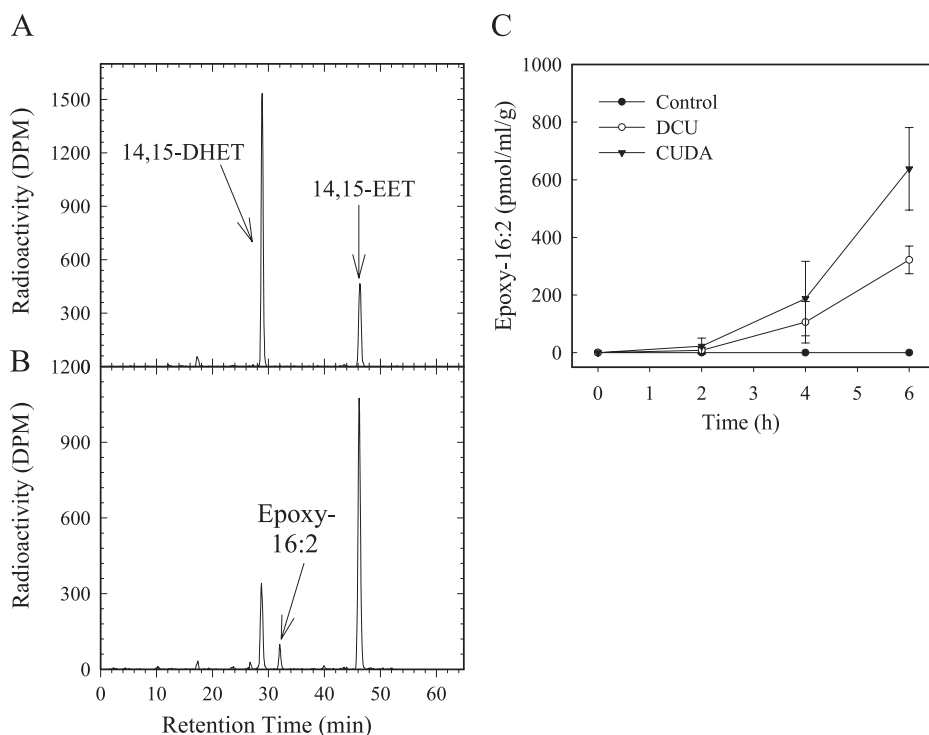

Fig. 4. Formation of epoxy-16:2 by HSVs in the presence of CUDA and DCU. HSV segments were incubated with 2 μmol/l 14,15-[^{3}H]EET for various times with or without CUDA or DCU. After incubation, the medium was assayed by HPLC. A: representative radiochromatogram from 6 h of incubation without any inhibitor; B: radiochromatogram from 6 h of incubation of HSVs treated with 10 μmol/l CUDA. The picomole values of epoxy-16:2 shown in C were calculated from the specific activity of 14,15-[^{3}H]EET added to the cultures and are normalized to tissue wet weight. Results shown in C are expressed as means ± SE; $n = 3$.
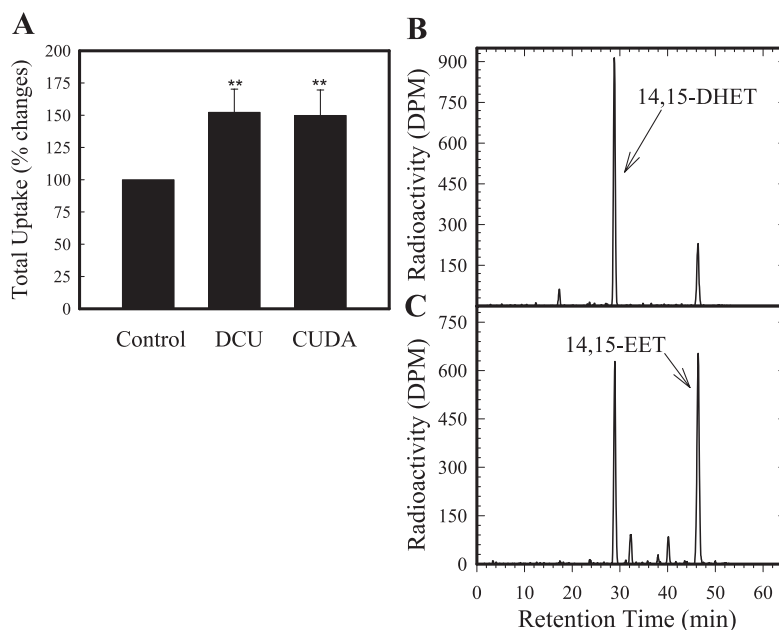
Fig. 5. Effects of CUDA and DCU on the incorporation of 14,15-EET into HSVs. HSV segments were incubated with 2 μmol/l 14,15-[³H]EET in the absence or presence of 10 μmol/l CUDA or DCU, as described in Fig. 3. After 6 h, the medium was removed, and the tissue was washed and homogenized. *A*: total uptake of radioactivity in tissue lipids. The picomole values were calculated from the specific activity of 14,15-[³H]EET added to the cultures and are normalized to tissue wet weight, and percent changes in total uptake were calculated. Results are expressed as means ± SE; $n = 5$. **$P < 0.01$ vs. control. Additional aliquots of the tissue lipid extract were hydrolyzed by saponification and analyzed for radioactivity by HPLC. *B*: representative radiochromatogram of the tissue lipid extract from a control incubation. *C*: radiochromatogram from an incubation with CUDA.

studies were performed only under static conditions. As observed with HSVs, 14,15-DHET was the major radiolabeled metabolite when HCAs or HAs were incubated with 14,15-[³H]EET. After 6 h of incubation with HCAs, 96% of the radioactivity present in the medium was converted to 14,15-DHET, whereas <4% remained as 14,15-EET (Fig. 6*A*). In the presence of 10 μmol/l CUDA, only 30% of the radioactivity present in the medium was converted to 14,15-DHET, whereas >65% remained as 14,15-EET (Fig. 6*B*). The formation of 14,15-DHET was time dependent when HCAs were incubated with 2 μmol/l 14,15-[³H]EET, and 10 μmol/l CUDA inhibited the formation of 14,15-DHET over at least a 9-h period (Fig. 6*C*). Under control conditions, ~7% of the total radioactivity was present in HCA lipids after a 9-h incubation. HPLC analysis of the hydrolyzed tissue lipid extract demonstrated a single radiolabeled product that coeluted with 14,15-DHET. In the presence of 10 μM CUDA, the amount of 14,15-DHET

present in tissues was reduced by 50%, and 14,15-EET accounted for the remainder of the tissue-associated radioactivity. Analysis of tissue lipids indicated that CUDA also enhanced the total uptake of radiolabeled material [3,850 (control) vs. 4,582 pmol/g tissue (CUDA)].

Similar to HCA, HA rapidly converted 14,15-[³H]EET to 14,15-DHET. After a 6-h incubation, 93% of the radioactivity present in the medium was converted to 14,15-DHET (Fig. 7*A*). Inhibition of 14,15-[³H]DHET formation by 10 μM CUDA was time dependent, and only 20% of the radioactivity in the medium was in the form of 14,15-DHET (Fig. 7, *B* and *C*). In addition, a small amount of 10,11-[³H]epoxy-16:2 accumulated in the medium during a 9-h incubation when CUDA was added (Fig. 7*D*). Analysis of the HA tissue lipids indicated that CUDA also enhanced the total uptake of radiolabeled material [2,449 (control) vs. 5,174 pmol/g tissue (CUDA)].
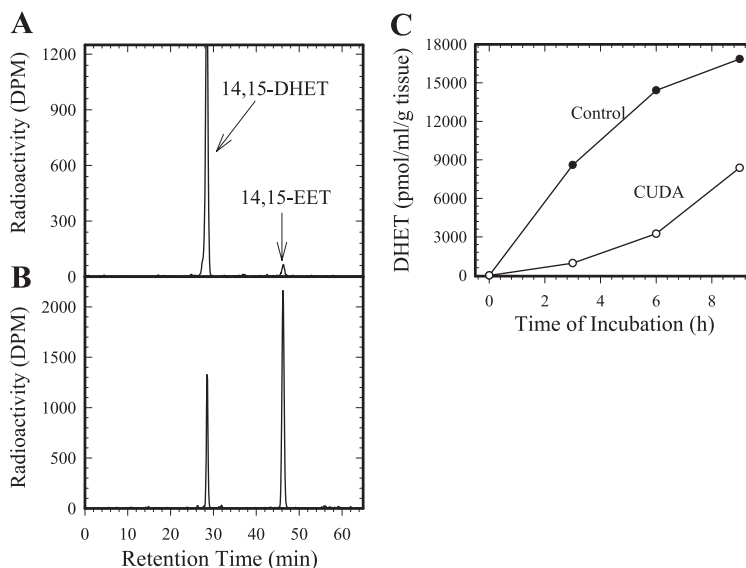

Fig. 6. Metabolism of 14,15-EET by intact human coronary arteries (HCAs): effect of CUDA. HCA segments were incubated with vehicle or 10 μmol/l CUDA for 45 min. This medium was removed and replaced with fresh medium containing vehicle (DMSO; *A*) or 10 μmol/l CUDA (*B*) along with 2 μmol/l 14,15-[³H]EET for 6 h. In a separate experiment, HCA segments were incubated with 10 μmol/l CUDA along with 2 μmol/l 14,15-[³H]EET for up to 9 h, and the time dependence of 14,15-DHET formation with or without CUDA is shown in *C*. At the indicated times, the medium was removed, and lipids were extracted and analyzed for radioactivity by HPLC. The picomole values were calculated from the specific activity of 14,15-[³H]EET added to the cultures and are normalized to tissue wet weight. Each point is the average of results obtained from two HCA segments from one patient.
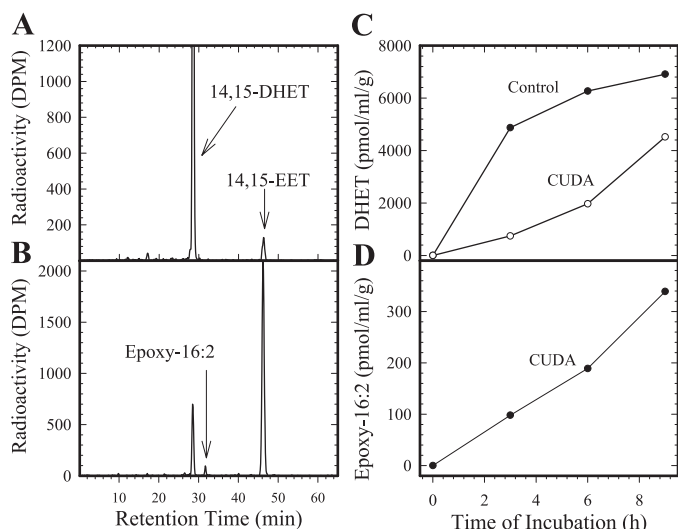
Fig. 7. Metabolism of 14,15-EET by intact human aortas (HAs): effect of CUDA. HA segments were incubated with vehicle (DMSO) or 10 μmol/l CUDA for 45 min. This medium was removed and replaced with fresh medium containing DMSO (*A*) or 10 μmol/l CUDA (*B*) along with 2 μmol/l 14,15-[³H]EET for 6 h. In a separate experiment, HA segments were incubated with 10 μmol/l CUDA and 2 μmol/l 14,15-[³H]EET for up to 9 h. *C*: time dependence of DHET formation with and without CUDA. *D*: time dependence of 10,11-epoxy-16:2 formation in the presence of CUDA. At the indicated times, the medium was removed, and lipids were extracted and analyzed for radioactivity by HPLC. The picomole values were calculated from the specific activity of 14,15-[³H]EET added to the cultures and are normalized to tissue wet weight. Each point is the average of results obtained from two HA segments from one patient; similar results were obtained from HA segments from another patient.

Metabolism of 14,15-EET by Cultured HSVECs and HSVSMCs

The results obtained in intact human blood vessels were different from those we observed previously with cultured human cells, where β-oxidation was found to play a major role in 14,15-EET metabolism (13). This suggested that the metabolism of 14,15-EET might be altered when vascular cells are removed from their native state and grown in culture. To test this possibility, HSVECs and HSVSMCs were cultured from HSV segments and incubated with 14,15-[³H]EET. When HSVECs were incubated with 2 μmol/l 14,15-[³H]EET for 3 h, only 12% of the total radioactivity in the medium was converted to 14,15-DHET (Fig. 8*A*), whereas the β-oxidation product 10,11-epoxy-16:2 accounted for 29%. A radiolabeled metabolite with a retention time of 39 min that coeluted with another β-oxidation product, 12,13-epoxy-18:2, also was detected, accounting for 7% of the total radioactivity in the medium (Fig. 8*A*). Likewise, HSVSMCs formed substantial amounts of radiolabeled chain-shortened epoxy fatty acids in the absence of sEH inhibition (Fig. 8*B*). These metabolites accumulated in the medium in a time-dependent manner (Fig. 8, *C* and *D*). CUDA inhibited the formation of 14,15-DHET when the HSVECs and HSVSMCs were incubated with 14,15-[³H]EET. After 6 h of incubation, 10 μM CUDA decreased 14,15-DHET formation by 61% and 67%, respectively, in HSVECs and HSVSMCs.

Detection of sEH Protein in HSVs, HSVECs, and HSVSMCs

We next determined whether there was any difference in expression of sEH protein between the intact vascular segments and cultured human vascular cells. A substantial amount of sEH protein was detected in HSV segments using a specific antibody against human sEH protein (Fig. 9*A*). However, a relatively small amount of sEH protein was detected in cultured HSVECs and HSVSMCs (Fig. 9*B*).

DISCUSSION

In the present study, we found that *1*) conversion to 14,15-DHET by sEH is the predominant pathway of 14,15-EET metabolism in the intact HSV, HCA, and HA; *2*) endothelial denudation did not affect the conversion of 14,15-EET to 14,15-DHET in the HSV; *3*) 14,15-DHET formed from 14,15-EET in the perfused HSV was released from both the luminal and adventitial surfaces of the blood vessel; *4*) both 14,15-EET and 14,15-DHET accumulated in the tissue when the HSV was
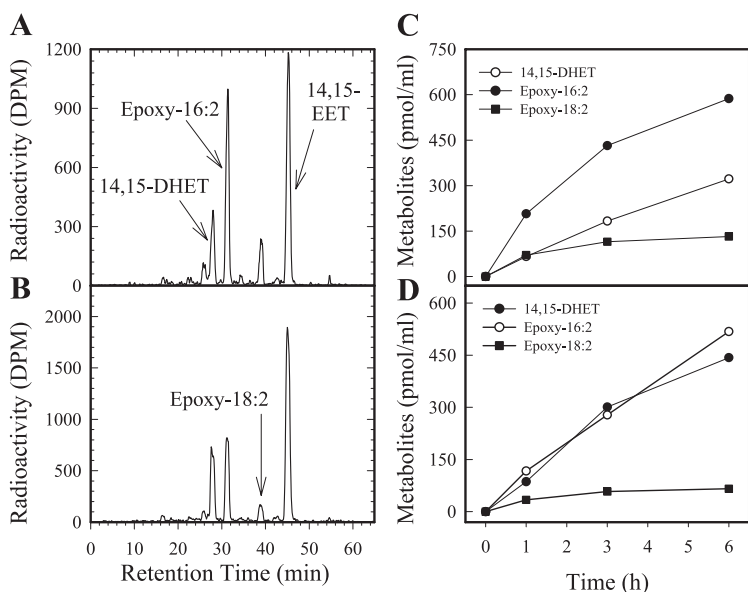

Fig. 8. Formation of 14,15-[³H]EET metabolites by HSV endothelial cells (ECs) and smooth muscle cells (SMCs). ECs (*A*) or SMCs (*B*) cultured from HSVs were incubated with 2 μmol/l 14,15-[³H]EET in M199 containing 0.1 μmol/l BSA for various times. After incubation, the medium was removed, and lipids were extracted and analyzed for radiolabeled metabolites as described in Fig. 1. *A* and *B*: radiochromatograms from 3-h incubations of ECs (*A*) and SMCs (*B*). *C* and *D*: time-dependent formation of 14,15-EET metabolites of ECs (*C*) and SMCs (*D*). The picomole values were calculated from the specific activity of 14,15-[³H]EET added to the cultures. Each point is the average of results obtained from two separate cultures, and both values agreed within 10%. Expoxy-16:2, 10,11-epoxy-16:2; Epoxy 18:2, 12,13-epoxy-18:2.
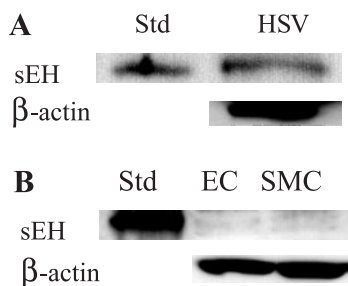
Fig. 9. The presence of soluble epoxide hydrolase (sEH) protein in HSVs and cultured HSV ECs and SMCs. Protein was extracted from HSVs (*A*) and HSV ECs and SMCs (*B*). Equal amounts of protein (50 µg) were loaded for all samples, and purified sEH protein (0.2 µg) was used as a standard (Std) for each membrane. The presence of sEH protein was detected by Western blot analysis. β-Actin was used as the control for protein loading and transfer.

either perfused or incubated with 14,15-EET; *5*) inhibition of sEH blocked the conversion of 14,15-EET to 14,15-DHET, enhanced tissue uptake of 14,15-EET, and increased the formation of chain-shortened epoxide fatty acids by the HSV; and *6*) in contrast to intact human blood vessels, cultured human vascular cells converted substantial amounts of 14,15-EET to chain-shortened epoxy fatty acids even in the absence of sEH inhibition, suggesting that phenotypic changes associated with cell culture lead to alterations in EET metabolism in human cells.

sEH is a cytosolic enzyme that is widely distributed in mammalian tissues, and high levels are present in the liver, kidney, and intestinal tissues (33). sEH activity also has been detected in cultured porcine and bovine arterial endothelial and smooth muscle cells, where it plays a major role in metabolizing EETs by converting them to the corresponding DHETs (8–10). Because EETs are potent vasodilators and potentiate endothelium-dependent dilation to bradykinin, conversion of EETs to DHETs by sEH may diminish the vasodilator potential of EETs (35, 36). In this regard, pharmacological inhibition of sEH decreases blood pressure in the spontaneously hypertensive rat (39), and it attenuates the pressor response elicited by infusion of angiotension II (18). Moreover, male sEH knockout mice have reduced blood pressure compared with their wild-type counterparts (31). These observations in rodent models and in cultured bovine and porcine vascular cells suggest that inhibition of sEH could represent a novel approach for the treatment of hypertension.

On the other hand, studies in cultured human coronary endothelial and smooth muscle cells and in human skin fibroblasts have suggested that β-oxidation rather than sEH might play a predominant role in metabolizing EETs in the human vasculature (7, 13). If so, the effectiveness of sEH inhibitors in humans might be diminished. Therefore, the present study was undertaken to determine the relative contributions of DHET formation and β-oxidation to EET metabolism in intact human vascular tissue. Our findings indicate that intact human veins and arteries contain substantial sEH activity and that it is primarily responsible for EET metabolism in blood vessels.

When the intact human vein and arterial segments were incubated with 14,15-[³H]EET, 14,15-DHET was the only predominant product that accumulated in the medium. Although 14,15-[³H]DHET formation was linear for at least 6 h in the HSV and HCA incubation, it deviated from linearity

after 3 h in incubations with HA segments. Therefore, the rates of DHET formation were compared in the three vascular preparations in 3-h incubations. The calculated values were 6,200 pmol·ml medium^{-1}·g tissue wet wt^{-1} for the HSV, 8,500 pmol·ml medium^{-1}·g tissue wet wt^{-1} for the HCA, and 4,870 pmol·ml medium^{-1}·g tissue wet wt^{-1} for the HA. These differences might be due to intrinsic variability in the three types of blood vessels. However, the preoperative treatment and procurement of the tissue varied to some extent in each case depending on the surgical procedure. As indicated by the large SEs in Fig. 3*A*, some variability was observed in the values obtained for the different HSV segments. Furthermore, the HCA and HA segments were obtained from diseased hearts during transplant surgery. Although the incubated vascular segments did not appear to be diseased, they most likely were affected to some extent by the clinical condition. Our results suggest that these factors did not have a major influence on the ability of these blood vessels to convert 14,15-EET to DHET. However, they may have had enough of an effect to produce the quantitative differences that were observed in the three types of vascular segments. Furthermore, this variation of sEH activity could be related to genetic polymorphisms of sEH that have been observed in humans (30).

Endothelial denudation of the HSV did not affect the conversion of 14,15-[³H]EET to 14,15-DHET, suggesting that vascular smooth muscle cells play an important role in metabolism of EETs. We previously reported that cultured porcine smooth muscle cells have a substantial ability to convert EETs to DHETs (9, 12). Consistent with this observation, significant amounts of sEH protein were detected in human vascular smooth muscle by immunohistochemistry (6, 38). Although substantial sEH activity also has been observed in cultured endothelial cells (8, 33–36), the mass of smooth muscle is greater than that of the endothelium in an intact vascular segment, and this may account for the fact that endothelial denudation did not appreciably reduce 14,15-[³H]DHET production.

There are several possible explanations for the differences in 14,15-EET metabolism in cultured human cells versus intact blood vessels. First, our data suggest that the switch from epoxide hydrolysis in intact blood vessels to β-oxidation in cultured cells is most likely not an effect of the reagents in the culture medium because HSV maintained in this medium for up to 4 days retained sEH activity. Thus phenotypic changes in human cells resulting from growth in culture are more likely to account for the difference. The Western blot analysis indicated that the expression of sEH protein is decreased in cultured HSVECs and HSVSMCs (Fig. 9), which is similar to our previous observation with cultured human coronary vascular cells (13). Indeed, sEH activity was found to be only 16 pmol·min^{-1}·mg protein^{-1} in cultured human coronary endothelial cells compared with 520 pmol·min^{-1}·mg protein^{-1} in cultured porcine coronary endothelial cells (13). These observations suggest that specific conditions or cofactors may be required for expression of sEH protein or maintaining sEH activity in human cells during culture. Alternatively, the β-oxidation activity of human cells might be upregulated by growth in culture. In support of this possibility, we detected a high level of acyl-CoA oxidase mRNA expression in cultured human coronary endothelial cells (unpublished observations).

As observed previously in cultured cells from several species (8, 10), 14,15-EET and 14,15-DHET were incorporated into human blood vessels. Moreover, in HSVs incubated with 14,15-[^{3}H]EET, sEH inhibition significantly enhanced the incorporation of radiolabeled EET into tissue lipids. This suggests that enhanced accumulation of EETs in blood vessels may contribute to the hypotensive effects of sEH inhibitors. In HSVs perfused with 14,15-EET in the absence of sEH inhibitors, the amount of 14,15-DHET incorporated into tissue lipids was very small compared with the amount that was released into the medium. Interestingly, although most of the 14,15-DHET was released into the luminal perfusion medium, some also was recovered in the organ bath medium. It is unlikely that the 14,15-DHET simply leaked into the organ chamber from the blood vessel because the HSV segments were carefully tested for leaks before experimentation and then tightly secured onto the tubing. Moreover, a leak also should have resulted in recovery of 14,15-EET in the organ bath medium. These results suggest that the transport of DHETs in intact blood vessels could be flow regulated and that the release of DHET from the adventitial surface of blood vessels in vivo could potentially modulate the function of cells in the interstitial space.

Urea derivatives, including DCU and CUDA, represent a new class of potent, selective and stable sEH inhibitors (21, 22). DCU is poorly soluble in water, and this may limit its potential therapeutic usefulness. On the other hand, CUDA, in which one of the cyclohexyl groups of DCU is replaced with a carboxylic acid chain, is more water soluble and at least as potent as DCU (22). CUDA inhibited the conversion of 14,15-EET to DHET by HSVs, HCAs, and HAs in a time-dependent manner, and it also inhibited the conversion of 11,12-EET to DHET in HSVs. CUDA also enhanced uptake of 14,15-EET by these human vessels. Considering that the conversion of small amount of 14,15-EET to 14,15-DHET was detected as early as 15 min in HSV, these results suggest that CUDA is readily taken up by intact human blood vessels and has a rapid onset of action. CUDA also appears to have a long duration of action because 14,15-DHET formation was effectively inhibited for at least 6 h. These pharmacokinetic characteristics suggest that CUDA could be useful for therapeutic application in humans.

EETs are vasodilators in various vascular beds (2, 29, 33, 40), and we have shown previously that 4-phenylchalcone, a sEH inhibitor, potentiates EET-mediated vasorelaxation in porcine coronary artery rings (36). Because of the limited amounts of human vascular tissue that was available, however, vasoactive responses could not be tested in the present study.

EETs are converted to chain-shortened β-oxidation products when sEH is inhibited in cultured porcine coronary endothelial cells (8). The present results show that intact HSV also have the ability to convert small amounts of 14,15-EETs to β-oxidation products, particularly 10,11-epoxy-16:2, but only when sEH is inhibited. However, the formation of chain shortened fatty acid in intact human rings is a slow process, and little epoxy-16:2 was detected until 4 h after the sEH inhibitors were added to the incubations (Fig. 4C). Like 14,15-EET, 10,11-epoxy-16:2 has been shown to dilate porcine and human coronary microvessels and to inhibit tumor necrosis factor-α-induced interleukin-8 production in human endothelial cells (13). Therefore, these chain-shortened epoxy fatty acids could

potentially contribute to the effects of sEH inhibition in human blood vessels.

In summary, these findings indicate that sEH-mediated conversion to DHET is the predominant pathway for 11,12- and 14,15-EET metabolism in intact human blood vessels. CUDA, a potent selective sEH inhibitor, decreased DHET formation and increased EET accumulation in the vascular tissue. Although β-oxidation plays a prominent role in 14,15-EET metabolism in cultured human vascular cells (13), this enzymatic pathway emerges only when sEH is inhibited in intact human blood vessels. These findings support the possibility that sEH inhibition might directly affect EET metabolism and retention in human blood vessels.

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