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Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BK_{Ca} channels: implications for soluble epoxide hydrolase inhibition

Brandon T. Larsen,[1,3] Hiroto Miura,[2,3] Ossama A. Hatoum,[2,3] William B. Campbell,[1] Bruce D. Hammock,[5] Darryl C. Zeldin,[6] John R. Falck,[7] and David D. Gutterman[1,2,3,4]

Departments of [1]Pharmacology and Toxicology, [2]Medicine, and the [3]Cardiovascular Center, Medical College of Wisconsin and [4]Veterans Affairs Medical Center, Milwaukee, Wisconsin; [5]Department of Entomology and Cancer Research Center, University of California, Davis, California; [6]Division of Intramural Research, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina; and [7]Departments of Biochemistry and Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas

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Larsen, Brandon T., Hiroto Miura, Ossama A. Hatoum, William B. Campbell, Bruce D. Hammock, Darryl C. Zeldin, John R. Falck, and David D. Gutterman. Epoxyeicosatrienoic and dihydroxyeicosatrienoic acids dilate human coronary arterioles via BK_{Ca} channels: implications for soluble epoxide hydrolase inhibition. *Am J Physiol Heart Circ Physiol* 290: H491–H499, 2006. First published October 28, 2005; doi:10.1152/ajpheart.00927.2005.—Epoxyeicosatrienoic acids (EETs) are metabolized by soluble epoxide hydrolase (sEH) to form dihydroxyeicosatrienoic acids (DHETs) and are putative endothelium-derived hyperpolarizing factors (EDHFs). EDHFs modulate microvascular tone; however, the chemical identity of EDHF in the human coronary microcirculation is not known. We examined the capacity of EETs, DHETs, and sEH inhibition to affect vasomotor tone in isolated human coronary arterioles (HCAs). HCAs from right atrial appendages were prepared for videomicroscopy and immunohistochemistry. In vessels preconstricted with endothelin-1, three EET regioisomers (8,9-, 11,12-, and 14,15-EET) each induced a concentration-dependent dilation that was sensitive to blockade of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels by iberiotoxin. EET-induced dilation was not altered by endothelial denudation. 8,9-, 11,12-, and 14,15-DHET also dilated HCA via activation of BK_{Ca} channels. Dilation was less with 8,9- and 14,15-DHET but was similar with 11,12-DHET, compared with the corresponding EETs. Immunohistochemistry revealed prominent expression of cytochrome *P*-450 (CYP450) 2C8, 2C9, and 2J2, enzymes that may produce EETs, as well as sEH, in HCA. Inhibition of sEH by 1-cyclohexyl-3-dodecylurea (CDU) enhanced dilation caused by 14,15-EET but reduced dilation observed with 11,12-EET. DHET production from exogenous EETs was reduced in vessels pretreated with CDU compared with control, as measured by liquid chromatography electrospray-ionization mass spectrometry. In conclusion, EETs and DHETs dilate HCA by activating BK_{Ca} channels, supporting a role for EETs/DHETs as EDHFs in the human heart. CYP450s and sEH may be endogenous sources of these compounds, and sEH inhibition has the potential to alter myocardial perfusion, depending on which EETs are produced endogenously.

endothelium-derived hyperpolarizing factor; endothelium; cytochrome *P*-450 epoxygenase; soluble epoxide hydrolase

EPOXYEICOSATRIENOIC ACIDS (EETs) are endogenous lipid mediators of vascular function that are derived through metabolism of arachidonic acid by cytochrome *P*-450 (CYP450) epoxygenase enzymes. EETs are potent vasodilators that have been proposed to function as an endothelium-derived hyperpolarizing factor (EDHF) in some tissues. EDHF modulates vasomotor tone when nitric oxide (NO)-mediated vasodilation is impaired, particularly in resistance vessels (32, 33, 48), and thus may regulate local tissue perfusion in cardiovascular disease states. Despite more than a decade of investigation, the chemical identity of EDHF remains controversial (6) and has been relatively unstudied in the human coronary circulation.

Hydrolysis of the epoxide group of EET regioisomers by soluble epoxide hydrolase (sEH) leads to the formation of corresponding vicinal diols, the dihydroxyeicosatrienoic acids (DHETs). EETs are generally more biologically active than their corresponding DHET metabolites and may function not only as vasodilators but also as vascular protective agents (42, 45). Indeed, EETs inhibit vascular smooth muscle migration (46), decrease inflammation (38), and inhibit platelet aggregation (15, 25), each an important component of atherogenesis. Pharmacological inhibition of sEH has been the focus of intense investigation as a potential approach to enhance EET-mediated vascular protection, and several compounds have appeared promising in recent animal studies (23, 36, 53) and in isolated human conduit arteries (13). However, the utility of sEH inhibition to enhance EET activity in the human coronary microcirculation has previously not been evaluated.

The present study investigates the role of EETs in the coronary microcirculation of patients with heart disease, where EDHF-mediated vasodilatory mechanisms are prominent (32,

Address for reprint requests and other correspondence: D. D. Gutterman, Northwestern Mutual Professor of Medicine, Senior Associate Dean for Research, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226 (e-mail: dgutt@mcw.edu).

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33). Specifically, we examined *1*) whether EETs dilate human coronary arterioles (HCA) via large-conductance calcium-activated potassium (BK_{Ca}) channels, *2*) whether CYP450 epoxygenases are expressed in the endothelium of HCA, and *3*) whether EET-induced vasodilation of HCA may be enhanced through inhibition of sEH.

MATERIALS AND METHODS

Tissue acquisition. All protocols were approved by the appropriate Institutional Review Boards on the use of human subjects in research at the Medical College of Wisconsin, Froedtert Memorial Lutheran Hospital, St. Joseph's Regional Medical Center, St. Luke's Medical Center, St. Mary's Hospital, and Sinai Samaritan Medical Center (all in Milwaukee, WI). Fresh human right atrial appendages were obtained as discarded surgical specimens from patients undergoing cardiopulmonary bypass procedures, as reported previously (29–34, 47). Each board determined that the project was classified as exempt; therefore, written informed consent was not required to use the discarded tissues.

Measurement of HCA dilation by videomicroscopy. Internal diameter measurement was performed on isolated, pressurized HCA by videomicroscopy as reported previously (29–34, 47). Briefly, isolated HCA were cannulated at both ends by glass micropipettes and secured in an organ chamber containing a physiological saline solution [PSS, consisting of (in mol/l) 123 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 16 $NaHCO_3$, 0.026 Na_2ETDA, 1.2 KH_2PO_4, and 11 glucose]. The micropipettes were connected to a single pressure reservoir containing PSS to ensure that no pressure gradient existed and that no intraluminal flow developed. The preparation was transferred to the stage of an inverted microscope (magnification ×200, Olympus CK2) coupled to a video camera (WV-BL200, Panasonic), a video monitor (Panasonic), and a calibrated videomicrometer (VIA-100K, Boeckeler Instruments). The vessels were then incubated at 20 mmHg for 30 min (37°C, pH = 7.40 ± 0.05, P_{O_2} = 140 ± 10 mmHg). Pressure was then slowly increased to 60 mmHg, and the vessels were incubated at 60 mmHg for an additional 30-min period.

After the initial equilibration period, vessels were constricted with KCl (55.9 mmol/l final concentration) to assess viability. Vessels that constricted more than 30% of the internal diameter observed at the time of pressurization to 60 mmHg were used for subsequent experiments (29–34). After washing with fresh buffer, endothelin-1 (ET-1, 5×10^{-10} M to 10^{-9} M) was added to constrict the vessel by 30–50% of its diameter observed at the time of pressurization to 60 mmHg. Internal diameter readings (0.4-μm resolution) were taken 3 min after each ET-1 application until the desired diameter was obtained and constriction was stable. Cumulative concentrations of EET or DHET (10^{-9} M to 10^{-5} M) were then added to the bath, and steady-state diameter readings were taken 3 min after each application. The tissue bath was then washed with fresh buffer, 10–15 min was allowed for equilibration, and iberiotoxin [IBTX, 10^{-7} mol/l, a specific inhibitor of BK_{Ca} channels (2)], 1-cyclohexyl-3-dodecylurea [CDU, 10^{-6} mol/l, an inhibitor of sEH (9, 35)], or vehicle was added. After incubation with inhibitor for 30 min, vessels were again constricted with ET-1 in the same manner, followed by a second application of the same EET or DHET concentrations. At the end of each experiment, papaverine (10^{-4} mol/l, an endothelium-independent vasodilator) was added to determine the maximal internal diameter for normalization of dilator responses. All drugs were added directly to the bath, and the change in bath volume over the course of experiments was <1%.

Endothelial denudation. To assess the role of the endothelium in the vasodilatory response to EET, in some vessels the endothelium was mechanically denuded by passing air through the lumen (32–34). Absence of dilation to ADP (10^{-4} mol/l, an endothelium-dependent vasodilator) was used to confirm adequacy of denudation (29), and full dilation to papaverine (10^{-4} mol/l) was used to confirm that the vascular smooth muscle cells (VSMCs) remained functionally intact following denudation.

Immunohistochemistry. Immunohistochemistry was performed to visualize CYP450 epoxygenase and sEH expression in human atrial tissue as described previously (34). Briefly, small pieces of pectinate muscle were fixed with 4% paraformaldehyde in phosphate-buffered saline, infiltrated with 20% sucrose-HEPES buffer solution, and snap-frozen in Tissue-Tek OCT compound (Ted Pella, Redding, CA). Sections (5 μm thickness) were immunolabeled with rabbit anti-human polyclonal antibodies against CYP2C8, 2C9, 2J2, or sEH (1:50, 1:1,000, 1:1,000, and 1:500 dilution, respectively). Immunostains were visualized by using an avidin-biotin horseradish peroxidase visualization system (Vectastain Universal Quick Kit, Vector Laboratories). Additional experiments were performed on sections in which the primary antibody was omitted as a control for nonspecific binding (34).

Measurement of EETs and DHETs by mass spectrometry. Isolated vessels from a single tissue sample were pooled in HEPES buffer [consisting of (in mol/l) 138 NaCl, 4 KCl, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 0.026 Na_2ETDA, 1.2 KH_2PO_4, 10 HEPES, and 6 glucose] and incubated in the absence or presence of CDU (10^{-6} mol/l) for 30 min at 37°C. 11,12- and 14,15-EET were added (10^{-7} mol/l final concentration each), and the vessels were incubated for 4 h at 37°C. Vessels were then removed, and EET regioisomers and their respective DHETs were quantified by using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), as described previously (37). Briefly, [$^2H_8$]EETs and [$^2H_8$]DHETs were added to the supernatant as internal standards, and fatty acids were immediately isolated by using solid-phase C_{18} Bond Elut SPE columns. The samples were dried under a stream of nitrogen gas, redissolved in 20 μl of acetonitrile, and analyzed by LC-ESI-MS (Agilent 1100 LC/MSD, Sl model). Detection was made in the negative ion mode. Mass spectra of the fatty acids revealed the $[M-1]^-$ as the major ion. The $[M-1]^-$ ions for DHETs and [$^2H_8$]DHETs [mass/charge (*m/z*) = 337 and 345] were monitored from 0 to 20 min, and $[M-1]^-$ ions for EETs and [$^2H_8$]EETs (*m/z* = 319 and 327) were monitored from 20 to 40 min. The regioisomeric DHETs and EETs resolved and eluted between 11–15 and 26–31 min, respectively. The ratios of the peak areas of the metabolites and their respective standards were used for quantification.

Materials and antibodies. Iberiotoxin was obtained from Alomone Labs. EET and DHET regioisomers were obtained from J. R. Falck (Univ. of Texas Southwestern, Dallas, TX). EET and DHET were dissolved in ethanol and further diluted in PSS. The final concentration of ethanol in the bath was 0.1%. CDU was obtained from B. D. Hammock (Univ. of California, Davis, CA). CDU was dissolved in DMSO and further diluted in PSS. The final concentration of DMSO in the bath was 0.05%. Vehicle control studies indicated that the final concentration of ethanol or DMSO had no effect on basal tone or function of arterioles. All other chemical reagents were obtained from Sigma Chemical. Rabbit anti-human polyclonal antibodies specific for CYP2C8 and CYP2C9 and rabbit preimmune serum were obtained from Serotec (Raleigh, NC). Rabbit anti-human polyclonal antibody selective for CYP2J2 (49) was a kind gift from D. C. Zeldin (NIH, Research Triangle Park, NC). Rabbit anti-human polyclonal antibody selective for sEH (13) was a kind gift from C. Morisseau (Univ. of California, Davis, CA).

Statistical analysis. Percent dilation was calculated as the percent change from the preconstricted diameter to the maximal diameter, which was generally the diameter following application of papaverine (10^{-4} mol/l). Values are represented as means ± SE. To compare concentration-response relationships between treatment groups, a two-factor repeated-measures ANOVA was used. When a significant difference was observed between concentration-response curves ($P < 0.05$), specific effect slices were done comparing individual concentrations between two treatment groups using a Holm-Sidak multiple comparison test. Multiple stepwise regression analyses were used to detect the influence of underlying diseases, age, and gender on vasodilation at various concentrations (34). Mass spectrometry data are expressed as means ± SE, and the significance of differences between the means was evaluated by using a paired Student's *t*-test. Significance was accepted at $P < 0.05$; *n* = number of patients.

RESULTS

Right atrial appendages were obtained from 93 patients, and 107 HCAs with a mean maximum internal diameter of 166 ± 6 μm were used. Patient demographic information is summarized in Table 1.

EET-induced dilation of HCA. To determine whether EETs dilate HCA, internal diameter was measured in isolated, pressurized HCAs using videomicroscopy following administration of three EET regioisomers. As shown in Fig. 1, each EET regioisomer tested elicited a concentration-dependent dilation of HCAs (maximum dilation 67 ± 7%, 67 ± 6%, and 45 ± 5% with 8,9-, 11,12-, and 14,15-EET at 10^{-5} mol/l, $n = 9$, 18, and 25, respectively). The maximum vasodilatory responses to 8,9-EET and 11,12-EET were similar, whereas the maximum response to 14,15-EET was significantly less compared with the other two [8,9- vs. 11,12-EET, P = not significant (NS); 8,9- vs. 14,15-EET, $P < 0.05$; 11,12- vs. 14,15-EET, $P < 0.05$]. EET-induced dilation was not influenced by sex, age, surgical procedure, or underlying disease (coronary artery disease, hypertension, hypercholesterolemia, atrial fibrillation, diabetes mellitus, congestive heart failure, myocardial infarction, or tobacco use). To assess the role of the endothelium in EET-mediated vasodilation, the concentration-dependent response to 14,15-EET was measured in vessels that were denuded of endothelium. No significant difference in the response to 14,15-EET was observed between intact and denuded vessels (maximum dilation 48 ± 11% intact vs. 51 ± 5% denuded at 10^{-5} mol/l, $n = 5$, P = NS), suggesting that the endothelium is not required for EET-mediated dilation and that EETs act directly on the VSMC.

Contribution of BK_{Ca} channels to EET-induced dilation. Previous studies using animal models (3, 5, 26) and human conduit arteries (1) indicate that EETs activate BK_{Ca} channels. To determine whether EETs dilate HCAs through activation of these channels, concentration-dependent responses of isolated HCAs to cumulative applications of EETs were tested with the use of videomicroscopy, first in the absence and then in the presence of IBTX. As shown in Fig. 2, *A–C*, dilation of HCA to all three EET regioisomers tested was significantly reduced in the presence of IBTX (maximum dilation 74 ± 10%, 67 ± 11%, and 49 ± 11% without IBTX vs. 17 ± 11%, 15 ± 10%, and 14 ± 7% in the presence of IBTX with 8,9-, 11,12-, and 14,15-EET at 10^{-5} mol/l, $n = 6$, 7, and 6, respectively, $P < 0.05$), suggesting that BK_{Ca} channels play a significant role in the response to each of these compounds. To investigate whether a general desensitization to EET contributed to this reduction following the initial concentration-dependent response, separate experiments were performed in which two sequential concentration-response curves to 8,9-EET were generated in vessels without intervening application of IBTX. In these vessels, there was no significant overall difference between the initial and subsequent concentration-response curves or between individual concentrations from these two curves

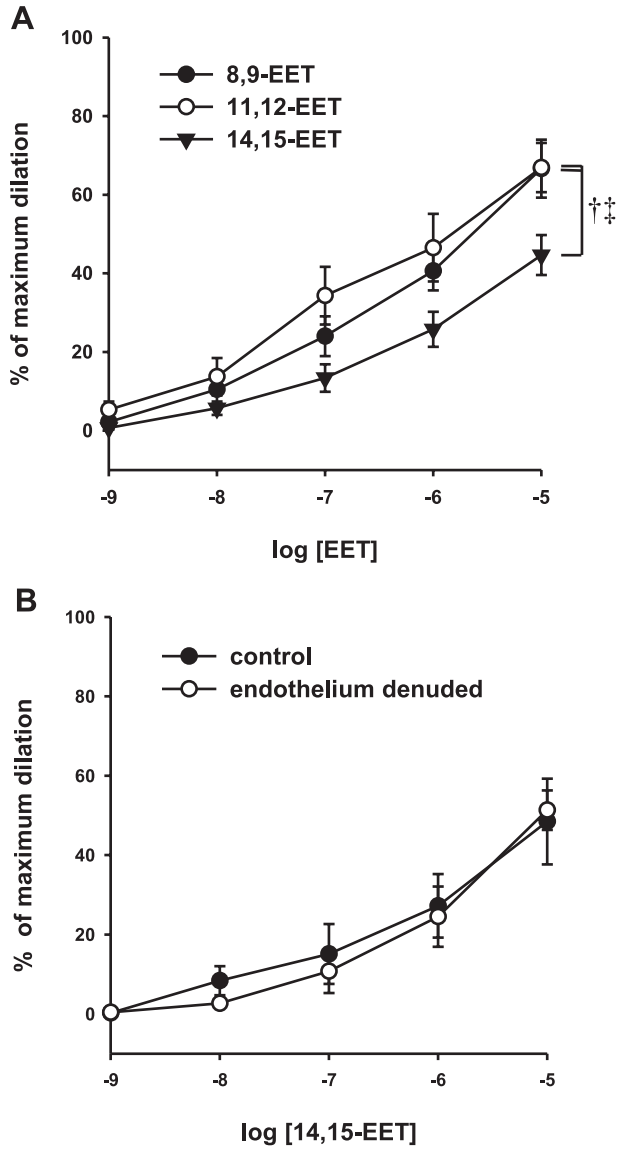

Fig. 1. Epoxyeicosatrienoic acid (EET)-induced dilation of human coronary arterioles (HCAs). *A*: 8,9-, 11,12-, and 14,15-EET each induces a dose-dependent relaxation of HCAs ($n = 9$, 18, and 25, respectively). 14,15-EET is a less potent dilator than 8,9- and 11,12-EET (†$P < 0.05$ vs. 8,9-EET curve, ‡$P < 0.05$ vs. 11,12-EET curve). *B*: endothelial denudation does not diminish or enhance vasodilation to 14,15-EET [$n = 5$, P = not significant (NS)]. n, No. of vessels.

Table 1. *Patient demographics*

Characteristics	n
Sex (male/female)	59/34
Age, yr (mean ± SD)	66 ± 13
Surgical procedure	
Coronary artery bypass graft	48 (52%)
Valve replacement	
Mitral	23 (25%)
Aortic	12 (13%)
Tricuspid	2 (2%)
Other	8 (9%)
Underlying diseases	
Coronary artery disease	54 (58%)
Hypertension	51 (55%)
Atrial fibrillation	23 (25%)
Hypercholesterolemia	21 (23%)
Diabetes mellitus	18 (19%)
Congestive heart failure	13 (14%)
Myocardial infarction	8 (9%)
(Tobacco use)	20 (22%)
None of the above	11 (12%)

$n = 93$.

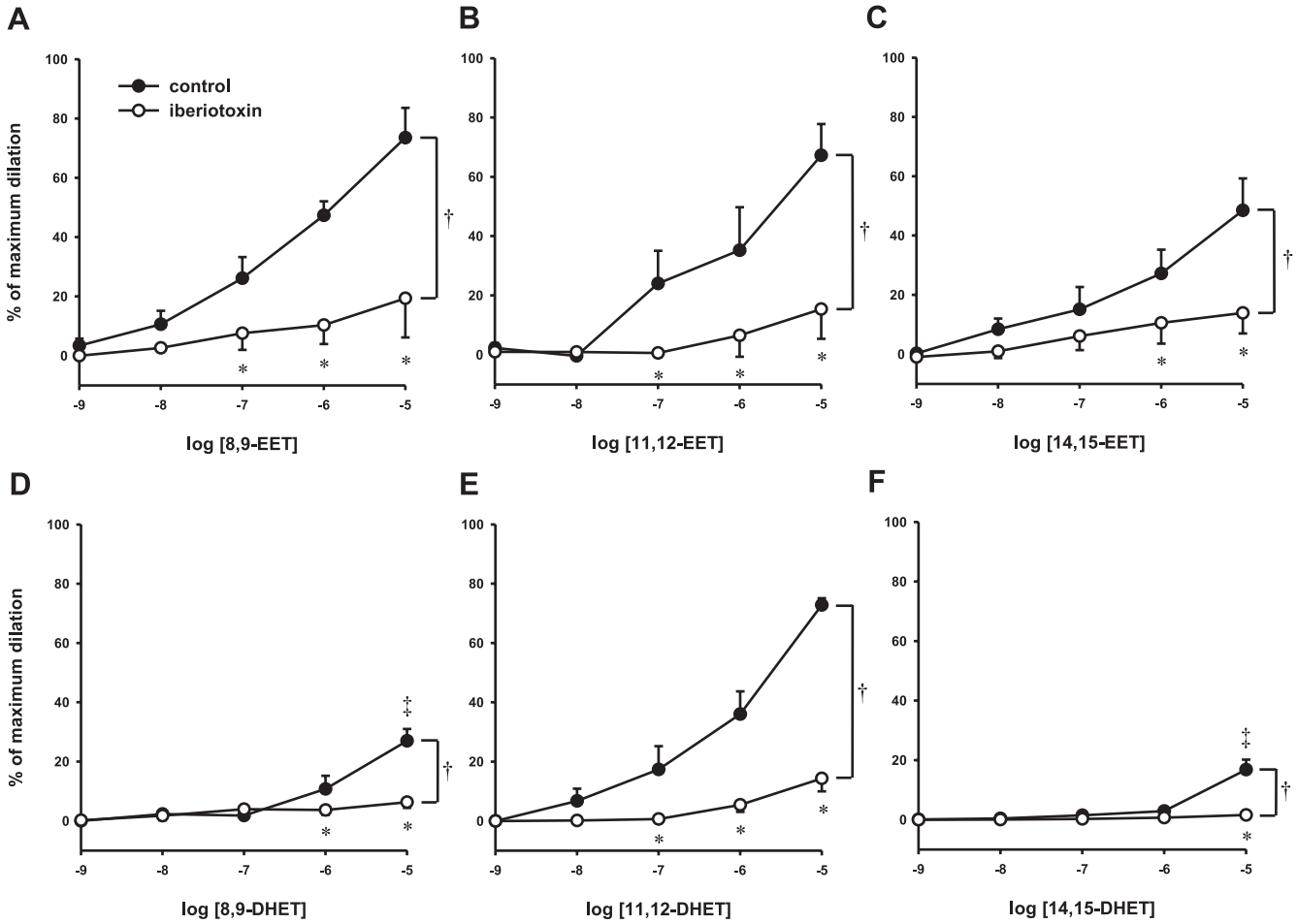

Fig. 2. Effect of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blockade on EET- and dihydroxyeicosatrienoic acids (DHET)-induced dilation of HCAs. *A–C*: dilation to 8,9-, 11,12-, and 14,15-EET is inhibited by iberiotoxin (100 nmol/l, $n = 6$, 7, and 6, respectively). *D–F*: 8,9-, 11,12-, and 14,15-DHET each induces a dose-dependent relaxation of HCAs that is sensitive to iberiotoxin (100 nmol/l, $n = 6$, 6, and 6, respectively). 8,9- and 14,15-DHET are less potent dilators than their respective EETs, whereas 11,12-DHET is similar to 11,12-EET. *n*, No. of vessels. †$P < 0.05$ vs. control curve, *$P < 0.05$ vs. control at specific dose, ‡$P < 0.05$ vs. respective EET curve.

($n = 3$, P = NS, data not shown), indicating that tachyphylaxis to EET did not develop. Application of IBTX had no significant effect on basal vascular tone or on the ability of the vessel to maximally dilate to papaverine.

DHET-induced dilation of HCA. To determine whether DHETs dilate HCAs with similar potency to EETs and whether this response is dependent on activation of BK_{Ca} channels, concentration-dependent responses of isolated HCAs to each corresponding DHET were tested, first in the absence and then in the presence of IBTX. As shown in Fig. 2, *D–F*, each DHET regioisomer elicited dilation of HCA in an IBTX-sensitive manner (maximum dilation 27 ± 4%, 73 ± 2%, and 17 ± 3% without IBTX vs. 6 ± 2%, 14 ± 4%, and 2 ± 1% in the presence of IBTX with 8,9-, 11,12-, and 14,15-DHET at 10^{-5} mol/l, $n = 6$, 6, and 6, respectively, $P < 0.05$). Dilation mediated by 8,9- and 14,15-DHET was significantly less than the dilation elicited by the corresponding EETs (27 ± 4% with 8,9-DHET vs. 67 ± 7% with 8,9-EET at 10^{-5} mol/l, $P < 0.05$; 17 ± 3% with 14,15-DHET vs. 45 ± 5% with 14,15-EET at 10^{-5} mol/l, $P < 0.05$). However, 11,12-DHET appeared to be just as potent as 11,12-EET (73 ± 2% with 11,12-DHET vs. 67 ± 6% with 11,12-EET at 10^{-5} mol/l, P = NS). HPLC analysis of our 11,12-DHET stock confirmed its purity, ruling out contamination with 11,12-EET as a possible cause for this robust response.

Expression of CYP450s in HCA. To investigate whether EETs and/or DHETs may be important endogenous endothelium-derived vasodilators in HCA, immunohistochemistry was used to determine whether the major CYP450 enzymes responsible for EET production (52) are expressed in HCA endothelium, as has been seen in human conduit arteries (38) and microvessels from uterine cervix (10). As shown in Fig. 3, *A–C*, CYP2C8 is expressed prominently in the endothelium of HCA, as well as in the VSMC and myocardium. CYP2C9 and -2J2 are expressed diffusely throughout the tissue, although expression is more prominent throughout the vascular wall and in the myocardium. No tissue staining occurred in the absence of primary antibody or in the presence of preimmune serum (Fig. 3, *E* and *F*). These findings provide indirect evidence that EETs and/or DHETs may be generated by the HCA itself and/or by the surrounding tissue.

Expression of sEH in HCA. It has been proposed that sEH may be an important modulator of EET activity in the vasculature, and its tissue-specific expression often coincides with

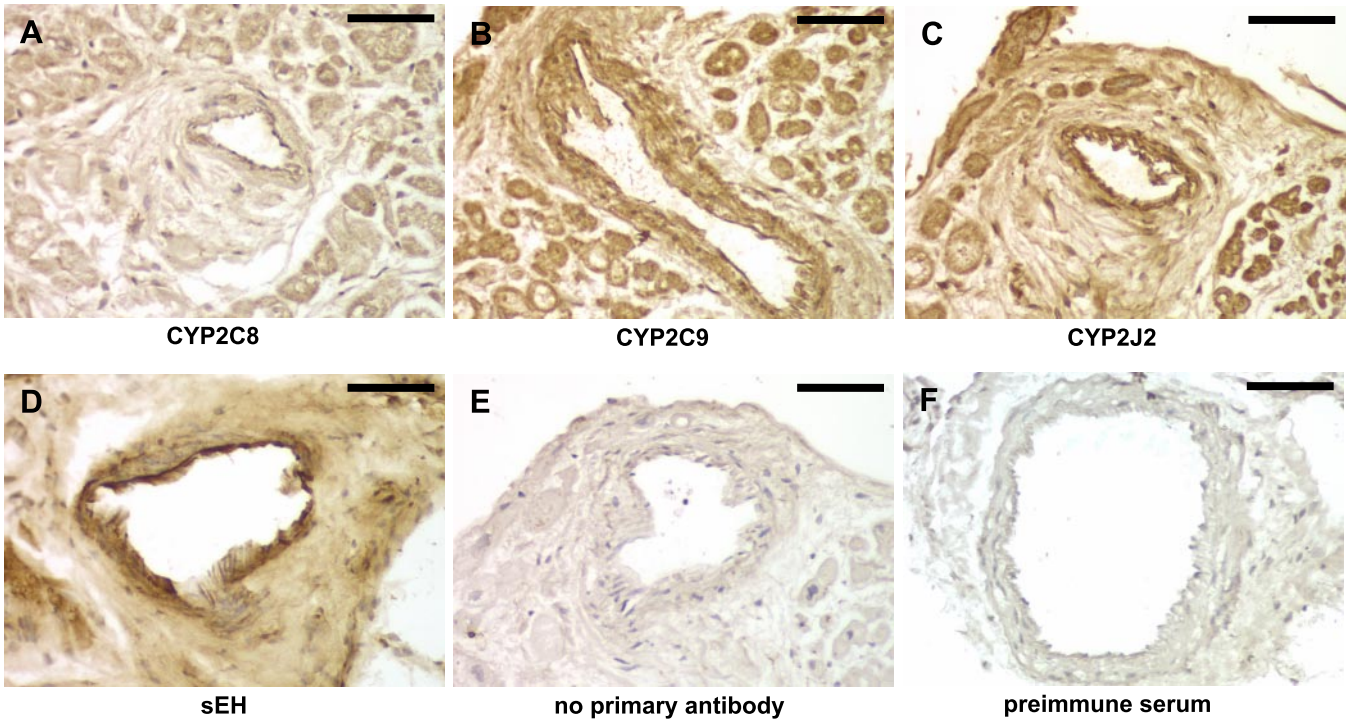

Fig. 3. Expression of cytochrome *P*-450 (CYP450) epoxygenases and soluble epoxide hydrolase (sEH) in the microvasculature of human right atrial appendage. Representative images of tissue sections (5 μm thick) following immunohistochemical staining. A positive reaction is indicated by the production of a brown pigment. *A–C*: CYP2C8, -2C9, and -2J2 are expressed in the endothelium of HCA, as well as in the vascular smooth muscle and surrounding tissues (n = 5, 6, and 4, respectively). *D*: sEH is expressed in HCA endothelium and in surrounding tissues (n = 4). *E* and *F*: staining was absent when each primary antibody was omitted or replaced by preimmune serum, ruling out nonspecific binding of the secondary antibody (n = 5, 6, 4, and 4, respectively). n, No. of patients. Bar, 100 μm.

the expression pattern of CYP450 epoxygenases, particularly CYP2J2 (10). To investigate whether sEH is available to modulate the activity of EET and/or DHET in the HCA, we next evaluated the expression of sEH in HCA. As shown in Fig. 3*D*, sEH is prominently expressed in HCA and is also expressed diffusely throughout the surrounding tissue. No tissue staining occurred in the absence of primary antibody or in the presence of preimmune serum (Fig. 3, *E* and *F*). As sEH expression mirrors the expression pattern of the CYP450 epoxygenases, it is possible that sEH plays a role in modulating the vascular effects of these eicosanoids.

Effect of sEH inhibition on EET-induced dilation of HCA. To evaluate whether sEH can modulate the response of HCA to EETs, we tested concentration-dependent responses of HCAs to EET, first in the absence and then in the presence of CDU. As shown in Fig. 4, *A–C*, treatment with CDU significantly enhanced the dilation response of HCA to 14,15-EET (maximum dilation 49 ± 13% without CDU vs. 87 ± 8% in the

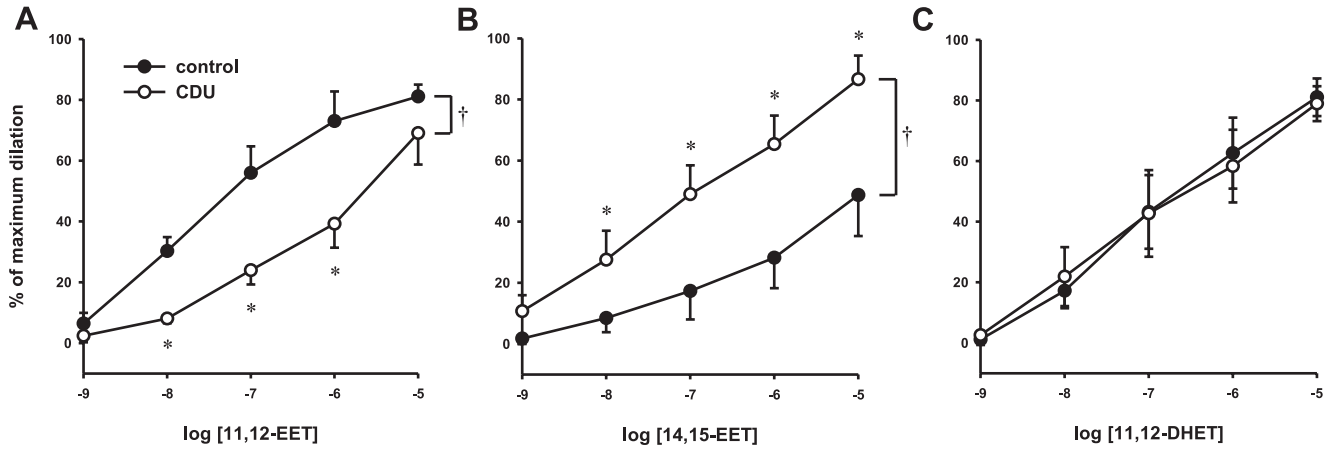

Fig. 4. Effect of sEH inhibition on EET-mediated dilation of HCA. *A*: 11,12-EET-mediated dilation is reduced in the presence of 1-cyclohexyl-3-dodecylurea (CDU; 1 μmol/l, n = 6). *B*: 14,15-EET-induced dilation is enhanced in the presence of CDU (n = 6). *C*: 11,12-DHET-induced dilation is not altered in the presence of CDU (n = 5, P = NS). n, No. of vessels. †$P < 0.05$ vs. control curve, *$P < 0.05$ vs. control at specific dose.

presence of CDU at 10^{-5} mol/l, $n = 6$, $P < 0.05$). However, dilation to 11,12-EET was significantly reduced in the presence of CDU (dilation 73 ± 10% without CDU vs. 39 ± 8% in the presence of CDU at 10^{-6} mol/l, $n = 6$, $P < 0.05$), suggesting that the overall consequence of sEH inhibition may depend on the particular EET regioisomer(s) present. Dilation to 11,12-DHET was not affected by CDU (dilation 81 ± 6% without CDU vs. 79 ± 6% in the presence of CDU at 10^{-5} mol/l, $n = 5$, P = NS), indicating that the effect of CDU on EET-mediated dilations was not due to nonspecific effects on vascular viability or function. Application of CDU had no significant effect on basal vascular tone, stability of preconstriction by ET-1, or the ability of the vessel to maximally dilate to papaverine (data not shown).

Effect of sEH inhibition on EET metabolism in HCA. To evaluate whether inhibition of sEH actually decreases the metabolism of EETs to DHETs in HCA, we quantified production of DHETs from vessels incubated with EETs in the presence and absence of CDU using LC-ESI-MS. As shown in Fig. 5, when 11,12- and 14,15-EET were incubated with HCAs, a significantly greater percentage of each was converted to its corresponding DHET compared with incubations of EETs in buffer alone (87 ± 4% and 84 ± 5% conversion with HCAs vs. 12 ± 4% and 15 ± 5% in buffer, respectively, $P <$ 0.05), suggesting that endogenous sEH activity may be sufficient to modulate the local bioactivity of endogenous EETs. When HCA were pretreated with CDU, however, metabolism of EETs to DHETs was significantly reduced (39 ± 5% and 40 ± 10% with CDU, respectively, $P < 0.05$), suggesting that pharmacological inhibition of sEH may potentially enhance endogenous EET bioavailability.

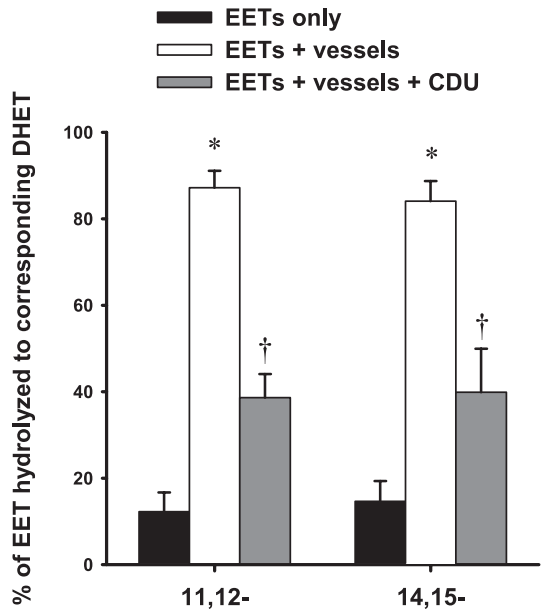

Fig. 5. Effect of sEH inhibition on the metabolism of EETs to DHETs by HCA. After incubations using 11,12- and 14,15-EET, EETs and DHETs were quantified by using liquid chromatography electrospray-ionization mass spectrometry. A greater percentage of EETs were hydrolyzed to their corresponding DHET derivatives in the presence of HCAs than in buffer alone ($n = 3$, $*P < 0.05$). However, production of DHETs was reduced when separate vessels from the same patient were pretreated with CDU ($\dagger P < 0.05$). *n*, No. of patients.

DISCUSSION

This study is the first to directly examine the capacity of EETs and DHETs to modulate vascular tone in the human coronary microvasculature. The novel findings of the present study are threefold. First, EETs and DHETs dilate HCA in subjects with heart disease, in a concentration-dependent manner through activation of BK_{Ca} channels, with variable potency among the regioisomers. Second, CYP450 epoxygenase isoforms 2C8, 2C9, and 2J2, as well as sEH, are prominently expressed in HCA, as well as in the tissue surrounding HCA. Third, pharmacological inhibition of sEH markedly reduces metabolism of EET regioisomers by HCA to their respective DHETs and significantly alters vasodilation of HCA to exogenous EETs. Taken together, these data indirectly suggest that EETs and DHETs may be important endogenous mediators of vascular function in human coronary resistance vessels and that they may function as EDHFs in this vascular bed. In addition, these data suggest that sEH inhibition has the potential to alter myocardial perfusion, depending on which of the EETs are produced endogenously.

EETs and DHETs as putative EDHFs. A large body of evidence points to EET as an EDHF (7, 14, 17, 22). In the coronary circulation of animal models, EETs have been implicated as an EDHF by evidence that indicates that *1*) they hyperpolarize VSMC membranes via activation of BK_{Ca} channels (5, 26); *2*) when the appropriate CYP450 enzyme is blocked or eliminated, EDHF-mediated dilation is abolished (7, 20, 40); and *3*) EET is produced in response to stimulated release of EDHF (5, 7, 19). The present study provides initial evidence that EETs and DHETs may function as EDHFs in the resistance vessels of the human heart, as they induce vasodilation through a BK_{Ca} channel-mediated mechanism. In addition, CYP450 epoxygenase enzymes are expressed in the endothelium of HCA, suggesting that these enzymes may be an endogenous source of these compounds. Prior studies from our laboratory indicate that flow-mediated dilation of human coronary resistance vessels is reduced by inhibiting CYP450 epoxygenases (33). EETs and DHETs may therefore be important modulators of human coronary vascular resistance and myocardial perfusion, as EDHF regulates tone of microvessels to a greater degree than in conduit arteries (44, 48). EETs and DHETs may be particularly important in the human heart in disease states, when NO-mediated vasodilation is impaired by oxidative stress (24) and vascular regulation becomes increasingly dependent on EDHF (33).

In addition to EETs, another prominent candidate for EDHF is hydrogen peroxide (H_2O_2) (28, 30). H_2O_2 likely arises by dismutation of superoxide, which may originate from numerous intracellular sources, including mitochondria (27) and CYP450 (16). Flow-induced dilation of HCA is sensitive to inhibition by catalase, implicating a role for H_2O_2 (30); however, as described above, flow-induced dilation of HCA is also blocked by inhibitors of CYP450 epoxygenases (33). It is not known whether CYP450 blockade impairs flow-induced dilation by diminishing H_2O_2 production, EET production, or both, and the possibility of multiple EDHFs in the HCA cannot be excluded. The relative contributions of each to EDHF-mediated vascular function and potential interactions between them, if any, remain unknown.

DHET compounds are generally considered to be inactivated by-products of EET following metabolism by sEH. However, it is interesting that 11,12-DHET and 11,12-EET appeared to be equipotent dilators of HCA. A similar phenomenon has been observed in animal studies in which 11,12-DHET was an equally potent dilator of porcine coronary arteries compared with 11,12-EET (12) and 11,12-DHET was 1,000 times more potent than its corresponding EET as a dilator of canine coronary microvessels (39). It is possible that 11,12-DHET is actually more potent than the corresponding 11,12-EET in HCA as well. It is possible that the potent dilation observed with 11,12-EET may have actually been due to rapid conversion to 11,12-DHET by sEH because sEH inhibition reduced dilation of HCA to 11,12-EET. However, the underlying reason for the difference in potency seen with 11,12-DHET compared with the other DHETs is unknown.

Although the endothelium is an important source of EETs in many animal models, its role in the vascular response to these eicosanoids is controversial (4). In cell culture, endothelium-derived EET acts in an autocrine manner on the endothelium to modulate store-operated Ca^{2+} influx (21), endothelial BK_{Ca} channel activity (3), and gap junctional communication (41), suggesting that the endothelium itself may play an important role in EET-mediated vascular effects. However, studies using isolated bovine coronary arteries indicate that the endothelium is not required for EETs to elicit vasodilation (5, 18). Our results indicate that EETs elicit their vasomotor effects on HCA via a direct interaction with the VSMC, as endothelial denudation had no effect on EET-induced dilation. Although the endothelium does not contribute significantly to vasomotor responses of HCA to exogenous eicosanoids, the possibility that the endothelium may modulate nonvasomotor effects of EETs should be considered.

Potential clinical utility of sEH inhibitors. In addition to their role as vasodilators, EETs and/or DHETs may also function as vascular protective agents, particularly in disease states when NO-mediated vascular protection is impaired. EETs may possess antiatherosclerotic properties similar to NO (15, 38, 46). Pharmacological inhibition of sEH has emerged as a potential therapeutic approach for hypertension and vascular inflammation by increasing the bioavailability of EETs (9, 13, 23, 53). Our results suggest that the potential clinical utility of sEH inhibitors may depend on the relative sensitivity of a particular vascular bed to EETs and/or their respective DHET metabolites. For example, in HCA, blockade of 11,12-EET metabolism by an sEH inhibitor would actually reduce myocardial perfusion, while action on other EETs could enhance perfusion. The effects of sEH inhibitors on vascular physiology may also depend on which CYP450 epoxygenase(s) is (are) expressed and/or enzymatically active in an individual patient, as each subtype produces its own unique profile of EET regioisomers (8, 43, 49), and expression of subtypes varies among different individuals and vascular beds (10).

It must be remembered that the EETs are more lipophilic than their corresponding diols and therefore will tend to accumulate in cells, whereas the DHETs will tend to remain outside the cell. This compartmentalization based on polarity may send the epoxides and diols down different metabolic pathways. Intracellular EETs may be degraded by β-oxidation to form chain-shortened epoxides, especially when metabolism to DHET by sEH is inhibited (13), whereas extracellular DHETs may be moved away from potential targets by the circulation and rapidly metabolized by conjugation and other reactions. The overall consequence of sEH inhibition may therefore depend on which particular metabolic pathways predominate inside and outside the cell.

Potential study limitations. An inherent limitation of the current study is the lack of heart tissue from subjects completely free of disease, as truly normal fresh tissue is rarely obtainable. Numerous animal studies have demonstrated that endothelial dilator function is dramatically altered in cardiovascular disease states. However, the use of human atrial tissue from subjects with disease also presents the unique advantage of being able to study the contribution of EET and DHET to vascular function in the setting of chronic cardiovascular disease and its risk factors, conditions that cannot be adequately mimicked in animal models. In HCA from subjects with coronary artery disease, endothelium-dependent dilation is mediated almost exclusively by EDHF (32, 33); therefore, such tissue facilitates evaluation of EET and DHET as EDHFs in a clinically relevant setting, and without the potentially confounding influence of NO or PGI_2.

In the present study, HCAs were isolated solely from the right atrial appendage. It is possible that endothelium-dependent regulation of HCA function may be different in the ventricular myocardium and other vascular beds. Although this possibility cannot be excluded, published data indicate that the vasodilatory responses of atrial and ventricular HCAs to intraluminal flow are similar (33). Furthermore, with the exception of acetylcholine, other vasodilator agents elicit similar responses in isolated human atrial and ventricular arterioles (29), including bradykinin, ADP, substance P, sodium nitroprusside, the calcium ionophore A23187, and arachidonic acid, the precursor of EETs.

In the present study, EETs and DHETs appear to be exogenous dilators of HCA; however, it remains to be determined whether these compounds are actually produced endogenously in this vascular bed. Animal studies indicate that 8,9- and 14,15-EET are released by the endothelium of coronary arterioles on stimulation by methacholine (7), and human left internal mammary arteries release 8,9-, 11,12-, and 14,15-EET in response to acetylcholine (1). In addition, flow-induced dilation of HCA is markedly reduced by multiple distinct inhibitors of CYP450 and is eliminated by endothelial denudation (33), suggesting that EETs and/or DHETs may be important endothelium-derived vasodilators in the human heart. It is possible that EETs may be derived from both endothelial and nonendothelial sources, as some CYP450 epoxygenases and sEH were observed in the endothelium as well as vascular smooth muscle and perivascular tissue; however, the physiological source(s) and the functional significance of nonendothelial EETs remain unknown.

In the present study, endothelial denudation had no apparent effect on EET-induced dilation. This result was surprising, as sEH is prominently expressed in the endothelium; therefore, we expected that a loss of endothelial sEH would affect the vascular response to EETs. However, sEH is also expressed in the VSMC, and it is possible that our exogenous EETs were metabolized predominantly by sEH in these cells and not by endothelial sEH, as they permeated the vessel from the adventitial side and not the luminal side. sEH localized to the VSMC may be an important modulator of EET activity in the vascu-

lature, because it is also highly expressed in the smooth muscle of human renal microvessels (51) and exhibits potent hydrolase activity in cultured porcine arterial VSMCs (11); however, the significance of sEH in HCA VSMCs as a modulator of EET-induced dilation in vivo is unknown. Although the endothelium does not contribute significantly to vasomotor responses of HCA to exogenous eicosanoids, the possibility that the endothelium may modulate vasomotor effects of endogenous EETs via endothelial sEH activity should be considered.

One regioisomer of EET, the 5,6- compound, has a remarkably short half-life of <10 min (50) and rapidly degrades to 5,6-DHET. Because of its chemical instability, its use is technically difficult in vasodilation studies in vitro, and no attempt was made to characterize the role of the 5,6- compounds in HCA in the present study. However, it is possible that 5,6-EET degradation may be mediated in part by sEH; therefore, the possibility that 5,6-EET or 5,6-DHET may also contribute to regulation of vascular function should be considered when evaluating the utility of sEH inhibition in a particular vascular bed.

Clinical implications. With the expanding body of evidence implicating EET as a potential EDHF, pharmacological inhibition of sEH has emerged as a potential therapeutic approach for several cardiovascular diseases (9, 13, 23, 53). The present study confirms that sEH inhibition is a feasible method for enhancing EET bioavailability in the human coronary microcirculation. This approach may be useful for improving myocardial perfusion in cardiovascular disease states, when NO-mediated vascular function is impaired by oxidative stress (24). However, our results suggest that sEH inhibition may not uniformly enhance blood flow and that the utility of sEH inhibitors may depend on the relative contribution of endogenous EETs and DHETs to vascular tone in a particular vascular bed.

In conclusion, CYP450 epoxygenases expressed in HCA may be an endogenous source of EETs and DHETs, which dilate HCA by a BK_{Ca} channel-mediated mechanism. These findings support a role for EETs and DHETs as EDHFs in the human coronary microcirculation. While sEH inhibition enhances EET bioavailability in HCA, its physiological importance remains to be determined.

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DISCLOSURES

D. C. Zeldin and B. D. Hammock are coinventors on a patent entitled "Inhibitors of Epoxide Hydrolases for the Treatment of Hypertension" [U.S. Patent No. 6,531,506 B1, issued March 11, 2003; coinventors: D. L. Kroetz (Univ. of California San Francisco), D. C. Zeldin (National Institute of Environmental Health Sciences), B. D. Hammock (Univ. of California Davis), and C. Morisseau (Univ. of California San Francisco)]. In December 2004, this patent was exclusively licensed to Arete Therapeutics, a company founded by B. D. Hammock to explore moving epoxide hydrolase inhibitors into clinical trials.

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