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Objective—An initial step in endothelium-derived hyperpolarizing factor-mediated responses is endothelial cell hyperpolarization. Here we address the mechanisms by which cytochrome P450 (CYP)-derived epoxyeicosatrienoic acids (EETs) contribute to this effect in native and cultured endothelial cells.

Methods and Results—In native CYP2C-expressing endothelial cells, bradykinin elicited a Ca^{2+} influx that was potentiated by the soluble epoxide hydrolase inhibitor, 1-adamantyl-3-cyclohexylurea (ACU), and attenuated by CYP inhibition. Similar effects were observed in cultured endothelial cells overexpressing CYP2C9, but not in CYP2C9-deficient cells, and were prevented by the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid as well as by the cAMP antagonist, Rp-cAMPS. The effects on Ca^{2+} were mirrored by prolongation of the bradykinin-induced hyperpolarization. Ruthenium red and the combination of charybdotoxin and apamin prevented the latter effect, suggesting that Trp channel activation increases Ca^{2+} influx and prolongs the activation of Ca^{2+}-dependent K^+ (K_{Ca}) channels. Indeed, overexpression of CYP2C9 enhanced the agonist-induced translocation of a TrpC6-V5 fusion protein to caveolin-1–rich areas of the endothelial cell membrane, which was prevented by Rp-cAMPS and mimicked by 11,12-EET.

Conclusions—Elevated EET levels regulate Ca^{2+} influx into endothelial cells and the subsequent activation of K_{Ca} channels, via a cAMP/PKA-dependent mechanism that involves the intracellular translocation of Trp channels. (***Arterioscler Thromb Vasc Biol*. 2007;27:2612-2618.)**

Key Words: caveolae ■ cytochrome P450 ■ endothelium-derived hyperpolarizing factor ■ protein kinase A

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Cytochrome P450 (CYP)-derived epoxyeicosatrienoic acids (EETs) have been proposed to act as endothelium-derived hyperpolarizing factors (EDHFs) (for review see reference 1). However, the fact that EETs activate iberiotoxin-sensitive BK_{Ca} channels[2] while most EDHF-dependent responses are sensitive to the combination of charybdotoxin and apamin that inhibit small (SK_{Ca}) and intermediate conductance (IK_{Ca}) K_{Ca} channels,[3] has frequently been used as an argument against the involvement of an EET in the EDHF-mediated response. Despite the confusion that exists in the literature related to this topic, it has become clear that probably the most important event in the initiation/generation of EDHF-mediated responses is the rapid hyperpolarization of endothelial cells, an event that precedes the hyperpolarization of vascular smooth muscle cells.

The EDHF-mediated relaxation of porcine coronary arteries that is sensitive to the CYP2C9 inhibitor sulfaphenazole and that can be attenuated by antisense oligonucleotides directed against CYP2C; is insensitive to iberiotoxin and sensitive to charybdotoxin and apamin[4] suggesting that the role of EETs in the EDHF phenomenon may not simply be related to the activation of BK_{Ca} channels. There is certainly circumstantial evidence linking EETs with the activation of SK_{Ca} and IK_{Ca} channels inasmuch as the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) attenuates the bradykinin-induced hyperpolarization of native porcine coronary artery endothelial cells.[5] The molecular mechanisms involved in mediating such effects are however unclear, but the activation of PKA by 11,12-EET is reported to be important for afferent arteriole vasodilatation[6] and EETs have been linked with a role in modulating intracellular Ca^{2+} levels in endothelial cells.[7]

The aim of the present study was to assess the role of CYP-derived EETs in the regulation of endothelial cell Ca^{2+} signaling and agonist-induced changes in membrane potential in native and cultured endothelial cells. To this end CYP expressing as well as CYP-deficient cells were studied as were the effects of specific inhibitors of the soluble epoxide hydrolase (sEH), which prevent the degradation of EETs to their respective dihydroxyeicosatrienoic acids (DHETs).[8]

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Materials and Methods

Materials

Fura-2-AM was from Molecular Probes and adenosine 3′,5′-cyclic monophosphothioate, Rp-Isomer (Rp-cAMPs) was purchased from Alexis Biochemicals. The sEH inhibitor 1-adamantyl-3-cyclohexylurea (ACU), the EET antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), and the microsomal EH antagonist elaidamide were synthesized as described[9–11] and incubated with cells for 15 minutes before analysis. The CYP2C9 antibody used for Western blotting was generated by immunizing rabbits with a CYP2C specific peptide (RRRKLPPGPTPLPIC; Eurogentec), and the antibody used for immunofluorescence was kindly provided by E. Morgan (Atlanta, Ga). All other chemicals were purchased from Sigma.

Cell Culture

Human umbilical vein endothelial cells for the measurement of membrane potential and $[Ca^{2+}]_i$ were isolated as described.[12] The transient transfection of TrpC6 was assessed in umbilical vein endothelial cells purchased from Cell Systems/Clonetics which are possible to transfect.

Isolation of RNA and RT-PCR

Total RNA was isolated using phenol and guanidine isothiocyanate (TriReagenz, Sigma). RT-PCR was performed using primers derived from the CYP2C sequences (2C forward: agacaacgagcaccactctg; reverse: ccttggggatgaggtagttt). The PCR fragments were size-separated on an agarose gel and visualized by Southern blot analysis using a ^{32}P-labeled full-length CYP2C9 cDNA probe.

Transfection and Adenoviral Infection of HUVECs

Subconfluent cells (Clonetics) were transfected with expression plasmids encoding TrpC6-V5 or TrpC3-myc fusion proteins using Superfect (Qiagen). Immediately after transfection the cells were infected with control or CYP2C9 overexpressing adenovirus as previously described.[13] The full-length TRPC6-V5-tagged expression plasmid for the generation of adenoviruses was obtained from Thomas Hofmann (University Marburg). The generation of the adenoviral expression vector (pAd-Track-CMV), the recombination with the adenoviral backbone and the amplification and propagation of the viruses was performed as described.[14] After 30 hours the cells were transferred to serum-free medium and stimulated as described in the results section.

Immunohistochemistry

Cultured endothelial cells were stimulated as described in the results section. Freshly isolated human umbilical veins were cut into rings, frozen in Tissue Tek (Sakura Finetec), cut into sections (10 μm), and placed on glass coverslips. Samples were fixed (4% paraformaldehyde in PBS), permeabilized with Triton X-100 and incubated with specific antibodies to CYP 2C, V5, or caveolin-1. The preparations were mounted and viewed using a confocal microscope (LSM 510 META, Zeiss).

Measurement of Intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Human umbilical veins were excised from the cord, cannulated at both ends, filled with culture medium containing fura 2-AM, and incubated at 37°C for 1 hour. Thereafter, the veins were rinsed and cut into 4 or more 20×10-mm segments, and the adventitial surface was attached to glass coverslips using liquid skin adhesive (Dermabond; Ethicon, Norderstedt). The coverslips were then washed in nominally Ca^{2+}-free HEPES-modified Tyrode's solution (mmol/L: NaCl, 132; KCl, 4; $CaCl_2$, 1; $MgCl_2$, 0.5; HEPES, 9.5 NaH_2PO_4, 0.36; glucose), and $[Ca^{2+}]_i$ was determined fluorometrically. At the end of each experiment, the background fluorescence was subtracted from the original signals as described.[15] Data are presented as changes in the 340/380-nm ratio. Cultured endothelial cells (first passage) were loaded with fura 2-AM (3 μmol/L, 37°C, 45 minutes) and $[Ca^{2+}]_i$ measured and calculated as described.[15]

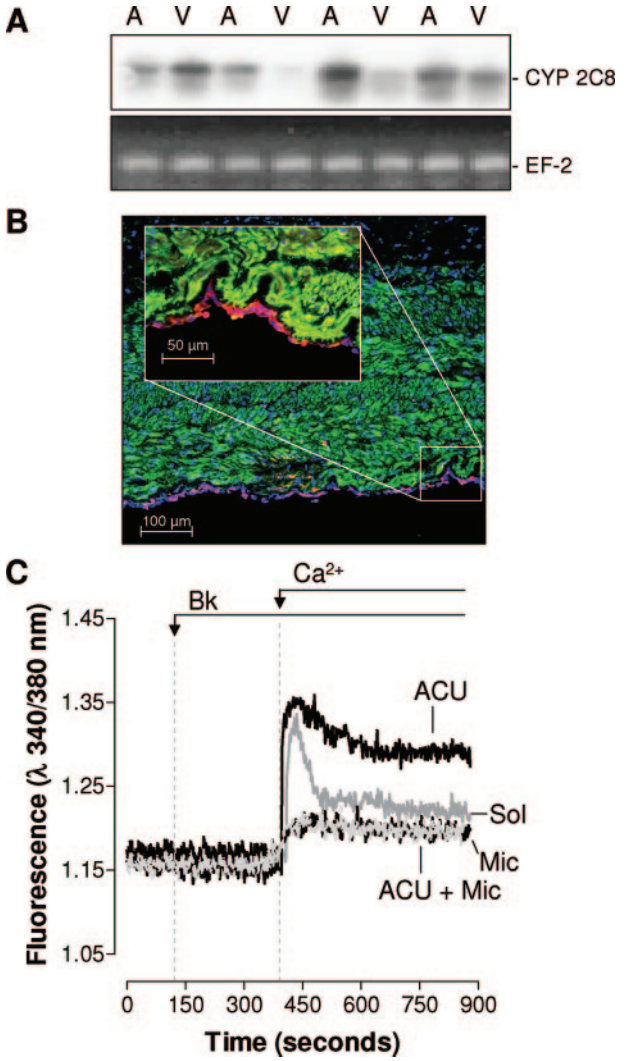

Figure 1. Expression of CYP 2C in human umbilical cords and effect of sEH inhibition on the bradykinin-induced Ca^{2+}-response in isolated umbilical veins. A, Expression of CYP2C8 and elongation factor 2 mRNA in umbilical cord arteries (A) and veins (V). B, Immunohistochemical staining of CYP2C (red), α-actin (green) and nuclei (blue) in the umbilical vein. C, effect of ACU and miconazole (Mic) on bradykinin-induced Ca^{2+} transients in an isolated human umbilical vein.

Cell Surface Biotinylation and Immunoblotting

Cell surface biotinylation was performed in HUVECs (Clonetics) 30 hours after transient transfection (TrpC6-V5) and infection (CYP2C9 or CTL virus). Cells were then incubated with ACU (10 μmol/L), followed by EZ-link-sulfo NHS-SS-Biotin (Pierce, 0,3 mg/mL) and ionomycin (0,1 μmol/L). Cells were incubated for 15 minutes at 37°C, then lysed in Triton X-100 buffer (in mmol/L: HEPES, 50; NaCl, 150; NaF, 100; EDTA, 1; Triton X-100, 1% [v/v], orthovanadate, 2 and 2 μg/mL each of leupeptin, pepstatin A, antipain, aprotinin, chymostatin and tyrpsin inhibitor, and PMSF, 40 μg/mL). Avidin agarose was added to the cell lysate, the mixture was incubated overnight at 4°C and washed 5 times with ice-cold lysis buffer, then immunoblotted with antibodies against V5 and PECAM-1.

For immunoblotting, cells were lysed in Triton X-100 buffer, and cell supernatants or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described.[16]

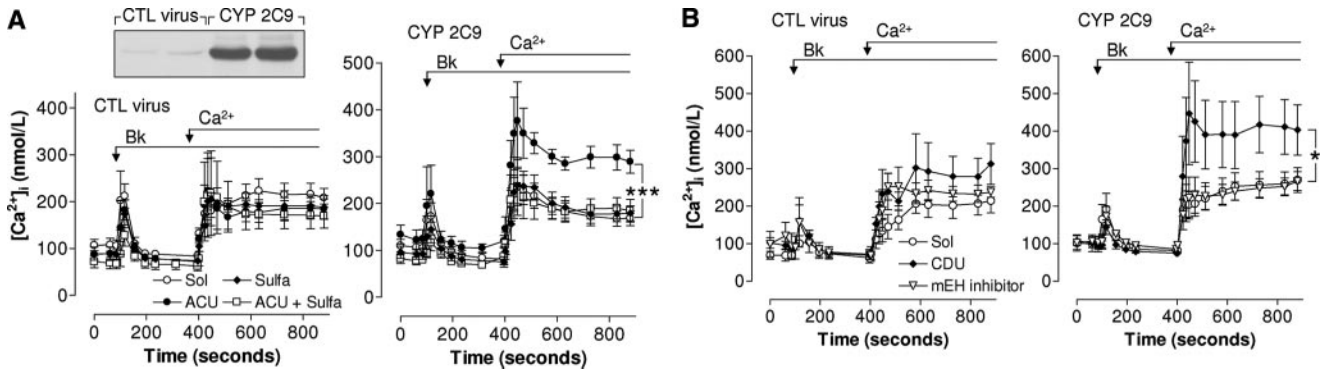

Figure 2. Inhibition of the sEH potentiates bradykinin-induced Ca^{2+} influx in CYP2C9-overexpressing cells. Endothelial cells were infected with CYP2C9 sense or antisense (CTL) adenoviruses. Experiments were performed in the absence and presence of (A) ACU, sulfaphenazole (Sulfa), (B) CDU or elaidamide (mEH inhibitor). Graphs summarize data from 5 to 8 experiments, $^{*}P<0.05$, $^{***}P<0.001$ vs solvent (Sol).

Electrophysiological Measurements

The endothelial cell membrane potential was recorded using the whole-cell configuration (current-clamp mode) of the patch-clamp technique as described.[17]

Statistical Analysis

Data are expressed as the mean±SEM. Statistical evaluation was performed with Student *t* test for unpaired data, 1-way ANOVA followed by a Bonferroni *t* test, or ANOVA for repeated measures where appropriate. Values of $P<0.05$ were considered statistically significant.

Results

CYP2C in Native Human Umbilical Vein Endothelial Cells Modulates the Ca^{2+} Response to Bradykinin

CYP2C RNA and protein were expressed in human umbilical veins (Figure 1A and 1B). Although RNA expression tended to be greater in umbilical arteries (Figure 1A), $[Ca^{2+}]_i$ was assessed in veins as the responses to bradykinin were more consistent. In nominally Ca^{2+}-free buffer, the application of bradykinin elicited a small transient increase in $[Ca^{2+}]_i$ that was generally masked by the background signal. However, the addition of $CaCl_2$ elicited a pronounced Ca^{2+} increase characterized by a transient peak followed by a plateau phase. No such responses were observed in the presence of the B_2 kinin receptor antagonist icatibant (data not shown). The plateau phase of the Ca^{2+} response was elevated in vessels treated with ACU, whereas the Ca^{2+} response was markedly attenuated in veins treated with ACU and the CYP inhibitor miconazole (Figure 1C).

Modulation of Endothelial Cell Responses to Bradykinin by the sEH Inhibitor ACU in CYP2C9-Overexpressing HUVECs

CYP2C protein and mRNA levels decline rapidly after endothelial cell isolation, making it difficult to determine the physiological role of CYP metabolites in the regulation of intracellular signaling. To improve the signal to noise ratio and to differentiate better between Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx, further experiments were performed using HUVECs (first passage) overexpressing CYP2C9. Although CYP2C8 was the more prominent endogenously expressed enzyme in the endothelial cells tested we chose to overexpress CYP2C9 as its activity can be selectively inhibited by sulfaphenazole.[13,18]

The overexpression of CYP2C9 had no significant effect on basal $[Ca^{2+}]_i$, or on the release of Ca^{2+} from intracellular stores (ie, the initial response to bradykinin in the absence of extracellular Ca^{2+}). The sEH inhibitor did not affect Ca^{2+} influx in CYP2C9-deficient cells but significantly increased Ca^{2+} influx in CYP2C9-expressing endothelial cells, an effect prevented by cotreatment with sulfaphenazole (Figure 2A). Similar results were obtained using ionomycin as an agonist (not shown) and a second sEH inhibitor, 1-cyclohexyl-3-dodecyl area (CDU), but not elaidamide, an inhibitor of the microsomal epoxide hydrolase (Figure 2B).

Overexpression of CYP2C9 slightly but significantly hyperpolarized the resting membrane potential of the endothelial cells studied (−39.6±1.2 mV versus −43.4±1.3 mV in CYP-deficient versus CYP-overexpressing cells; n=14, $P<0.05$) and increased the bradykinin-induced hyperpolarization (Figure 3A and 3B). ACU was without effect on the resting membrane potential (−42.4±2.8 versus −40.4±2.8 in CYP-expressing cells in the absence and presence of ACU, n=13), or on the magnitude of the bradykinin-induced hyperpolarization but significantly prolonged the duration of hyperpolarization (Figure 3A and 3C).

The EET-antagonist 14,15-EEZE (10 μmol/L) was without effect on $[Ca^{2+}]_i$ in CYP-deficient endothelial cells either in the absence (supplemental Figure I, available online at http://atvb.ahajournals.org) or presence of ACU (Figure 4A). However, in CYP2C9-overexpressing endothelial cells 14,15-EEZE prevented the sEH-inhibitor-induced increase in Ca^{2+} influx (Figure 4A). Comparable results were obtained when agonist-induced alterations in membrane potential were assessed inasmuch as the EET antagonist attenuated the amplitude and shortened the duration of the bradykinin-induced hyperpolarization of CYP-overexpressing cells treated with the sEH inhibitor (Figure 4B). No effect of 14,15-EEZE on the membrane potential was detected in CYP-deficient cells.

Role of cAMP/PKA in CYP-Dependent Responses in CYP2C9-Overexpressing Cells

Several of the cellular effects of EETs have been linked to the activation of adenylyl cyclase and PKA.[19] Therefore,

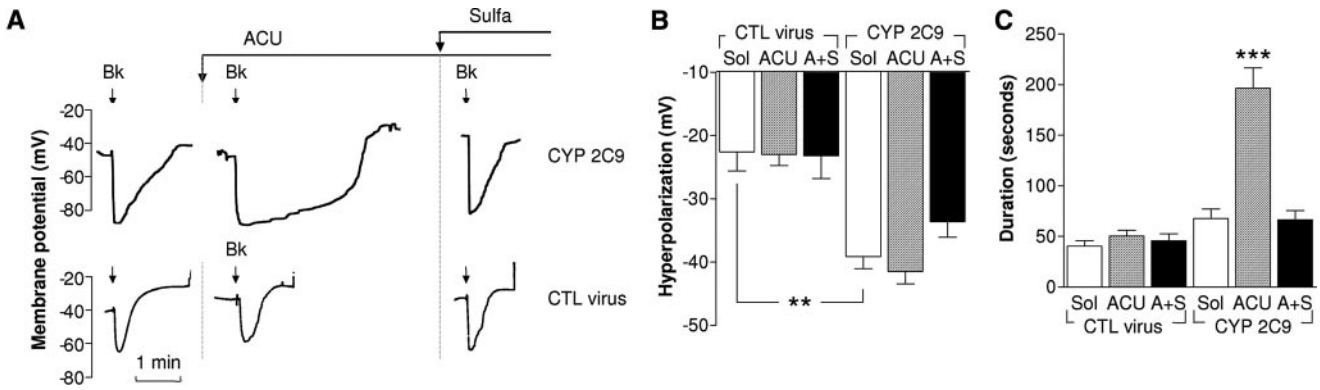

Figure 3. Effect of CYP2C9 on the bradykinin-induced hyperpolarization of human endothelial cells. Original traces (A) and bar graphs (B&C) summarizing the effect of ACU either alone or in combination with sulfaphenazole (Sulfa; A+S) on the magnitude (B) and duration (C) of the bradykinin-induced hyperpolarization. Graphs summarize data from 7 to 9 experiments; $^{**}P<0.01$, $^{***}P<0.001$ vs solvent (Sol)-treated cells.

we assessed the consequences of a cAMP antagonist on the CYP-dependent alterations in the Ca^{2+} response to bradykinin.

RpcAMPs (10 μmol/L) completely prevented the effect of ACU on the plateau phase of the Ca^{2+} transient (Figure 4A) but had no effect in the absence of the sEH inhibitor (supplemental Figure I). The inhibitor was without effect on either the release of intracellularly stored Ca^{2+} or on Ca^{2+} influx into CYP-deficient cells in either the absence or presence of the sEH inhibitor (Figure 4A). RpAMPs also attenuated the amplitude and shortened the duration of the bradykinin-induced hyperpolarization of CYP-overexpressing cells treated with the sEH inhibitor but had no effect in CYP-deficient cells (Figure 4B).

Role of EETs in the Translocation of Trp Channels

EETs are reported to alter the open probability of large conductance K_{Ca} channels[20] as well as of K_{ATP}[21] and Trp channels.[22] Therefore, we assessed the consequences of inhibiting these on the CYP-dependent potentiation of the bradykinin-induced hyperpolarization in the presence of the sEH inhibitor.

In CYP-deficient cells the sEH inhibitor, alone or in combination with the K_{ATP} inhibitor glibenclamide (10 μmol/L), did not significantly affect endothelial cell hyperpolarization to bradykinin. The combination of charybdotoxin and apamin (each 100 nmol/L) significantly decreased the bradykinin-induced endothelial cell hyperpolarization whereas the nonselective Trp channel blocker ruthenium red (1 μmol/L) exerted a nonsignificant effect (supplemental Figure II).

The amplitude of the hyperpolarization elicited by bradykinin was greater in CYP2C9-overexpressing than in CYP-deficient cells. The sEH inhibitor ACU did not further potentiate the magnitude of this response but significantly prolonged the hyperpolarization. The latter effect was unaffected by glibenclamide but was abrogated by charybdotoxin and apamin as well as by ruthenium red (supplemental Figure II). Neither ACU alone nor together with glibenclamide, or ruthenium red affected the resting membrane potential of CYP-expressing endothelial cells (data not shown). The combination of charybdotoxin and apamin however reduced the resting membrane potential by 10.7±3.5 mV and 9.7±3.2 mV in CYP-deficient and in CYP-expressing cells, respectively. Consistent with its effect on the bradykinin-induced hyperpolarization of CYP2C9-expressing cells, ruthenium red also attenuated the plateau phase of the Ca^{2+} response and abrogated the effects of ACU (supplemental Figure III).

As Trp channel activity can be determined by its intracellular localization,[23] we assessed the consequences of CYP activation and sEH inhibition on the localization of a

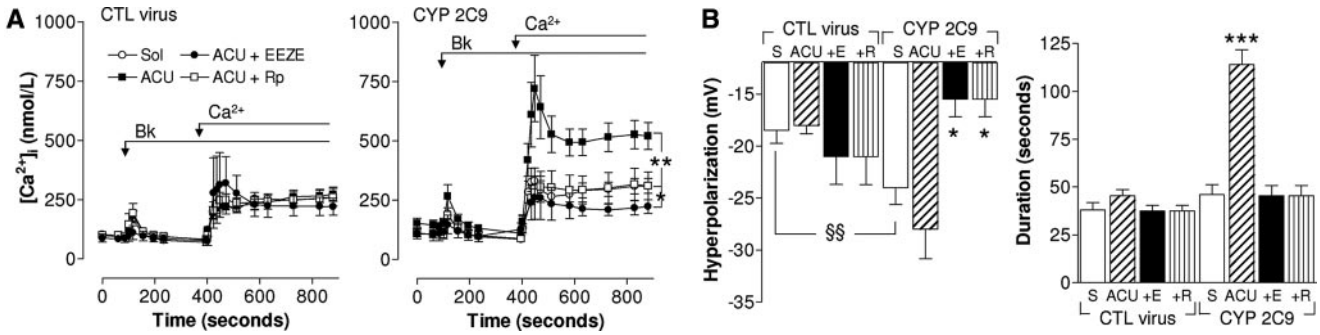

Figure 4. Effect of 14,15-EEZE and RpAMPs on bradykinin-induced responses. Endothelial cells were infected with either CYP2C9 or control (CTL) adenoviruses. The Ca^{2+} transient (A) or changes in membrane potential in response to bradykinin (BK; B) were assessed in the presence of ACU, as well as ACU and 14,15-EEZE (+EEZE/E) or RpAMPs (+Rp/R). Graphs summarize data from 6 to 11 experiments, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs solvent (Sol), §§$P<0.01$ vs CTL virus.

TrpC6-V5 fusion protein. TrpC6 was chosen because it has been implicated in agonist-induced Ca^{2+} influx into human endothelial cells.[24] We used 2 approaches to overexpress TrpC6-V5 and compared the effects of bradykinin on TrpC6-V5 in primary cultures of human umbilical vein endothelial cells treated with bradykinin with the effect of ionomycin in commercially available endothelial cells transfected with the TrpC6-V5 fusion protein (these cells no longer express the B_2 kinin receptor). In ACU-treated CYP2C9-deficient cells under basal conditions, the fusion protein was localized around to the nucleus with little or no membrane staining. In CYP2C9-overexpressing cells, there was a slight increase in the plasma membrane staining of V5. After stimulation with bradykinin (10 nmol/L, 30 seconds, Figure 5A) or ionomycin (0.1 μmol/L; Figure 5B), the fusion protein translocated from the perinuclear region to the cell membrane. The translocation of TrpC6-V5 was much more pronounced in cells overexpressing CYP2C9, was potentiated by ACU (Figure 5A), and was sensitive to Rp-cAMPs (Figure 5B). Similar results were obtained using a TrpC3-myc fusion protein (data not shown).

To demonstrate that the translocation observed reflected membrane insertion of the channels we repeated the experiments described above in endothelial cells transfected with the TrpC6-V5 fusion protein, and subjected the cells to surface biotinylation, then isolated the biotinylated proteins with avidin-agarose beads and detected TrpC6 by Western blotting for its V5 tag. Consistent with the immunofluorescence data, ionomycin elicited the translocation of TrpC6 to the plasma membrane and this effect was significantly enhanced in CYP-expressing cells in the presence of the sEH inhibitor and attenuated by Rp-cAMPs (Figure 5C).

We next determined whether 11,12-EET was able to enhance the Ca^{2+} response to bradykinin and elicit the translocation of the TrpC6-V5 fusion protein. Bradykinin elicited the expected biphasic Ca^{2+} response in (nontransfected/uninfected) primary cultures of human umbilical vein endothelial cells and 11,12-EET (1 μmol/L) significantly increased the plateau phase of this response when added concomitantly with extracellular Ca^{2+} (Figure 6A). We failed to detect any effect of 14,15-EET on Ca^{2+} influx (data not shown). 11,12-EET was also able to elicit the rapid (within 30 seconds) intracellular translocation of TrpC6-V5 to the plasma membrane, more specifically to membrane domains enriched with the caveolae marker, caveolin-1 (Figure 6B).

Discussion

The results of the present investigation demonstrate that CYP2C-derived EETs modulate the agonist-induced hyperpolarization of endothelial cells by a mechanism related to cAMP/PKA, the translocation of Trp channels to the plasma membrane, and an increase in capacitative Ca^{2+} entry into native and cultured endothelial cells. The effects were most pronounced in cells treated with sEH inhibitors to prevent the rapid metabolism of EETs to biologically less active DHETs, thus highlighting the importance of the latter enzyme in the regulation of endothelial and vascular responses.

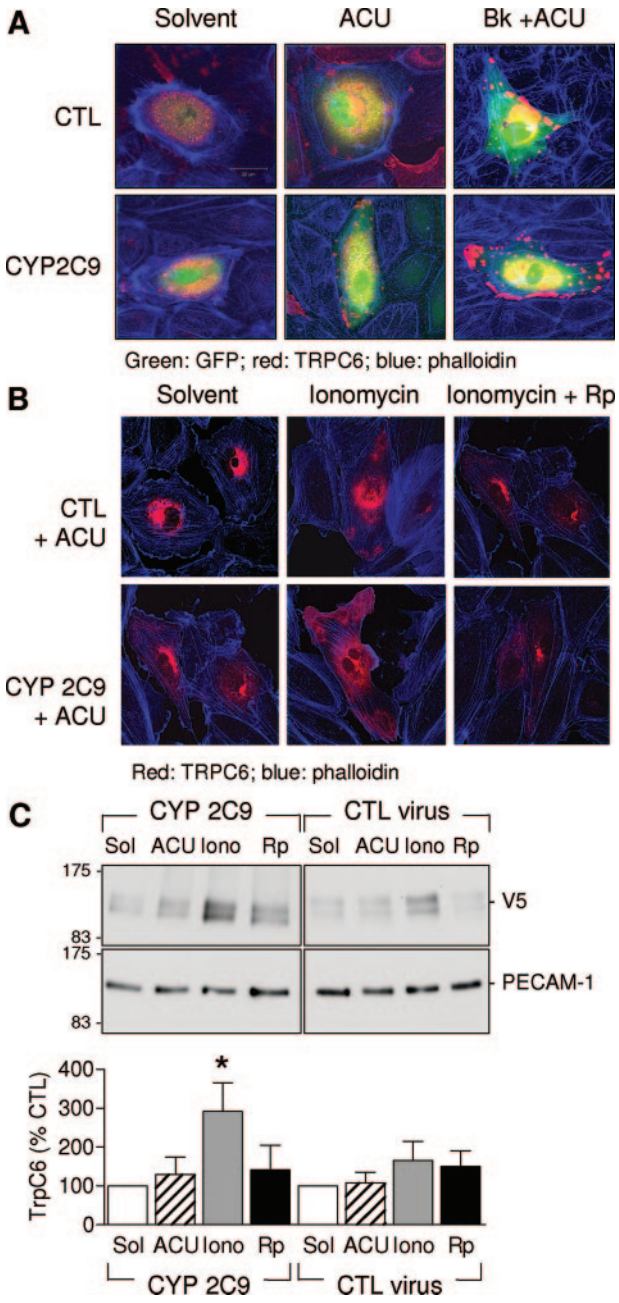

Figure 5. Effect of CYP2C9 on the translocation of TrpC6-V5 channels. Translocation of TrpC6-V5 in (A) bradykinin-stimulated primary endothelial cells and (B) ionomycin-stimulated multipassaged endothelial cells; infected with CYP2C9 or control viruses (CTL/GFP) in the absence and presence of ACU and Rp-cAMPs. C, Recovery of the surface-biotinylated TrpC6-V5 fusion protein; n=4, *$P<0.05$ vs the solvent group.

There is circumstantial evidence indicating that endogenously generated EETs affect $[Ca^{2+}]_i$ mainly from studies in cultured endothelial cells treated with CYP inducers such as β-naphthoflavone or dexamethasone.[7,25] Our data using a combination of native CYP2C-expressing cells or adenoviral overexpression and pharmacological inhibitors of CYP2C activity as well as EET degradation provide convincing evidence for a role of CYP2C-derived EETs in the regulation of capacitative Ca^{2+} entry into endothelial cells. We found similar effects in native endothelial cells, which express

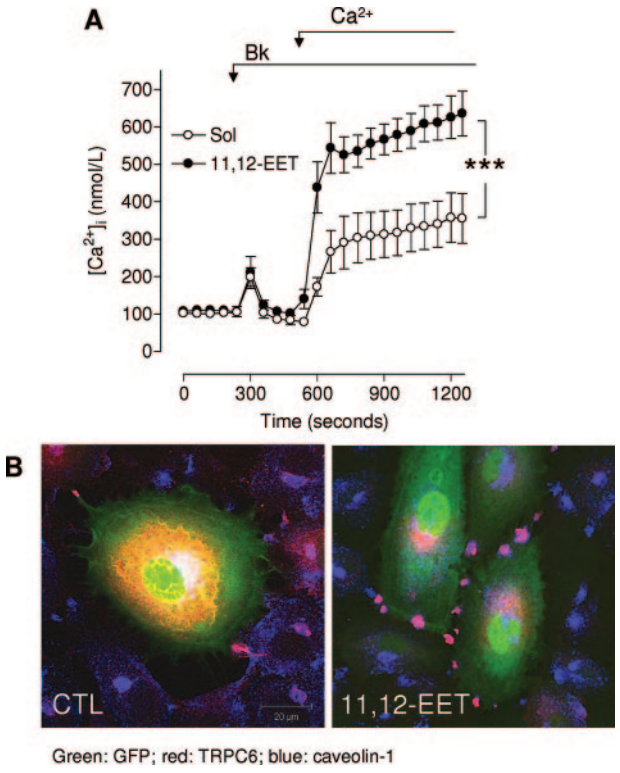

Figure 6. Effect of 11,12-EET on the Ca^{2+}-response to bradykinin and the intracellular translocation of TrpC6-V5 in primary cultures of human endothelial cells. A, The bradykinin-induced Ca^{2+} transient in the absence and presence of 11,12-EET; n=7 to 9, ***$P<0.001$ vs solvent. B, Effect of 11,12-EET (30 seconds) on the translocation of TrpC6-V5. Identical results were obtained in 3 additional experiments.

CYP2C8 and in cultured endothelial cells overexpressing CYP2C9. Although both of these CYP's synthesize 11,12- and 14,15-EET, the latter being ineffective in our hands, the stereochemistry of the EETs produced by these 2 enzymes is quite different. It will be important to determine whether different effects are observed using different EET enantiomers. Our findings also indicate that the CYP-dependent regulation of Ca^{2+} signaling is largely dependent on cAMP as well as of Trp channels. Such results imply that CYP2C-derived EETs are intracellular amplifiers of K_{Ca} channel activation and endothelial hyperpolarization and that EETs potentiate these responses by affecting nonselective cation channels of the Trp family. This hypothesis is also supported by recent data showing that both CYP induction and sEH inhibition increase TrpV4 activity in freshly isolated murine endothelial cells, and that the arachidonic acid-induced activation of TrpV4 can be attributed to a CYP-dependent process.[26] Little is known about the molecular mechanisms involved in this process although EETs have been speculated to act as endogenous ligands for Trp channels,[22] some of which possess an arachidonic acid binding site and can be activated by EETs (for review see [27]). Our data clearly indicate that the effect of EETs on Ca^{2+} influx and Trp channel translocation within endothelial cells is cAMP-dependent.

The function of Trp channels ultimately depends on their insertion into the plasma membrane, and recent data indicate that membrane insertion of the channels is a rapid and dynamic process. Indeed, in different cell lines[23] as well as in endothelial cells,[28] Trp channels insert into the plasma membrane in response to stimulation. Little is currently known about the trafficking of the different Trp channels or the mechanisms involved in its regulation, but the Rho kinase[28] is thought to be involved. Our data indicate that EETs are also able to stimulate the translocation of TrpC6 to the plasma membrane and that cAMP plays a key role in this process.

On the basis of the data presented it is clear that EETs can act intracellularly to modulate endothelial cell hyperpolarization, and it is not essential that EETs diffuse to vascular smooth muscle cells to activate BK_{Ca} channels to induce EDHF-dependent vasodilatation. Indeed, although CYP metabolites can be released from the cells in which they are generated to exert paracrine effects,[29] the majority is either not usually present in a free form or is rapidly metabolized by the sEH,[8] hinting that EETs may play an important role as intracellular second messengers. Our results also highlight the importance of the sEH in regulating cellular responses to EETs. As the sEH is highly expressed in human endothelial cells, alterations in its expression or activity, as are reported in hypertension,[30] are likely to have significant consequences on Trp-dependent Ca^{2+} signaling and autacoid production in endothelial cells and thus on vascular homeostasis. Moreover, the effects of sEH inhibitors on Ca^{2+} influx into endothelial cells may at least partly account for the depressor effects of these compounds in vivo.[31]

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Disclosures

B.D.H. Founded Arete Therapeutics to develop sEH inhibitors.

References

1. Michaelis UR, Fleming I. From endothelium-derived hyperpolarizing factor (EDHF) to angiogenesis: epoxyeicosatrienoic acids (EETs) and cell signaling. *Pharmacol Ther*. 2006;111:584–595.
2. Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res*. 1996;78:415–423.
3. Zygmunt PM, Högestätt ED. Role of potassium channels in endothelium-dependent relaxation resistant to nitroarginine in the rat hepatic artery. *Br J Pharmacol*. 1996;117:1600–1606.
4. Fisslthaler B, Popp R, Kiss L, Potente M, Harder DR, Fleming I, Busse R. Cytochrome P450 2C is an EDHF synthase in coronary arteries. *Nature*. 1999;401:493–497.
5. Weston AH, Feletou M, Vanhoutte PM, Falck JR, Campbell WB, Edwards G. Bradykinin-induced, endothelium-dependent responses in porcine coronary arteries: involvement of potassium channel activation and epoxyeicosatrienoic acids. *Br J Pharmacol*. 2005;145:775–784.
6. Imig JD, Inscho EW, Deichamnn PC, Reddy KM, Falck JR. Afferent arteriolar vasodilation to the sulfonimide analog of 11,12-epoxyeicosatrienoic acid involves protein kinase A. *Hypertension*. 1999; 33:408–413.

7. Hoebel BG, Kostner GM, Graier WF. Activation of microsomal cytochrome P450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells. *Br J Pharmacol*. 1997;121:1579–1588.
8. Newman JW, Morisseau C, Hammock BD. Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog Lipid Res*. 2005; 44:1–51.
9. Morisseau C, Goodrow MH, Newman JW, Wheelock CE, Dowdy DL, Hammock BD. Structural refinement of inhibitors of urea-based soluble epoxide hydrolases. *Biochem Pharmacol*. 2002;63:1599–1608.
10. Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondlela M, Falck JR, Campbell WB. 14,15-Epoxyeicosa-5(Z)-enoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. *Circ Res*. 2002;90: 1028–1036.
11. Morisseau C, Newman JW, Dowdy DL, Goodrow MH, Hammock BD. Inhibition of microsomal epoxide hydrolases by ureas, amides, and amines. *Chem Res Toxicol*. 2001;14:409–415.
12. Busse R, Lamontagne D. Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol*. 1991;344:126–129.
13. Michaelis UR, Falck JR, Schmidt R, Busse R, Fleming I. Cytochrome P4502C9-derived epoxyeicosatrienoic acids induce the expression of cyclooxygenase-2 in endothelial cells. *Arterioscler Thromb Vasc Biol*. 2005;25:321–326.
14. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A*. 1998;95:2509–2514.
15. Randriamboavonjy V, Busse R, Fleming I. 20-HETE-induced contraction of small coronary arteries depends on the activation of Rho-kinase. *Hypertension*. 2003;41:801–806.
16. Fleming I, Fisslthaler B, Dixit M, Busse R. Role of PECAM-1 in the shear-stress-induced activation of Akt and the endothelial nitric oxide synthase (eNOS) in endothelial cells. *J Cell Sci*. 2005;118:4103–4111.
17. Popp R, Fleming I, Busse R. Pulsatile stretch in coronary arteries elicits release of endothelium-derived hyperpolarizing factor: a modulator of arterial compliance. *Circ Res*. 1998;82:696–703.
18. Mancy A, Dijols S, Poli S, Guengerich P, Mansuy D. Interaction of sulfaphenazole derivatives with human liver cytochromes P450 2C: molecular origin of the specific inhibitory effects of sulfaphenazole on CYP 2C9 and consequences for the substrate binding site topology of CYP 2C9. *Biochemistry*. 1996;35:16205–16212.
19. Wong PY, Lai PS, Shen SY, Belosludtsev YY, Falck JR. Post-receptor signal transduction and regulation of 14(R),15(S)-epoxyeicosatrienoic acid (14,15-EET) binding in U-937 cells. *J Lipid Mediat Cell Signal*. 1997;16:155–169.
20. Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR, Roman RJ. Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K^+-channel activity. *Am J Physiol Renal Physiol*. 1996;270:F822–F832.
21. Lu T, Hoshi T, Weintraub NL, Spector AA, Lee HC. Activation of ATP-sensitive K^+ channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol (Lond)*. 2001;537:811–827.
22. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature*. 2003;424:434–438.
23. Bezzerides VJ, Ramsey IS, Kotecha S, Greka A, Clapham DE. Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol*. 2004;6:709–720.
24. Gias UA, Asrar BM. Functional role of TRPC channels in the regulation of endothelial permeability. *Pflügers Archiv Eur J Physiol*. 2005;451:131–142.
25. Graier WF, Simecek S, Sturek M. Cytochrome P450 mono-oxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells. *J Physiol (Lond)*. 1995;482:259–274.
26. Vriens J, Owsianik G, Fisslthaler B, Suzuki M, Janssens A, Voets T, Morisseau C, Hammock BD, Fleming I, Busse R, Nilius B. Modulation of the Ca^{2+} permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res*. 2005;97:908–915.
27. Clapham DE. TRP channels as cellular sensors. *Nature*. 2003;426: 517–524.
28. Mehta D, Ahmmed GU, Paria BC, Holinstat M, Voyno-Yasenetskaya T, Tiruppathi C, Minshall RD, Malik AB. RhoA interaction with inositol 1,4,5-trisphosphate receptor and transient receptor potential channel-1 regulates Ca^{2+} entry: role in signaling increased endothelial permeability. *J Biol Chem*. 2003;278:33492–33500.
29. Gauthier KM, Edwards EM, Falck JR, Reddy DS, Campbell WB. 14,15-Epoxyeicosatrienoic acid represents a transferable endothelium-dependent relaxing factor in bovine coronary arteries. *Hypertension*. 2005;45:666–671.
30. Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler MM, Hammock BD, Zeldin DC, Kroetz DL. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ Res*. 2000;87:992–998.
31. Jung O, Brandes RP, Kim IH, Schweda F, Schmidt R, Hammock BD, Busse R, Fleming I. Soluble epoxide hydrolase is a main effector of angiotensin II-induced hypertension. *Hypertension*. 2005;45:759–765.