Epoxyeicosatrienoic Acids Regulate Trp Channel Dependent Ca 2+ Signaling and Hyperpolarization in Endothelial Cells

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Conclusions
and apamin that inhibit small (SKCa) and intermediate responses are sensitive to the combination of charybdotoxin C
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-epoxyeicos-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\(^{+}\) (KCa) 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 KCa 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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Cytchrome 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 BKCa channels while most EDHF-dependent responses are sensitive to the combination of charybdotoxin and apamin that inhibit small (SKCa) and intermediate conductance (IKCa) KCa channels, 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 suggesting that the role of EETs in the EDHF phenomenon may not simply be related to the activation of BKCa channels. There is certainly circumstantial evidence linking EETs with the activation of SKCa and IKCa channels inasmuch as the EET antagonist 14,15-epoxyeicos-5(Z)-enoic acid (14,15-EEZE) attenuates the bradykinin-induced hyperpolarization of native porcine coronary artery endothelial cells. 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 vasodilation and EETs have been linked with a role in modulating intracellular Ca\(^{2+}\) levels in endothelial cells.

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).
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 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\(^{2+}\)]. were isolated as described. The transient transfection of TrpC6 was assessed in umbilical vein endothelial cells purchased from Cell Systems/Clonetics which are possible to transfec.

Isolation of RNA and RT-PCR
Total RNA was isolated using phenol and guanidine isothiocyanate (TriReagent, Sigma). RT-PCR was performed using primers derived from the CYP2C sequences (2C forward: agacaagcagacccactgcttg; reverse: cctgggtagtgggtggtt). 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. 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. 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 \(\mu\)m), 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\(^{2+}\)])
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 (Derma bond; Ethicon, Norderstedt). The preparations were then washed in nominally Ca\(^{2+}\)-free HEPES-modified Tyrode’s solution (mmol/L: NaCl, 152; KCl, 4; CaCl\(_2\), 1; MgCl\(_2\), 0.5; HEPES, 9.5 NaH\(_2\)PO\(_4\), 0.36; glucose), and [Ca\(^{2+}\)] was determined fluorometrically. At the end of each experiment, the background fluorescence was subtracted from the original signals as described. Data are presented as changes in the 340/380-nm ratio. Cultured endothelial cells (first passage) were loaded with fura 2-AM (3 \(\mu\)mol/L, 37°C, 45 minutes) and [Ca\(^{2+}\)], measured and calculated as described.

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 \(\mu\)mol/L), followed by EZ-link-sulfo NHS-SS-Biotin (Pierce, 0.3 mg/mL) and ionomycin (0.1 \(\mu\)mol/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 \(\mu\)g/mL each of leupeptin, pepstatin A, antipain, aprotonin, chymostatin and trypsin inhibitor, and PMSF, 40 \(\mu\)g/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.
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\textsuperscript{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\textsuperscript{2+}], was assessed in veins as the responses to bradykinin were more consistent. In nominally Ca\textsuperscript{2+}-free buffer, the application of bradykinin elicited a small transient increase in [Ca\textsuperscript{2+}], that was generally masked by the background signal. However, the addition of CaCl\textsubscript{2} elicited a pronounced Ca\textsuperscript{2+} increase characterized by a transient peak followed by a plateau phase. No such responses were observed in the presence of the B\textsubscript{2} kinin receptor antagonist icatibant (data not shown). The plateau phase of the Ca\textsuperscript{2+} response was elevated in vessels treated with ACU, whereas the Ca\textsuperscript{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\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores and Ca\textsuperscript{2+} influx, further experiments were performed using HUVECs (first passage) overexpressing CYP2C9. Although CYP2C8 was the more prominent endog-

Figure 2. Inhibition of the sEH potentiates bradykinin-induced Ca\textsuperscript{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).

EETs expressively 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\textsuperscript{2+}], or on the release of Ca\textsuperscript{2+} from intracellular stores (ie, the initial response to bradykinin in the absence of extracellular Ca\textsuperscript{2+}). The sEH inhibitor did not affect Ca\textsuperscript{2+} influx in CYP2C9-deficient cells but significantly increased Ca\textsuperscript{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\textsuperscript{2+}], 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\textsuperscript{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,
we assessed the consequences of a cAMP antagonist on the CYP-dependent alterations in the Ca^{2+} response to bradykinin.

RpAMPs (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 KCa channels as well as of KATP and Trp channels. 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_{CYP} 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, we assessed the consequences of CYP activation and sEH inhibition on the localization of a
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 \(\mu\)mol/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 \(\mu\)mol/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.

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 \(\beta\)-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...
Ca2+ cultures of human endothelial cells. A, The bradykinin-induced nin and the intracellular translocation of TrpC6-V5 in primary omers. Our findings also indicate that the CYP-dependent signaling is largely dependent on cAMP as well as of Trp channels. Such results imply that CYP2C-derived EETs are intracellular amplifiers of Kca 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 smooth muscle cells to activate BKca 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 Ca2+ signaling and autacoid production in endothelial cells and thus on vascular homeostasis. Moreover, the effects of sEH inhibitors on Ca2+ 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.

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