Epoxideicosatrienoic Acids Increase Intracellular Calcium Concentration in Vascular Smooth Muscle Cells

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Abstract—Epoxideicosatrienoic acids (EETs) are cytochrome P450–derived metabolites of arachidonic acid. They are potent endogenous vasodilator compounds produced by vascular cells, and EET-induced vasodilation has been attributed to activation of vascular smooth muscle cell (SMC) K+ channels. However, in some cells, EETs activate Ca2+ channels, resulting in Ca2+ influx and increased intracellular Ca2+ concentration ([Ca2+]i). We investigated whether EETs also can activate Ca2+ channels in vascular SMC and whether the resultant Ca2+ influx can influence vascular tone. The 4 EET regioisomers (1 μmol/L) increased porcine aortic SMC [Ca2+]i, by 52% to 81%, whereas arachidonic acid, dihydroxyicosatetraenoic acids, and 15-hydroxyicosatetraenoic acid (1 μmol/L) produced little effect. The increases in [Ca2+]i, produced by 14,15-EET were abolished by removal of extracellular Ca2+ and by pretreatment with verapamil (10 μmol/L), an inhibitor of voltage-dependent (L-type) Ca2+ channels. 14,15-EET did not alter Ca2+ signaling induced by norepinephrine and thapsigargin. When administered to porcine coronary artery rings precontracted with a thromboxane mimic, 14,15-EET produced relaxation. However, when administered to rings precontracted with acetylcholine or KCl, 14,15-EET produced additional contractions. In rings exposed to 10 mmol/L KCl, a concentration that did not affect resting ring tension, 14,15-EET produced small contractions that were abolished by EGTA (3 mmol/L) or verapamil (10 μmol/L). These observations indicate that 14,15-EET enhances [Ca2+]i, influx in vascular SMC through voltage-dependent Ca2+ channels. This 14,15-EET–induced increase in [Ca2+]i can produce vasosstriction and therefore may act to modulate EET-induced vasorelaxation. (Hypertension. 1999;34:1242-1246.)

Key Words: calcium channels • epoxyeicosatrienoic acid • endothelium-derived factor • vasoconstriction • vasorelaxation

Arachidonic acid (AA) is converted by cytochrome P-450 epoxygenases to 4 regioisomeric epoxyeicosatrienoic acids (EETs): 5,6-, 8,9-, 11,12-, and 14,15-EET. EETs are produced by blood vessels and are considered important regulators of vascular tone.1 EETs produce relaxation in the renal, cerebral, and coronary circulation,2–4 and this has largely been attributed to activation of Ca2+-activated K+ channels in the smooth muscle.5,6 A number of recent studies suggest that endothelium-derived hyperpolarizing factor, an unidentified substance of endothelial origin that also relaxes blood vessels by activating K+ channels, may be an epoxide metabolite(s) of AA.6–9

EETs have also been hypothesized to produce biological responses by affecting intracellular Ca2+ concentration ([Ca2+]i) in different types of cells, including endothelium,10,11 myocytes,12,13 anterior pituitary cells,14 glomerular mesangial cells,15 epithelial cells,16 and astrocytes.17 Recent reports suggest that EETs increase [Ca2+]i, and cause contraction of ventricular myocytes, probably through activation of L-type Ca2+ currents.13 EETs also increase [Ca2+]i, in vascular endothelial cells, and this may cause production of autacoids and activation of tyrosine kinases.11,18 To our knowledge, the effects of EETs on Ca2+ influx and [Ca2+]i in vascular smooth muscle cells (SMC) have not been previously reported. Since vascular SMC [Ca2+]i is an important determinant of vascular tone and SMC proliferation, EET-induced alterations in SMC [Ca2+]i could contribute importantly to vascular properties and function.

Methods

Cell Culture
Porcine thoracic arterial SMC were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO). The cells were developed from primary cultures isolated from explants and characterized as previously described.19 Cultures were maintained until confluent at 37°C in a humidified atmosphere containing 5% CO2. Stocks were subcultured weekly by trypsinization, and the cultures were used for experiments between passages 5 and 12.

Estimation of [Ca2+]i
SMC maintained in culture as described above were plated onto fibronectin-coated glass coverslips. On reaching confluence, the SMC were loaded with 2 μmol/L fura 2-acetoxymethyl ester (fura 2-AM; Sigma) in serum-free medium containing 0.1 μmol/L bovine serum albumin for 1 hour. [Ca2+]i was measured with a SPEX ARCM-COM-Z-A cation measurement system or Photoscan II spectrofluorometer (Photon Technologies International) with a Ni-
kon microscope in Hanks’ balanced salt solution (HBSS),19,20 EETs, dihydroxyicosatetraenoic acids (DHETs), AA, and 15-hydroxyicosatetraenoic acid (15-HETE) were purchased from Cayman Chemical. Lipid compounds were dissolved in ethanol; stock solutions were diluted 1:1000 in buffer or serum-free medium for experiments (final ethanol concentration, 0.1%). Verapamil, norepinephrine, thapsigargin, and ionomycin were obtained from Sigma.

**Incorporation of [3H]Dextran**

Cell permeability was measured by incorporation of [3H]dextran. Confluent SMC cultures were incubated with [3H]dextran (molecular weight=70,000 Da; 0.5 μCi per well) in the presence or absence of 1 μmol/L 14,15-EET for 1 hour in DMEM. The medium was removed, the cells were washed twice with cold phosphate-buffered saline, and cold 5% trichloroacetic acid was added for 1 hour. Trichloroacetic acid–precipitable material was solubilized in 0.25N NaOH for 30 minutes at 37°C and then neutralized with 6N HCl. Radioactivity was measured in a Packard 4640 liquid scintillation spectrometer (Canberra Corp).

**Measurement of Ring Tension**

Coronary arteries were dissected from pig hearts (n=4 animals) immediately after removal at a local slaughterhouse. The arteries were placed in modified Krebs-Ringer bicarbonate solution, transported to the laboratory, cut into rings, and studied under conditions of isometric tension, as described previously.21 The rings were repetitively contracted with KCl (60 mmol/L) until tension stabilized. 14,15-EET or 11,12-EET was administered either to rings equilibrated at resting tension or to rings submaximally precontracted (ie, to 40% to 80% of the tension produced by 60 mmol/L KCl) with U46619 (a thromboxane mimetic; 7 to 50 nmol/L), KCl (20 to 35 mmol/L), or acetylcholine (0.1 to 1 μmol/L). In some experiments, the effects of verapamil (10 μmol/L) or EGTA (3 mmol/L) on 14,15-EET–induced tension were determined.

**Statistical Analysis**

All data are expressed as mean±SE. Calculated levels of [Ca2+]i, before and after administration of EETs or vehicle were compared by Student’s t tests for paired data. Differences between mean values of 2 groups were analyzed by unpaired t tests. Probability values of ≤0.05 were considered statistically significant.

**Results**

**Effect of EETs on SMC [Ca2+]i**

Figure 1 shows that 5,6-, 8,9-, 11,12-, and 14,15-EET, at a concentration of 1 μmol/L, produced rapid increases in SMC [Ca2+]i (P<0.05 versus pretreatment values for each EET regioisomer). [Ca2+]i began to decline within 60 seconds after EET administration but remained above baseline for at least 200 seconds. No significant change in [Ca2+]i was observed with the use of vehicle control (0.1% ethanol; P>0.05 versus pretreatment value) (Figure 1). The maximal percent increases in [Ca2+]i, over pretreatment values were 52±10% for 5,6-EET, 68±7% for 8,9-EET, 81±11% for 11,12-EET, and 71±8% for 14,15-EET. Because all 4 EETs produced similar results, most of the subsequent experiments were conducted only with 14,15-EET. The effects of 14,15-EET on [Ca2+]i were dose dependent, with increases in [Ca2+]i ranging from ~17% to 94% after administration of 0.3 to 3 μmol/L 14,15-EET.

**Source of EET-Induced Increase in [Ca2+]i**

We investigated whether EETs increase SMC [Ca2+]i by enhancing the influx of extracellular Ca2+. In the presence of HBSS containing 1.3 mmol/L Ca2+, 14,15-EET (1 μmol/L) produced an increase in [Ca2+]i (Figure 2A). The EET-induced increase in [Ca2+]i was not observed when Ca2+ was omitted from the HBSS (Figure 2B); however, readdition of 3 mmol/L Ca2+ to the HBSS 100 seconds after application of 14,15-EET restored the response (Figure 2C). In some experiments, the HBSS Ca2+ concentration was varied from 0.3 to 6 mmol/L. Under these conditions, the magnitude of the increase in SMC [Ca2+]i, produced by 1 μmol/L 14,15-EET was dependent on the concentration of Ca2+ in the HBSS (Figure 3). 8,9-EET also produced only a minimal increase in [Ca2+]i in Ca2+-free HBSS (data not shown). These observations suggest that the increase in SMC [Ca2+]i, produced by EETs is due, at least in part, to enhanced influx of extracellular Ca2+.

To investigate whether 14,15-EET might enhance Ca2+ influx by nonspecifically increasing SMC membrane permeability, we determined the effects of 14,15-EET on uptake of [3H]dextran into SMC. Treatment of SMC with 14,15-EET (1 μmol/L) did not enhance [3H]dextran uptake into SMC (162±6 [control] versus 159±6 cpm per well [14,15-EET]; n=6; P>0.05).

We used verapamil to investigate the role of Ca2+ channels (L-type) in the 14,15-EET–induced increase in SMC [Ca2+]i. Verapamil (10 μmol/L), an inhibitor of voltage-dependent Ca2+ channels (L-type), eliminated the increase in SMC [Ca2+]i, induced by KCl (30 mmol/L), confirming that functional L-type Ca2+ channels are present in our cells and can be blocked by verapamil (data not shown). Verapamil markedly attenuated the increase in SMC [Ca2+]i, produced by 14,15-EET (Figure 2D), suggesting that Ca2+ influx through
voltage-dependent Ca^{2+} channels mediates the 14,15-EET–induced increase in SMC [Ca^{2+}] _i_.

To determine whether 14,15-EET can interfere with Ca^{2+} store capacity, cells were pretreated with vehicle or 5 μmol/L 14,15-EET for 30 minutes and then stimulated by 10 μmol/L norepinephrine or 2 μmol/L thapsigargin in the presence of 1.3 mmol/L extracellular Ca^{2+}. Pretreatment with 14,15-EET did not significantly affect the increases in [Ca^{2+}] _i_ induced by either of these agonists (maximal percent increase in [Ca^{2+}] _i_ over baseline: 25 ±5% [norepinephrine + vehicle; n=5] versus 34 ±9% [norepinephrine + 14,15-EET; n=6], P<0.05; 130 ±19% [thapsigargin + vehicle; n=7] versus 101 ±14% [thapsigargin + 14,15-EET; n=6], P>0.05).

**Importance of the Epoxide Groups for EET-Induced Increases in SMC [Ca^{2+}] _i_**

To determine whether the effect of EETs on SMC [Ca^{2+}] _i_ is functionally related to the epoxide groups, we examined SMC [Ca^{2+}] _i_ responses to 1 μmol/L AA, 15-HETE, and all 4 DHET regioisomers. These compounds, which are structurally similar to 14,15-EET but do not possess epoxide groups, produced only minimal increases in [Ca^{2+}] _i_ (data not shown).

**Effects of 14,15-EET on Isolated Arterial Ring Tension**

When administered to porcine coronary artery rings precontracted with U46619, 14,15-EET produced relaxation. However, when administered to rings precontracted with acetylcholine (Figure 4, top) or KCl (Figure 4, bottom), 14,15-EET produced additional contractions. 11,12-EET also produced additional contractions of rings precontracted with KCl (142±9% of KCl-induced tension at 5 μmol/L 11,12-EET).

We attempted to investigate the role of extracellular Ca^{2+} in mediating the effects of 14,15-EET on ring tension. However, removal of Ca^{2+} from the Krebs-Ringer bicarbonate solution or pretreatment with verapamil (10 μmol/L) strongly attenuated the contractions to U46619, KCl, and acetylcholine. Therefore, under these conditions, it was impossible to investigate the role of extracellular Ca^{2+} in the effects produced by 14,15-EET. We also administered 14,15-EET to nonprecontracted porcine coronary artery rings. A
measurable change in ring tension did not occur in most instances, although occasional contractions were observed. However, when nonprecontracted rings were exposed to 10 mmol/L KCl, a concentration that did not affect ring tension, and 5 µmol/L 14,15-EET was administered at 0 minutes while ring tension was monitored. Top, 14,15-EET-induced contraction was abolished by the administration of 10 µmol/L verapamil. Bottom, 14,15-EET–induced contraction was abolished by the administration of 3 mmol/L EGTA and subsequently restored by 3 mmol/L CaCl₂.

Discussion

The role of EETs in the regulation of vascular tone has recently been the subject of intensive investigation. The potent vasodilatory effects produced by EETs in a variety of blood vessels have been attributed to the activation of Ca²⁺-dependent K⁺ channels in vascular SMC.1,5,6 Activation of K⁺ channels results in hyperpolarization of vascular SMC, which in turn leads to closure of voltage-dependent Ca²⁺ channels, reduced Ca²⁺ influx, decreased [Ca²⁺], and consequently vasorelaxation.24 In the present study we report that EETs can also increase [Ca²⁺], in vascular SMC, an effect that appears to be largely mediated by enhanced influx of extracellular Ca²⁺. Although modest in magnitude, the EET-induced increases in [Ca²⁺], were, under certain conditions, capable of producing vasoconstriction. This observation was somewhat surprising because EETs have been observed to produce relaxation of bovine and porcine coronary arteries.6,9 Our findings therefore suggest that the effects of EETs on vascular tone may be variable and dependent on the experimental or physiological conditions.

The observation that EETs increase [Ca²⁺], in vascular SMC is consistent with reports of studies with a variety of other cells. For example, 5,6-EET mediates Ca²⁺ influx through verapamil- and nifedipine-sensitive channels in renal proximal tubule epithelial cells.16 5,6- and 11,12-EET significantly increased cell shortening and [Ca²⁺], in ventricular myocytes.12 In vascular endothelial cells, 5,6-EET increases [Ca²⁺], by enhancing the influx of extracellular Ca²⁺.10 Likewise, 11,12-EET enhances the L-type Ca²⁺ current in adult rat ventricular myocytes, although the Ca²⁺ influx was not blocked by a voltage-dependent Ca²⁺ channel antagonist.10,13 However, in a recent study EETs were reported to inhibit cardiac L-type Ca²⁺ channel activity.25 Thus, EETs appear to produce diverse effects on L-type Ca²⁺ channel activity and Ca²⁺ influx.

Previously it was reported that EETs can interfere with Ca²⁺ influx induced by thapsigargin, a receptor-independent Ca²⁺ agonist, and thrombin, a receptor-dependent agonist.17,26,27 However, 14,15-EET did not alter thapsigargin- or norepinephrine-induced Ca²⁺ signaling in our cells. Thus, under the experimental conditions employed in the present study, EETs do not appear to interfere with Ca²⁺ store capacity in vascular SMC.

The observation that compounds that are structurally similar to EETs but lack epoxide groups produced only minimal increases in [Ca²⁺], suggests an obligatory role for the epoxide group in stimulating Ca²⁺ influx. Interestingly, the magnitude of the EET-induced increases in [Ca²⁺], was similar for all 4 EET regioisomers, suggesting that the location of the epoxide group is not critical to the activation of Ca²⁺ influx. Although enhancement of SMC membrane permeability can result in increased Ca²⁺ influx, 14,15-EET did not increase [³H]-dextran uptake into SMC, indicating that the compound did not nonspecifically enhance membrane permeability. The pronounced inhibition of EET-induced increases in SMC [Ca²⁺], by verapamil suggests that the Ca²⁺ influx resulted from activation of voltage-dependent Ca²⁺ channels, although the mechanism by which this occurs is unknown. This EET-induced activation of voltage-dependent Ca²⁺ channels probably does not result from the effects of EETs on SMC membrane potential, since EETs reportedly hyperpolarize vascular SMC, an action that results in closure of voltage-dependent Ca²⁺ channels.24 A more likely explanation is that EETs stimulate the production of a second messenger(s) capable of modulating voltage-dependent Ca²⁺ channel activity.28 EETs are rapidly (within 30 seconds) incorporated into cellular phospholipids, primarily phosphatidylcholine and phosphatidylinositol.23 Since phosphatidylcholine and phosphatidylinositol are involved in membrane signal transduction,29 the presence of EETs in membrane phospholipids could conceivably alter vascular SMC signaling, including Ca²⁺ transport. For example, lipids released from phosphatidylcholine or phosphatidylinositol as a result of phospholipase activation may serve as signal molecules, or the phospholipids containing EET may interact with membrane ion channels.

The physiological importance of the EET-induced Ca²⁺ influx remains to be determined. One possibility is that Ca²⁺ influx is required for EET-induced activation of Ca²⁺-dependent K⁺ channels in vascular SMC, implying a role for Ca²⁺ influx in EET-induced vasorelaxation. This seems unlikely for 2 reasons. First, we found that micromolar concentrations of EETs were required to stimulate Ca²⁺ influx into vascular SMC, whereas others have reported that nanomolar concentrations of EETs activate Ca²⁺-dependent K⁺ channels in vascular smooth muscle.1,6 Second, although the increase in [Ca²⁺], produced by 11,12-DHET was far less than that produced by 11,12-EET, the 2 compounds produced equivalent amounts of relaxation in porcine coronary artery

Figure 5. Effect of 14,15-EET on nonprecontracted porcine coronary artery rings. Porcine coronary artery rings were treated with 10 mmol/L KCl (a concentration that did not affect ring tension), and 5 µmol/L 14,15-EET was administered at 0 minutes while ring tension was monitored. Top, 14,15-EET–induced contraction was abolished by the administration of 10 µmol/L verapamil. Bottom, 14,15-EET–induced contraction was abolished by the administration of 3 mmol/L EGTA and subsequently restored by 3 mmol/L CaCl₂.
rings. On the other hand, activation of K+ channels and SMC relaxation is coupled with Ca2+ "sparks," but this cannot be detected by the methods used in this study. It is therefore possible that nanomolar concentrations of EETs could produce Ca2+ sparks, which in turn mediate relaxation.

Our observations that the Ca2+ influx induced by micromolar concentrations of 14,15-EET is associated with vasoconstriction suggest that the Ca2+ influx may, under some circumstances, act to modulate EET-induced vasorelaxation. Thus, while nanomolar concentrations of EETs could initiate vasorelaxation, perhaps by producing Ca2+ sparks, higher concentration of EETs (micromolar) may yield a larger magnitude of Ca2+ influx and hence contraction. In addition to regulating vascular tone, the EET-mediated intracellular calcium mobilization may also modulate smooth muscle growth. Graber et al recently reported that 8.9- and 11,12-EET may restore intracellular Ca2+ pools and growth responses in thapsigargin-treated SMC lines.

In conclusion, the 4 EET regioisomers can produce an increase in vascular SMC [Ca2+]i, that is functionally dependent on the presence, but not the location, of the epoxide group on the fatty acyl hydrocarbon chain. The increase in [Ca2+]i, produced by 14,15-EET is largely mediated by an enhanced influx of extracellular Ca2+ through voltage-dependent Ca2+ channels. Under certain conditions, the 14,15-EET–induced influx of extracellular Ca2+ is associated with vasoconstriction, suggesting that the increases in [Ca2+]i may serve to modulate EET-induced vasodilation.

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