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*Hypertension*. 1999;34:1242-1246

doi: 10.1161/01.HYP.34.6.1242

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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# Epoxyeicosatrienoic Acids Increase Intracellular Calcium Concentration in Vascular Smooth Muscle Cells

Xiang Fang, Neal L. Weintraub, Lynn L. Stoll, Arthur A. Spector

**Abstract**—Epoxyeicosatrienoic 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  $Ca^{2+}$  channels, resulting in  $Ca^{2+}$  influx and increased intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ). We investigated whether EETs also can activate  $Ca^{2+}$  channels in vascular SMC and whether the resultant  $Ca^{2+}$  influx can influence vascular tone. The 4 EET regioisomers (1  $\mu\text{mol/L}$ ) increased porcine aortic SMC  $[Ca^{2+}]_i$  by 52% to 81%, whereas arachidonic acid, dihydroxyeicosatrienoic acids, and 15-hydroxyeicosatetraenoic acid (1  $\mu\text{mol/L}$ ) produced little effect. The increases in  $[Ca^{2+}]_i$  produced by 14,15-EET were abolished by removal of extracellular  $Ca^{2+}$  and by pretreatment with verapamil (10  $\mu\text{mol/L}$ ), an inhibitor of voltage-dependent (L-type)  $Ca^{2+}$  channels. 14,15-EET did not alter  $Ca^{2+}$  signaling induced by norepinephrine and thapsigargin. When administered to porcine coronary artery rings precontracted with a thromboxane mimetic, 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  $\mu\text{mol/L}$ ). These observations indicate that 14,15-EET enhances  $[Ca^{2+}]_i$  influx in vascular SMC through voltage-dependent  $Ca^{2+}$  channels. This 14,15-EET–induced increase in  $[Ca^{2+}]_i$  can produce vasoconstriction 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.<sup>1</sup> EETs produce relaxation in the renal, cerebral, and coronary circulation,<sup>2-4</sup> and this has largely been attributed to activation of  $Ca^{2+}$ -activated  $K^+$  channels in the smooth muscle.<sup>5,6</sup> 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.<sup>6-9</sup>

EETs have also been hypothesized to produce biological responses by affecting intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) in different types of cells, including endothelium,<sup>10,11</sup> myocytes,<sup>12,13</sup> anterior pituitary cells,<sup>14</sup> glomerular mesangial cells,<sup>15</sup> epithelial cells,<sup>16</sup> and astrocytes.<sup>17</sup> Recent reports suggest that EETs increase  $[Ca^{2+}]_i$  and cause contraction of ventricular myocytes, probably through activation of L-type  $Ca^{2+}$  currents.<sup>13</sup> EETs also increase  $[Ca^{2+}]_i$  in vascular endothelial cells, and this may cause production of autacoids and activation of tyrosine kinases.<sup>11,18</sup> To our knowledge, the effects of EETs on  $Ca^{2+}$

influx and  $[Ca^{2+}]_i$  in vascular smooth muscle cells (SMC) have not been previously reported. Since vascular SMC  $[Ca^{2+}]_i$  is an important determinant of vascular tone and SMC proliferation, EET-induced alterations in SMC  $[Ca^{2+}]_i$  could contribute importantly to vascular properties and function.

## Methods

### Cell Culture

Porcine thoracic aortic 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.<sup>19</sup> Cultures were maintained until confluent at 37°C in a humidified atmosphere containing 5%  $CO_2$ . Stocks were subcultured weekly by trypsinization, and the cultures were used for experiments between passages 5 and 12.

### Estimation of $[Ca^{2+}]_i$

SMC maintained in culture as described above were plated onto fibronectin-coated glass coverslips. On reaching confluence, the SMC were loaded with 2  $\mu\text{mol/L}$  fura 2-acetoxymethyl ester (fura 2-AM; Sigma) in serum-free medium containing 0.1  $\mu\text{mol/L}$  bovine serum albumin for 1 hour.  $[Ca^{2+}]_i$  was measured with a SPEX ARCM-COM-Z-A cation measurement system or Photoscan II spectrofluorometer (Photon Technologies International) with a Ni-

Received April 8, 1999; first decision April 29, 1999; revision accepted August 6, 1999.

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kon microscope in Hanks' balanced salt solution (HBSS).<sup>19,20</sup> EETs, dihydroxyeicosatrienoic acids (DHETs), AA, and 15-hydroxyeicosatetraenoic 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 [<sup>3</sup>H]Dextran

Cell permeability was measured by incorporation of [<sup>3</sup>H]dextran. Confluent SMC cultures were incubated with [<sup>3</sup>H]dextran (molecular weight=70 000 Da; 0.5  $\mu$ Ci per well) in the presence or absence of 1  $\mu$ mol/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.<sup>21</sup> 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  $\mu$ mol/L). In some experiments, the effects of verapamil (10  $\mu$ mol/L) or EGTA (3 mmol/L) on 14,15-EET-induced tension were determined.

### Statistical Analysis

All data are expressed as mean $\pm$ SE. Calculated levels of [ $\text{Ca}^{2+}$ ]<sub>i</sub> 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  $\leq 0.05$  were considered statistically significant.

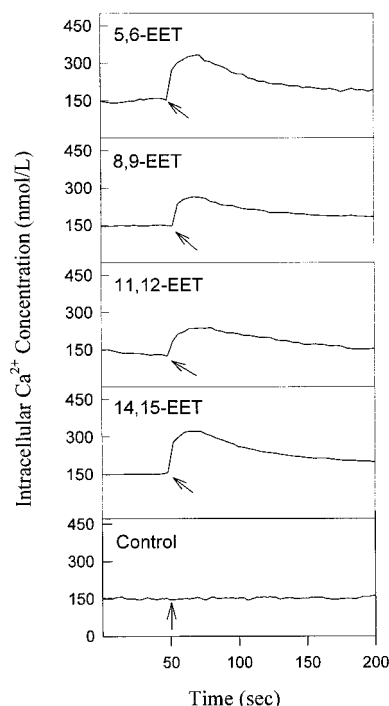
## Results

### Effect of EETs on SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub>

Figure 1 shows that 5,6-, 8,9-, 11,12-, and 14,15-EET, at a concentration of 1  $\mu$ mol/L, produced rapid increases in SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub> ( $P < 0.05$  versus pretreatment values for each EET regioisomer). [ $\text{Ca}^{2+}$ ]<sub>i</sub> began to decline within 60 seconds after EET administration but remained above baseline for at least 200 seconds. No significant change in [ $\text{Ca}^{2+}$ ]<sub>i</sub> was observed with the use of vehicle control (0.1% ethanol;  $P > 0.05$  versus pretreatment value) (Figure 1). The maximal percent increases in [ $\text{Ca}^{2+}$ ]<sub>i</sub> over pretreatment values were  $52 \pm 10\%$  for 5,6-EET,  $68 \pm 7\%$  for 8,9-EET,  $81 \pm 11\%$  for 11,12-EET, and  $71 \pm 8\%$  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 [ $\text{Ca}^{2+}$ ]<sub>i</sub> were dose dependent, with increases in [ $\text{Ca}^{2+}$ ]<sub>i</sub> ranging from  $\approx 17\%$  to 94% after administration of 0.3 to 3  $\mu$ mol/L 14,15-EET.

### Source of EET-Induced Increase in [ $\text{Ca}^{2+}$ ]<sub>i</sub>

We investigated whether EETs increase SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub> by enhancing the influx of extracellular  $\text{Ca}^{2+}$ . In the presence of HBSS containing 1.3 mmol/L  $\text{Ca}^{2+}$ , 14,15-EET (1  $\mu$ mol/L) produced an increase in [ $\text{Ca}^{2+}$ ]<sub>i</sub> (Figure 2A). The EET-induced increase in

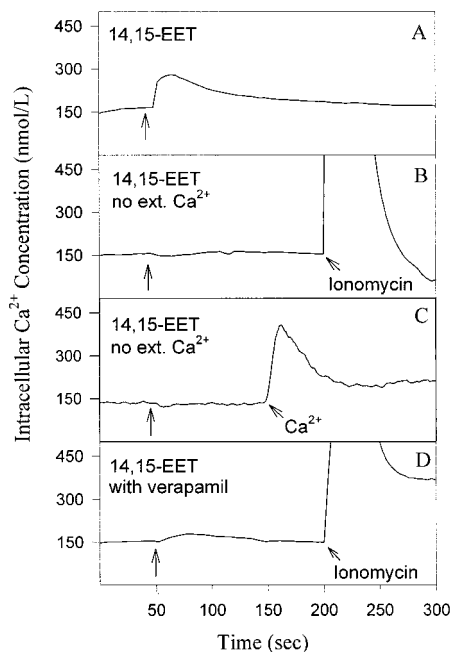


**Figure 1.** Effects of 5,6-, 8,9-, 11,12-, 14,15-EET on [ $\text{Ca}^{2+}$ ]<sub>i</sub> in porcine aortic SMC. SMC grown on glass coverslips were loaded with 2  $\mu$ mol/L fura 2-AM in serum-free DMEM for 1 hour. Fluorescence was measured with the use of the SPEX ARCM-COM-Z-A cation measurement system or Photoscan II spectrofluorometer, with excitation preset at 340 and 380 nm and emission at 505 nm. The tracing in each panel is representative of  $\geq 6$  individual coverslips in at least 4 separate experiments.

[ $\text{Ca}^{2+}$ ]<sub>i</sub> was not observed when  $\text{Ca}^{2+}$  was omitted from the HBSS (Figure 2B); however, readdition of 3 mmol/L  $\text{Ca}^{2+}$  to the HBSS 100 seconds after application of 14,15-EET restored the response (Figure 2C). In some experiments, the HBSS  $\text{Ca}^{2+}$  concentration was varied from 0.3 to 6 mmol/L. Under these conditions, the magnitude of the increase in SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub> produced by 1  $\mu$ mol/L 14,15-EET was dependent on the concentration of  $\text{Ca}^{2+}$  in the HBSS (Figure 3). 8,9-EET also produced only a minimal increase in [ $\text{Ca}^{2+}$ ]<sub>i</sub> in  $\text{Ca}^{2+}$ -free HBSS (data not shown). These observations suggest that the increase in SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub> produced by EETs is due, at least in part, to enhanced influx of extracellular  $\text{Ca}^{2+}$ .

To investigate whether 14,15-EET might enhance  $\text{Ca}^{2+}$  influx by nonspecifically increasing SMC membrane permeability, we determined the effects of 14,15-EET on uptake of [<sup>3</sup>H]dextran into SMC. Treatment of SMC with 14,15-EET (1  $\mu$ mol/L) did not enhance [<sup>3</sup>H]dextran uptake into SMC ( $162 \pm 6$  [control] versus  $159 \pm 6$  cpm per well [14,15-EET]; n=6;  $P > 0.05$ ).

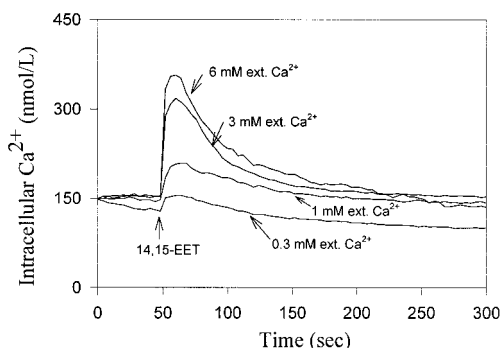
We used verapamil to investigate the role of  $\text{Ca}^{2+}$  channels (L-type) in the 14,15-EET-induced increase in SMC [ $\text{Ca}^{2+}$ ]<sub>i</sub>. Verapamil (10  $\mu$ mol/L), an inhibitor of voltage-dependent  $\text{Ca}^{2+}$  channels (L-type), eliminated the increase in SMC [ $\text{Ca}^{2+}$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



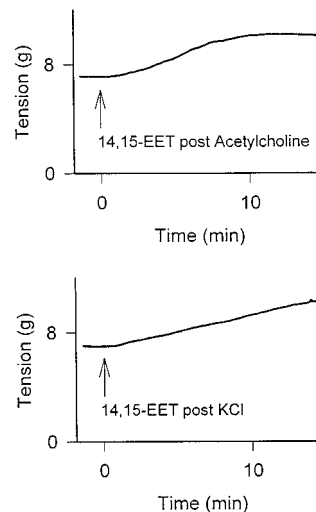
**Figure 2.** Effect of removal of external calcium and treatment with verapamil on 14,15-EET-induced increases in SMC  $[Ca^{2+}]_i$ . SMC were loaded with fura 2-AM and assayed as described in the legend to Figure 1. A,  $1 \mu\text{mol/L}$  14,15-EET in the presence of  $1.3 \text{ mmol/L}$   $Ca^{2+}$  ext. B,  $1 \mu\text{mol/L}$  14,15-EET in the absence of extracellular (ext) calcium;  $5 \mu\text{mol/L}$  ionomycin was added at 200 seconds (arrow). C,  $1 \mu\text{mol/L}$  14,15-EET in the absence of extracellular  $Ca^{2+}$ ;  $3 \text{ mmol/L}$  extracellular  $Ca^{2+}$  was added at 150 seconds (arrow). D,  $1 \mu\text{mol/L}$  14,15-EET in the presence of extracellular  $Ca^{2+}$  and the  $Ca^{2+}$  channel blocker verapamil ( $10 \mu\text{mol/L}$ );  $5 \mu\text{mol/L}$  ionomycin was added at 200 seconds (arrow). The tracing in each panel is representative of  $\geq 4$  separate assays in at least 3 separate experiments.

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  $5 \mu\text{mol/L}$  14,15-EET for 30 minutes and then stimulated by  $10 \mu\text{mol/L}$  norepinephrine or  $2 \mu\text{mol/L}$  thapsigargin in the presence of  $1.3 \text{ mmol/L}$  extracellular  $Ca^{2+}$ . Pretreatment with 14,15-EET did not significantly affect the increases in  $[Ca^{2+}]_i$  induced by



**Figure 3.** Dependence of 14,15-EET-induced increases in SMC  $[Ca^{2+}]_i$  on extracellular (ext) calcium concentration. SMC were loaded with fura-2 AM as described previously and then placed in HBSS in which the extracellular calcium concentration ranged from  $0.3$  to  $6 \text{ mmol/L}$ . The data are representative of 3 separate experiments at each calcium concentration.



**Figure 4.** Effect of 14,15-EET on tension in submaximally precontracted porcine coronary artery rings. Representative tracings depict the effects of 14,15-EET on porcine coronary artery rings ( $3$  to  $5 \text{ mm}$  in diameter) submaximally precontracted with acetylcholine (top) or KCl (bottom). Tension was continuously monitored with an isometric force transducer coupled to a polygraph. In each experiment, 14,15-EET (final concentration,  $5 \mu\text{mol/L}$ ) was introduced into the organ chamber at 0 minutes.

either of these agonists (maximal percent increase in  $[Ca^{2+}]_i$  over baseline:  $25 \pm 5\%$  [norepinephrine+vehicle;  $n=5$ ] versus  $34 \pm 9\%$  [norepinephrine+14,15-EET;  $n=6$ ],  $P>0.05$ ;  $130 \pm 19\%$  [thapsigargin+vehicle;  $n=7$ ] versus  $101 \pm 14\%$  [thapsigargin+14,15-EET;  $n=6$ ],  $P>0.05$ ).

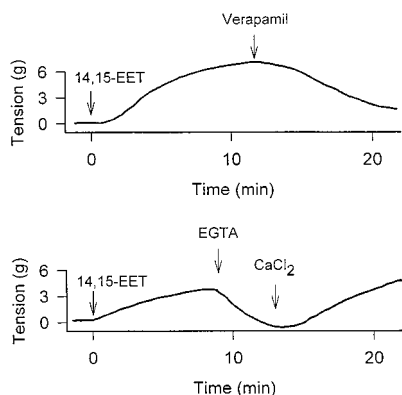
### Importance of the Epoxide Groups for EET-Induced Increases in SMC $[Ca^{2+}]_i$

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  $1 \mu\text{mol/L}$  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.<sup>22,23</sup> 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 ( $142 \pm 9\%$  of KCl-induced tension at  $5 \mu\text{mol/L}$  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 ( $10 \mu\text{mol/L}$ ) 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



**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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$  verapamil. Bottom, 14,15-EET-induced contraction was abolished by the administration of 3 mmol/L EGTA and subsequently restored by 3 mmol/L  $\text{CaCl}_2$ .

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 resting ring tension, 14,15-EET produced small contractions that were abolished by 10  $\mu\text{mol/L}$  verapamil (Figure 5, top) or 3 mmol/L EGTA (Figure 5, bottom). The effects of EGTA were reversed by adding 3 mmol/L  $\text{CaCl}_2$  to the organ bath (Figure 5, bottom).

## 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  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels in vascular SMC.<sup>1,5,6</sup> Activation of  $\text{K}^+$  channels results in hyperpolarization of vascular SMC, which in turn leads to closure of voltage-dependent  $\text{Ca}^{2+}$  channels, reduced  $\text{Ca}^{2+}$  influx, decreased  $[\text{Ca}_i^{2+}]$ , and consequently vasorelaxation.<sup>24</sup> In the present study we report that EETs can also increase  $[\text{Ca}_i^{2+}]$  in vascular SMC, an effect that appears to be largely mediated by enhanced influx of extracellular  $\text{Ca}^{2+}$ . Although modest in magnitude, the EET-induced increases in  $[\text{Ca}_i^{2+}]$  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.<sup>6,9</sup> 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  $[\text{Ca}_i^{2+}]$  in vascular SMC is consistent with reports of studies with a variety of other cells. For example, 5,6-EET mediates  $\text{Ca}^{2+}$  influx through verapamil- and nifedipine-sensitive channels in renal proximal tubule epithelial cells.<sup>16</sup> 5,6- and 11,12-EET significantly increased cell shortening and  $[\text{Ca}_i^{2+}]$  in ventricular myocytes.<sup>12</sup> In vascular endothelial cells, 5,6-EET increases  $[\text{Ca}_i^{2+}]$  by enhancing the

influx of extracellular  $\text{Ca}^{2+}$ .<sup>10</sup> Likewise, 11,12-EET enhances the L-type  $\text{Ca}^{2+}$  current in adult rat ventricular myocytes, although the  $\text{Ca}^{2+}$  influx was not blocked by a voltage-dependent  $\text{Ca}^{2+}$  channel antagonist.<sup>10,13</sup> However, in a recent study EETs were reported to inhibit cardiac L-type  $\text{Ca}^{2+}$  channel activity.<sup>25</sup> Thus, EETs appear to produce diverse effects on L-type  $\text{Ca}^{2+}$  channel activity and  $\text{Ca}^{2+}$  influx.

Previously it was reported that EETs can interfere with  $\text{Ca}^{2+}$  influx induced by thapsigargin, a receptor-independent  $\text{Ca}^{2+}$  agonist, and thrombin, a receptor-dependent agonist.<sup>17,26,27</sup> However, 14,15-EET did not alter thapsigargin- or norepinephrine-induced  $\text{Ca}^{2+}$  signaling in our cells. Thus, under the experimental conditions employed in the present study, EETs do not appear to interfere with  $\text{Ca}^{2+}$  store capacity in vascular SMC.

The observation that compounds that are structurally similar to EETs but lack epoxide groups produced only minimal increases in  $[\text{Ca}_i^{2+}]$  suggests an obligatory role for the epoxide group in stimulating  $\text{Ca}^{2+}$  influx. Interestingly, the magnitude of the EET-induced increases in  $[\text{Ca}_i^{2+}]$  was similar for all 4 EET regioisomers, suggesting that the location of the epoxide group is not critical to the activation of  $\text{Ca}^{2+}$  influx. Although enhancement of SMC membrane permeability can result in increased  $\text{Ca}^{2+}$  influx, 14,15-EET did not increase [ $^3\text{H}$ ]-dextran uptake into SMC, indicating that the compound did not nonspecifically enhance membrane permeability. The pronounced inhibition of EET-induced increases in SMC  $[\text{Ca}_i^{2+}]$  by verapamil suggests that the  $\text{Ca}^{2+}$  influx resulted from activation of voltage-dependent  $\text{Ca}^{2+}$  channels, although the mechanism by which this occurs is unknown. This EET-induced activation of voltage-dependent  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels.<sup>24</sup> A more likely explanation is that EETs stimulate the production of a second messenger(s) capable of modulating voltage-dependent  $\text{Ca}^{2+}$  channel activity.<sup>28</sup> EETs are rapidly (within 30 seconds) incorporated into cellular phospholipids, primarily phosphatidylcholine and phosphatidylinositol.<sup>23</sup> Since phosphatidylcholine and phosphatidylinositol are involved in membrane signal transduction,<sup>29</sup> the presence of EETs in membrane phospholipids could conceivably alter vascular SMC signaling, including  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  influx remains to be determined. One possibility is that  $\text{Ca}^{2+}$  influx is required for EET-induced activation of  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels in vascular SMC, implying a role for  $\text{Ca}^{2+}$  influx in EET-induced vasorelaxation. This seems unlikely for 2 reasons. First, we found that micromolar concentrations of EETs were required to stimulate  $\text{Ca}^{2+}$  influx into vascular SMC, whereas others have reported that nanomolar concentrations of EETs activate  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels in vascular smooth muscle.<sup>1,6</sup> Second, although the increase in  $[\text{Ca}_i^{2+}]$  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

rings.<sup>21-23</sup> On the other hand, activation of K<sup>+</sup> channels and SMC relaxation is coupled with Ca<sup>2+</sup> "sparks,"<sup>30</sup> but this cannot be detected by the methods used in this study. It is therefore possible that nanomolar concentrations of EETs could produce Ca<sup>2+</sup> sparks, which in turn mediate relaxation.

Our observations that the Ca<sup>2+</sup> influx induced by micromolar concentrations of 14,15-EET is associated with vasoconstriction suggest that the Ca<sup>2+</sup> influx may, under some circumstances, act to modulate EET-induced vasorelaxation. Thus, while nanomolar concentrations of EETs could initiate vasorelaxation, perhaps by producing Ca<sup>2+</sup> sparks, higher concentration of EETs (micromolar) may yield a larger magnitude of Ca<sup>2+</sup> influx and hence contraction.<sup>17,31</sup> In addition to regulating vascular tone, the EET-mediated intracellular calcium mobilization may also modulate smooth muscle growth. Graber et al<sup>32</sup> recently reported that 8,9- and 11,12-EET may restore intracellular Ca<sup>2+</sup> pools and growth responses in thapsigargin-treated SMC lines.

In conclusion, the 4 EET regioisomers can produce an increase in vascular SMC [Ca<sup>2+</sup>]<sub>i</sub> that is functionally dependent on the presence, but not the location, of the epoxide group on the fatty acyl hydrocarbon chain. The increase in [Ca<sup>2+</sup>]<sub>i</sub> produced by 14,15-EET is largely mediated by an enhanced influx of extracellular Ca<sup>2+</sup> through voltage-dependent Ca<sup>2+</sup> channels. Under certain conditions, the 14,15-EET-induced influx of extracellular Ca<sup>2+</sup> is associated with vasoconstriction, suggesting that the increases in [Ca<sup>2+</sup>]<sub>i</sub> may serve to modulate EET-induced vasodilation.

### Acknowledgments

These studies were supported by research grant HL-49264 from the National Institutes of Health and by an American Heart Association Clinician-Scientist Award (96004540) and Grant-in-Aid (9650661A). We thank Dr Gerene M. Denning for assistance with the Phototscan II spectrofluorometer calcium measurement system. We also thank Dr Gary D. Snyder for valuable discussions during the preparation of this manuscript. The authors appreciate the assistance provided by Cell Fluorescence Core Facility of the Veterans Affairs Medical Center (Iowa City, Iowa).

### References

- Harder DR, Campbell WB, Roman RJ. Role of cytochrome P-450 enzymes and metabolites of arachidonic acid in the control of vascular tone. *J Vasc Res*. 1995;32:79-92.
- Ellis EF, Police RJ, Yancey L, McKinney JS, Amruthesh SC. Dilatation of cerebral arterioles by cytochrome P-450 metabolites of arachidonic acid. *Am J Physiol*. 1990;259:H1171-H1177.
- Katoh T, Takahashi K, Capdevila J, Karara A, Falck JR, Jacobson HR, Badr KF. Glomerular stereospecific synthesis and hemodynamic actions of 8,9-epoxyeicosatrienoic acid in rat kidney. *Am J Physiol*. 1991;261:F578-F586.
- Oyekan AO, McGiff JC, Rosencrantz-Weiss P, Quilley J. Relaxant responses of rabbit aorta: influence of cytochrome P450 inhibitor. *J Pharmacol Exp Ther*. 1994;268:262-269.
- Gebremedhin D, Ma YH, Flack JR, Roman RJ, VanRollins M, Harder DR. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am J Physiol*. 1992;263:H519-H525.
- Campbell WB, Gebremedhin D, Pratt PF, Harder DR. Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res*. 1996;78:415-423.
- Bauersachs J, Hecker M, Busse R. Display of the characteristics of endothelium-derived hyperpolarizing factor by a cytochrome P450-derived arachidonic acid metabolite in the coronary microcirculation. *Br J Pharmacol*. 1994;113:1548-1553.
- Fulton D, Mahboubi K, McGiff JC, Quilley J. Cytochrome P450-dependent effects of bradykinin in the rat heart. *Br J Pharmacol*. 1995;114:99-102.
- Hecker M, Bara AT, Bauersachs J, Busse R. Characterization of endothelial-derived hyperpolarizing factor as a cytochrome P450-derived arachidonic acid metabolite in mammals. *J Physiol (Lond)*. 1994;481:407-414.
- Graier WF, Simecek S, Sturek M. Cytochrome P450 monooxygenase regulated signaling of endothelial Ca<sup>2+</sup> entry. *J Physiol (Lond)*. 1995;482:259-274.
- Hoebel BG, Steyrer E, Graier WF. Origin and function of epoxyeicosatrienoic acids in vascular endothelial cells: more than just endothelial-derived hyperpolarizing factor? *Clin Exp Pharmacol Physiol*. 1998;25:826-830.
- Moffat MP, Ward CA, Bend JR, Mock T, Farhangkhoe P, Karmazyn M. Effects of epoxyeicosatrienoic acids on isolated hearts and ventricular myocytes. *Am J Physiol*. 1993;264:H1154-H1160.
- Xiao YF, Huang L, Morgan JP. Cytochrome P450: a novel system modulating Ca<sup>2+</sup> channels and contraction in mammalian heart cells. *J Physiol (Lond)*. 1998;508:777-792.
- Snyder G, Lattanzio F, Yadagiri P, Flack JR, Capdevila J. 5,6-Epoxyeicosatrienoic acid mobilizes Ca<sup>2+</sup> in anterior pituitary cells. *Biochem Biophys Res Commun*. 1986;139:1188-1194.
- Force T, Hyman G, Hajjar R, Sellmayer A, Bonventre JV. Noncyclooxygenase metabolites of arachidonic acid amplify the vasopressin-induced Ca<sup>2+</sup> signal in glomerular mesangial cells by releasing Ca<sup>2+</sup> from intracellular stores. *J Biol Chem*. 1991;266:4295-4302.
- Madhun ZT, Goldthwait DA, McKay D, Hopfer U, Douglas JG. An epoxygenase metabolite of arachidonic acid mediates angiotensin II-induced rises in cytosolic calcium in rabbit proximal tubule epithelial cells. *J Clin Invest*. 1991;88:456-461.
- Rzagalinski BA, Willoughby KA, Hoffman SW, Falck JR, Ellis EF. Calcium influx factor, further evidence it is 5,6-epoxyeicosatrienoic acid. *J Biol Chem*. 1999;274:175-182.
- Hoebel BG, Graier WF. 11,12-Epoxyeicosatrienoic acid stimulates tyrosine kinase activity in porcine aortic endothelial cells. *Eur J Pharmacol*. 1998;346:115-117.
- Stoll LL, Spector AA. Lysophosphatidylcholine causes cGMP-dependent verapamil-sensitive Ca<sup>2+</sup> influx in vascular smooth muscle cells. *Am J Physiol*. 1993;264:C885-C893.
- Denning GM, Railsback MA, Rasmussen GT, Cox CD, Britigan BE. Pseudomonas pyocyanine alters calcium signaling in human airway epithelial cells. *Am J Physiol*. 1998;274:L893-L900.
- Weintraub NL, Fang X, Kaduce TK, VanRollins M, Charterterjee P, Spector AA. Potentiation of endothelium-dependent relaxation by epoxyeicosatrienoic acids. *Circ Res*. 1997;81:258-267.
- Fang X, Kaduce TL, Weintraub NL, VanRollins M, Spector AA. Functional implications of a newly characterized pathway of 11,12-epoxyeicosatrienoic acid metabolism in arterial smooth muscle. *Circ Res*. 1996;79:784-793.
- Fang X, Kaduce TK, Weintraub NL, Spector AA. Cytochrome P450 metabolites of arachidonic acid: rapid incorporation and hydration of 14,15-epoxyeicosatrienoic acid in arterial smooth muscle cells. *Prostaglandins Leukot Essent Fatty Acids*. 1997;57:367-371.
- Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol*. 1995;268:C799-C822.
- Chen J, Capdevila JH, Zeldin DC, Rosenberg RL. Inhibition of cardiac L-type calcium channels by epoxyeicosatrienoic acids. *Mol Pharmacol*. 1999;55:288-295.
- Malcolm KC, Fitzpatrick FA. Epoxyeicosatrienoic acids inhibit Ca<sup>2+</sup> entry into platelets stimulated by thapsigargin and thrombin. *J Biol Chem*. 1992;267:19854-19858.
- Mombouli JV, Holzmann S, Kostner GM, Graier WF. Potentiation of Ca<sup>2+</sup> signaling in endothelial cells by 11,12-epoxyeicosatrienoic acid. *J Cardiovasc Pharmacol*. 1999;33:779-784.
- 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.
- Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science*. 1992;258:607-614.
- Nelson TM, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ, Lederer WJ. Relaxation of arterial smooth by calcium sparks. *Science*. 1995;270:633-637.
- Leffler CW, Fedinec AL. Newborn piglet cerebral microvascular responses to epoxyeicosatrienoic acids. *Am J Physiol*. 1997;273:H333-H338.
- Graber MN, Alfonso A, Gill DL. Recovery of Ca<sup>2+</sup> pools and growth in Ca<sup>2+</sup> pool-depleted cells is mediated by specific epoxyeicosatrienoic acids derived from arachidonic acid. *J Biol Chem*. 1997;272:29546-29553.