12-Lipoxygenase Products Modulate Calcium Signals in Vascular Smooth Muscle Cells

Fumio Saito, Mark T. Hori, Yasufumi Ideguchi, Morris Berger, Michael Golub, Naftali Stern, and Michael L. Tuck

Previous studies have shown that inhibition of the lipoxygenase pathway of arachidonic acid metabolism can prevent the development of elevated blood pressure in renin-dependent models of hypertension. Agents that inhibit the lipoxygenase pathway such as phenidone and the flavonoid baicalein can selectively attenuate contractile responses to angiotensin II in vivo as well as in isolated vascular tissue. In the present study, the effects of lipoxygenase inhibitors on pressor-induced changes in cytosolic calcium were examined in cultured rat vascular smooth muscle cells using the fluorescent dye fura-2. Two structurally unrelated lipoxygenase inhibitors, baicalein and 5,8,11-eicosatriynoic acid, attenuated angiotensin II–stimulated increases in cytosolic calcium in both normal and calcium-poor buffer. The addition of 5-, 12-, or 15(S)-hydroxyeicosatetraenoic acid alone to the cells had no acute effect on intracellular calcium concentration. However, the addition of 12(S)-hydroxyeicosatetraenoic acid but not 5- or 15(S)-hydroxyeicosatetraenoic acid restored the initial calcium response to angiotensin II in vascular smooth muscle cells pretreated with both inhibitors; 5,8,11-eicosatriynoic acid also reduced [Arg1]-vasopressin and endothelin-stimulated increases in intracellular calcium. The attenuation of vasopressor-induced calcium transients by agents that inhibit lipoxygenase may explain their observed hypotensive effects in vivo. Moreover, lipoxygenase products, in particular 12(S)-hydroxyeicosatetraenoic acid, may act as mediators for the intracellular actions of angiotensin II and possibly other pressor hormones in vascular tissue by regulation of intracellular calcium metabolism. (Hypertension 1992;20:138–143)

KEY WORDS • lipoxygenase • angiotensin II • endothelin • vasopressins • calcium • muscle, smooth, vascular

It is well recognized that many hormone-coupled events are mediated by changes in intracellular calcium concentration ([Ca2+]i), either by release from intracellular stores or as a result of influx from the extracellular space. In vascular smooth muscle, an important pathway that follows pressor hormone–receptor coupling is activation of phospholipase C. This results in the hydrolysis of phosphatidylinositol diphosphate and the production of inositol trisphosphate (IP3) and diacylglycerol (DG). IP3 affects intracellular calcium release from the sarcoplasmic reticulum. DG couples membrane-associated protein kinase C and serves as a sustained source of arachidonic acid, presumably by activation of DG lipase. Arachidonic acid can be metabolized via several enzyme systems to biologically important compounds.

One of these enzyme complexes, the lipoxygenase (LO) pathway, may contribute to the effector mechanisms of angiotensin II (Ang II). We have shown that inhibition of the LO pathway can reduce the in vivo and in vitro vascular responses to Ang II, but not norepinephrine and potassium chloride. In bovine and human endothelial cells, Ang II–induced chemotactic generation is blocked by LO pathway inhibition but not by cyclooxygenase (CO) inhibition. Ang II–dependent inhibition of renin secretion can be reversed by LO but not CO inhibition. Additionally, 12- and 15(S)-hydroxyeicosatetraenoic acids (HETE), which are products of LO pathway metabolism, can inhibit renal renin secretion, emulating the action of Ang II. Previous observations have also implicated LO products as mediators of Ang II effects on the adrenal cortex, since various LO blockers selectively inhibit aldosterone responses to Ang II but not to adrenocorticotropic hormone or potassium.

It is not known, however, what mechanisms in hormone–response coupling might mediate the attenuation of vascular responses to Ang II during LO pathway inhibition. Changes in [Ca2+]i affect multiple intracellular steps in Ang II action, and [Ca2+]i can be altered by LO products. The LO inhibitor baicalein attenuates degranulation and Ca2+ mobilization from intracellular stores in human polymorphonuclear leukocytes. Moreover, 12-HETE enhances Ca2+ uptake in rabbit neutrophils and mobilizes calcium from liver mitochondria. LO but not CO products have been implicated as the major intermediates of arachidonic acid–induced contraction of canine cerebral arteries. Since changes in [Ca2+]i are essential for Ang II–induced...
contractile responses in vascular smooth muscle cells (VSMC), we examined the effect of LO pathway inhibition on Ang II– and other agonist-induced calcium transients in cultured VSMC.

Methods

Culture of Vascular Smooth Muscle Cells

VSMC were isolated from rat thoracic aorta (250–300 g, male Sprague-Dawley rats, Bantin and Kingman, Freemont, Calif.) by enzymatic dispersion as previously described. The harvested cells were grown in Dulbecco's modified Eagle's medium (Sigma Chemical Company, St. Louis, Mo.) supplemented with 10% fetal calf serum (HyClone Laboratory, Logan, Utah), 50 units/ml penicillin, and 50 μg/ml streptomycin (Sigma). Cells, from passage 5 to 20, were seeded onto 25-mm round glass coverslips and grown to confluence in 4–6 days. Before experimentation, cell lines were randomly screened for smooth muscle actin expression as assayed by immunofluorescent staining with anti-rat smooth muscle actin (Enzo Diagnostics Inc., New York), visualized with fluorescein conjugated rabbit anti-mouse immunoglobulin G (IgG) (Cappel Laboratories, Organon Teknika Corp., Westchester, Pa.).

Measurement of [Ca2+]i

Confluent cells attached to coverslips were deprived of serum for 24 hours before the experiments and then incubated with 4 μM fura 2-acetoxymethylester (fura 2/AM) (Molecular Probes, Eugene, Ore.) for 40 minutes at 37°C in balanced salt solution (BSS) (mM: NaCl 145, KCl 5, CaCl2 2, MgSO4•7H2O 1, Na • PO4•7H2O 0.5, glucose 6, and HEPES 10, pH 7.4) without added surfactants. Loaded cells were washed three times for 2–3 minutes and then incubated with fresh BSS for 15 minutes at 37°C to allow hydrolysis of the entrapped ester. Coverslips were rinsed in fresh BSS, fixed to a specially designed incubation well, and placed in a thermoregulated holder (maintained at 37°C) on the microscopic stage. Photon emission was monitored with a Deltascan spectrofluorometer (Photon Technology International, South Brunswick, N.J.) at 510 nm with excitation wavelength alternating between 350 (F350) and 380 (F380) nm. Changes in fura 2/AM lot number excitation wavelength alternating between 350 (F350) and 380 (F380) nm. Changes in fura 2/AM lot number

where Kd represents the dissociation constant of fura-2 for calcium (224 nM), R represents R350/380 collected in real time at 0.5-second intervals, and b represents the ratio of F380 measured in EGTA plus ionomycin to that of F380 in buffer with excess Ca2+ plus ionomycin. All data were individually corrected for autofluorescence by Mn2+ quenching, and each coverslip was calibrated separately. Autofluorescence was typically 3 to 4×10^4 counts per second and did not vary significantly during the experimental time period. This methodology does not totally exclude errors due to the development of bleached intermediates or other non-calcium-sensitive fluorescent products. Intracellular calcium values, therefore, must be interpreted as approximations. However, the control and experimental cells should have been similarly influenced. Thus, the relative changes reported would not be affected by these potential artifacts.

Experimental Protocol

The agonist-induced [Ca2+]i transients were measured after preincubation with either 0.0623 μl/ml DMSO (vehicle), 1.0 μM baicalein (a bioflavonoid inhibitor of 15- and 12-LO), or 2.5 μM 5,8,11-eicosatriynoic acid (ETI) (a substrate analogue for 5- and 12-LO)[22,23] for 20 minutes. To evaluate if HETEs could restore [Ca2+]i responses, 400 nM 12(S)-HETE, 5(S)-HETE, or 15(S)-HETE (Biomol Research Laboratories, Plymouth Meeting, Pa.) or 0.25% ethanol (vehicle) was added for 1 minute before the addition of Ang II (10^{-8} M), arginine vasopressin (AVP) (10^{-7} M), or endothelin (2×10^{-7} M). In all cases, the observed rise in calcium was immediate and rapidly reached peak levels within 20 seconds of agonist challenge (see Figure 1 for illustration). 5(S)-HETE and 15(S)-HETE, which are naturally occurring products of alternate LO pathways in some tissues, were used as controls for 12-HETE add-back experiments for ETI and baicalein, respectively, because of the reported effects of these inhibitors on these pathways, as a test of specificity.

Statistics

Results are expressed as mean±SEM. Data were evaluated by analysis of variance with subset analysis by Tukey's group-to-group comparison.

Results

Effect of Baicalein and ETI on Angiotensin II–Induced [Ca2+]i Transients

Basal [Ca2+]i levels were not significantly different among the VSMC of the four experimental protocols (Table 1). The addition of baicalein inhibited (p<0.05) 100 nM Ang II–induced increments in [Ca2+]i. However, the initial phase of the calcium response to Ang II could be restored by addition of 12(S)-HETE to VSMC pretreated with baicalein. Restoration of [Ca2+]i responses was not observed with addition of 15(S)-HETE to baicalein-blocked cells. The order of additions appeared important for the restoration experiments. The calcium signals could be consistently restored in treated cells when 12(S)-HETE was added 1 minute before the addition of Ang II. However, when Ang II and 12(S)-HETE were added simultaneously or when 12(S)-HETE was added after the application of Ang II,
calcium responses to Ang II could not be restored. These results suggest that LO products must be available in the cell in sufficient concentration to transmit the Ang II–induced calcium signals. ETI also inhibited the peak Ang II–induced [Ca\textsuperscript{2+}] responses in VSMC as shown in Table 2. This inhibition was likewise restored by the addition of 12(S)-HETE but not by 5(S)-HETE. The averaged traces from 1 day of experiments showing ETI inhibition of calcium transients and restoration by 12(S)-HETE are shown in Figure 1. In all cases, 0.25% ethanol vehicle with or without 5-, 12-, or 15(S)-HETE caused a small (10–15 nM), transient increase in [Ca\textsuperscript{2+}]. These increments rapidly returned to baseline and stabilized in approximately 30 seconds. There were no significant differences in this effect between treatments.

To evaluate the contribution of extracellular calcium, 4 mM EGTA was added to the medium 19 minutes after the addition of ETI and 1 minute before the addition of Ang II. In this set of experiments addition of EGTA alone decreased basal [Ca\textsuperscript{2+}] by 12%. Subsequent Ang II–stimulated transients were preserved, although they were slightly (−5%) smaller than in the 2 mM calcium buffer. In this calcium-poor buffer, there was no significant difference in basal [Ca\textsuperscript{2+]}, between control and ETI-treated cells. However, the Ang II–stimulated increase in [Ca\textsuperscript{2+}] was smaller in the ETI-treated cells than in the control cells (Table 3).

ETI also inhibited AVP and endothelin-stimulated increase in [Ca\textsuperscript{2+}] in the buffer with 2 mM Ca\textsuperscript{2+}. The incremental changes in [Ca\textsuperscript{2+}], were: for 100 nM AVP, 143.3±19.0 versus 56.0±16.0 nM, in the absence or presence of ETI, respectively, n=4 for each protocol, p<0.01; and for 200 nM endothelin, 393.9±97.7 versus 123.5±37.0 nM, in the absence and presence of ETI, respectively, n=8 for each treatment, p<0.05 (Figures 2 and 3). The same effects were observed in the calcium-poor medium (changes in [Ca\textsuperscript{2+]}: 100 nM AVP, 343.2±86.8 versus 95.9±4.9 nM, in the absence or presence of ETI, n=6 for each treatment, p<0.05; 200 nM endothelin, 158.5±6.7 versus 75.8±18.8 nM, in the absence or presence of ETI, n=4 for each treatment, p<0.01) (Figure 4).

**Discussion**

We reported that the nonspecific LO/CO pathway blocker phenidone attenuated the development of hypertension in the Goldblatt two-kidney, one clip renovascular hypertensive rat model\textsuperscript{22} but not in the deoxycorticosterone acetate–salt model of hypertension.\textsuperscript{23} In addition, intraperitoneal pretreatment with LO pathway blockers such as phenidone, baicalein, and esculetin can attenuate pressor responses to Ang II infusion but not to norepinephrine in Sprague-Dawley rats.\textsuperscript{6} Phenidone and baicalein also inhibited contractile responses to Ang II but not to norepinephrine or potassium chloride in isolated rat femoral artery segments.\textsuperscript{6} These studies offered preliminary support for a selective inhibitory effect of LO pathway blocking agents on the vascular actions of Ang II and provide strong evidence that LO pathway products are involved in the mediation of the actions of Ang II in vascular tissue. Since changes in intracellular calcium represent a major step in the cellular actions of Ang II in vascular tissue, it is possible that LO pathway products alter this function in VSMC.

**Table 1. Effect of Baicalein on Angiotensin II–Induced Calcium Transients**

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal</th>
<th>Peak</th>
<th>Change</th>
<th>Change (%)</th>
</tr>
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<tbody>
<tr>
<td>Control (n=14)</td>
<td>103.8±9.9</td>
<td>250.3±17.2</td>
<td>146.6±11.7</td>
<td>147.0±17.1</td>
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<tr>
<td>Baicalein (1×10^{-4} M)</td>
<td>121.6±9.1</td>
<td>199.5±13.6*</td>
<td>4.9±5.0*</td>
<td>64.6±2.7*</td>
</tr>
<tr>
<td>ETI (2.5×10^{-7} M)</td>
<td>125.5±12.2</td>
<td>256.6±22.9†</td>
<td>131.1±16.0†</td>
<td>107.8±13.8§</td>
</tr>
<tr>
<td>ETI+5(S)-HETE (2×10^{-7} M)</td>
<td>86.6±27.6</td>
<td>145.6±26.9*</td>
<td>58.9±30.2*</td>
<td>88.1±62.9*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal</th>
<th>Peak</th>
<th>Change</th>
<th>Change (%)</th>
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</thead>
<tbody>
<tr>
<td>Control (n=11)</td>
<td>104.3±6.7</td>
<td>435.2±69.7</td>
<td>330.9±71.7</td>
<td>346.1±79.9</td>
</tr>
<tr>
<td>ETI (2.5×10^{-4} M)</td>
<td>98.2±10.5</td>
<td>233.2±38.4*</td>
<td>124.6±36.0*</td>
<td>152.8±37.7*</td>
</tr>
<tr>
<td>ETI+12(S)-HETE (4×10^{-7} M)</td>
<td>93.8±13.9</td>
<td>387.7±62.1†</td>
<td>293.9±60.8†</td>
<td>365.0±88.2†</td>
</tr>
<tr>
<td>ETI+5(S)-HETE (4×10^{-7} M)</td>
<td>112.2±19.7</td>
<td>175.5±46.6*</td>
<td>63.3±53.9*</td>
<td>85.5±75.0*</td>
</tr>
</tbody>
</table>

Results (mean±SEM) showing the effect of 20-minute treatment with 2.5 μM 5,8,11-eicosatriynoic acid, a suicide substrate analogue for 5- and 12-lipoxygenase, on 100 nM angiotensin II–mediated intracellular calcium mobilization. ETI, 5,8,11-eicosatriynoic acid; [Ca\textsuperscript{2+}], intracellular calcium concentration; HETE, hydroxyeicosatetraenoic acid.

\*p<0.01 compared with control.

\†p<0.05 compared with control.

\§p<0.05 compared with baicalein.

\$p<0.01 compared with baicalein and baicalein plus 15(S)-HETE.

\&p<0.05 compared with control.
FIGURE 1. Effect of the lipoxygenase blocker 5,8,11-eicosatriynoic acid (ETI) on 100 nM angiotensin II (Ang II)-induced calcium mobilization in vascular smooth muscle cells. Representative cytosolic calcium concentration ([Ca$^{2+}$]$_i$) tracings of the three different treatments for a single day of experiments. Each line was generated by the graphic averaging of the final data for three experimental runs. DMSO vehicle or 2.5 µM ETI in vehicle was added 19 minutes before tracing. For add-back experiments, either 0.25% ethanol vehicle, or 400 nM 12(S)-hydroxyeicosatetraenoic acid (HETE) was added at time 0. Ang II (100 nM) was added at 60 seconds in all three traces. Note attenuation of initial calcium response with ETI and restoration by 12(S)-HETE.

We now report that two structurally unrelated inhibitors of the LO pathway, baicalein and ETI, attenuate Ang II-induced calcium transients as studied in cultured rat VSMC. Moreover, the finding that addition of the LO pathway product 12-HETE can restore the initial Ang II-induced [Ca$^{2+}$]$_i$ responses in LO-blocked VSMC provides evidence that this LO product may participate in the modulation of Ang II-dependent calcium transients in VSMC. In contrast, the absence of an effect with 5-HETE and 15-HETE on LO-blocked VSMC demonstrates the specificity of 12-HETE in the vascular actions of Ang II. The fact that direct addition of 12-HETE to cultured VSMC did not affect [Ca$^{2+}$], suggests that this LO product may act more as a cofactor in the action of Ang II in vascular tissue. However, the current studies do not resolve this issue.

An important assumption in the proposed series of events for LO pathway involvement in Ang II action in the vasculature is that the LO pathway products are present in sufficient amounts in vascular cells and can respond to Ang II. There is some controversy regarding the levels and biosynthetic origin of HETE production in vascular tissue. Takayama et al. reported finding only minute amounts of LO pathway products in vascular tissue. Bailey et al. provided evidence for 11- and 15-HETE synthesis in cultured smooth muscle cells but attributed their production to the CO rather than LO pathway. In contrast, Greenwald et al. showed signifi-

### TABLE 3. Effect of Calcium-Deficient Buffer on 5,8,11-Eicosatriynoic Acid Attenuation of Angiotensin II-Induced Calcium Transients

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal (nM)</th>
<th>Change (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=6)</td>
<td>70.2±13.9</td>
<td>113.3±13.9</td>
</tr>
<tr>
<td>ETI (2.5 µM) (n=6)</td>
<td>63.8±11.6</td>
<td>22.4±3.6*</td>
</tr>
</tbody>
</table>

Results (mean±SEM) of the effect of 2.5 µM ETI on 100 nM angiotensin II induced calcium transients in calcium-deficient buffer. ETI, 5,8,11-eicosatriynoic acid; [Ca$^{2+}$], intracellular calcium concentration. *p<0.05 vs. control.

FIGURE 2. Bar graph shows effect of 5,8,11-eicosatriynoic acid (ETI) on arginine vasopressin (AVP)- and endothelin (ENDT)-stimulated increases in intracellular calcium concentration ([Ca$^{2+}$]$_i$) in a buffer containing 2 mM calcium. Peak calcium responses to 100 nM AVP and 200 nM ENDT-I with and without 2.5 µM ETI are shown. Due to variability in calcium responses from cell line to cell line, changes are expressed as percentage of basal [Ca$^{2+}$]$_i$ levels for comparison. ETI-treated cells have mean maximal [Ca$^{2+}$]$_i$ that is reduced by 60.9% for AVP (**p<0.01 vs. AVP alone) and 68.6% for ENDT (*p<0.05 vs. ENDT alone).

FIGURE 3. Effect of 5,8,11-eicosatriynoic acid (ETI) on arginine vasopressin (AVP) and endothelin 1-stimulated cytosolic calcium concentration ([Ca$^{2+}$]$_i$). Representative real time tracings of endothelin (panel A) and AVP (panel B) stimulated calcium transients showing the effect of 20-minute pretreatment with 2.5 µM ETI. Addition of the agonist occurred at 60 seconds as indicated by the arrows.
cant LO activity in freshly prepared rabbit aortic rings and found that HETE production could be reduced by LO pathway inhibitors. Larrue et al identified considerable basal activity of the 5-, 12-, and 15-LO pathways in cultured rabbit aortic smooth muscle cells and reported that activated cells produced predominantly 12-HETE. We have also observed significant 12-HETE production in de-endothelialized aortic tissue that was reduced by LO pathway inhibitors. Significant amounts of 5-, 12-, and 15-HETE products have also been noted in cultured endothelial cells taken from bovine coronary arteries. More recently, Moore and coworkers described LO activity in brain microvessels that produced 12(S)-HETE when stimulated by addition of exogenous arachidonic acid or the calcium ionophore A23187.

The correlation between vascular contraction and a rise in [Ca^{2+}], is well documented. However, the mechanism of calcium mobilization is influenced by multiple factors and varies with different agonists. Noradrenaline and potassium chloride addition produces a sustained contraction that is dependent on extracellular calcium. When added to vascular tissue, Ang II causes a transient increase in [Ca^{2+}], and a corresponding brief contractile response. Ang II–induced calcium transients are maintained in a calcium-free medium, suggesting that mobilization of intracellular calcium stores is primarily responsible for the observed changes in [Ca^{2+}]. The selective effects of LO inhibitors on Ang II responses compared with other vascular agonists in vivo and in vitro may be due to differences in the cellular mechanism leading to the [Ca^{2+}] response. This assumption is supported by our results in the calcium-poor buffer, in which ETI also blocked Ang II–stimulated increases in [Ca^{2+}]. In cultured VSMC, both AVP- and endothelin-stimulated [Ca^{2+}], transients are reported to be preserved in the absence of extracellular calcium. In our experiments, AVP- and endothelin-induced [Ca^{2+}], transients were inhibited by ETI in both 2 mM and calcium-poor buffer. The fact that the inhibitory effect of ETI on Ang II is also observed with AVP- and endothelin-mediated [Ca^{2+}], indicates that LO inhibition alters the mobilization of [Ca^{2+}] from intracellular sites via the same mechanism.

Considerable evidence indicates that IP_{3}-mediated [Ca^{2+}] release from sarcoplasmic reticulum is responsible for transient increase in [Ca^{2+}]. IP_{3} and DG are derived by hydrolysis of phosphatidylinositol 4,5-di-phosphate by a specific receptor-linked phospholipase. Further studies into interactions between LO products and IP_{3}-mediated calcium release are needed to elucidate possible interactions in this pathway.

In summary, we have shown that LO pathway inhibition in VSMC also leads to a reduction in Ang II–stimulated [Ca^{2+}]. When 5-, 12-, and 15-HETE are added alone to cultured VSMC, they have little effect on [Ca^{2+}]. However, under the experimental conditions where VSMC are pretreated with LO inhibitors, the inhibitory effect on Ang II calcium transients can be reversed by addition of 12-HETE, but not by 5-HETE or 15-HETE. Thus, the LO pathway of arachidonic acid metabolism, in particular 12-HETE, may participate in the contractile actions of Ang II through modulation of the intracellular calcium responses to Ang II in VSMC. The observation that LO inhibitors also block AVP- and endothelin-stimulated calcium signals suggests that LO products might be involved in effector mechanisms of other vasoactive peptide agonists that use the calcium-DG messenger system.

Acknowledgments

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