Inhibitors of Arachidonic Acid Metabolism Have Variable Effects on Calcium Signaling Pathways

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The metabolic pathways of arachidonic acid (AA) have been shown to be important in the regulation of cellular function. Several studies have demonstrated both direct and indirect effects of products of these pathways in the regulation of vascular actions, and in particular on signaling pathways. Because intracellular calcium concentration is a significant mediator of stimulus-coupled signal transduction, we investigated the effects of AA pathway inhibitors on angiotensin II (Ang II)-induced calcium mobilization in cultured rat vascular smooth muscle cells (VSMC). Thus, specific calcium pools were examined for differential effects resulting from inhibitors of the three major pathways of AA metabolism. Inhibition of lipoygenase (LO) with 2.5 μmol/L of 5,8,11 eicosatriynoic acid (ETI), cyclooxygenase (CO) with 2 μmol/L of ibuprofen (IBU), and cytochrome P-450 (P-450) with 1 μmol/L of 7-ethoxyresorufin (7ER) all reduced total Ang II-induced intracellular calcium transients ([Ca²⁺])₂ in fura 2-loaded cultured rat VSMC. However, the sites of action affected were unique for each inhibitor. Pretreatment of VSMC with either ETI or IBU inhibited thapsigargin (TG) (1 μmol/L)-sensitive calcium increments (control; 118.0 ± 33.1 nmol/L, n = 9, ETI; 34.7 ± 4.8 nmol/L, n = 9, IBU; 40.3 ± 8.8 nmol/L, n = 8, P < .05 v control). Both caffeine (CAF) and ryanodine (RY) treatment attenuated Ang II-induced [Ca²⁺]; however, pretreatment with ETI, IBU, or 7ER did not alter this effect. In other studies, the LO inhibitor ETI attenuated Ang II-induced Ca²⁺ influx, whereas inhibitors of CO and P-450 pathways had no effect. These data show that 1) ETI and IBU affect TGSensitive Ca pools; 2) ETI, but not IBU nor 7ER, inhibited calcium influx; and 3) ETI, IBU, and 7ER affect similar intracellular calcium stores as CAF and RY. Thus, agonist-specific differences in the effects of LO, CO, and P450 inhibition on vascular actions may be due to site-specific effects of these inhibitors on calcium mobilization. Am J Hypertens 2001;14:248–253 © 2001 American Journal of Hypertension, Ltd.

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Investigations into the physiologic importance of the eicosanoids have revealed a complex of multiple pathways. Arachidonic acid (AA), the principal substrate for these products, is a 20-carbon fatty acid that is incorporated in the phospholipids of the cellular plasma membranes and is released by the action of stimulus-coupled phospholipase enzymes. The activation of the phospholipase is a critical step, which can be triggered by various physical or hormonal stimuli, including angiotensin II (Ang II). In several tissues, phospholipase A₂ is activated by a calcium/calmodulin complex, which is sensitive to changes in intracellular calcium concentration. The action of phospholipase C, which acts on the substrate phosphatidylinositol, produces the important intracellular messengers inositol triphosphate (IP₃) and diacylglycerol (DAG). DAG is acted upon by lipases to release AA, one of several enzyme systems. The cyclooxygenase (CO) enzyme complex generates the prostaglandins and thromboxanes. The lipoygenase (LO) enzymes form the hydroperoxyeicosatetraenoic acids (HPETE), which are then rapidly converted to the more stable hydroxyeicosatetraenoic acids (HETE). Various cytochrome P-450 enzyme systems can use AA as a substrate to form HETE, epoxides, and dihydroxy-HETE.

Several studies have described elements of action for the eicosanoids in intracellular signaling pathways. Many hormone receptor-coupled events are mediated by changes in cytosolic calcium ([Ca²⁺]), either by release from intracellular stores, or by influx from the extracellular space. In vascular tissue, pressor agents such as potassium chloride (KCl) and norepinephrine (NE) produce both a sustained contraction and a parallel increase in [Ca²⁺] that is primarily dependent on extracellular calci-
whereas Ang II contractions are characterized by a calcium profile that includes an initial calcium spike, followed by a smaller, sustained elevation in $[Ca^{2+}]_i$. We have shown that pressor agonists such as Ang II, arginine vasopressin (AVP), and endothelin-1, induce increases in $[Ca^{2+}]_i$ in vascular smooth muscle cells (VSMC) that are markedly attenuated by agents that inhibit the LO pathway.

These data provide the basis for studies to determine how eicosanoids regulate various $Ca^{2+}$ sources in VSMC in response to agonist stimulation. Potential sites for these effects include inositol triphosphate-induced $Ca^{2+}$ mobilization, $Ca^{2+}$ fluxes, $Ca^{2+}$-sensitive calcium release, and the Ca-ATPase pump. Because Ang II-induced calcium signals involve participation by multiple calcium pathways, we use this model to investigate the effects of inhibitors of eicosanoid metabolism on the regulation of these pathways.

**Methods**

Vascular smooth muscle cells were isolated from the thoracic aorta of male Sprague-Dawley rats (250 to 300g, Bantin and Kingman, Freemont, CA) by enzymatic dispersion as previously reported. Cells, from passage 4 to 12, were seeded onto 25-mm round glass coverslips and grown to confluence. After overnight serum deprivation, cells were loaded with 4 $\mu$mol/L fura 2-acetoxymethyl-ester (Molecular Probes, Eugene, OR) for 40 min at 37°C in a HEPES buffered balanced salt solution, washed, and then incubated for an additional 20 min to allow hydrolysis of the entrapped acetyl methyl ester. Intracellular calcium changes were monitored using a Deltascan Spectrofluorometer (Photon Technology International, South Brunswick, NJ) at 510 nm with excitation wavelengths alternating between 340 (F340) and 380 (F380) nm. At the end of each experiment, the minimum ($R_{min}$) and maximum ($R_{max}$) ratio of F340 and F380 ($R_{350/380}$) was determined in buffers containing 0 $\mu$mol/L $Ca^{2+}$ plus 2 $mmol/L$ EGTA and 10 $mmol/L$ $[Ca^{2+}]_i$, respectively, and the intracellular calcium concentration was then calculated from the resulting data by ratio analysis according to the method of Grynkiewicz et al. All data were individually corrected for autofluorescence by Mn$^{2+}$ quenching and each coverslip was calibrated separately.

To evaluate the effect of AA pathway inhibition historic ED$_{50}$ doses of 2.5 $\mu$mol/L of 5,8,11 eicosatriynoic acid (ETI), 2 $\mu$mol/L of ibuprofen (IBU), or 1 $\mu$mol/L of 7-ethoxyresorufin (7ER) were used to inhibit lipoxigenase, cyclooxygenase, and cytochrome P-450, respectively. For total intracellular calcium determinations, loaded cells were treated with inhibitors for 20 min, and then challenged by 100 $nmol/L$ of Ang II. Thapsigargin (TG) is a sesquiterpene lactone that is a potent inhibitor of microsomal $Ca^{2+}$-ATPase and has been used to evaluate inositol triphosphate (IP$_3$)-sensitive calcium stores without activating IP$_3$ receptors. In these studies, VSMC were loaded with fura 2 as described above, treated with AA inhibitors, and challenged with 1 $\mu$mol/L of TG. Coverslips were then calibrated and $[Ca^{2+}]_i$ calculated as before.

Ang II-induced calcium influx was estimated by using the fura 2 quenching characteristics of ionic manganese (Mn). $MnCl_2$ (250 $\mu$mol/L) as a surrogate for calcium was added 30 sec before the addition of 10 $nmol/L$ of Ang II. Total fluorescence data, calculated as either the sum of F340 and F380, or as the fluorescence emission after excitation at 360 nm (the isosbestic point for fura 2) were collected for 2 min after Ang II addition. The total fluorescence emission in photons per second was then converted to the percent decay using the Ang II addition point as 100%. Finally, the maximum quench rate was determined using the first order derivative of the resulting quench curve. This rate was defined as the momentary maximum point of percent change in total fluorescence.

Caffeine (CAF)- and ryanodine (RY)-sensitive calcium pools were also evaluated for their sensitivity to AA pathway inhibitors. In these experiments, cells were treated with either 10 $mmol/L$ of CAF or 10 $\mu$mol/L of RY in 0.05% dimethyl sulfoxide (DMSO) vehicle for 5 min before the addition of 100 $nmol/L$ of Ang II.

**Animal Care**

All animals were housed according to institutional VA Medical Center and VA Central Office guidelines, and the protocols approved by the Animal Studies Subcommittee of the Research and Development Committee, Veterans Affairs Greater Los Angeles Healthcare System, Sepulveda, California.

**Statistics**

Results are expressed as mean ± SEM. Group comparisons were evaluated by analysis of variance with subsequent subcomparisons by Tukey analysis. In the case of comparisons between treated and untreated patients within the same treatment group (ie, with or without ibuprofen or before and after Ang II) comparisons were done by paired Student’s $t$ test.

**Results**

Resting intracellular calcium levels for the total calcium determinations were 109.4 ± 14.7 $nmol/L$. Treatment of cultured rat VSMC with each of the inhibitors of AA metabolism had no effect on basal levels of intracellular calcium, but attenuated the maximal increment of Ang II-induced calcium mobilization by 62.3% ± 11.3%, 58.6% ± 5.4%, and 68.4% ± 4.3% v control for ETI, IBU, and 7ER, respectively (Fig. 1). Further experiments, however, revealed that the specific pools affected were unique to each inhibitor.

Thapsigargin treatments of VSMC cause a rapid increase in the intracellular calcium concentration, which is sustained over several minutes. For logistic reasons, the experiments to investigate the effects of metabolic AA
inhibitors on TG-sensitive calcium pools were conducted in two stages. In the first stage, the effects of LO inhibition with ETI was compared to CO inhibition with IBU. In these experiments both ETI and IBU inhibited TG-mediated calcium release (Fig. 2A). The increment of calcium for controls in these studies was 118.0 ± 33.0 nmol/L; however, pretreatment of the cells with 2.5 μmol/L ETI resulted in a significant reduction in the [Ca2+]i response to TG to 34.7 ± 4.9 nmol/L and 2 μmol/L IBU treatment reduced the TG response to 40.3 ± 8.8 nmol/L (P < .05 for each experimental group). In a separate study, ETI again attenuated the response to TG compared to vehicle controls; however, pretreatment with 1 μmol/L 7ER had no effect (Fig. 2B).

Using ionic Mn as a surrogate for calcium, experiments were undertaken to evaluate the sensitivity of calcium influx to eicosanoid inhibitors. After the Mn2+ -mediated fluorescence decay initiated by Ang II addition, we found that none of the inhibitors tested had any effect on basal levels of calcium influx (Fig. 3). Pretreatment of VSMC with 2.5 μmol/L of ETI attenuated the maximal Ang II-induced rate of influx by 52% compared to control (Fig. 4); however, IBU and 7ER had no effect on either total influx or maximal influx rate.

Caffeine- and ryanodine-sensitive calcium pools are typically released by initial increases in intracellular calcium released from other sources (ie, IP3-sensitive pools). As expected, CAF and RY pretreatment inhibited Ang II-induced calcium transients; however, pretreatment of VSMC with ETI, IBU, or 7ER before the addition of CAF or RY did not alter these effects (data not shown).

**Discussion**

This study demonstrates for the first time that inhibitors of eicosanoid metabolism can affect calcium increments in vascular cells at several sites. Thus, the effects of the inhibitors on the resting calcium state was nil in all cases. Moreover, all three AA pathway inhibitors attenuated the total Ang II-induced intracellular calcium signals, and none altered the CAF or RY effects on Ang II calcium mobilization. Experiments into the mechanism of action of each inhibitor, however, showed unique effects of each on the calcium pools affected. Thus, the LO inhibitor ETI attenuated calcium mobilization from both TG-sensitive and Ang II-induced influx, whereas the CO inhibitor IBU reduced calcium released by TG, but had no effect on calcium influx. The cytochrome P-450 inhibitor 7-ethoxyresorufin attenuated total Ang II-induced intracellular calcium concentration, but did not demonstrate measurable effects on the isolated pools tested.
The importance of the regulation of intracellular calcium concentration on vascular action is demonstrated by the positive correlation between vascular contraction and an increase in \([\text{Ca}^{2+}]_{i}\). Because changes in \([\text{Ca}^{2+}]_{i}\) are an essential element for many contractile events in VSMC, the effect of eicosanoids on calcium trafficking is of considerable interest. Changes in intracellular calcium can affect multiple pathways, and the mechanisms that regulate calcium responses are influenced by several factors that vary with different stimuli. Norepinephrine and KCl have been shown to produce a sustained contraction that is dependent on extracellular calcium. When added to vascular tissue, Ang II causes a transient increase in \([\text{Ca}^{2+}]_{i}\) 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 are primarily responsible for the observed changes in \([\text{Ca}^{2+}]_{i}\). In cultured VSMC, arginine vasopressin-(AVP) and endothelin-stimulated \([\text{Ca}^{2+}]_{i}\) transients are also reported to be preserved in the absence of extracellular calcium. Ang II-, AVP-, and endothelin-induced increases in \([\text{Ca}^{2+}]_{i}\) are inhibited by ETI in both 2 mmol/L calcium and calcium-poor buffer.

Stimulus–receptor interactions in smooth muscle cells that result in contractions activate G protein-coupled receptors that result in activation of phospholipase C or in the activation of membrane calcium channels. Prostaglandins and P-450 epoxide products can have effects on enzymatic systems, such as the sodium–potassium pump, that can alter intracellular sodium and calcium, and thus affect basal tone and vascular reactivity. Other studies also suggest that AA itself, as well as AA metabolites and other fatty acids, can have direct effects on membrane channels.

The potential physiologic consequences of these eicosanoid-modified responses to cellular stimuli are many and varied. Most investigations into the nature of eicosanoid action have focused on their production or direct effects. Thus, the production and actions of the vasodilator, prostacyclin, a CO product produced in both endothelial and VSMC, are well described. The vasoconstrictive actions of the CO products thromboxanes, and prostaglandin F2α in vascular tissues appear to be mediated by a membrane endoperoxide/thromboxane receptor. These interactions, in turn, have been correlated with blood pressure regulation. Lin and coworkers have demonstrated that a thromboxane antagonist can lower blood pressure in the coarctation model of hypertension in rats. In the Lyon rat, a genetic model of hypertension, elevated thromboxane in the urine was decreased by thromboxane antagonists and synthesis inhibitors. In these studies the blood pressure was lowered gradually after 3 weeks, suggesting a mechanism separate from the direct vasoconstriction due to thromboxane/endoperoxide receptor activation.

Several cytochrome P-450 metabolites of AA have also been shown to have important biologic functions. As is the case with CO, this enzyme system can produce both vasodilatory and vasoconstrictive compounds. Epoxycosatriynoic acids (EETs) produced by P-450 have direct activity on potassium channels opening, as demonstrated in patch clamp studies. The production of EETs by VSMC has been documented. P-450 isozymes also produce the powerful vasoconstrictor, 20-hydroxyecosatetraenoic acid in cat cerebral microvessels and in rat kidney.

There is also considerable documentation of LO activity in the vascular tissues. Piomelli and coworkers demonstrated that canine and human coronary arteries generate the LO products 5-HETE, 12-HETE, and 15-HETE in addition to leukotrienes C4, D4, and E4. These products have also been identified in endothelial cell cultures from bovine coronary arteries. Greenwald et al. showed significant LO activity in freshly prepared rabbit aortic rings and found that HETE production could be reduced by LO pathway inhibitors. In cultured rabbit aortic smooth mus-
cellular responses. Our experiments with ETI also suggests that LO inhibition may have differing effects dependent on the contractile agonist. In the femoral artery preparation, the inhibitor markedly decreased contractile responses to some agonists (U46619, Ang II, vasopressin, and norepinephrine) had little effect on some (potassium chloride, serotonin) and potentiated endothelin-1 responses. These observations are in keeping with the concept that pathway specificity is a potential method of eicosanoid regulation of cellular responses.

Previous investigations of elevated [Ca$^{2+}$], and phospholipase C activity in VSMC and other cells from spontaneously hypertensive rats suggest enhanced postreceptor signaling in tissues that regulate blood pressure. Pharmacologic experiments demonstrated that LO inhibition attenuated Ang II-induced intracellular calcium transients in cultured VSMC. Moreover, this attenuation could be reversed by preincubating the cells with exogenously added 12-HETE. These results suggested that 12-HETE may play a role in modulating Ang II-mediated signal transduction. Additional experiments in cultured VSMC revealed that although in vitro pretreatment with 12-HETE alone had no effect on basal calcium levels, subsequent stimulation with Ang II resulted in augmented intracellular calcium responses.

In summary, this study demonstrates that inhibitors of the three major pathways of arachidonic acid metabolism have differential effects on calcium mobilization. We conclude that arachidonic acid pathways may be important modifiers of agonist-induced calcium transients. The three pathways may have vascular actions by modulating calcium responses at different sites.

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