Arachidonic Acid-Induced Vasodilation of Rat Small Mesenteric Arteries Is Lipoxygenase-Dependent

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ABSTRACT

We examined the mechanism of arachidonic acid-induced vasodilation in rat small mesenteric arteries and determined the primary arachidonic acid metabolites produced by these arteries. Responses to arachidonic acid in small mesenteric arteries from Sprague-Dawley rats were investigated in vitro in the presence or absence of endothelium or after pretreatment with inhibitors of nitric oxide (NO), cyclooxygenase, cytochrome P450, lipoxygenase, or K+ channels. In addition, the metabolism of arachidonic acid was examined by incubating arteries with [3H]arachidonic acid in the presence and absence of cyclooxygenase, cytochrome P450, lipoxygenase inhibitors. Finally, the vascular response to both 12(S)-hydroxyeicosatetraenoic acid (HETE) and 12(S)-hydroperoxyeicosatetraenoic acid (HPETE) was determined. Arachidonic acid induced an endothelium-dependent vasodilation that was abolished by lipoxygenase inhibitors [cin-namyl-3,4-dihydroxy-cyanocinnamate (CDC) or 5,8,11-eicosatriynoic acid (ETI)] and KCl, whereas it was partially inhibited by either tetraethy lammonium or ibetoxicin. In contrast, neither NO nor cytochrome P450 enzyme inhibitors affected arachidonic acid-mediated dilation, whereas inhibition of cyclooxygenase enhanced dilation. Biochemical analysis revealed that small mesenteric arteries primarily produce 12-HETE, a lipoxygenase metabolite. Moreover, CDC and ETI inhibited the production of 12-HETE. Finally, both 12(S)-HETE and 12(S)-HPETE induced a concentration-dependent vasodilation in mesenteric arteries. These findings provide functional and biochemical evidence that the lipoxygenase pathway mediates arachidonic acid-induced vasodilation in rat small mesenteric arteries through a K+ channel-dependent mechanism.

Endothelial cells play an integral role in maintenance of vascular tone through the production of vasoactive substances. The three primary vasoactive substances produced by the endothelium are nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF) (Rubanyi, 1993). In most arterial beds, particularly conduit arteries, NO is the primary endothelium-derived relaxing factor. However, in some resistance artery beds, it is thought that EDHF may be a more important mediator of vasodilation (Hwa et al., 1994). The identity of EDHF is currently unknown, but is likely several different substances dependent on the vascular bed and model studied. Many investigators have suggested that noncyclooxygenase metabolites of arachidonic acid meet the criteria for an EDHF because they are produced by the endothelium and can induce vascular smooth muscle hyperpolarization and thus relaxation through the activation of K+ channels (Garland et al., 1995; Fisslthaler et al., 1999, 2000). Endothelial cells can metabolize arachidonic acid through several different noncyclooxygenase pathways, most notably the cytochrome P450 and lipoxygenase pathways. In endothelial cells, cytochrome P450 enzymes metabolize arachidonic acid to four regioisomeric epoxyeicosatrienonic acids, which can be rapidly hydrolyzed to dihydroxyeicosatrienoic acids (Oltman et al., 1998; Weintraub et al., 1999). Both of these groups of metabolites have been shown to induce vascular smooth muscle hyperpolarization and are putative EDHFs (Oltman et al., 1998; Campbell et al., 2001). Moreover, three lipoxygenases have been described in endothelial cells: 5-, 12-, and 15-lipoxygenase. Each of these enzymes generates a stereospecific hydroperoxyicosatetraenoic acid (HPETE), which are highly unstable compounds that are rapidly reduced by cellular peroxidases to the corresponding hydroxyeicosatetraenoic acid (HETE) (Soberman et al., 1985). These metabolites have

ABBREVIATIONS: NO, nitric oxide; EDHF, endothelium-dependent hyperpolarizing factor; HPETE, hydroperoxyicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; PE, phenylephrine; CDC, cin-namyl-3,4-dihydroxy-cyanocinnamate; ETI, 5,8,11-eicosatriynoic acid; KCa, calcium-dependent potassium channel; HPLC, high-performance liquid chromatography.
been reported to have various vasoactive properties, encompassing both vasodilation and vasoconstriction (Utila et al., 1987; Uski and Hogestatt 1992; Pfiester et al., 1998; DelliPizzi et al., 2000; Faraci et al., 2001; Zink et al., 2001). Recently, a 12-lipoxygenase metabolite, 12(S)-HETE, has been shown to possess EDHF-like characteristics (Pfiester et al., 1998; Faraci et al., 2001; Zink et al., 2001).

Previous studies in small rat mesenteric arteries have demonstrated that endothelium-dependent vasodilation is primarily mediated by nitric oxide-independent vasodilators (Chen and Cheung, 1997; Doughty et al., 1999; Katakam et al., 1999). Thus, the role of arachidonic acid and its metabolites in this vascular bed is physiologically relevant. However, the mechanism by which arachidonic acid induces relaxation in rat mesenteric arteries is currently unclear. Therefore, the purpose of the current study was to determine the mechanism of arachidonic acid-induced vasodilation in small rat mesenteric arteries and to determine the primary arachidonic acid metabolites produced by these arteries.

Materials and Methods

The Animal Care Committees at the Wake Forest University School of Medicine and the University of Iowa College of Medicine approved the current protocol. Ten-week-old male Sprague-Dawley rats (n = 54) were anesthetized with pentobarbital (50 mg/kg i.p.) and anticoagulated with heparin (500 units i.p.). A midline incision was made and a section of the small intestine was clamped, removed, and anticoagulated with heparin (500 units i.p.). A midline incision was made and a section of the small intestine was clamped, removed, and anticoagulated with heparin (500 units i.p.). A midline incision was made and a section of the small intestine was clamped, removed, and anticoagulated with heparin (500 units i.p.).

Mechanisms of Vascular Responsiveness to Arachidonic Acid. Small mesenteric arteries (~2 mm in length) isolated from the mesenteric vascular bed were transferred to a vessel chamber and mounted and secured between two glass micropipettes with 0.08 mm ophthalmic suture. The vessel chamber was transferred to an inverted light microscope stage coupled to a video dimension analyzer (Living Systems Instrumentation, Burlington, VT). The video dimension analyzer was connected to both a video monitor (for visualization of the vessel) and to a strip chart recorder for constant recording of the intraluminal diameter of the vessel. Oxygenated (20% O2, 5% CO2) Krebs’ solution maintained at 37°C was continuously circulated through the vessel bath. In addition, the lumen of the vessel was filled with Krebs’ solution through the micropipettes and maintained at a constant pressure of 60 mm Hg. Only one concentration-response experiment was performed per artery; however, several arteries were taken from each rat.

Mesenteric arteries were allowed to equilibrate for 30 min and subsequently preconstricted to approximately 40% of their resting diameter with phenylephrine (PE), an α1 receptor agonist. Concentration-response experiments to arachidonic acid (10-8–5 x 10-5 mol/l) were performed in control (intact endothelium) arteries, arteries pretreated with 100 μM N-nitro-l-arginine, and arteries denuded of endothelium. Endothelial denudation was performed by perfusing air through the lumen of the artery and was verified by the absence of a dilator response to acetylcholine and viability was tested by vasodilator response to sodium nitroprusside. We have previously documented this method of endothelium removal by electron microscopy (Miller et al., 2001).

Because arachidonic acid can be metabolized by three different enzyme systems to generate vasoactive products, we inhibited each of these systems individually in endothelium-intact arteries to determine the mechanism of vasodilation by arachidonic acid. To determine the role of cyclooxygenase products arteries were pretreated with 10 μM indomethacin, whereas the role of cytochrome P450 products was determined by pretreatment with either 10 μM miconazole or 10 μM 17-octadecynoic acid. Moreover, to assess the role of the lipoxygenase products we pretreated arteries with either 100 μM cin-namyl-3,4-dihydroxy-cyanocinnamate (CDC) or 10 μM 5,8,11-eicosatriynic acid (ETI).

Vasodilatory metabolites of arachidonic acid are often reported to induce vascular relaxation through their ability to activate potassium channels (Adeagbo and Malik, 1991; Faraci et al., 2001). Moreover, EDHF is characterized as inducing vascular smooth muscle hyperpolarization and vasodilation through the activation of potassium channels (Peletou and Vahvoutte, 1999, 2000). Thus, to assess whether K+ channels contribute to arachidonic acid-induced vasodilation, 50 mM KCl rather than PE was used to constrict arteries. Furthermore, to assess the role of KCa or ATP-dependent K+ channels in arachidonic acid-induced vasodilation we pretreated arteries with either 1 mM tetrathyammonium or 10 μM glibenclamide before PE constriction. To specifically assess the role of the large conductance KCa, we pretreated the arteries with 0.1 μMiberiotoxin before PE constriction.

Metabolism of Radiolabeled Arachidonic Acid by Mesenteric Arteries. Rat small mesenteric arteries were harvested and pooled from three animals for each incubation experiment. The arteries were placed in a test tube containing 1 ml of Krebs-Ringer bicarbonate supplemented with 0.1 μM fatty acid-free bovine serum albumin and maintained in a 5% CO2 incubator (37°C). After 1 h, vehicle (dimethyl sulfoxide), the combination of indomethacin + miconazole (10 μM each), 10 μM CDC, or 10 μM ETI was added, and the incubations were continued for 30 min. The Krebs-Ringer bicarbonate solution was then removed, and fresh Krebs-Ringer bicarbonate solution containing 1.7 μM [3H]arachidonic acid was then added along with vehicle or inhibitor compounds. One hour later, 2 μM A23187 was added to induce arachidonic acid mobilization and metabolism, and after 30 min, the incubation was terminated by removal of the Krebs-Ringer bicarbonate solution.

The radioactivity present in the Krebs-Ringer bicarbonate solution after the incubation was measured by liquid scintillation counting. Lipids contained in the medium were extracted twice with 4 volumes of ice-cold ethyl acetate saturated with water, the extracts were combined, the solvent was evaporated under N2, and the residue was dissolved in acetonitrile. The lipids were separated by reverse-phase HPLC using a Gilson dual pump gradient system equipped with model 306 pumps, a model 117 dual wavelength UV detector, a model 231 XL automatic sample injector (Gilson Medical Electronics, Middleton, WI), and a Discovery C18 column (5 μm, 4.6 x 150 mm) obtained from Supelco (Bellefonte, PA). The elution profile consisted of water adjusted to pH 4.0 with formic acid and an acetonitrile gradient that increased from 30 to 57% over 60 min and then from 57 to 65% over 25 min, at which time the acetonitrile was taken to 100% and held constant for 15 min. The distribution of radioactivity was measured by combining the column with scintillation cocktail. The data were analyzed using the program BioRad (American Scientific, TX) to estimate the percentage distribution of the radioactivity.

Vascular Response to Metabolic Products. Based on the above-mentioned biochemical studies we assessed the vascular response to both exogenous 12(S)-HETE and 12(S)-HPETE in small mesenteric arteries. Arteries were isolated and prepared as described previously. After preconstriction with PE to 40% of baseline,
concentration-response experiments \((10^{-10} - 10^{-6} \text{ M})\) were determined for both of the above-mentioned lipoxigenase metabolites.

**Data Analysis.** Data from vascular reactivity studies are expressed as percentage of relaxation after preconstriction. All data are expressed as mean ± S.E.M. All concentration-response curves were evaluated for changes in maximal response and differences at each concentration using analysis of variance with repeated measures followed by a Fisher's pairwise least significant difference test for multiple comparisons. The criterion for significance was \(p < 0.05\).

**Results**

Resting intraluminal diameter of small mesenteric arteries was 210 ± 6 \(\mu\)m. Percentage of arterial constriction after phenylephrine or KCl was similar with 39 ± 2% for PE and 36 ± 2% for KCl. Neither endothelial denudation nor pharmacological inhibition significantly altered the resting diameter compared with the arteries in the control (without intervention) group. The percentage of constriction in experiments with endothelial denudation or pharmacological inhibition also did not differ compared with control arteries, although the concentration of PE used to induce preconstriction was decreased by approximately one-half to induce the same amount of tone in these arteries. For example, arteries that were denuded of endothelium required 1.08 ± 0.07 \(\mu\)M PE to induce preconstriction, whereas arteries with intact endothelium required 2.17 ± 0.03 \(\mu\)M.

**Mechanisms of Vascular Responsiveness to Arachidonic Acid.** Arachidonic acid produced a concentration-dependent vasodilation in control mesenteric arteries with a maximal relaxation of 89 ± 5% (Fig. 1). This vasodilation was not significantly altered in the presence of the NO synthase inhibitor \(N\)-nitro-L-arginine (maximal relaxation 83 ± 6%; Fig. 1). In contrast, arachidonic acid-induced vasodilation was nearly abolished after endothelium denudation (maximal relaxation 13 ± 3%; \(p < 0.01\)) (Fig. 1), illustrating that the vascular responsiveness to arachidonic acid in rat mesenteric arteries is primarily endothelium-dependent.

Pretreatment of endothelium intact arteries with indomethacin shifted the midportion of the dose-response curve to the left with a similar maximal relaxation to control arteries (Fig. 2A). Although the EC\(_{50}\) value for these two dose-response curves was not statistically different (2.7 ± 1.4 \(\mu\)M for control versus 0.67 ± 0.4 \(\mu\)M with indomethacin) vasodilation was significantly greater in the presence of indomethacin at two of the concentrations administered (Fig. 2A). These data suggest that either cyclooxygenase metabolites normally exert a mild vasoconstriction in this vascular bed or that the inhibition of cyclooxygenase shifts metabolism of arachidonic acid toward greater production of vasodilatory eicosanoids.

In contrast, treatment with miconazole or 17-octadecynoic...
acid, cytochrome P450 inhibitors, had no significant effect on arachidonic acid-induced vasodilation. Maximal relaxation to arachidonic acid after miconazole pretreatment was 82 ± 8 and 73 ± 6% after pretreatment with 17-octadecynoic acid (Fig. 2A).

Conversely, application of either ETI or CDC, inhibitors of lipoxygenase, abolished vasodilation to arachidonic acid (maximal relaxation 0.4 ± 6% for ETI, 5 ± 5% for CDC; \( p < 0.01 \) versus control) (Fig. 2B). These latter data suggest that lipoxygenase participates in arachidonic acid-induced dilation in rat small mesenteric arteries.

When KCl was used to constrict arteries instead of PE, vasodilation to arachidonic acid was almost completely eliminated [maximal dilation 12 ± 6% after KCl; \( p < 0.01 \) versus PE-constricted control arteries (Fig. 3)]. Likewise, vasodilation was markedly inhibited after pretreatment of arteries with tetraethylammonium [maximal vasodilation 21 ± 2% after tetraethylammonium; \( p < 0.01 \) versus control] or iberiotoxin [maximal vasodilation 33 ± 10% after iberiotoxin; \( p < 0.01 \) versus control] (Fig. 3). Conversely, glibenclamide had no effect on vasodilation to arachidonic acid (maximal relaxation 86 ± 9% with glibenclamide; data not shown). These data suggest that arachidonic acid induces vasodilation primarily through the activation of \( K_{\text{Ca}} \) channels.

**Metabolism of \([3H]\)Arachidonic Acid by Mesenteric Arteries.** To examine the profile of arachidonic acid metabolites produced by rat small mesenteric arteries, pooled arteries (three animals per experiment) were incubated with \([3H]\)arachidonic acid, followed by extraction of lipids from the incubation solution and separation by reverse-phase HPLC. The main radiolabeled products detected in the incubation solution were arachidonic acid and an unknown metabolite that comigrated with authentic 12-HETE standard (Fig. 4). In control experiments (vehicle-treated) there was a 4.5 ± 1.5% conversion (\( n = 8 \)) to the unknown metabolite. In one experiment, tissue-associated lipids were extracted using chloroform/methanol, saponified, and separated by reverse-phase HPLC. The only radiolabeled peak detected was arachidonic acid, suggesting that the unknown product was preferentially released into the incubation solution rather than retained in cell lipids (data not shown). Pretreatment with indomethacin + miconazole did not inhibit the formation of the unknown metabolite (\( n = 3 \)) (Fig. 4). In contrast, production of the unknown was abolished by pretreatment of arteries with either CDC (\( n = 2 \); Fig. 4) or ETI (\( n = 1 \); data not shown).

Further information about the identity of the unknown metabolite was obtained by performing normal-phase HPLC.

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**Fig. 3.** Cumulative concentration-response experiments to arachidonic acid in rat small mesenteric arteries alone (control, PE-preconstricted) or after KCl depolarization or pretreatment with tetraethylammonium or iberiotoxin (IBTX). Asterisk (*) indicates statistical significance (\( p < 0.05 \)) compared with the response in control arteries without treatment.

**Fig. 4.** Metabolism of arachidonic acid by rat small mesenteric arteries. Arteries were pretreated with vehicle (top), 10 \( \mu M \) CDC (middle), or with indomethacin + miconazole (10 \( \mu M \) each; bottom) and then incubated with 1.7 \( \mu M \) [3H]arachidonic acid for 1 h. A23187 (2 \( \mu M \)) was then added for 30 min, after which lipids contained in the Krebs-Ringer bicarbonate solution were extracted, separated by reverse-phase HPLC, and assayed for radioactivity. Authentic radiolabeled eicosanoid standards are shown by the dashed line for comparison of retention times with products detected in the incubation solution. The main radiolabeled products in the incubation solution are arachidonic acid (AA) (retention time 57.1 min) and an unknown (Unk) metabolite that comigrated with authentic 12-HETE standard (top and bottom). The unknown metabolite was not produced by vessels treated with CDC (middle). PGE\(_2\), prostaglandin E\(_2\); DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid.
The unknown metabolite was resolved into a single peak that comigrated with the authentic 12-HETE standard (Fig. 5). Taken together, these findings strongly suggest that under the defined experimental conditions, 12-HETE is a major product of arachidonic acid metabolism in rat small mesenteric arteries.

**Vascular Response to Metabolic Products.** Both 12(S)-HETE and 12(S)-HPETE induced a concentration-dependent vasodilation of small mesenteric arteries, with maximal dilations of 50 ± 10 and 59 ± 10%, respectively (Fig. 6). These experiments demonstrate that the primary metabolite produced by small rat mesenteric arteries (12-HETE) and its immediate precursor (12-HPETE) are indeed vasodilators in this arterial bed.

**Discussion**

There are three major new findings from this study. First, arachidonic acid induces an endothelium-dependent dilation of small rat mesenteric arteries that is abolished by inhibitors of lipoxygenase, but not by inhibitors of NO synthase, cyclooxygenase, or cytochrome P450. Second, biochemical studies suggest that lipoxygenase-derived 12-HETE is a major product of arachidonic acid metabolism in rat small mesenteric arteries. Moreover, vascular studies with exogenous 12(S)-HETE demonstrate that it acts as a vasodilator in this vascular bed. Third, arachidonic acid-mediated vasodilation was also inhibited by depolarization with KCl or pretreatment with either tetraethylammonium or iberiotoxin. Together, these findings suggest that arachidonic acid produces dilation of rat small mesenteric arteries through a mechanism dependent upon lipoxygenase and activation of KCa channels.

To our knowledge only one prior study has assessed arachidonic acid-induced vasodilation in rat mesentery, whereas no studies have evaluated arachidonic acid metabolism using biochemical techniques in this vascular bed. In an isolated perfused mesenteric arterial preparation, vascular dilation via arachidonic acid was shown to be primarily endothelium-dependent (Adeagbo and Malik, 1991). Moreover, cyclooxygenase inhibitors enhanced arachidonic acid-induced dilation, whereas cytochrome P450 inhibitors had no effect (Adeagbo and Malik, 1991). All of these findings are very consistent with those in the present study. In contrast, however, the previous study evaluated one lipoxygenase enzyme inhibitor, 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodeca-diynyl)-1,4-benzoquinone (AA861), that did not alter vasodilation to arachidonic acid (Adeagbo and Malik, 1991). However, biochemical assessment of the efficacy of lipoxygenase inhibition by AA861, which is reported to be more selective for 5-lipoxygenase, was not demonstrated. Therefore, the role of the lipoxygenase enzyme system was not fully evaluated in the prior study (Adeagbo and Malik, 1991). Regarding potassium channel activation, the prior study, like our own, showed that arachidonic acid decreased vascular resistance through the activation of KCa channels (Adeagbo and Malik, 1991).

**Lipoxygenase as a Vasodilator Pathway.** In addition to the current data, recent findings from other species and vascular beds have also suggested that lipoxygenase mediates arachidonic acid-induced vasodilation in some preparations. Studies in rabbit and rat aorta have demonstrated that
arachidonic acid produces relaxation by activation of the lipoxygenase pathway (Uttilla et al., 1987; Pfister et al., 1998). Moreover, Zink and colleagues have shown that porcine coronary microvascular endothelial cells metabolize arachidonic acid to 12(S)-HETE. Furthermore, these investigators went on to show that this lipoxygenase product induced potent vasodilation of porcine coronary microvessels via vascular smooth muscle hyperpolarization (Zink et al., 2001). Finally, a recent study completed in rat basilar artery demonstrated, similar to our study, that arachidonic acid-induced vasodilation was abrogated by lipoxygenase enzyme inhibitors and that the major product detected by HPLC analysis of \(^{3}H\)arachidonic acid metabolism was indeed 12(S)-HETE (Faraci et al., 2001).

Although biochemical and functional data in this study suggest that the rat mesenteric artery metabolizes arachidonic acid via the lipoxygenase pathway, which lipoxygenase metabolite(s) mediates this response is unclear. Lipoxygenases convert arachidonic acid into HPETEs that can then be metabolized into various compounds, including HETEs and trihydroxyeicosatrienoic acids, all of which have vasoactive properties (Soberman et al., 1985; Pfister et al., 1998; Zink et al., 2001). Moreover, lipoxygenase enzymatic activity can lead to generation of reactive oxygen species, which themselves can induce vasodilation by activation K\(^+\) channels (Fleming et al., 2001). Thus, although we detected 12-HETE in our assay system and have shown that both exogenous 12(S)-HETE and 12(S)-HPETE induce vasodilation in this arterial bed, our results do not exclude the possibility that trihydroxyeicosatrienoic acids or other lipoxygenase products are mediators of dilation to arachidonic acid in rat mesenteric arteries.

**Role of Potassium Channels.** The current study showed that arachidonic acid metabolites produced by the mesenteric arterial endothelium induce vasodilation through potassium channels. Specifically, we demonstrated that either depolarization with KCl or pretreatment with either tetraethylammonium or iberiotoxin markedly inhibited the vascular responses to arachidonic acid, whereas glibenclamide pretreatment had no effect. These findings suggest that lipoxygenase metabolites of arachidonic acid induce vasodilation through K\(_{Ca}\) but not ATP-dependent K\(^+\) channels. These data are consistent with recent findings in rat basilar arteries and rabbit aorta (Pfister et al., 1998; Faraci et al., 2001). Moreover, in rat basilar arteries, investigators not only showed that arachidonic acid-mediated dilation could be inhibited by tetraethylammonium or iberiotoxin but also demonstrated that the hyperpolarization of vascular smooth muscle was abolished in the presence of tetraethylammonium (Faraci et al., 2001). Finally, in porcine coronary microvessels, the effect of 12(S)-HETE was specifically evaluated and was not only shown to induce vasodilation but also induced vascular smooth muscle hyperpolarization through activation of the large conductance K\(_{Ca}\) channel (Zink et al., 2001). These results are supportive of our findings and suggest that lipoxygenase metabolites likely induce vasodilation by activating vascular smooth muscle K\(_{Ca}\) channels.

**Summary.** The present study is the first to elucidate a specific mechanism by which arachidonic acid produces dilation of rat small mesenteric arteries. Both pharmacological and biochemical studies suggest that the lipoxygenase pathway is a functionally active mechanism for control of vascular motor tone in this vascular bed. Based on these findings, lipoxygenase may function as a putative EDHF synthase in small rat mesenteric arteries.

**References**


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