Intestinal Vasodilation by Epoxyeicosatrienoic Acids: Arachidonic Acid Metabolites Produced by a Cytochrome P450 Monoxygenase

Kenneth G. Proctor, J. R. Falck, and Jorge Capdevila

Purified synthetic products from the cytochrome P450 pathway of arachidonate metabolism were applied to the intestinal serosa. Arteriolar blood flow was calculated using video microscopy. After a steady-state baseline, a bolus containing 10–60 µg 14,15-epoxyeicosatrienoic acid/ml (14,15-EET) had no detectable effect on blood flow. However, 25 ± 3 µg 11,12-EET/ml and 36 ± 2 µg 8,9-EET/ml caused increases (134 ± 8% and 127 ± 6%) that were similar to those elicited by 8 ± 2 µg adenosine/ml (138 ± 12%). Furthermore, the increases (275 ± 38%) produced by 32 ± 6 µg 5,6-EET/ml exceeded those elicited (160 ± 10%) by a similar concentration (27 ± 3 µg/ml) of adenosine. Thus, a structure-activity relationship is suggested. Nevertheless, these values probably underestimate the potency of the EETs because the vasoactivity was reduced by contact with water. The activity of the cytochrome P450 pathway seemed to limit the formation of vasoactive quantities of EETs, or other nonprostaglandins, from exogenous arachidonate in the serosa but not the mucosa. A bolus (1.3 ± 0.2 mg/ml) or continuous application (122 ± 45 µg/ml) of arachidonate caused blood flow increases (236 ± 14% or 229 ± 27%) that were almost eliminated (129 ± 5% or 121 ± 9%) by a cyclooxygenase inhibitor; the residual response was abolished by a cytochrome P450 inhibitor. However, cytochrome P450 inhibitors alone did not attenuate the arachidonate response. In contrast, a continuous application of 194 µg arachidonate/ml to the mucosa caused a markedly smaller blood flow increase (119 ± 8%) and cyclooxygenase inhibitors potentiated (132 ± 8%) rather than reduced, this response. We conclude that EETs are a labile class of vasodilators with a potency comparable to adenosine in the intestinal microcirculation. Indirect evidence suggests regional differences in the formation of vasoactive quantities of arachidonate metabolites within the intestinal wall. (Circulation Research 1987;60:50–59)

Arachidonic acid can be oxidized by at least three distinct enzymatic pathways. The physiological and pathophysiological significance of products from the cyclooxygenase (e.g., prostaglandins and thromboxane) and lipooxygenase (e.g., leukotrienes) pathways is now well documented. In contrast, relatively little information is available on the biological actions of epoxyeicosatrienoic acids (EETs), which are produced by a cytochrome P450 mixed-function monoxygenase acting on arachidonate.1,2

Although no physiologic role for EETs has been defined, 5,6-EET is a potent in vitro stimulus for the release of somatostatin from the hypothalamic median eminence.2 In isolated kidney cells, induction of cytochrome P450 increases the formation of nonprostaglandin metabolites of arachidonate, which inhibit Na+, K-ATPase and relax isolated vascular smooth muscle.3–5 In isolated vascular smooth muscle, arachidonic acid stimulates the release of a labile, nonprostaglandin vasorelaxing factor from endothelial cells6 whose action is blocked by inhibitors of cytochrome P450.7,8

Arachidonic acid metabolites are thought to play an important role in the control of intestinal blood flow during nutrient absorption because the response is potentiated by cyclooxygenase inhibitors9,10 and inhibited by arachidonic acid.11 At least two mechanisms could explain these observations: direct evidence has shown that cyclooxygenase products limit the food-induced increase in intestinal oxygen uptake9,11 while indirect evidence suggests that novel vasodilator substances may be produced from arachidonate when cyclooxygenase is inhibited.10 The purpose of this study was to test whether metabolites derived from the cytochrome P450 pathway of arachidonic acid metabolism were vasoactive in the intestinal microcirculation.

Materials and Methods

General

Male rats (120–250 g) were premedicated with atropine (4 µg/100 g, i.p.) and anesthetized with an aqueous solution of 13% urethane and 1% chloralose (1.2 ml/100 g, i.p.). Supplemental anesthetic was adminis-
tered temperature when needed through a jugular venous cannula. Rectal temperature was continuously monitored (YSI 401, Yellow Springs, Ohio) and maintained at 36–38°C by a heat lamp. Respiration was spontaneous on room air through a tracheal cannula. In these conditions, arterial blood PO₂, PCO₂, and pH were typically >70 mm Hg, <40 mm Hg, and 7.35–7.45, respectively, as measured on a Radiometer (Oxnard, Calif.) analyzer. Carotid arterial blood pressure was continuously measured with a Gould-Statham (Copenhagen) P23ID transducer and typically ranged between 80–100 mm Hg. An experiment was terminated if arterial blood pressure decreased below 70 mm Hg.

The microcirculation of the small intestine was surgically prepared with methods identical to those previously described. The mucosal and serosal surfaces were suffused with bicarbonate-buffered Ringer’s solutions that contained ascorbic acid (0.5 mM) as an antioxidant. Suffusate gas tensions and pH were maintained by equilibration with a mixture of 5% CO₂, 0–5% O₂, and balance N₂ in glass reservoirs encased in water jackets. These reservoirs were connected to the mucosal and serosal suffusate chambers with thick-walled rubber tubing.

Tissue temperature was continuously monitored (YSI LN 3207) and maintained at 36–37°C by varying the flow rates of heated solutions that suffused the mucosa (average = 3–7 ml/min) and serosa (average = 1–3 ml/min). In all experiments, isoproterenol (Sigma Chemical Co., St. Louis, Mo.) was added to the serosal suffusate to suppress spontaneous intestinal motility. The threshold isoproterenol concentration for causing vasodilation was 1 µM, which is 25× higher than the maximum concentration used in these studies.

The intestinal microcirculation was transfused with color video microscopy, as previously described. Submucosal arterioles were observed with long working distance optics (objective = Leitz L20 X or L32 X; condenser = Leitz L11), which were aligned in accordance with Kohler’s principle to enhance contrast. Submucosal arterioles were studied because the serosal and mucosal layers have parallel vascular circuits that are supplied by these vessels and calculated blood flow in submucosal arterioles is representative of total tissue blood flow. Arteriolar diameter was measured online with a video-micrometer (For-A IV-550, Tokyo) by positioning two parallel lines on a microvessel’s image. An analog voltage proportional to the distance separating the two lines was continuously measured. The system was calibrated with a stage micrometer to an accuracy of 1 µm.

Red blood cell velocity was measured with a custom-designed rotating prism velocimeter (Micro Precision Co., Norcross, Ga.), which is based on a mechanical image-streaking principle discussed earlier. This instrument was calibrated by moving a clear plastic film smeared with dried red cells past the detector at known rates.

Arteriolar blood flow was calculated online by a Commodore microcomputer. The product of vessel cross-sectional area (computed from digitized diameter data) and mean blood flow velocity (computed from digitized red blood cell velocity data divided by a proportionality factor) was displayed at 5-second intervals on a Dec writer along with mean arterial blood pressure, arteriolar diameter, and red blood cell velocity. In addition, the analog signals were continuously recorded on a six-channel polygraph (Gould Model 2600).

**Synthesis and Storage of Eicosanoids**

Arachidonic acid was purchased from Nu-Chek Prep (Elysian, Minn.) and stored at -60°C. On the day of an experiment, a stock arachidonic acid solution was made in ethanol (40 mg/ml). The stock ethanol-arachidonic acid solution was protected from light at -12°C and used within 1 hour.

In some experiments, different aliquots of the stock solution were added to either the mucosal or serosal suffusate chambers to final concentrations of 50–200 µg/ml (167–667 µM). These solutions were continuously suffused over the tissue.

In other experiments, different aliquots of the stock solution were diluted to 2 ml with temperature- and gas-equilibrated buffered Ringer’s to final concentrations of 1–4 mg/ml (3–12 mM). These solutions were immediately applied as a bolus into the flowing serosal or mucosal suffusates. To determine the mechanical and/or metabolic effect of the bolus application, a 2 ml bolus of 2–10% ethanol in Ringer’s was infused into the serosal suffusate.

Four regioisomeric epoxyeicosatrienoic acids (EETs) were synthesized at the University of Texas Health Science Center, Dallas, Tex. 5,6-EET was synthesized according to the method of Corey et al. Each compound was shipped to the University of Tennessee-Memphis on dry ice.

The purity of these compounds was verified by high performance liquid chromatography. Details of the method have been previously published.

Because of its volatility, 5,6-EET was used within 24 hours of its synthesis. An original sample of this compound was reconstituted with deoxygenated ethanol on the day of shipment to a concentration of 1–2 µg/µl, diluted to 2 ml with temperature- and gas-equilibrated buffered Ringer’s to final concentrations of 5–50 µg/ml (17–170 µM) and immediately infused into the serosal suffusate.

Other EETs were more stable and were used within 1 week of synthesis. Individual samples were diluted with deoxygenated ethanol to 1–2 µg/µl and stored in several vials at -60°C under N₂. On the day of an experiment, aliquots from a vial were diluted to 2 ml with Ringer’s to final concentrations of 10–80 µg/ml (27–270 µM) and immediately infused into the serosal suffusate. A vial was stored at -11°C between dilutions and used within 1 hour of removal from long-
Inhibitors on Arachidonic Acid-Induced Hyperemia

Effect of EETs

Thereafter, a 2-ml solution of 1-4 mg arachidonic acid/ml was manually infused at a rate of approximately 1 ml/min. To determine the mechanical effect of the bolus, an equal volume of the vehicle (ethanol) was applied to the tissue. Depending on availability, several different doses of a given EET were administered in random order to a given animal as long as the baseline was restored after each bolus. The mucosal and serosal suffusates were equilibrated with 5% CO$_2$/90% N$_2$, which were the same conditions in the above described EET experiments.

Effect of Cyclooxygenase and Cytochrome P450 Inhibitors on Arachidonic Acid-Induced Hyperemia

Hypothesis 2: If products from the cytochrome P450 pathway of arachidonic acid metabolism mediate all or part of the vasodilation caused by exogenous arachidonic acid, then a residual hyperemia should persist in the presence of cyclooxygenase inhibitors.

The mucosal and serosal suffusates were equilibrated with 5% CO$_2$/5% O$_2$/90% N$_2$, until baseline hemodynamic variables were maintained for 10-30 minutes. Thereafter, a 2-ml solution of 1-4 mg arachidonic acid/ml was manually infused at a rate of approximately 1 ml/min into the serosal suffusate. The flow rate of the serosal suffusate ranged from 1-3 ml/min, depending on tissue temperature and experimental conditions, so the final dilution factor of the bolus varied from day-to-day; the actual suffusate concentration was always lower than that in the bolus. Hemodynamic data were continuously monitored after the bolus infusion until a new steady state appeared. This procedure was repeated at several different arachidonic acid concentrations applied in random sequence as long as the baseline was restored after each bolus.

To examine whether cyclooxygenase products could account for the effects of arachidonic acid, the above protocol was repeated with structurally dissimilar inhibitors of cyclooxygenase in the suffusate solutions (10 μg/ml). Either meclofenamate (Warner-Lambert, Ann Arbor, Mich.) or sodium indomethacin trihydrate (Merck Sharpe & Dohme, West Point, Penn.) were used on alternating days. At least 10 minutes was allowed for an inhibitor to equilibrate with the tissue, and it remained in the suffusates for the duration of an experiment.

To examine whether cytochrome P450 products could account for the effects of arachidonic acid, structurally dissimilar inhibitors of cytochrome P450 were added to the mucosal and serosal suffusate solutions. Either SKF-525A (10 μg/ml; Smith Kline & French, Philadelphia, Penn.) or metyrapone (100 μg/ml; 2-methyl 1,2-di-3-pyridyl 1-propanone, Sigma) were used on alternating days. In most experiments, a cytochrome P450 inhibitor was added to solutions that already contained a cyclooxygenase inhibitor. At least 10 minutes was allowed for the drug combination to equilibrate with the tissue, and the drug combination remained in the suffusates for the duration of an experiment.

To examine whether the cyclooxygenase and/or cytochrome P450 inhibitors could nonspecifically prevent vasodilation, the steady state vascular response to adenosine was examined. The mucosal and serosal surfaces were continuously suffused with buffered Ringer’s equilibrated with 5% O$_2$/5% CO$_2$/90% N$_2$. After a steady state was maintained for 10-30 minutes, adenosine was added to the serosal suffusate to produce 3-4 different concentrations from 3-300 μg/ml. Data were collected after 5-10 minutes at each suffusate adenosine concentration. The protocol was repeated with either SKF-525A alone (10 μg/ml) or SKF-525A + either meclofenamate or indomethacin (10 μg/ml) in both suffusates. Data were rejected if a complete set of paired measurements was not obtained with buffered Ringer’s and the drugs.

To compare the effect caused by bolus applications to the effect caused by steady-state applications, various concentrations of arachidonic acid were added to the suffusate chambers and then continuously suffused over the mucosa or serosa in the presence or absence of cyclooxygenase inhibitors. In some of these experiments, the mucosal suffusate also contained 10 mM bile salt (Sigma). In these experiments, the mucosal and serosal suffusates were equilibrated with 5% CO$_2$/95% N$_2$, which were the same conditions in the above described EET experiments.

Statistical Analysis

All values were expressed as mean ± standard error of the mean and normalized to “% control.” The con-
Control was a 10–60-minute steady-state baseline. All measurements obtained during a bolus application of arachidonic acid or an EET were paired with a steady-state baseline. If the baseline was not restored after a bolus, the data were rejected and the experiment was terminated. Multiple data points were obtained from individual animals, and it was assumed that all treatments were independently assorted. Data were compared using paired $t$ tests or linear regression. Significance for all tests was assessed at the 95% confidence interval.

**Results**

**Effect of Bolus Applications of EETs to the Serosa**

Figure 1 shows the vascular effects produced by the topical application of four different EETs to the intestinal serosa.

In pilot experiments, various concentrations of 8,9-EET were added to the mucosal and serosal suffusates. The increase in blood flow evoked by 8,9-EET was greater when the substance was applied to the serosa. Since the availability of the EETs was limited, the compounds were applied only to the serosa.

A 2-ml bolus containing 5–50 $\mu$g 5,6-EET/ml produced a peak blood flow change that typically exceeded 150% of control (Figure 1). In 6 animals, calculated blood flow averaged $28 \pm 5$ nl/sec in steady-state control conditions. There was no systematic effect caused by a bolus application of the ethanol vehicle only. The best-fit regression for the data shown in the top panel of the figure is described as:

$$Y = (3.86 \pm 0.98)X + 142; \ r = 0.680, \ n = 20 \quad (1)$$

where $Y =$ calculated blood flow (% control) and $X =$ 5,6-EET concentration ($\mu$g/ml). The regression was significantly linear and the slope significantly differed from 0.

A 2-ml bolus containing 10–80 $\mu$g 8,9-EET/ml produced peak responses that were lower than those elicited by 5,6-EET (Figure 1). The increases typically ranged from 120–170% of control. In 4 animals, calculated blood flow averaged $31 \pm 9$ nl/sec in steady-state control conditions. There was no systematic effect caused by bolus application of vehicle only. The best-fit regression for the data shown in the second panel of the figure is described as:

$$Y = (0.81 \pm 0.15)X + 91; \ r = 0.836, \ n = 15 \quad (2)$$

where $Y =$ calculated blood flow (% control) and $X =$ 8,9-EET concentration ($\mu$g/ml). The regression was significantly linear and the slope significantly differed from 0.

The calculated blood flow changes caused by a bolus application of 11,12-EET were similar to those by a bolus application of the ethanol vehicle only. The best-fit regression for the data shown in the top panel of the figure is described as:

$$Y = (3.86 \pm 0.98)X + 142; \ r = 0.680, \ n = 20 \quad (1)$$

where $Y =$ calculated blood flow (% control) and $X =$ 5,6-EET concentration ($\mu$g/ml). The regression was significantly linear and the slope significantly differed from 0.

A 2-ml bolus containing 10–80 $\mu$g 8,9-EET/ml produced peak responses that were lower than those elicited by 5,6-EET (Figure 1). The increases typically ranged from 120–170% of control. In 4 animals, calculated blood flow averaged $31 \pm 9$ nl/sec in steady-state control conditions. There was no systematic effect caused by bolus application of vehicle only. The best-fit regression for the data shown in the second panel of the figure is described as:

$$Y = (0.81 \pm 0.15)X + 91; \ r = 0.836, \ n = 15 \quad (2)$$

where $Y =$ calculated blood flow (% control) and $X =$ 8,9-EET concentration ($\mu$g/ml). The regression was significantly linear and the slope significantly differed from 0.
elicited by 8,9-EET (Figure 1). In 4 animals, baseline blood flow averaged 31 ± 8 nl/sec. There was no systematic effect caused by a bolus of vehicle only. In 2 animals, the increase in calculated blood flow ranged from 140–150% of control after a bolus containing 20–40 μg/ml was applied. In 2 other animals, the response was biphasic. In the whole population, calculated blood flow averaged 145 ± 8% of control (n = 7) at 12 ± 2 μg/ml and was significantly elevated above the baseline. However, at 48 ± 4 μg/ml, calculated blood flow (114 ± 8% of control, n = 4) did not significantly differ from the paired baseline. Thus, the best-fit regression for the data shown in the third panel of the figure is described by the hyperbolic function:

\[ Y = \frac{(107X)}{(X - 2)}; r = 0.981, n = 17 \]  

where Y = calculated blood flow (% control) and X = 11,12-EET concentration (μg/ml). These data are difficult to interpret, but suggest that at low doses, 11,12-EET causes a greater vasodilation than at high doses.

In contrast to the effects of the other cytochrome P450 products, 14,15-EET caused no detectable effect on calculated intestinal blood flow (Figure 1). In 3 animals, blood flow in steady-state control conditions averaged 39 ± 9 nl/sec. Neither the ethanol vehicle alone nor 14,15-EET produced any systematic change in calculated blood flow at doses up to 60 μg/ml, even though the topical application of adenosine produced vasodilation.

Table 1 shows the peak blood flow changes elicited by bolus applications of similar amounts of adenosine or the various EETs. It must be emphasized that these data are an approximate index of vasodilator potency because of the transient nature of the evoked response. At concentrations of 25–50 μg/ml, the order of potency for causing vasodilation was 5,6-EET>adenosine>8,9-EET=11,12-EET>14,15-EET. Thus, a structure activity relationship is suggested.

In 5 animals, the stability of EETs in aqueous solution was tested and the results are shown in Figure 2. When 5,6-EET (20 μg/ml; n = 1), 8,9-EET (35 μg/ml; n = 2) or 11,12-EET (12.5 μg/ml; n = 2) were added to the aqueous vehicle and then immediately applied to the tissue, calculated blood flow significantly increased to 146 ± 13% of control. When the EET was added to the aqueous solution and 20–30 minutes elapsed before it was applied to the tissue, the blood flow increase averaged only 118 ± 9% of control. These data suggest that EETs are autooxidized or hydrolyzed to less active compounds in aqueous solution.

The nature of the breakdown product is suggested by the profile of products obtained with high performance liquid chromatography shown in Figure 3. The top panel shows the standard retention time for purified synthetic [14C]5,6-EET in well-defined conditions.12 The bottom panel shows the changes after the compound has been exposed to the bicarbonate-buffered Ringer’s solution for 1 minute. More than 80% of the radioactivity was recovered in fractions that corresponded to 5,6-diHETE. The purpose of this measurement was to duplicate the conditions when the compound was applied to the microcirculation.

In summary, Figure 1 shows that EETs cause vasodilation. Table 1 compares the vasoactive potency of EETs to that of adenosine. Figure 2 shows that exposure of purified EET to water reduces the vasodilator potency. Figure 3 suggests that a large fraction of 5,6-EET was converted to 5,6-diHETE after 1 minute in aqueous solution.

Table 1. Blood Flow (BF) Changes Evoked by Various Substances (μg/ml) Topically to the Intestinal Serosa by a Bolus Method

<table>
<thead>
<tr>
<th>Dose</th>
<th>% BF</th>
<th>Dose</th>
<th>% BF</th>
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<th>% BF</th>
<th>Dose</th>
<th>% BF</th>
<th>Dose</th>
<th>% BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,6-EET</td>
<td>0</td>
<td>116 ± 4</td>
<td>10 ± 1</td>
<td>191 ± 19</td>
<td>32 ± 6</td>
<td>275 ± 38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,9-EET</td>
<td>0</td>
<td>99 ± 13</td>
<td>15 ± 5</td>
<td>98 ± 12</td>
<td>36 ± 5</td>
<td>127 ± 7</td>
<td>72 ± 4</td>
<td>148 ± 9</td>
<td></td>
</tr>
<tr>
<td>11,12-EET</td>
<td>0</td>
<td>89 ± 5</td>
<td>12 ± 2</td>
<td>145 ± 8</td>
<td>25 ± 3</td>
<td>134 ± 8</td>
<td>48 ± 4</td>
<td>114 ± 8</td>
<td></td>
</tr>
<tr>
<td>14,15-EET</td>
<td>0</td>
<td>98 ± 8</td>
<td>15 ± 5</td>
<td>101 ± 1</td>
<td>10 ± 6</td>
<td>160 ± 10</td>
<td>244 ± 14</td>
<td>260 ± 23</td>
<td></td>
</tr>
<tr>
<td>Adenosine</td>
<td>0</td>
<td>103 ± 4</td>
<td>8 ± 2</td>
<td>138 ± 12</td>
<td>27 ± 3</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Bolus was added to solution flowing at 1–3 ml/min over tissue. Therefore final concentration was always lower than nominally indicated. All blood flow changes were expressed relative to a steady-state baseline and normalized to "% control." n = number of observations at each dose. Multiple measurements were obtained in each animal, but measurements at all doses were not obtained in each animal.
Proctor et al  Epoxide-Induced Vasodilation

55

H

R MINU

O

100

800

120

1000

800

600

200

-200

Solvent A Acetoni1rite/H2O/HoAc (50:50:0.1)
Solvent B Acetoni1rite/H2O/HOAc (100:0:0.1)
Program: A to 100% B
1 hour, 1 ml/minute, Novapak C18 column

FIGURE 3. Profile of products obtained with high performance liquid chromatography. After one minute in a buffered Ringer's solution, most of the radioactivity of [14C]5,6-EET was recovered in the fraction associated with [14C]5,6-diHETE. This confirms previous studies that the free acid is extremely labile in aqueous solution.

Effect of Cyclooxygenase and Cytochrome P450 Inhibitors on the Response Elicited by Bolus Applications of Arachidonic Acid to the Serosa

A 2-ml bolus containing ≤ 2 mg arachidonic acid/ml manually infused into a flowing serosal suffusate produced a transient hyperemia, but higher concentrations produced irreversible vasodilation. If a cyclooxygenase inhibitor was added to the mucosal and serosal suffusates, the vasodilation elicited by ≤ 2 mg arachidonic acid/ml was significantly attenuated and the response elicited by 2–4 mg arachidonic acid/ml was reversible (Figures 4 and 5). The residual hyperemia elicited by arachidonic acid in the presence of a cyclooxygenase inhibitor was eliminated by a cytochrome P450 inhibitor (Figures 4 and 5). These data suggest that the major fraction of hyperemia evoked by the serosal application of arachidonic acid is caused by a cyclooxygenase product, but that a product derived from the cytochrome P450 pathway might cause vasodilation when cyclooxygenase is inhibited.

In 2 animals, a bolus containing arachidonic acid was infused into either the serosal or mucosal suffusate. Two different concentrations (1 and 2 mg arachidonic acid/ml) were applied in random sequence. If arachidonic acid was applied to the serosal side, calculated blood flow increased to 257 ± 20% of control, but the same dose applied to the mucosal side caused no significant change in blood flow. A similar regional difference has been reported for the vasodilator effects of adenosine. Thus, the serosa was apparently a more sensitive assay tissue for the vasodilator effects of adenosine and arachidonic acid.

In 7 animals, calculated blood flow averaged 21 ± 3

nl/sec in steady-state control conditions with no inhibitors in the suffusates. A 2-ml bolus containing ethanol vehicle alone produced no systematic effect on blood flow. This demonstrates that the mechanical effect of the bolus and the metabolic effect of the ethanol was minimal in these conditions (Figure 4, top left). A bolus containing ≤ 2 mg arachidonic acid/ml produced transient increases in blood flow that usually exceeded 200% of control. The baseline was restored within 5–10 minutes after each arachidonic acid application.

If more than 2 mg arachidonic acid/ml was infused into the suffusate, the vasodilation was sustained for > 30 minutes. In addition, the topical application of adenosine produced no further vascular effect, which suggested that the arterioles were maximally dilated.

The top right panel of Figure 4 illustrates responses in 7 animals in which ≤ 2 mg arachidonic acid/ml was applied to the serosa in the presence of a cyclooxygenase inhibitor. Calculated blood flow averaged 15 ± 2

nl/sec in steady-state control conditions with the serosal and mucosal suffusates containing meclofenamate (n = 4) or indomethacin (n = 3). A bolus containing the vehicle produced no systematic effect on baseline blood flow. Arachidonic acid in the presence of cyclooxygenase inhibitors caused variable increases in calculated blood flow that ranged from 125–150% of control. Responses with indomethacin or meclofenamate were virtually identical.

The bottom left panel of Figure 4 shows responses from 6 animals in which 2–4 mg arachidonic acid/ml was applied to the serosa in presence of a cyclooxygenase inhibitor. Calculated blood flow averaged 14 ± 2

nl/sec with either meclofenamate (n = 4) or indometh-
The effect of cyclooxygenase inhibitors and cytochrome P450 inhibitors on peak changes in calculated blood flow (% control) in individual animals caused by a bolus application of arachidonic acid into the serosal suffusate. The control was a 10–30-minute steady-state baseline before the application of arachidonic acid. The baseline was restored after each bolus administration or the data were rejected. The value at 0 in each of the four panels represents the response to a bolus of 5–10% ethanol in Ringer’s, which was the vehicle for arachidonic acid. In the top left panel, the experiment was performed with the tissue suffused with buffered Ringer’s solution, which was the vehicle for all inhibitors of arachidonic acid oxidation. In the top right and bottom left panels, meclofenamate or indomethacin (cyclooxygenase inhibitors) were added to the suffusates. In the bottom right panel, either SKF-525A or metyrapone (cytochrome P450 inhibitors) were combined with either meclofenamate or indomethacin.

At least three explanations could account for the residual hyperemia elicited by arachidonic acid in the presence of a cyclooxygenase inhibitor: a nonspecific artifact of high doses of arachidonic acid (e.g., detergent effect on cells, oxygen-free radical formation); a breakthrough of cyclooxygenase inhibition; or vasodilation caused by a noncyclooxygenase metabolite of arachidonic acid. In support of the latter idea, the response was blocked by adding structurally dissimilar inhibitors of cytochrome P450 to both suffusates.

In 2 animals, neither metyrapone nor SKF-525A altered the magnitude of the hyperemia induced by the serosal application of arachidonic acid. These observations suggested that cytochrome P450 products were formed in the serosa only when cyclooxygenase was inhibited.

The bottom right panel of Figure 4 shows responses from 6 animals treated with a cyclooxygenase inhibitor.
Elicited by Continuous Applications of Arachidonic Acid to the Mucosa and Serosa

The serosal application of arachidonic acid caused a relatively large steady-state blood flow increase, whereas the mucosal application of arachidonic acid alone, even at higher concentrations, had no observable effect. Even in the presence of bile, mucosal arachidonic acid caused a relatively small blood flow increase. Cyclooxygenase inhibitors markedly reduced the response to serosal arachidonic acid, but had no effect on the increase caused by mucosal arachidonic acid plus bile.

Figure 6 shows that a continuous topical application of 122 ± 45 μg arachidonic acid/ml (n = 4 animals) to the serosa caused a steady-state blood flow increase (299 ± 27% of control) that was significantly reduced (121 ± 9% of control) by meclofenamate (n = 2) or indomethacin (n = 2). In contrast, the mucosal application of 194 ± 6 μg arachidonic acid/ml (n = 5) elicited no observable response when the mucosa was suffused with a bicarbonate-buffered Ringer’s. If 10 mM bile salt was added to the mucosal suffusate, then arachidonic acid elicited a blood flow increase that averaged 119 ± 8% of control. The addition of meclofenamate (n = 3) or indomethacin (n = 2) to the solution did not attenuate this response; instead, the blood flow increase averaged 132 ± 8% of control during application of arachidonic acid plus cyclooxygenase inhibitors.

These results suggest that a vasodilator derived from a noncyclooxygenase pathway might mediate arachidonic-acid–induced hyperemia in the mucosa but not the serosa.
steady-state blood flow changes (% control) elicited by continuous application of arachidonic acid to the mucosa or serosa. The serosal response was similar to that elicited by a bolus application of arachidonate, but the mucosal response was smaller in magnitude and not altered by cyclooxygenase inhibitors.

**Discussion**

Purified synthetic compounds derived from the cytochrome P450 pathway of arachidonic acid metabolism (EETs) caused vasodilation in the intestinal microcirculation (Figure 1). At least one of these compounds, 5,6-EET, was a more potent vasodilator than adenosine in comparable conditions (Table 1) even though its vascular effect was probably underestimated because of its instability (Figures 2 and 3). Previous studies have demonstrated the rapid hydrolysis of EETs in aqueous solution. Therefore, it is reasonable to speculate that endogenous production of EETs could be an important vasodilator influence on the microcirculation. Unfortunately, we could not convincingly demonstrate that vasoactive quantities of EETs were produced in vivo from exogenous arachidonic acid.

Since cytochrome P450 is distributed within blood vessels and in intestinal extravascular tissue, we designed experiments to test whether some or all of the microcirculatory response evoked by exogenous arachidonic acid would be mediated by EETs. Interpretation of these results (Figures 4–6) is limited by the specificity of the various inhibitors of arachidonic acid oxidation and by a regional difference in the response evoked by arachidonic acid within the intestinal wall. Cyclooxygenase inhibitors reduced most of the blood flow increase evoked by bolus or continuous applications of arachidonic acid to the serosa (Figures 4–6), so most of that response could be attributed to vasoactive prostanoids. A small residual response was eliminated by metyrapone or SKF-525A, which are putative inhibitors of cytochrome P450. However, these drugs might have nonspecifically inhibited lipoxigenase, so the participation of a vasodilator derived from a lipoxigenase pathway cannot be excluded. It is unlikely that this residual hyperemia is a nonspecific artifact or a breakthrough of cyclooxygenase inhibition because structurally dissimilar compounds (metyrapone or SKF-525A) had similar effects (Figures 4, 5) without systematically altering baseline blood flow or the vasodilation caused by adenosine. Nevertheless, these data imply that an EET might be produced from exogenous arachidonate only when cyclooxygenase is inhibited. Even then, the vascular response was small compared to that elicited by cyclooxygenase products.

On the other hand, cyclooxygenase inhibitors actually potentiated the vasoactive effect of arachidonic acid when it was applied to the mucosa (Figure 6), which confirms previous results. There was a striking difference in the magnitude of the blood flow increases evoked by the application of arachidonic acid to the mucosa or serosa (Figure 6). These data suggest that noncyclooxygenase products account for most of the vasodilation caused by mucosal arachidonic acid, but this response is relatively small. A corollary is that there may be a regional difference in arachidonic acid metabolism within the intestinal wall.

It was assumed that the response to an arachidonic acid metabolite was reversible and that the basal metabolic status of the tissue was unaltered if the baseline blood flow was restored after a bolus application and if the vasodilator response to adenosine was preserved. However, it is conceivable that an EET, or arachidonic acid in the presence of the different inhibitors, altered some aspect (e.g., capillary permeability, oxygen consumption, intestinal secretion, etc.) that was not directly measured. To minimize these possible artifacts on blood flow changes, the sequence of application of the various doses was randomized.

**Interpretation**

Previous reports have suggested that arachidonic acid metabolites participate in the regulation of intestinal blood flow during absorption. The effect of arachidonic acid or cyclooxygenase inhibitors has been attributed to alterations in the food-induced increase in jejunal oxygen uptake or to the action of a nonprostaglandin vasoactive metabolite that was unmasked by inhibiting cyclooxygenase. These present experiments do not directly relate to the physiologic regulation of intestinal blood flow because the metabolism of exogenous arachidonic acid is both qualitatively and quantitatively different from the metabolism of endogenous arachidonic acid in most tissues.

At least three actions of certain fats could theoretically promote EET synthesis during absorption: 1) inhibition of cyclooxygenase; 2) activation of phospholipase to mobilize arachidonic acid from membrane phospholipids; and 3) de novo synthesis of arachidonic acid from precursors in the meal. Thus, fats could promote EET synthesis by increasing the available substrate (arachidonic acid) and by partially inhibiting a competing enzyme system (cyclooxygenase).

A growing body of evidence has implicated the cytochrome P450 pathway as a source of arachidonic acid metabolites with potentially important physiologic roles.
from endothelial cells that is an extremely labile, non-
cyclooxygenase product whose action is blocked by
putative cytochrome P450 inhibitors. These present
results may not be directly related to previous in vitro
studies on vascular smooth muscle. Nevertheless, 5,6-
EET is derived from arachidonic acid, is extremely
potent as a vasodilator, and has a very short half-life in
aqueous solution, so it might be considered a possible
candidate as an endothelial-derived relaxing factor.

In conclusion, the results of this study showed that
EETs are a labile class of vasodilators with a potency
comparable to adenosine in the intestinal microcircula-
tion. In conditions where arachidonic acid availability
is increased, cyclooxygenase is inhibited, and/or cy-
tochrome P450 is activated, EETs might be produced
in vivo and could be important determinants of vaso-
motor tone. However, expression of the vasoactive
effects of EETs in physiologic conditions is probably
limited by substrate availability and by the activity of
the cyclooxygenase system.

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