14,15-Epoxyeicosatrienoic Acid Inhibits Platelet Aggregation in Mouse Cerebral Arterioles

Marcia L. Heizer, BS; Jerry S. McKinney, BS; and Earl F. Ellis, PhD

Background and Purpose: Epoxygenase metabolites of arachidonic acid are produced by several tissues and have been shown to inhibit in vitro platelet aggregation. The purpose of the present investigation was to determine whether 14,15- or 8,9-epoxyeicosatrienoic acid, epoxygenase derivatives of arachidonic acid, affect the speed of platelet aggregation in in vivo mouse cerebral arterioles.

Methods: We performed a craniectomy in 116 anesthetized male mice and observed the pial arterioles by microscopy. We induced in situ platelet aggregation using a mercury light and intravascularly injected fluorescein dye.

Results: Indomethacin (0.5 mg/kg i.p.), a known cyclooxygenase inhibitor, and 14,15-epoxyeicosatrienoic acid (0.3 mg/kg i.v.) increased the time necessary for the light plus dye to induce the first arterial platelet aggregate by 35% and 26%, respectively, whereas 8,9-epoxyeicosatrienoic acid (0.3 mg/kg i.v.) had no effect. Analysis of mouse serum by radioimmunoassay showed that the degree of inhibition of platelet aggregation by indomethacin and epoxyeicosatrienoic acids correlated with the degree of inhibition of thromboxane production.

Conclusions: We conclude that 14,15-epoxyeicosatrienoic acid is a potent inhibitor of in vivo platelet aggregation but cannot conclusively confirm that its effect on aggregation occurs via its reduction of platelet thromboxane A2. Because epoxyeicosatrienoic acids are produced by several tissues, including brain and vascular tissue, they may be important in vivo modulators of platelet aggregation and hemostasis. (Stroke 1991;22:1389–1393)

The cyclooxygenase and lipoxygenase enzyme pathways of arachidonic acid metabolism were the first and second pathways of arachidonic acid metabolism to be discovered. The cyclooxygenase pathway produces prostaglandins and thromboxane A2 (TXA2), the latter of which is produced by platelets and is an important in vivo stimulator of platelet aggregation. It is well known that inhibition of platelet cyclooxygenase by aspirin or other cyclooxygenase inhibitors will increase bleeding time. In more recent years, a third pathway of arachidonic acid metabolism, the cytochrome P-450/monooxygenase pathway, has been described. This pathway is also referred to as the “third pathway” of arachidonic acid metabolism, or “epoxygenase” pathway. In this third pathway, epoxides and hydroxylated products are formed. Four different epoxides can be synthesized, each having an epoxide ring in the 5,6, 8,9, 11,12, or 14,15 position of the arachidonic acid molecule. Third pathway metabolites have been shown to be formed enzymatically by a number of tissues, including liver,2,3 kidney,4,5 cornea,6 vascular,7 and nervous tissue.8 The function of epoxides is not completely understood; however, investigations in the last few years have shown that they can inhibit Na+,K+-ATPase,9 affect blood flow,10 and also affect hormone and calcium disposition.8,11

Studies by Fitzpatrick et al12 have shown that certain of the epoxides can also inhibit in vitro platelet aggregation. Their experiments showed that epoxides were capable of inhibiting purified seminal vesicle cyclooxygenase; however, their capacity to inhibit thromboxane B2 (TXB2) production did not always completely correlate with their capacity to inhibit in vitro arachidonic acid–induced platelet aggregation. We have recently shown that mouse brain slices can synthesize 14,15-epoxyeicosatrienoic acid (14,15-EET).13 Because epoxyeicosatrienoic acids...
are produced by the brain they may, under certain circumstances, be released and influence the vascular wall or circulating blood cells. The purpose of the present studies was to determine whether epoxides are capable of inhibiting in vivo platelet aggregation in the cerebral microcirculation. Our results indicate that 14,15-EET is approximately equipotent to indomethacin in slowing in vivo aggregation in the cerebral microcirculation and that 14,15-EET inhibition of serum thromboxane production correlates with the effects of this epoxide on in vivo platelet aggregation. However, 8,9-epoxyeicosatrienoic acid (8,9-EET) affects neither in vivo aggregation nor TXB2 formation.

**Materials and Methods**

In vivo platelet aggregation studies were conducted in a total of 116 Institute of Cancer Research male mice weighing an average of 33±0.5 (mean±SE) g. Initially, the mice were anesthetized with 2 mg/g urethane i.p. and intubated to facilitate respiration. Next, a craniectomy was made and the dura removed for visualization of the pial arterioles by in vivo microscopy, as previously described by Rosenblum and El-Sabban.14 To induce platelet aggregation, 0.2 ml of a sodium fluorescein solution was injected into the tail vein. To initiate platelet aggregation, the light illuminating the cranial surface was switched from a tungsten light source to a mercury lamp with a heat filter and filters to provide a 300–500-nm wavelength of light. The light interacts with fluorescein dye to produce platelet aggregates in the venous and arterial microcirculation. As previously reported, aggregation first occurs in the slower flowing venules, followed by aggregation in the arterioles.14 To demonstrate that we had accurately reproduced the technique in our laboratory, we first showed that indomethacin, a cyclooxygenase inhibitor and inhibitor of platelet thromboxane formation, would cause a slowing of platelet aggregation. The sodium salt of indomethacin, a cyclooxygenase inhibitor and inhibitor of platelet thromboxane formation, would cause a slowing of platelet aggregation. The sodium salt of indomethacin (Sigma Chemical Co., St. Louis, Mo.) was administered intraperitoneally (0.5 mg/kg) in a total volume of 0.2 ml saline in 18 rats 30 minutes before inducing aggregation. Controls (n=17) received 0.2 ml of saline with an appropriate amount of sodium carbonate solution.

We next examined the effects of 14,15-EET and 8,9-EET, which were obtained from Biomol (Plymouth Meeting, Pa.). The epoxyeicosatrienoic acids obtained from Biomol were concentrated such that the total dose was in 10 μl ethanol. This 10-μl solution was then diluted to 0.1 ml with saline and administered intravenously 1 minute before inducing aggregation. Controls received only the vehicle.

To correlate the effects of indomethacin and epoxide administration on in vivo platelet aggregation with their respective effects on the proaggregatory TXA2, we also analyzed TXB2 formation in mouse blood. Indomethacin or the individual epoxyeicosatrienoic acids were administered in a manner identical to that for studies of in vivo platelet aggregation. The carotid arteries of the mouse were cut, and blood was collected into a 1-ml syringe. The blood was then immediately transferred to a plastic tube and allowed to clot. The tubes were then spun in a centrifuge and the serum collected. As previously reported,15,16 aliquots of the serum were extracted using Baker reverse phase solid phase extraction columns and analyzed for TXB2 using specific antibodies and the method obtained from Advanced Magnetics (Boston, Mass.). All radioimmunoassays were run in duplicate and the results calculated using the computerized program of M.L. Jaffe and Associates (Silver Spring, Md.).

To determine whether 14,15-EET affected cerebral blood flow and therefore might affect aggregation by mechanisms other than action on platelets, we examined the effect of 14,15-EET on the local cerebral microcirculation using a laser–Doppler flowmeter (model BPM 403a, TSI, St. Paul, Minn.). We have previously shown in detail that laser–Doppler flowmetry can reproducibly and linearly measure changes in flow in the microcirculation of the brain cortical surface.17,18 Briefly, the laser–Doppler probe was placed over the cerebral microcirculation adjacent to the microscopic field of view such that the edge of the laser–Doppler probe could be seen through the microscope. The TSI laser–Doppler flowmeter measures velocity and volume, which are multiplied electronically to produce an indication of flow. The control laser–Doppler output for flow was recorded and 14,15-EET (0.3 mg/kg) was injected intravenously and the effect on flow monitored.

Previous studies of the cerebral microcirculation in mice have shown that there is some day-to-day variation between control platelet aggregation times. The cause or reason for the changing day-to-day control aggregation times is uncertain but has been a consistent report in the literature. Therefore, to analyze platelet aggregation times and determine the effects of various experimental drugs, we examined both control and experimental animals on each day of investigation. Thus, for the in vivo platelet aggregation experiments, two-way analysis of variance was performed using treatment and day as the two factors. For examination of thromboxane results, analysis of variance and a multiple range test were performed. A value of p≤0.05 was considered significant.

**Results**

The studies with laser–Doppler flowmetry showed that 14,15-EET (0.3 mg/kg i.v.) had no effect on cerebral blood flow in the cerebral microcirculation (data not shown). These findings agree with our previous studies using the cranial window technique, which showed that topically applied 14,15-EET does not affect rabbit pial arteriolar diameter.19

As shown in Table 1, indomethacin caused a significantly delayed time to first platelet aggregate, as previously reported by Rosenblum and El-Sabban.14 Table 1 also shows that the 0.3-mg/kg dose of 14,15-EET significantly increased the time to first aggregate. Lower doses of 14,15-EET did not produce a statistically significant effect on platelet aggre-

---

**Table 1**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time to First Aggregate (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.8</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>6.2</td>
</tr>
<tr>
<td>8,9-EET</td>
<td>5.1</td>
</tr>
</tbody>
</table>

---
Table 1. Effect of Epoxyeicosatrienoic Acids and Indomethacin on Time to First Platelet Aggregate in Cerebral Arterioles

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>n</th>
<th>Arteriolar diameter (µm, ±SE)</th>
<th>Time to first aggregate (sec±SE)</th>
<th>Experimental control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (0.5 mg/kg)</td>
<td>18</td>
<td>35±1</td>
<td>127±7</td>
<td>1.35</td>
<td>0.002</td>
</tr>
<tr>
<td>Control</td>
<td>17</td>
<td>34±1</td>
<td>95±7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14,15-EET 0.3 mg/kg</td>
<td>10</td>
<td>42±1</td>
<td>118±4</td>
<td>1.26</td>
<td>0.003</td>
</tr>
<tr>
<td>Control</td>
<td>11</td>
<td>43±1</td>
<td>94±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 mg/kg</td>
<td>10</td>
<td>37±1</td>
<td>105±4</td>
<td>1.06</td>
<td>0.19</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>38±1</td>
<td>99±4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03 mg/kg</td>
<td>10</td>
<td>39±1</td>
<td>105±5</td>
<td>1.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>38±1</td>
<td>99±5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,9-EET (0.3 mg/kg)</td>
<td>10</td>
<td>39±2</td>
<td>97±6</td>
<td>1.07</td>
<td>0.52</td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>39±2</td>
<td>91±6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

EET, epoxyeicosatrienoic acid. Because previous investigations have shown there is day-to-day variability among mice, both control and experimental mice were examined each day and two-way analysis of variance was used. Both indomethacin and 0.3-mg/kg dose of 14,15-EET significantly delayed in vivo platelet aggregation in pial arterioles of mice. Lower doses of 14,15-EET were without statistically significant effect; however, average time to aggregation is longer and probability value for 14,15-EET is smaller as dose is increased. 8,9-EET at 0.3-mg/kg dose had no effect on speed of in vivo aggregation.

Discussion

The epoxyeicosatrienoic acid derivatives of arachidonic acid have been shown to inhibit in vitro platelet aggregation; however, average aggregation times increased compared with control, and the probability values became progressively smaller as the dose increased. Table 1 also shows that 8,9-EET had no effect on time to first aggregate.

To determine whether the slowing of platelet aggregation by indomethacin and 14,15-EET and the lack of effect by 8,9-EET might correlate with their effects on platelet production of the proaggregatory TXA2, we measured TXB2 formation in blood collected from mice treated with the various agents. As shown in Figure 1, animals given indomethacin intraperitoneally 30 minutes before collection of blood had a significantly reduced formation of TXB2. However, 8,9-EET, which had no effect on in vivo aggregation, also had no effect on thromboxane formation. Interestingly, however, 14,15-EET (0.3 mg/kg), which slowed in vivo platelet aggregation almost as much as indomethacin, also inhibited TXB2 almost as effectively as indomethacin.

Fitzpatrick et al12 showed that inhibition of in vitro platelet aggregation by epoxyeicosatrienoic acids was not always associated with a reduction of TXB2 formation. Evidence in the present study that slowing of platelet aggregation by 14,15-EET may not be due to an effect on TXB2 formation may be derived from...
our recent studies of the effect of ω-3 fish oils on in vivo platelet aggregation.20 We found that chronic dietary enrichment with fish oil reduced serum TXB2 formation by 40% but did not cause an alteration in platelet aggregation, as studied by the light-plus-dye method. Therefore, the currently reported 50% decrease in TXB2 formation may not be the cause of the 14,15-EET effect on platelet aggregation.

Wong et al21 have recently shown in rats that intra-arterially administered 14,15-EET causes a greater blood pressure decrease than intravenously administered 14,15-EET. However, effects on blood pressure are not likely to influence aggregation in the mouse brain since our laser-Doppler studies showed no effect of 14,15-EET on cerebral blood flow. Additionally, a decrease in blood pressure should, if anything, transiently slow cerebral blood flow only until cerebral autoregulation occurs. A decrease in cerebral microcirculatory flow would cause a speeding, and not the observed slowing, of platelet aggregation.

The potency of 14,15-EET in inhibiting platelet aggregation is similar to that of indomethacin because, if one transforms doses we administered into nanomoles per mouse, the amount of indomethacin given is 45 nmol and that of 14,15-EET is 31 nmol. Therefore, it can be concluded that at pharmacological doses, 14,15-EET is a potent inhibitor of in vivo platelet aggregation and platelet thromboxane formation.

The effect of indomethacin on platelet cyclooxygenase is reversible whereas that of aspirin is irreversible and results in inhibition of platelet cyclooxygenase for the circulating life of the platelet. With respect to 14,15-EET, it is unknown how long the inhibition of platelet aggregation lasts. Because 14,15-EET has been shown to be made by the vasculature7 and brain,13 we speculate that local formation by the vasculature may contribute to modulation of platelet aggregation. However, it might be suggested that the concentration produced by the brain and vasculature is not likely to be as great as the concentration obtained when 0.3 mg/kg i.v. 14,15-EET is given to a mouse. This suggestion may be true, but it may also be countered since it is known that 14,15-EET is metabolized by epoxide hydrolase to form a 14,15-dihydroxylated eicosatrienoic acid.1 Therefore, the 0.3-mg/kg dose of 14,15-EET may be rapidly degraded, which results in much lower circulating levels of 14,15-EET. Whether the 14,15-dihydroxylated eicosatrienoic acid also inhibits platelet aggregation is also unknown but is an important question for future investigations.

It has been shown that 14,15-EET is an inhibitor of Na+,K+-ATPase, and its excessive production has been implicated in the hypertension found in an animal model of hypertension, the spontaneously hypertensive rat. Previous investigations by Sacerdotti et al22 have shown that developing hypertensive rats produce more P-450 metabolites of arachidonic acid and that inhibiting formation of these P-450 metabolites is associated with a decrease in blood pressure.

More recently, Catella et al23 have shown that in human pregnancy-induced hypertension, increased levels of 14,15-EET are excreted in the urine. Because pregnancy-induced hypertension is known to be associated with increased platelet activation, the increased formation of 14,15-EET may be a compensatory mechanism to counteract increased platelet activity in these patients. This, however, is speculative and will need to be examined.

In conclusion, the current studies show that 14,15-EET is a potent inhibitor of in vivo platelet aggregation and causes a reduction in platelet thromboxane production. Our results suggest that studies of 14,15-EET in subjects with increased or decreased hemostatic mechanisms may be useful. Additionally, because epoxyeicosatrienoic acids are produced by brain and vascular tissue,13,19 14,15-EET may be an important endogenous substance for regulation of in vivo platelet aggregation in cerebral and other vasculatures.

Acknowledgments

We thank Dr. W.I. Rosenblum for teaching us the in vivo platelet aggregation technique. We are grateful to S.A. Holt for her excellent administrative assistance.

References


Key Words • arachidonic acid • microcirculation • platelet aggregation • mice
14,15-Epoxyeicosatrienoic acid inhibits platelet aggregation in mouse cerebral arterioles.
M L Heizer, J S McKinney and E F Ellis

Stroke. 1991;22:1389-1393
doi: 10.1161/01.STR.22.11.1389
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/22/11/1389

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in
Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office.
Once the online version of the published article for which permission is being requested is located, click Request
Permissions in the middle column of the Web page under Services. Further information about this process is
available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/