Ibuprofen Protects Platelet Cyclooxygenase from Irreversible Inhibition by Aspirin

Gundu H. R. Rao, Gerhard G. Johnson, K. Ratnammal Reddy, and James G. White

Previous investigations have shown that ibuprofen inhibits the second wave of platelet aggregation and blocks the conversion of 14C-arachidonic acid to thromboxane. However, the influence of the drug on platelet function and cyclooxygenase is transitory, lasting only 24 hours. The present study has taken advantage of the short-lived influence of ibuprofen to study its interaction with the long-term effects of aspirin. As expected, both aspirin and ibuprofen suppressed platelet cyclooxygenase activity and function, but addition of aspirin to ibuprofen-treated platelets did not increase the degree of inhibition in vitro. Platelet function and prostaglandin synthesis recovered completely 26 hours following ingestion of ibuprofen, but remained compromised 26 hours after taking aspirin. When 650 mg of aspirin was administered after ibuprofen, platelet function and cyclooxygenase activity recovered as completely at 26 hours as did platelets which had been exposed to ibuprofen alone. Thus, prior exposure to ibuprofen in vivo completely protected cyclooxygenase from the irreversible effects of aspirin. Our findings indicate that ibuprofen-like indomethacin and other nonsteroidal antiinflammatory drugs react with the heme group of cyclooxygenase to prevent arachidonic acid conversion. Since ibuprofen completely blocks the effects of aspirin in platelets in vitro and in vivo, aspirin’s primary influence on inhibition of cyclooxygenase must also be through action on the heme portion of the enzyme, rather than acetylation of the protein.

(Arteriosclerosis 3:383-388, July/August 1983)

Aspirin is a powerful inhibitor of platelet function and prostaglandin synthesis.\(^1^\)\(^2\) Investigations into its mechanism of action several years ago\(^3^\)\(^5\) suggested that it suppressed conversion of arachidonic acid by acetylating the enzyme cyclooxygenase. Other potent inhibitors of prostaglandin formation, however, are incapable of acetylating proteins and must block cyclooxygenase in some other manner.\(^5^\)\(^-^\)\(^1^\)\(^4\) Studies in our laboratory using a cell-free system demonstrated that inhibitors of prostaglandin synthesis (including aspirin, indomethacin, tolmelin, and ibuprofen) prevented arachidonic acid oxidation by interfering with the action of ferrous iron or heme.\(^1^\)\(^5^\)\(^-^\)\(^1^\)\(^8\) Additional experiments with iron-chelating agents (such as aminotriazole, sodium cyanide, and dipyridyl) on intact platelets supported the concept that the redox state of heme plays an important role in prostaglandin synthesis and the mechanism of action of its inhibitors.\(^1^\)\(^9^\)\(^-^\)\(^2^\)\(^1\)

Ibuprofen (Motrin) is a nonsteroidal and antiinflammatory agent with antipyretic and antiplatelet properties.\(^2^\)\(^2^\)\(^-^\)\(^2^\)\(^5\) Like aspirin, ibuprofen inhibits platelet function by blocking prostaglandin synthesis,\(^1^\)\(^1\)\(^\)\(^1\)\(^4\)\(^\)\(^2^\)\(^4\)\(^\)\(^2^\)\(^5\) and, as a result, inhibits the second wave of platelet aggregation caused by epinephrine and adenosine diphosphate (ADP).\(^1^\)\(^1\) Unlike aspirin, the effect of ibuprofen on platelet cyclooxygenase is short-lived, and platelets are no longer affected 24 hours after administration of the drug in vivo.\(^1^\)\(^1\) Studies in vitro suggest that the agent may compete with aspirin for the active site on the enzyme.\(^1^\)\(^4\)

The reported differences between the effects of aspirin and ibuprofen suggested that in vivo studies might help to clarify the mechanism or mechanisms by which the different agents inhibit prostaglandin synthesis. Ibuprofen was given alone or before administration of aspirin to human and canine subjects.
As reported earlier, the effects of the drug on platelet prostaglandin synthesis and function revealed that the ibuprofen-induced suppression of enzyme activity was short-lived, recovery occurring within 24 hours after drug ingestion. Aspirin given after ibuprofen failed to influence the rapid recovery pattern observed with ibuprofen alone. Although acetylation may make the influence of aspirin irreversible, the agent appears to inhibit platelet cyclooxygenase through its action on ferrous heme in the same manner as ibuprofen.

**Methods**

**Materials**

Arachidonic acid as the sodium salt was obtained from Parke Davis Company, Detroit, Massachusetts. Commercially available aspirin and motrin were used for all the in vivo studies. The sodium salt of ibuprofen and an injectable preparation of the drug were a gift from the Upjohn Company, Kalamazoo, Michigan. Injectable adrenaline was obtained from Parke Davis Company, Detroit, Michigan. Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Company, St. Louis, Missouri. Blood drawn into plastic syringes was mixed immediately with trisodium citrate-citric acid-dextrose (CCD) buffer, (0.1 M citrate, mM citric acid, 0.14 M dextrose, pH 6.5) in a ratio of 9 parts blood to 1 part anticoagulant. Platelet-rich plasma (PRP) was separated by centrifugation at 100 × g for 20 minutes at room temperature. The recovery of aggregation response to arachidonate and other agents following drug ingestion was followed by testing the platelets obtained at various time periods on a dual channel Payton aggregometer or on a lumiaggregometer.

For measurement of arachidonic acid metabolism, reaction mixtures (1 ml) containing 1.5 × 10⁶ cells were stirred on an aggregometer for 5 minutes at 37°C with 1 μg of labeled arachidonic acid. At the end of an experiment, 1 ml of ethyl acetate was added to each reaction mixture and acidified to pH 3.5 with 0.5 M citric acid. After thorough mixing, the ethyl acetate layer was separated and the reaction mixture was reextracted once more with an equal volume of ethyl acetate. Fractions of the organic phase were pooled, concentrated over nitrogen, and plated on a silica gel G plate. The solvent system used for the separation of thromboxane was ether/methanol/ acetic acid (135:3:3, vol/vol/vol). Radioactive spots were monitored with a Berthold radiolabel scanner and quantitation was achieved by separation of the spots and scintillation counting. Thin layer plates were incubated with X-ray films (x-Omat, RXR-S Kodak) for 2 days and then developed for localization of radioactive spots. Standard statistical procedures were used to calculate standard deviation and Student's *t* test of significance. Procedures used in these studies have been approved by the committee on the use of animals and human subjects.

**Subjects and Experimental Animals**

Blood for these studies was obtained from normal human donors after informed consent, and from mongrel dogs. Human subjects who agreed to participate in this study were divided into four groups. The first group, considered "normal controls," had not taken aspirin or any other medication for 2 weeks before the study. A second drug-free group was given a single dose of aspirin (650 mg). A third group received a single dose of ibuprofen in the amount of 5 mg/kg. The fourth group received 5 mg/kg of ibuprofen and, after 90 minutes, 650 mg of aspirin. Blood was drawn for biochemical and physiological studies before the administration of the drugs and 1.5 and 24 hours after drug ingestion. Salicylate levels were determined in the plasma of all subjects who received aspirin to establish that the drug has been absorbed.

For studies on canine platelets, mongrel dogs were chosen whose platelets had been previously found to aggregate and produce thromboxane A₂ when stirred with arachidonic acid. All the dogs used in these studies were healthy and were maintained in the animal facilities several weeks before the study. The effects of ibuprofen and aspirin on canine platelet function were evaluated using the same protocol as that used for human platelet studies. Platelets separated from blood samples were tested for their aggregation response to arachidonate and for their ability to convert 1⁴C-arachidonic acid to thromboxane. All studies were repeated several times and the values presented represent a mean of six replicates.

**Results**

**Salicylate Levels After a Single Dose of Aspirin**

Levels of salicylate were measured in plasma samples obtained from blood drawn 1 hour after aspirin ingestion. Levels of salicylate in human volunteers that received 650 mg of aspirin was 2.8 ± 0.4 mg%. Those who received ibuprofen followed by aspirin had a salicylate level of 7.4 ± 0.6 mg%. In dogs the salicylate levels were 3.4 ± 0.6 and 7.1 ± 0.8 mg%, respectively.

**Aggregation Response of Platelets after a Single Dose of Aspirin**

Normal human platelets and the platelets from the dogs chosen for these studies developed irreversible aggregation when stirred with arachidonate and they released significant quantities of ATP. After oral aspirin, human and dog platelets did not aggregate when stirred with arachidonate and remained unresponsive to this agent for 24 hours after drug inges-
IBUPROFEN-INDUCED PROTECTION OF CYCLOOXYGENASE  Rao et al. 385

Figure 1. Effect of a single oral dose of ibuprofen (5 mg/kg) on the response of canine and human platelets to the action of 0.45 mM arachidonate (AA). At the dose of 5 mg/kg, ibuprofen effectively blocked arachidonate-induced platelet aggregation.

IBUPROFEN (5mg/kg)  Aspirin (650mgs)  IBUPROFEN + ASPIRIN

ΔT*-change in light transmission

Figure 2. Effect of ibuprofen and aspirin on the response of human platelets to arachidonate (AA). Samples of platelets obtained from blood drawn 24 hours after ibuprofen ingestion aggregated irreversibly and released significant quantities of ATP when stirred with arachidonate. However, both human and canine platelets recovered from this inhibitory effect and developed aggregation and the release reaction in response to the action of arachidonate by 24 hours after ibuprofen ingestion.

Recovery of Aggregation Response after Ingestion of Both Ibuprofen and Aspirin

Platelets obtained from human and canine blood samples 1 hour after the administration of ibuprofen showed no aggregation in response to the action of arachidonate. The release reaction was also completely inhibited in these platelets. These patients then received aspirin, and 1 hour later blood was drawn. Samples obtained after aspirin administration showed no synergistic effect. However, unlike patients given aspirin alone, samples from those receiving both drugs aggregated in response to arachidonate 24 hours after drug ingestion. These samples also released significant quantities of ATP when stirred with arachidonate, showing total recovery of functional capabilities (Figure 2).

Effect of Single Doses of Aspirin on the Thromboxane Synthesis

The ability of human and dog platelets to convert 14C-arachidonic acid to thromboxane was monitored before and after the drug administration. Normal control platelets converted 32% of the substrate arachidonic acid to thromboxane (Table 1). Platelets obtained from subjects after a single dose of aspirin converted 3% to 4% of the substrate to thromboxane at 1.5 and 24 hours.
Table 1. Effect of In Vivo Ibuprofen (IB) and/or Aspirin (AS) on $^{14}$C-Arachidonic Acid Conversion to Thromboxane (TXB$_2$) by Canine and Human Platelets

<table>
<thead>
<tr>
<th></th>
<th>CPM $\times 10^3$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total counts</td>
<td>Counts recovered</td>
<td>% Conversion</td>
</tr>
<tr>
<td></td>
<td>plated</td>
<td>as TXB$_2$</td>
<td>Canine</td>
</tr>
<tr>
<td>Control</td>
<td>41.7±4.6</td>
<td>13.9±2.3</td>
<td>33.3±1.9</td>
</tr>
<tr>
<td>Aspirin 1.5 hrs</td>
<td>35.5±2.4</td>
<td>1.3±0.4</td>
<td>3.6±0.6†</td>
</tr>
<tr>
<td>Aspirin 24 hrs</td>
<td>37.3±2.0</td>
<td>1.4±0.4</td>
<td>3.7±0.5‡</td>
</tr>
<tr>
<td>Ibuprofen 1.5 hrs</td>
<td>31.2±2.4</td>
<td>1.9±0.6</td>
<td>6.1±0.5†</td>
</tr>
<tr>
<td>Ibuprofen 24 hrs</td>
<td>33.8±2.5</td>
<td>9.8±1.2</td>
<td>29.0±2.6*</td>
</tr>
<tr>
<td>IB and AS 1.5 hrs</td>
<td>34.6±2.4</td>
<td>1.8±0.6</td>
<td>5.2±0.8†</td>
</tr>
<tr>
<td>IB and AS 24 hrs</td>
<td>32.3±1.8</td>
<td>8.9±1.5</td>
<td>27.5±2.6*</td>
</tr>
</tbody>
</table>

Values are means ± standard error (n = 6). For human studies, only percent conversion values are reported. Ibuprofen and aspirin significantly inhibited the ability of platelets to convert arachidonic acid to thromboxane. Platelets exposed to ibuprofen alone recovered their function significantly by 24 hours. Platelets exposed to aspirin alone remained functionally impaired at 24 hours after drug treatment. Platelets exposed to ibuprofen first and then aspirin recovered their ability to generate thromboxane by 24 hours in the same manner as platelets exposed to ibuprofen alone.

Recovery of Thromboxane Synthesis after Single Doses of Ibuprofen

Ibuprofen at a concentration of 5 mg/kg significantly inhibited the thromboxane synthesis in both human and dog platelets (7.1 and 6.2). However, both human and canine platelets recovered from the inhibitory effect and converted 29% and 25% of the arachidonic acid respectively, to thromboxane at 24 hours after drug ingestion.

Recovery of Thromboxane Synthesis after Ingestion of both Ibuprofen and Aspirin

Ibuprofen administered to these subjects effectively inhibited the conversion of arachidonic acid to thromboxane by platelets. Aspirin administration after ibuprofen did not confer any synergistic effect on the action of ibuprofen. Platelets which were exposed to the action of two potent inhibitors converted approximately 5% to 6% of the substrate to thromboxane. However, both human and canine platelets recovered from the inhibitory effect of these drugs by 24 hours and converted 27% and 25% of arachidonic acid to thromboxane, respectively.

Discussion

The present investigation has evaluated the influence of aspirin on blood platelet function and prostaglandin synthesis when given after the nonsteroidal antiinflammatory drug (NSAID), ibuprofen. Previous studies have shown that ibuprofen, like aspirin, inhibits platelet cyclooxygenase and blocks the second wave of aggregation stimulated by ADP or epinephrine. In contrast to aspirin, however, the action of ibuprofen is short-lived. Concentrations of the drug, which block conversion of arachidonic acid completely and inhibit platelet response to agonists shortly after ingestion, completely lose their influence within 24 hours.

The characteristic differences in the duration of the influence of aspirin and ibuprofen on platelet function were confirmed in our investigation. Platelet samples obtained shortly after ingestion of ibuprofen developed only single waves of aggregation in response to epinephrine and low concentrations of thrombin and ADP (data not shown). Sodium arachidonate failed to cause aggregation or secretion in platelet samples from ibuprofen-treated individuals, and thromboxane B$_2$ generation was markedly reduced. The results closely resembled findings obtained an hour or two after taking single doses of aspirin.

Twenty-four hours after ingesting ibuprofen, however, the effects of the drug on platelet function and prostaglandin synthesis had disappeared. The response of platelets to aggregating agents, including arachidonate, was similar to the reaction of control platelets. No differences in the generation of thromboxane B$_2$ or the secretion measured on the lumiaggregometer could be identified in ibuprofen compared to untreated platelet samples. In contrast, the function and biochemistry of aspirin-treated platelets were essentially as compromised at 24 hours as at 1 hour after taking the agent.

The major question explored in this study was whether ibuprofen and aspirin taken by the same volunteer would cause a more severe and long lasting inhibition of platelet physiology. For this purpose platelet samples were obtained from individuals taking ibuprofen first and then aspirin. One hour after the second drug, platelet samples were no different biochemically or functionally than platelets examined 1 to 2 hours following exposure to either drug alone.
If the separate influences of ibuprofen and aspirin were additive, the function and biochemical responses of platelets could have been more seriously affected than after either drug alone. However, synergism was not observed. The finding suggested that both drugs primarily influence platelet prostaglandin synthesis at the same site. The exposure of platelets to the reversible inhibitor at a threshold concentration first appears to block the inhibitory effect of the second drug on cyclooxygenase.

There was a significant difference between platelet samples taken 24 hours after ingestion of ibuprofen followed by aspirin, compared to those obtained 24 hours after aspirin alone. Platelets had fully recovered in individuals taking ibuprofen before aspirin. Function, secretion, and prostaglandin synthesis in ibuprofen-aspirin-treated platelets 24 hours after exposure to the drugs were essentially identical to cells exposed to ibuprofen alone for a similar interval. On the other hand, the platelets exposed to aspirin alone were completely compromised in their functional capabilities 24 hours after drug ingestion. The findings are consistent with the lack of synergism observed at the earlier time period. Prior exposure to ibuprofen clearly blocked the irreversible inactivation of cyclooxygenase by aspirin. As a result, the platelets treated with both drugs reacted as if they had been exposed to ibuprofen alone.

The ability of ibuprofen to protect platelet cyclooxygenase from the influence of aspirin in vivo is similar to results obtained with other NSAID.29-31 O-phenanthroline, a ferrous iron chelator, has been shown to block the inhibition of shear vesicular gland cyclooxygenase by indomethacin. Vargaftig using intact platelets demonstrated that salicylic acid and 1,10- and 1,7-phenanthroline prevent inhibition of platelet aggregation by aspirin, as well as generation of thromboxane A2 from arachidonic acid.29 Although early studies suggested that indomethacin acted on cyclooxygenase at a site on the molecule different from that influenced primarily by the aspirin, more recent investigations have shown that the agent prevents the long-lasting effect of aspirin on cyclooxygenase activity in vivo.29 Thus, many, if not all, of the nonsteroidal antiinflammatory agents appear to act at the same site on cyclooxygenase and, when given before aspirin, block the irreversible effect of that drug.30-32

The common primary site of action of aspirin and the NSAID may be the site sensitive to metal chelators.7 Studies from our laboratory using an iron-reactive drug, dexamethasone, demonstrated the localization of the prostaglandin synthesizing enzymes in the dense tubular system of platelets and established the association of heme groups with this enzyme.16 Further studies using a cell-free system demonstrated that ferrous iron or heme in the presence of oxygen could cause peroxidation of arachidonic acid and generate thiobarbituric acid-reactive metabolites.16 NSAID such as aspirin, indomethacin, tolmetin, and ibuprofen prevented the peroxidation of arachidonic acid by ferrous iron or heme by complexing with this metal.16 Subsequent studies demonstrated that chelating agents such as dipyriddy, or agents like sodium cyanide and aminotriazole that complex with ferrous or ferrichrome, inhibit cyclooxygenase activity in intact platelets.19,20 Our findings suggest that ibuprofen, metal chelators, and NSAID compete with aspirin on the site of cyclooxygenase, which is metal-sensitive, and that the metal-sensitive site is heme protein. Therefore, most inhibitors of prostaglandin synthesis appear to exert their inhibitory effect by interfering with the redox state of the heme, important in determining the activity of the enzyme. Additional groups or other sites of the enzyme may confer long-lasting inhibitory effects observed with drugs such as aspirin.7,10,33

References
16. Peterson DA, Gerrard JM, Rao GHR, White JG. Inhibition of
17. Peterson DA, Gerrard JM, Rao GHR, White JG. Salicylic acid inhibition of the irreversible effect of acetylsalicylic acid on prostaglandin synthetase may be due to competition for the enzyme cationic binding site. Prostaglandins Med 1981; 6:161–164


Index Terms: ibuprofen • platelets • cyclooxygenase • aspirin • prostaglandins
Ibuprofen protects platelet cyclooxygenase from irreversible inhibition by aspirin.

G H Rao, G G Johnson, K R Reddy and J G White

doi: 10.1161/01.ATV.3.4.383

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/3/4/383

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology 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 Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/