Sex Difference in the Effect of Aspirin on Rat Platelet Aggregation and Arachidonic Acid Metabolism

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Abstract—The rat platelet aggregation induced by collagen was stronger in males than in females. The platelet malondialdehyde (MDA) production was more in males than in females, and the platelet cyclooxygenase activity was higher in males than in females. Aspirin at a dose of 10 mg/kg inhibited the collagen-induced aggregation in males, but not in females. Aspirin at a dose of 5 mg/kg blocked the MDA production only in males, but aspirin at a dose of 10 mg/kg inhibited the MDA production in both sexes. The effect of aspirin on the cyclooxygenase activity was only in males, but aspirin at a dose of 10 mg/kg inhibited the MDA production in both sexes. The effect of aspirin on the cyclooxygenase activity was similar to that on the MDA production. In gonadectomized rats, the MDA production and the cyclooxygenase activity were decreased by castration, and they were increased by ovariectomy. Aspirin at a dose of 5 mg/kg failed to inhibit them in castrated rats. Besides, in in vitro experiments, aspirin also inhibited the MDA production and the aggregation. Nevertheless, there was no sex difference in the content of arachidonic acid, a substrate of platelet cyclooxygenase. It is suggested that there is a sex difference in rat platelet cyclooxygenase activity, and it is closely related to the sex difference in the antiplatelet effect of aspirin.

Aspirin is effective as an antithrombotic agent in clinical trials, as Fields et al. (1) reported that aspirin significantly suppressed cerebral ischemic attack. The balance between thromboxane generated in platelets and prostacyclin generated in vessel wall is important in the process of thrombosis. Aspirin inhibited the prostanooid production (2), but the inhibitory effect of aspirin on the prostanooid production was more effective in platelets than in vessel wall (3). The antithrombotic effect of aspirin is known to relate to the depression of platelet functions. However, the Canadian Cooperative Study Group (4) reported that aspirin was an efficacious drug for men with threatened stroke in randomized clinical trials, and Kelton et al. (5) showed that aspirin significantly lowered the frequency of experimental venous thrombosis only in male rabbits, but not in female ones. These results show that there is a sex-related difference in the antithrombotic effect of aspirin. Moreover, it has been demonstrated that men and male animals suffered from thrombosis more frequently than women and female animals (6). So it would be necessary to examine the difference in the antiplatelet effect of aspirin between males and females.

In this report, we investigated the sex difference in platelet aggregation, MDA production, cyclooxygenase activity, and arachidonic acid content, and also the effect of aspirin on these items, in rat platelets. In addition, we studied the influence of gonadectomy on the effect of aspirin on MDA production and cyclooxygenase activity.

Materials and Methods

Materials: Collagen (Type I) and sodium arachidonate were obtained from Sigma, aspirin from Sanko Seiyaku Co., thrombin
(human plasma thrombin) from Midori Cross, ADP from Boehringer Mannheim GmbH, and 1,1,3,3-tetraethoxychloroacetic acid and boron trifluoride methanol complex from Wako Pure Chemical Industry. All reagents not specified above were of analytic grade.

**Rats:** Wistar strain rats of both sexes, aged 10 weeks, were used. In gonadectomized animals, at 6 weeks of age, female rats were bilaterally ovariectomized via the back route, and male rats were bilaterally castrated via the ventral route under ether anesthesia.

**Drug:** Aspirin was suspended in 0.1% carboxymethylcellulose sodium salt solution for p.o. administration. The dose levels were 5, 10 and 20 mg/kg. Ex vivo experiments, rat blood was collected at an hour after the administration of aspirin.

**Platelet aggregation:** Blood obtained from the abdominal aorta was collected into a siliconized tube containing 3.13% citrate (9:1). Platelet-rich plasma (PRP) was obtained by centrifugation at 170 x g for 10 min, and platelet-poor plasma (PPP) obtained by centrifugation at 1700 x g for 10 min. Then the PRP was diluted with PPP to a platelet count of about 1.0 x 10^8 cells/ml. For the in vitro experiments, PRP was incubated in 1/15 M phosphate buffer (pH 7.4) with or without aspirin at 37°C for 5 min. The platelet aggregation was initiated by collagen or ADP and measured by the method of Born (7).

**Platelet MDA production:** The platelet pellet was obtained from PRP by centrifugation at 550 x g for 15 min and incubated with or without aspirin. Then, platelets were stimulated by thrombin (1, 2.5, 5, 10, 20 and 25 U/ml). Thrombin increased the MDA production dose-dependently from 1 to 20 U/ml, so platelets were stimulated by 20 U/ml thrombin. MDA was measured for the fluorescent intensity of thiobarbituric acid reacting substance, principally according to the method of Yagi (8).

**Platelet cyclooxygenase activity:** The platelet cyclooxygenase activity was measured by the amount of MDA produced using arachidonate (0.2 mM) as a substrate.

**Platelet arachidonic acid content:** Phospholipids of platelets were extracted by the method of Folch et al. (9) and separated by two-dimensional thin layer chromatography (10). The fatty acid of each phospholipid was methylated with boron trifluoride methanol complex and analyzed by a Shimadzu GC-6A GLC using a Unisol 3000 column with temperature 240°C and by a hydrogen flame detector.

**Results**

**Effect of aspirin on rat platelet aggregation:** The rat platelet aggregation induced by collagen or ADP was examined. Figure 1

![Fig. 1. Effect of aspirin on rat platelet aggregation induced by collagen in A) Males and B) Females. Aspirin-treated rats (control, ○: 5 mg/kg, △: 10 mg/kg, □: and 20 mg/kg, ●). The values given are the means±S.D. (n=5). *1: Significant difference from the value of control females at 8 μg/ml collagen (P<0.05). *2: Significant difference from the control value for each sex (P<0.05). *3: Significant difference from the value of 10 mg/kg aspirin-treated females at 10 μg/ml collagen (P<0.01).](image-url)
shows the result of the rat platelet aggregation induced by collagen. Using collagen (8 µg/ml), the platelet aggregation in male rats was stronger than that in females. Aspirin at a dose of 5 mg/kg hardly inhibited the collagen-induced aggregation in both sexes. Aspirin at a dose of 10 mg/kg suppressed the aggregation only in males, but at a dose of 20 mg/kg, it inhibited the aggregation both in males and females. However, the increase of collagen decreased the inhibitory effect of aspirin on platelet aggregation. There was a sex difference in the inhibitory effect of aspirin on rat platelet aggregation induced by collagen. However, there was no sex difference in the ADP-induced platelet aggregation, and aspirin did not influence that aggregation.

Figure 2 shows that in vitro experiments, aspirin dose-dependently inhibited the collagen-induced platelet aggregation. When 2 µg/ml aspirin was added, the platelet aggregation in males was inhibited more strongly than that in females. On the other hand, the platelet aggregation was inhibited by 5 µg/ml aspirin in both sexes.

**Effect of aspirin on MDA production of thrombin-stimulated platelet:** The MDA production of thrombin-stimulated rat platelets was chosen as an index of the cyclooxygenase pathway in arachidonic acid metabolism, and the formed MDA was measured as a thiobarbituric acid reacting substance. The MDA production was significantly more in males than in females (Table 1). Aspirin at a dose of 5 mg/kg significantly inhibited the MDA production in both sexes. The inhibitory effect of aspirin was stronger in male rats than in female ones. Figure 3 shows that aspirin inhibited the MDA production dose-dependently in the in vitro experiment. The MDA production in males was inhibited by aspirin of over 0.25 µg/ml, but that in females was inhibited by aspirin of more than 0.5 µg/ml.

As mentioned above, the inhibitory effect of aspirin on the MDA production of thrombin-stimulated platelets was shown

![Graph showing the effect of aspirin on rat platelet aggregation induced by collagen in the in vitro experiment.](image)

**Table 1. Effect of aspirin on MDA production of thrombin-stimulated platelets in intact and gonadectomized rats**

<table>
<thead>
<tr>
<th></th>
<th>Control MDA (nmol/10⁶ platelets)</th>
<th>ASA-5 MDA (nmol/10⁶ platelets)</th>
<th>ASA-10 MDA (nmol/10⁶ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact (I)</td>
<td>1.23±0.30*¹</td>
<td>0.61±0.15*²</td>
<td>0.30±0.08*²</td>
</tr>
<tr>
<td>Castration (C)</td>
<td>0.89±0.11</td>
<td>0.84±0.17</td>
<td></td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact (I)</td>
<td>0.83±0.21</td>
<td>0.72±0.10</td>
<td>0.35±0.10*²</td>
</tr>
<tr>
<td>Ovariectomy (O)</td>
<td>1.14±0.36</td>
<td>0.79±0.45</td>
<td></td>
</tr>
</tbody>
</table>

*¹: Intact; *²: Castration; O: Ovariectomy, ASA-5: Aspirin 5 mg/kg, ASA-10: Aspirin 10 mg/kg.

Not done. The values are given as the mean±S.D. (n=6). Platelets were stimulated by thrombin (20 U/ml). *¹: Significant difference from the value of intact females (P<0.05). *²: Significant difference from the control value for each sex (P<0.05).
both ex vivo and in vitro. Although aspirin efficiently inhibited the MDA production in both sexes, the inhibitory effect of aspirin was stronger in males than in females.

Influence of gonadectomy on MDA production of thrombin-stimulated platelet: Table 1 shows the influence of gonadectomy on the MDA production of thrombin-stimulated platelets. In castrated male rats, the MDA production had a tendency to decrease, and the inhibitory effect of aspirin was weakened. However, in ovariectomized female rats, the MDA production had a tendency to increase, and the effect of aspirin was strengthened. Aspirin at a dose of 5 mg/kg did not inhibit the MDA production in gonadectomized rats.

Effect of aspirin on platelet cyclooxygenase activity: After addition of arachidonate (0.2 mM) in rat platelets, the amount of produced MDA was measured as a parameter of the platelet cyclooxygenase activity (Table 2). The platelet cyclooxygenase activity in male rats was higher than that in female rats. Aspirin at a dose of 5 mg/kg significantly depressed the platelet cyclooxygenase activity in both sexes. However, the inhibitory effect of aspirin on platelet cyclooxygenase activity was stronger in males than in females.

In gonadectomized rats, castration significantly suppressed platelet cyclooxygenase activity, but ovariectomy strengthened it. The inhibitory effect of aspirin on platelet cyclooxygenase activity was weakened by castration, and aspirin at a dose of 5 mg/kg did not significantly depress platelet cyclooxygenase activity. There was a significant difference in the effect of aspirin on platelet cyclooxygenase activity between castrated and intact rats. Ovariectomy increased the effect of aspirin on platelet cyclooxygenase activity, and aspirin at a dose of 5 mg/kg significantly inhibited platelet cyclooxygenase activity. Platelet cyclooxygenase activity was more strongly influenced by castration than by ovariectomy.

Content of platelet arachidonic acid: Arachidonic acid is a substrate of cyclooxygenase and is almost released from membrane phospholipids. Table 3 shows the content of arachidonic acid in the main phos-

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**Table 1. Influence of gonadectomy on the MDA production of thrombin-stimulated rat platelets**

<table>
<thead>
<tr>
<th>Confidence Level</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.32±0.29</td>
<td>1.33±0.23</td>
</tr>
<tr>
<td>ASA-5</td>
<td>1.63±0.26</td>
<td>0.87±0.23</td>
</tr>
</tbody>
</table>

1: Intact, C: Castration, O: Ovariectomy, ASA-5: Aspirin-5 mg/kg. The values are given as means±S.D. (n=6). Significant difference from the control value for each sex (P<0.05).

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**Table 2. Influence of gonadectomy on effect of aspirin on rat platelet cyclooxygenase activity**

<table>
<thead>
<tr>
<th>Cyclooxygenase activity (MDA nmol/10⁹ platelets)</th>
<th>Control</th>
<th>ASA-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>2.32±0.29</td>
<td>1.63±0.26*1</td>
</tr>
<tr>
<td>C</td>
<td>1.63±0.34*3</td>
<td>1.39±0.23</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1.33±0.23*5</td>
<td>0.87±0.23*2</td>
</tr>
<tr>
<td>O</td>
<td>1.68±0.43</td>
<td>1.10±0.20*2</td>
</tr>
</tbody>
</table>

I: Intact, C: Castration, O: Ovariectomy, ASA-5: Aspirin-5 mg/kg. The values are given as means±S.D. (n=6). Significant difference from the control value for each sex (*1: P<0.001, *2: P<0.05). *3: Significant difference from the intact male (P<0.05).
phospholipids of rat platelets. There was no sex difference in the arachidonic acid content of rat platelets.

**Discussion**

Epidemiological studies have demonstrated that men are at higher risk for thrombus formation than women, and aspirin is an efficacious drug for men with threatened stroke in randomized clinical trials (4). Moreover, in experimental studies, Uzunova et al. (6) reported that there was a sex difference in the experimental thrombus formation in rats and rabbits, and Kelton et al. (5) showed that aspirin significantly lowered the frequency of experimental venous thrombosis only in male rabbits. It has been demonstrated that platelets are closely connected with thrombus formation. So, it is worthwhile to examine the sex difference in platelet aggregation, arachidonic acid metabolism, and antiplatelet effect of aspirin in rat platelets.

In this study, there was a sex difference in rat platelet aggregation and platelet cyclooxygenase activity. Moreover, platelets from males were more sensitive to aspirin than those from females.

Orchard and Botting (11) have shown that rat platelet sensitivity to ADP was influenced by sex hormones. In our study, there was no sex difference in the ADP-induced aggregation, but the collagen-induced aggregation was stronger in platelets from males than in those from females. Johnson et al. (12) have reported that testosterone enhanced rat platelet aggregation, and Pilo et al. (13) have reported that testosterone raised rat platelet aggregation in an in vitro study. Their results agreed with our result. Kelton et al. (14) indicated that the sex difference in platelet aggregation resulted from a difference in hematocrit. However, in this study, PRP used was fixed at about 1.0×10⁸ cells/ml of the platelet count with PPP, so the factor of hematocrit was excluded. The sex difference in platelet aggregation seemed to come from the platelet itself.

In this study, platelet aggregation and platelet cyclooxygenase activity were stronger in male than in female rats. It was thought that thromboxane A₂ was not important in rat platelet aggregation (15, 16). Recently, it has been shown that thromboxane A₂ directly induced platelet activation in human platelets (17, 18). It is likely that the arachidonic acid metabolism in rat platelets would modulate the platelet aggregation as well as that in human platelets.

It was reported that MDA production was useful as an index of the arachidonic acid metabolism (19). Under the conditions of our experiment, the MDA was also mainly formed by the platelet cyclooxygenase pathway (20). Added arachidonate itself was the substrate for the platelet cyclooxygenase pathway. The platelet cyclooxygenase activity in male rats was stronger than that in females. Platelet cyclooxygenase activity was depressed by castration, but ovarectomy raised it. The inhibitory effect of aspirin on platelet cyclooxygenase activity was stronger in males than in females. In castrated male rats, aspirin did not suppress platelet cyclooxygenase activity. It may be that the arachidonic acid content is involved in the sex differ-

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**Table 3. Content of arachidonic acid in each phospholipid of rat platelets**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0.61±0.05</td>
<td>0.56±0.30</td>
</tr>
<tr>
<td>PE</td>
<td>3.15±1.50</td>
<td>3.03±0.41</td>
</tr>
<tr>
<td>PI</td>
<td>4.69±1.09</td>
<td>4.78±1.53</td>
</tr>
<tr>
<td>PS</td>
<td>0.48±0.30</td>
<td>0.53±0.18</td>
</tr>
</tbody>
</table>

PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PI: Phosphatidylinositol, PS: Phosphatidylserine. The values are given as the mean±S.D. (n=5)
ence in the arachidonic acid metabolism, but the contents of arachidonic acid in male rat platelet was the same as that in female. It is possible that sex hormones may influence platelet cyclooxygenase activity. Chang et al. (21) suggested that estradiol stimulated the cyclooxygenase in rat aortic smooth cells, but did not participate in the thromboxane production in rat platelets. The characteristics of aortic cyclooxygenase seem to be different from those of platelet cyclooxygenase. It may be that sex hormones influence aortic and platelet cyclooxygenases in different manners. On the other hand, Johnson et al. (12) and Pilo et al. (13) have reported that testosterone influenced platelet aggregation. Our study suggested that castration influenced arachidonic acid metabolism more strongly than ovariectomy. From this point of view, our study agreed with their reports (12, 13).

There is a possibility that sex hormones modulate the production of platelets, as it has been demonstrated that sex hormones regulated the production of erythrocytes. It is likely that platelet activations in male rats are higher than that in females; therefore, male rat platelets are more sensitive to aspirin than female rat platelets.

There are sex differences in rat platelet aggregation and arachidonic acid metabolism. These sex differences must be one of the causes of the sex difference in the antiplatelet effect of aspirin in rats, and the differences in the platelet aggregation and the arachidonic acid metabolism may be related to the sex difference in the anti-thrombotic effect of aspirin in humans. It will be necessary to clarify the sex difference of the cyclooxygenase activity of platelets and aorta at the enzyme level.

References
7 Born, G.V.R. and Gross, M.J.: Platelet aggregation in male rats are higher than that in females; therefore, male rat platelets are more sensitive to aspirin than female rat platelets.


