Effects of Low-to-High Doses of Aspirin on Platelet Aggregability and Metabolites of Thromboxane A2 and Prostacyclin

Hideo Tohgi, MD; Shu Konno, MD; Kenichi Tamura, MD; Bunsho Kimura, MD; and Katsumi Kawano, PhD

Background and Purpose: The purpose of this study was to compare the effects of low-to-high doses of aspirin on platelet aggregability determined by different methods and on the metabolism of thromboxane A2 and prostacyclin.

Methods: We administered increasing doses (40, 320, and 1,280 mg/day) of aspirin to 19 poststroke patients and studied the differences in 1) the changes in platelet aggregability depending on the methods of evaluation and 2) the concentrations of prostaglandin metabolites in the blood and urine.

Results: Aggregation of platelet-rich plasma induced by a strong stimulus (10 μM ADP) was significantly reduced after 40 mg/day aspirin (p<0.005), and this reduction was similar to that after higher aspirin doses. In contrast, aggregation of platelet-rich plasma induced by weaker stimuli (1 and 5 μM ADP) decreased less significantly after 40 mg/day aspirin compared with that after higher aspirin doses. The serum thromboxane B2 generated after ex vivo incubation was reduced significantly (by 85%) after 40 mg/day aspirin and decreased further after 320 mg/day (by 96%) and 1,280 mg/day (by >99%) of aspirin. The urinary 11-dehydro-thromboxane B2 concentration decreased less significantly after 40 mg/day aspirin (by 42%) compared with that after 320 mg/day (by 78%) and 1,280 mg/day (by 91%) aspirin doses. The urinary concentration of 2, 3-dinor-6-keto-prostaglandin F1α did not decrease after 40 mg/day aspirin but decreased significantly after higher doses of aspirin.

Conclusions: These findings suggest that different doses of aspirin may be necessary to prevent thrombogenesis induced by different triggers of different strengths and that 40 mg/day aspirin is able to inhibit a large proportion of maximum thromboxane A2 release provoked acutely, with the prostaglandin I2 synthesis being little affected; however, higher doses of aspirin are required to attain further inhibition.

KEY WORDS • aspirin • platelet aggregation • thromboxane B2

Aspirin, a potent antiplatelet agent, is widely used for prevention of atherothrombotic diseases. It exerts antiplatelet action by inhibiting cyclooxygenase, thus reducing the synthesis of thromboxenic thromboxane (TX) A2 in platelets. However, it also inhibits cyclooxygenase in vascular endothelial cells and reduces their production of prostaglandin (PG) I2, which has an antithrombotic action. It has been demonstrated that at low doses (less than 100 mg/day), the reduction in TXB2 (the metabolite of TXA2) was remarkable, and the production of 6-keto-PGF1α (the metabolite of PGI2) was little affected. Clinical trials also have shown that 30 mg/day aspirin was as effective as 283 mg/day aspirin for the prevention of vascular events and that 75 mg/day aspirin significantly reduced the risk of stroke or death compared with a placebo. However, there are still some problems that need further study. First, the amount of reduction in platelet aggregability required to prevent atherothrombotic disease has not yet been established. This is largely because the degree of platelet aggregation inhibition differs depending on the method of determination (e.g., whole-blood impedance or light transmission) and the aggregating agents and their concentrations. Second, recent studies have indicated that 11-dehydro-TXB2 (a metabolite of TXB2) and 2,3-dinor-6-keto-PGF1α (a metabolite of 6-keto-PGF1α) are more stable and reliable indicators than TXB2 and 6-keto-PGF1α, respectively. Few systematic data, however, are available concerning the effects of aspirin doses on these metabolites.

We therefore administered low-to-high doses of aspirin to poststroke patients in the chronic phase and compared platelet aggregability measured by different methods, induced by different aggregating agents and concentrations; we also compared the concentrations of serum TXB2, urinary 11-dehydro-TXB2, and urinary 2,3-dinor-PGF1α.
Subjects and Methods
We studied 19 patients (7 men and 12 women, 71.9±6.3 years) in the chronic phase of cerebral thrombosis. Nine were hypertensive, eight had diabetes, and four were smokers. Since we did not find a significant influence of the baselines on their results, we combined these patients for analysis. None of the patients had taken nonsteroidal anti-inflammatory medication for more than 1 month before the study. The study period was 7 weeks; the patients received 40 mg/day aspirin for the first week and did not receive aspirin for the second and third weeks (washout period); they then received 320 mg/day aspirin for the fourth week, followed by a washout period during the fifth and sixth weeks; and they received 1,280 mg/day for the seventh week. We determined all the parameters described below before treatment and on the last days of the first, fourth, and seventh weeks of aspirin administration for each dose. Informed consent was obtained from all patients.

For platelet aggregation studies, venous blood was taken by venipuncture and put into tubes containing sodium citrate for platelet aggregation. Aggregation in both platelet-rich plasma (PRP) and whole blood were studied. The PRP aggregation was measured by percent maximum change in light transmission using a Born aggregometer (Niko Bioscience, Tokyo). We also inspected aggregation curves and determined whether the primary aggregation only or both primary and secondary aggregation occurred. Aggregating reagents used were 2 μg/ml collagen (Chrono-Log, Havertown, Pa.) and 1, 5, and 10 μM adenosine diphosphate (ADP; Sigma Chemical Co., St. Louis, Mo.). Whole-blood platelet aggregation was estimated using a Chrono-Log Model 540 whole-blood aggregometer (Coulter Electronics Ltd., Luton, UK); aggregating reagents were 2 μg/ml collagen. Rate of aggregation was assessed by the change in impedance (ohms) 6 minutes after adding the reagents. The linear relation between changes in impedance at 6 minutes and the maximum change have been confirmed.

For determination of serum TXB2, nonanticoagulated blood was allowed to clot at 37°C for 90 minutes to permit maximal generation of TXA2 by platelets in response to endogenously produced thrombin. The separated sera were kept at –20°C until assayed. The TXA2 production was studied indirectly by measuring the concentration of TXB2 in serum. Urine was collected for 24 hours.

The serum concentration of TXB2 and the urinary concentrations of 11-dehydro-TXB2 and 2,3-dinor-6-keto-PGF1α were determined according to Kawano et al. Prostaglandins were extracted from acidified sample by octadecysilsilica (Fuji Gel, Tokyo) suspension. Deproteinization and delipidization were performed, and prostaglandins were eluted by ethyl acetate. The dried residue containing prostaglandins was dissolved in eluent 1 (acetoni trile:chloroform:acetic acid, 10:90:0.5) and applied to a silica open minicolumn BOND ELUT Si (Analytic Chem International, Harbor City, Calif.). The column was washed with 10 ml eluent 1, then 11-dehydro-TXB2 and 2,3-dinor-6-keto-PGF1α were eluted with 5 ml eluent 2 (acetoni trile:chloroform:acetic acid, 20:80:0.5), and TXB2 and 6-keto-PGF1α were eluted with 5 ml eluent 3 (acetoni trile:chloroform:acetic acid, 50:50:0.5). We assayed TXB2 by [125I] radioimmunoassay kit (NEN Research Products, Du Pont, Boston, Mass.), 11-dehydro-TXB2 using 11-dehydro-thromboxane B2 ([3] radioimmunoassay kit (NEN Research Products), and 2,3-dinor-6-keto-PGF1α using [25]I-6-keto-PGF1α, radioimmunoassay kit (Amersham International, Amersham, UK), based on the 31.8% cross-reactivity. We used 2,3-dinor-6-keto-PGF1α (Cayman Chemical Co., Ann Arbor, Mich.) as a standard compound. The detection limit was less than 3 pg/ml, and the variation was less than 15% for all substances measured. Our control values of urinary 2,3-dinor-6-keto-PGF1α were consistent with the reported values determined by gas chromatography/mass spectros copy or high-performance liquid chromatography and radioimmunoassay.

Results
The 1-μM ADP-induced aggregation of platelet-rich plasma did not change after 40 mg/day aspirin but was significantly reduced after 320 mg/day and 1,280 mg/day aspirin compared with both the pretreatment and treatment (40 mg/day aspirin) conditions (p<0.005) (Table 1). The 5-μM ADP-induced aggregation of platelet-rich plasma was significantly reduced after 40 mg/day aspirin (p<0.05) and was significantly further reduced dose dependently with increasing doses of aspirin (320 mg/day and 1,280 mg/day, both p<0.005). The 10-μM ADP-induced aggregation of platelet-rich plasma was significantly reduced after 40 mg/day aspirin (p<0.005), and the degree of reduction did not change with increasing doses of aspirin. The 2-μg/ml collagen–induced aggregation of platelet-rich plasma was significantly reduced after 40 mg/day aspirin (p<0.005) and was further significantly reduced dose dependently with increasing doses of aspirin (320 mg/day and 1,280 mg/day, p<0.005). The 2-μg/ml collagen–induced whole-blood aggregation did not change after 40 mg/day aspirin but was significantly reduced after higher doses of aspirin (320 mg/day and 1,280 mg/day, p<0.005).

Inspection of the aggregation curves (light transmission method) (Table 2) before aspirin medication revealed that both the primary and secondary aggregation occurred in 47% of patients after stimulation with 1 μM ADP and in 95% after stimulation with 5 μM or 10 μM ADP. With the aggregation induced by 1 μM ADP, the rate of patients displaying both the primary and secondary phases decreased remarkably after 40 mg/day aspirin and decreased further after higher aspirin doses. However, with 5-μM ADP–induced aggregation, both phases occurred in 47% of patients after 40 mg/day aspirin, and the rate of patients displaying both phases decreased dose dependently. For 10-μM ADP–induced aggregation, the rate of patients displaying both phases of aggregation was 32% after 40 mg/day aspirin and did not change substantially after higher aspirin doses. Thus, the findings based upon inspection of the aggregation curves were correlated with those of quantitative estimation (% light transmission).

The serum TXB2 concentration and the urinary 11-dehydro-TXB2 concentration decreased significantly after 40 mg/day aspirin (by 85%) (p<0.005) (Table 1) but significantly decreased further dose dependently with increasing doses of aspirin (by 96% after 320 mg/day, p<0.005; and by >99% after 1,280 mg/day, p<0.005). The urinary 11-dehydro-TXB2 concentration decreased...
Table 1: Platelet Aggregation and Prostaglandin Concentrations After Different Doses of Aspirin Compared With Premedication

<table>
<thead>
<tr>
<th>Light transmission (%)</th>
<th>Before aspirin (n=19)</th>
<th>Aspirin dose (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM ADP</td>
<td>46.5±23.7</td>
<td>45.6±16.4</td>
</tr>
<tr>
<td>5 μM ADP</td>
<td>72.1±12.3</td>
<td>64.9±5.8</td>
</tr>
<tr>
<td>10 μM ADP</td>
<td>76.2±11.5</td>
<td>66.4±5.5</td>
</tr>
<tr>
<td>2 μg/ml collagen</td>
<td>73.4±8.6</td>
<td>59.7±16.3</td>
</tr>
</tbody>
</table>

Whole-blood aggregation (ohm)

| Collagen (2 μg/ml)     | 23.2±3.4              | 23.8±5.0             |
| Serum TXB₂ (ng/ml)    | 48.12±40.4            | 7.13±10.31†          |
| Urinary 11-dehydro-TXB₂ (pg/ml) | 994±620 | 217±174† $\pm$ 0.28** |
| Urinary 2,3-dinor-6-keto-PGF₁α (pg/ml) | 213±96 | 196±99 $\pm$ 511** |

Values are mean±SD. n, Number of patients; ADP, adenosine diphosphate; TX, thromboxane; PG, prostaglandin.

* (p<0.05), † (p<0.005) compared with pretreatment values.
§ (p<0.05), ‡ (p<0.005) compared with values after 40 mg/day aspirin treatment.
¶ (p<0.05) compared with values after 320 mg/day aspirin treatment.

Discussion

Our main findings were that 1) the changes in platelet aggregability after different doses of aspirin were different depending upon the concentrations of ADP used to induce platelet aggregation; 2) serum TXB₂ levels and the urinary 11-dehydro-TXB₂ concentration decreased dose dependently with increasing doses of aspirin, the rate of reduction being greater in the former than in the latter after the same aspirin doses; and 3) the urinary concentration of 2,3-dinor-PGF₁α was not reduced after 40 mg/day aspirin but was reduced significantly after higher doses of aspirin.

The significant decreases in the 10-μM ADP–induced aggregation of platelet-rich plasma after 40 mg/day aspirin as well as after higher doses suggest that the inhibition of an irreversible platelet aggregation induced by strong stimuli appears to be maximum with low-dose aspirin. On the other hand, we found that aggregation of platelet-rich plasma induced by weaker stimuli (1 and 5 μM ADP) could not be inhibited or was less inhibited by 40 mg/day aspirin compared with higher doses. These apparently paradoxical findings may be explained by the fact that 1 μM ADP induces mainly the primary aggregation and that aspirin essentially inhibits the secondary aggregation. Our findings based on inspection of the aggregation curves may support this assumption. Our results also demonstrated that 40 mg/day aspirin is insufficient to attenuate whole-blood aggregation induced by 2 μg/ml collagen (i.e., approximately 3–4 μg/ml in plasma, if we consider the hematocrit), suggesting that a low dose of aspirin appears to be less effective for inhibition of platelet aggregation when both red and white blood cells are present than in the solution consisting exclusively of

Table 2. Number of Patients with Aggregation Curves Indicating Primary Aggregation Alone or Both Primary and Secondary Aggregation

<table>
<thead>
<tr>
<th>Aspirin dosage</th>
<th>Before</th>
<th>40 mg</th>
<th>320 mg</th>
<th>1,280 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM ADP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>10</td>
<td>15</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Primary and secondary</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 μM ADP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>1</td>
<td>10</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Primary and secondary</td>
<td>18</td>
<td>9</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>10 μM ADP</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>1</td>
<td>6</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Primary and secondary</td>
<td>18</td>
<td>13</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

n, Number of patients; ADP, adenosine diphosphate.

* p<0.005, † p<0.05 compared with pretreatment values by $x^2$ test.
platelets. However, further studies are needed on whole-blood aggregation induced by higher concentrations of collagen to evaluate this assumption.

The remarkable (85%) reduction in serum TXB₂ generation in vitro after 40 mg/day aspirin, which is consistent with previous reports, indicates that the inhibition of a large proportion of the maximum capacity of acutely induced TXB₂ release from platelets is achieved by low-dose aspirin. Although we do not know whether the rate of reduction is sufficient to inhibit thrombogenesis in vivo, our results for urinary 2,3-dinor-6-keto-PGF₁α indicate that PG₁₂ synthesis in endothelial cells is not inhibited after 40 mg/day aspirin, which is consistent with the conclusion derived from the determination of the 6-keto-PGF₁α concentration. Thus, our findings may provide the basis for the reported effects of low-dose aspirin for stroke prevention. However, because there is an individual variation in the platelet inhibition with low-dose aspirin, moderately higher doses of aspirin may be required for relatively low responders. In such cases, 10-μM ADP-induced aggregation of platelet-rich plasma and serum TXB₂ determinations may be appropriate indices for monitoring the effects of aspirin.

The observed dose-dependent decrease of urinary 11-dehydro-TXB₂ with the increasing doses of aspirin suggests that TXA₂ secretion of platelets in baseline or ordinary conditions is inhibited less significantly with 40 mg/day aspirin compared with higher aspirin doses. The maximum capacity of platelets for TXA₂ production that still remains uninhibited with 40 mg/day aspirin (15% of the pretreatment value) but is inhibited only by higher doses of aspirin may have an important significance for baseline TXA₂ secretion as reflected in urinary 11-dehydro-TXB₂. Because the important role of platelets in the pathogenesis of atherosclerosis has been established, such a sustained level of TXA₂ may contribute to further progression of atherosclerosis. It has been reported that the plasma 11-dehydro-TXB₂ level is a useful parameter to discriminate between stroke patients and controls.

In conclusion, for average subjects 40 mg/day aspirin is able to significantly reduce the maximum capacity of TXA₂ formation of platelets and aggregation induced by strong stimuli, without the inhibition of PG₁2 production. However, the reduction in baseline TXA₂ production of platelets, aggregation induced by weak stimuli, and whole-blood aggregation were achieved only with higher aspirin doses, the clinical significance of which needs further scrutiny.

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References

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