

# Antiplatelet Drugs and Generation of Thrombin in Clotting Blood

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Platelets participate in formation of thrombin through secretion of coagulation factors and by providing a catalytic surface on which prothrombinase complex is assembled. We studied the effects of four antiplatelet drugs on thrombin formation in healthy volunteers. Thrombin generation was monitored both *in vitro*—in recalcified plasma—and *ex vivo*—in blood emerging from a standardized skin microvasculature injury, which also served to determine bleeding time. A mathematical model has been developed to describe the latter reaction. It is based on estimation of the rate of increase in fibrinopeptide A (FPA), a specific marker of thrombin activity, in blood emerging from skin incisions. Two hours after the ingestion of 500 mg of aspirin, thrombin

formation became significantly impaired both *in vitro* and *ex vivo*. In contrast, 2 hours after the oral administration of placebo, indomethacin 50 mg, or OKY-046 (a thromboxane synthase inhibitor) 400 mg, thrombinogenesis remained unaltered. Ticlopidine, studied either 3 hours after 500 mg oral administration, or after 5 days of intake at a daily dose of 500 mg, had no effect on thrombin generation. Thus, aspirin, contrary to other antiplatelet drugs, depresses thrombin formation in clotting blood, a phenomenon that might be of clinical relevance. It is suggested that aspirin exerts this effect by acetylating prothrombin and/or macromolecules of platelet membrane.

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**P**LATELETS PROVIDE the initial hemostatic plug at sites of vascular injury. They also participate in reactions that lead to atherosclerosis and pathologic thrombosis.<sup>1,2</sup> Antagonists of platelet function, or antiplatelet drugs, have been used in attempts to prevent thrombosis and to alter the natural history of atherosclerotic vascular disease. Their value has been proven recently by large clinical trials. Aspirin, the most widely used drug, is now firmly indicated in secondary prophylaxis and treatment of unstable angina, myocardial infarction, and transient ischemic attacks.<sup>3-5</sup>

Although it is possible that other pharmacologic properties of aspirin are relevant, its efficacy has been explicable in terms of its action as an inhibitor of platelet cyclooxygenase, and, consequently, as a depressant of thromboxane A<sub>2</sub> (TXA<sub>2</sub>) production.<sup>6</sup> However, it is recognized that cyclooxygenase blockade is not the optimal means of platelet inhibition. For example, endogenous compounds other than TXA<sub>2</sub>, including thrombin, collagen, epinephrine, and adenosine phosphate (ADP), are recognized to aggregate platelets. Furthermore, coincident inhibition of the vascular synthesis of platelet inhibitory prostaglandins, such as prostacyclin, may limit the efficacy of even low doses of aspirin.<sup>7</sup> These observations have prompted the evaluation of alternative strategies, including inhibition of phosphodiesterase and thromboxane synthase enzymes, blockade of thromboxane and serotonin receptors, inhibition of the ADP pathway of platelet aggregation, and infusion of platelets inhibitory prostaglandins. Evidence has been quickly accumulating pointing to clinical efficacy of some of these strategies, with ticlopidine being, perhaps, the best example.<sup>4,5-8</sup>

Platelets participate in formation of thrombin, the key enzyme of blood coagulation, through secretion of coagulation factors. They also provide a catalytic surface on which prothrombinase complex is assembled.<sup>1,9</sup> We wondered whether the drugs that inhibit platelet function might affect thrombin generation. The results presented here indicate that this, indeed, is true in the case of aspirin, but not in the case of some other antiplatelet agents. Furthermore, our results suggest that delay of thrombin generation by aspirin seems unrelated to its inhibition of platelet cyclooxygenase.

## METHODS AND SUBJECTS

**Study design.** We studied thrombin generation, both *in vitro* and *ex vivo*, before and after oral administration of an antiplatelet drug or placebo (lactose). Except for ticlopidine, all of the studies were performed 2 hours after a single ingestion of a drug at the following concentrations: aspirin (Polfa, Stargard, Poland) 500 mg, indomethacin (Polfa) 50 mg, OKY-046 (Ono, Osaka, Japan) 400 mg. In the case of ticlopidine (Sanofi, Gentilly, France), the test was performed 3 hours after oral administration of 500 mg and on the morning of the sixth day of the continuous drug administration at a daily dose of 500 mg. All experiments were started between 8 and 9 AM and were concluded within 3 to 4 hours. The study was not planned to be blind, although the technician performing the final measurements of thrombin activity was usually not aware of kind of the drug tested.

**Subjects.** In the *in vitro* study, 54 healthy volunteers took part. There were 50 men and four women, aged 19 to 63 years (mean, 28 years). The inclusion criteria called for lack of disorders of hemostasis or atopy, and lack of intake of aspirin or any other drugs known to affect platelet function for 14 days preceding the study. Platelet aggregation by arachidonic acid<sup>10</sup> was measured in all subjects; if platelets did not respond to 1,200 μmol/L arachidonic acid, the subjects were rescheduled for the study 10 to 14 days later.

In the *ex vivo* study, 58 healthy subjects were included. There were 49 men and nine women, aged 21 to 65 years (mean, 31 years). The inclusion criteria were the same as above. Of this number, 18 healthy volunteers participated in both *in vitro* and *ex vivo* studies.

In both *in vitro* and *ex vivo* studies, men markedly outnumbered women. Women were less willing to participate in the study, because they feared developing scars across the forearm, as well as repeated venipunctures.

**Generation of thrombin *in vitro*.** Generation of thrombin in recalcified plasma was monitored according to the method of Pitney and Dacie<sup>11</sup> with modifications previously described.<sup>12</sup> In essence, at intervals of 1 to 2 minutes, two equal samples were withdrawn from the incubation mixture consisting of platelet-rich

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plasma, containing 200,000 to 300,000 platelets/mm<sup>3</sup> and diluted 1:1 with saline. One of the samples was assayed for fibrinogen clotting time, the other was assayed for the amidolytic activity toward a chromogenic substrate, CBZ-Gly-Gly-Arg-2NA (Sigma Chemical, St Louis, MO). The clotting activity was expressed in terms of thrombin NIH units. The amidolytic activity was expressed in international units (IU) as the number of micromoles of 2-naphthylamine liberated in 1 minute and was calculated for 1,000 mL of clotting plasma. Effects of drugs were assessed by comparing times at which thrombin reached peak activity.

*Generation of thrombin ex vivo.* The method was based on a quantitative analysis of successive aliquots of blood obtained at the site of bleeding time wound.<sup>13</sup> Thrombin generation in blood emerging from a standardized skin microvasculature injury, which also served to determine bleeding time, was monitored as described previously.<sup>14</sup> Activity of thrombin was assayed by measurements of fibrinopeptide A (FPA) and the results were expressed in nanograms of FPA per milliliter of plasma.

Data were evaluated by comparing FPA levels at the same time intervals, before and after the drug administration. Sensitivity of such an evaluation is blunted by not taking into account data from preceding time intervals. We therefore developed a mathematical model for analysis of the rates of increase in FPA concentrations. It was based on the following assumptions: (1) in blood emerging from a skin wound, thrombin is generated continuously; and (2) the rate of hydrolysis of fibrinogen and hence FPA production is proportional to the concentration of thrombin.<sup>15</sup> If the reaction's environment contains large amounts of substrates and little products, the rate of thrombin generation at time  $n$  should be directly proportional to its concentration at time  $n - 1$ . We therefore assumed that the concentration of thrombin in blood changes for short time as

$$c(t) = c(0)e^{\lambda t}, \tag{1}$$

where  $c(0)$  is the initial concentration of thrombin expressed as the amount of FPA (product of thrombin action). In the experiment, some mean concentration of thrombin over time  $T$  has been measured. If the velocity of bleeding is assumed to be constant in each interval  $T$ , this mean concentration can be related to the integral of (1) over the interval  $T$ :

$$c_n = \int_{(n-1)T}^{nT} c(0)e^{\lambda t} dt = \frac{c(0)}{\lambda} (1 - e^{-\lambda})e^{n\lambda T}. \tag{2}$$

For simplicity, we take  $T$  as a unit time (which we can always do) and arrive at

$$c_n = \alpha e^{n\lambda}, \tag{3}$$

where

$$\alpha = \frac{c(0)}{\lambda} (1 - e^{-\lambda}), \tag{4}$$

$c_n$  is the measured concentration of thrombin in the  $n$ th interval, and  $\lambda$  is the velocity with which the concentration increases.

Each  $c_n$  has been measured with an error proportional to the value of  $c_n$ , so that

$$\sigma_{c_n}^2 = d^2(c_n)^2. \tag{5}$$

The value of  $d$  depends on the experimental setup and the measurement techniques; in the actual experiment, this value has been set to 10%.

If we take the logarithm of (3), we obtain

$$y_n = n\lambda + \beta, \tag{6}$$

with

$$y_n = \ln c_n, \quad \beta = \ln \alpha, \tag{7}$$

and

$$\sigma_{y_n}^2 = \frac{1}{(c_n)^2} \sigma_{c_n}^2 = d^2, \tag{8}$$

$$\sigma_{\beta}^2 = \frac{1}{\alpha^2} \sigma_{\alpha}^2.$$

The equation (6) is linear, so if we take the parameters  $\lambda$  and  $\beta$  as independent (which is a rough approximation), we can fit them by the linear least squares method.<sup>16</sup>

Having computed the values of  $\lambda$  and  $\beta$  (or  $\alpha$ ), we can compute the initial concentration  $c(0)$ :

$$c(0) = \alpha \frac{\lambda}{1 - e^{-\lambda}}, \tag{9}$$

$$\sigma_{c(0)}^2 = \left[ \frac{\lambda}{1 - e^{-\lambda}} \right]^2 \sigma_{\alpha}^2 + \alpha^2 \left[ \frac{1 - e^{-\lambda} + \lambda e^{-\lambda}}{(1 - e^{-\lambda})^2} \right]^2 \sigma_{\lambda}^2.$$

In the final analysis of the data, the parameters  $c(0)$  and  $\lambda$ —before and after the drug—were compared.

*Statistical methods.* Statistical analysis was performed on a personal computer using Complete Statistical System with graphics and data management (Ver. B640, release 2.1, 1988; StatSoft, Rockville, MD). Paired Student's  $t$ -test was used for evaluation of in vitro results, as well as for comparison of FPA concentrations at the same time periods in the ex vivo study. Wilcoxon two-sample paired test was used for comparing  $c(0)$  and  $\lambda$  parameters, characterizing the rate of increase in FPA concentrations.

## RESULTS

*Studies in vitro.* In recalcified plasma, both clotting and amidolytic activities of thrombin appeared at the same time, increased at a similar rate, and simultaneously reached peak values. Clotting activity disappeared shortly thereafter, while the amidolytic activity decreased by a few percentage points and remained essentially unchanged (Fig 1).

Two hours after aspirin administration, peaks of thrombin activity, both clotting and amidolytic, became shifted toward the right. The mean time at which thrombin reached the peak of its clotting activity increased significantly from  $10.1 \pm 0.54$  minutes to  $12.3 \pm 0.45$  minutes ( $n = 12$ ;  $P = .03$ ). The same parameter for amidolytic activity increased insignificantly from  $14.1 \pm 1.0$  minutes to  $15.0 \pm 0.5$  ( $n = 12$ ;  $P = .28$ ). Following aspirin ingestion, peaks of thrombin clotting activity showed a tendency toward higher values than before aspirin; this phenomenon did not reach the level of statistical significance ( $P = .07$ , paired  $t$  test). Other drugs or placebo had no effect on maximum thrombin values when thrombin reached the peak of its activity (Fig 2, Table 1).

*Studies ex vivo.* In blood emerging from the standardized skin incisions, mean FPA levels increased exponentially, reached a plateau at approximately 4 minutes, when blood flow decreased. On average, six FPA measurements were performed in each subject, both before and after the drug. When the results were fed into the theoretical formula (6) developed, high correlations between experi-

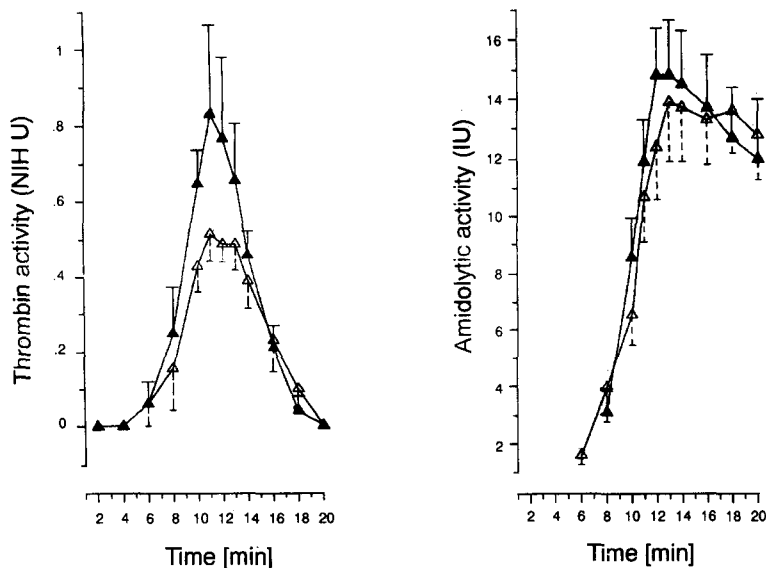


Fig 1. Clotting and amidolytic activities of thrombin generated in recalcified plasma ( $\Delta$ ) before and ( $\blacktriangle$ ) 2 hours after placebo. Data represent means  $\pm$  SE, recorded in five subjects.

mental and theoretical data were obtained ( $r = .86$  to  $1.00$ ,  $P = .020$  to  $<.001$ ).

Two hours after ingestion of 500 mg aspirin, thrombin generation became markedly depressed. At all time intervals measured, the mean FPA values were higher before than after the drug administration. These differences of means were significant at 120, 150, and 180 seconds ( $P < .05$ ), but not at the remaining blood sampling times (Fig 3 and Table 2).

The course of the mathematical curves describing the reaction of thrombinogenesis also changed markedly after the administration of aspirin. The rate of increase in FPA concentration, expressed by parameter  $\lambda$  of the exponential curve, decreased from  $0.51 \pm 0.04$  to  $0.36 \pm 0.04$  ( $n = 16$ ;  $P = .002$ ). The initial mean thrombin (FPA) concentration, expressed by  $c(0)$ , remained unchanged (Table 3).

Indomethacin, OKY-046, ticlopidine, and placebo had no effect on thrombin generation in blood emerging from

skin incision. No significant changes ( $P > .2$ ) were observed in either mean FPA concentrations at any time of blood sampling or in parameters  $\lambda$  and  $c(0)$ .

Two hours after administration of aspirin and indomethacin, and 5 days after regular ticlopidine intake, bleeding times became significantly increased. Placebo, OKY-046, and single dose of ticlopidine produced no changes in bleeding time (Table 4).

Prothrombin time and thrombin time were measured in 14 patients before and after a 500-mg single dose of aspirin. The mean values remained essentially unchanged ( $P > .20$ , paired  $t$ -test).

## DISCUSSION

Contrary to other antiplatelet drugs studied, aspirin in a single dose of 500 mg impaired generation of thrombin in clotting blood. In vitro studies showed a significant delay in

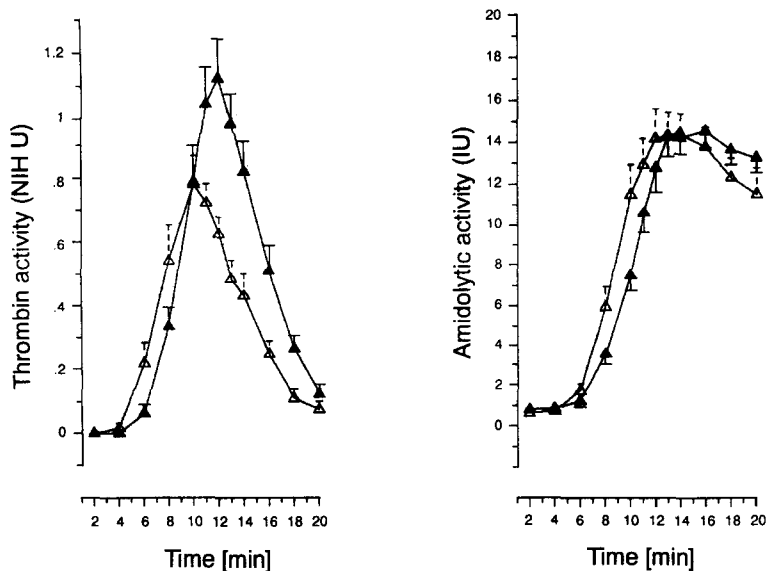


Fig 2. Clotting and amidolytic activities of thrombin generated in recalcified plasma ( $\Delta$ ) before and ( $\blacktriangle$ ) 2 hours after 500 mg aspirin ingestion. Data represent means  $\pm$  SE recorded in 12 subjects.

**Table 1. Thrombinogenesis In Vitro**

Drug	n	Mean ± SE Before Drug	Mean ± SE After Drug	P*
Aspirin	12	10.1 ± 0.543	12.3 ± 0.449	.03
OKY-046	7	11.7 ± 0.421	12.0 ± 0.535	.63
Indomethacin	10	11.6 ± 0.371	11.9 ± 0.348	.34
Ticlopidine 3 h	14	11.1 ± 0.450	12.1 ± 0.490	.41
Ticlopidine 5 d	7	10.6 ± 0.730	9.7 ± 0.781	.41
Placebo	5	11.6 ± 0.510	11.2 ± 0.490	.18

Comparison of mean times at which thrombin reached peak of its clotting activity, before and after drug's administration.

\*Paired Student's *t*-test.

peak thrombin clotting activity. A similar tendency was observed for thrombin amidolytic activity, although it did not reach statistical significance. This could be explained by difficulties in defining the maximum of the curve. In contrast to clotting activity, the peak of amidolytic activity was not sharp, but broad. The amidolytic activity of thrombin after reaching the peak did not decrease abruptly, but remained at the level close to maximum in a complex with  $\alpha_2$ -macroglobulin.<sup>12</sup>

The above observations were paralleled by studies ex vivo. Data were analyzed in two ways: by direct testing of differences between the means at specific time intervals, and by analyzing the curves of thrombinogenesis using a mathematical model. Both approaches revealed a significant impairment of thrombin generation after aspirin, but not after other drugs.

The ex vivo method sampled blood emerging from standardized skin incision. The technique was introduced into coagulation studies by Thorngren et al,<sup>13</sup> and was later used with some modifications by other investigators.<sup>7,13,17,18</sup> Kyrle et al<sup>17</sup> studied effects of aspirin on FPA formation in bleeding time wounds. They reported that after 7 days of treatment with 30 mg aspirin, generation of thrombin became significantly depressed in a few (four of seven) volunteers studied. The concentration of FPA recorded by

**Table 2. Thrombinogenesis Ex Vivo**

Time Interval (s)	n Before/After Drug	Mean FPA Before/After Drug	SE Before/After Drug	P*
0-30	2/3	25.8/24	15.4/12.3	.33
30-60	15/13	136.3/131.1	25.0/26.5	.71
60-90	17/13	197.5/169.4	33.8/40.7	.994
90-120	16/13	355.7/250.9	74.2/49.7	.05
120-150	6/6	943.4/431.3	220.8/94.4	.04
150-180	16/14	699.1/418.4	94.4/74.5	.001
180-210	7/12	1,229.6/791.4	226.3/200.7	.38
210-240	10/9	1,067.8/465.2	264.3/106.3	.007

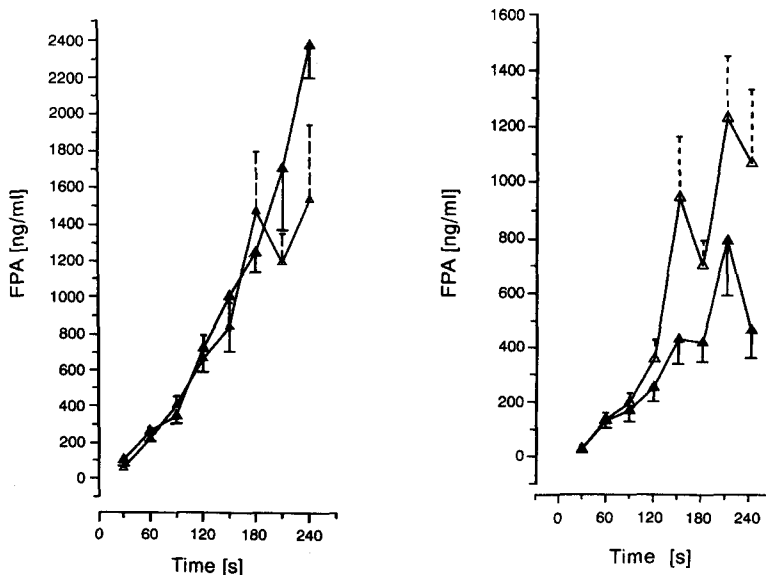
Mean FPA concentrations (ng/mL) in blood sampled every 30 seconds from skin wounds; n = number of blood aliquots collected before and after drug's administration.

\*Paired Student's *t*-test.

Kyrle et al was substantially higher than in our experiments, possibly because they used nonheparinized capillaries for blood collection. Similar FPA levels to those recorded by us were reported by Weiss and Lages,<sup>18</sup> who also used heparinized capillaries in a study of tissue factor activation in blood obtained from the bleeding time wounds.

We have developed a mathematical model that describes the reaction of thrombinogenesis ex vivo. The model assumes that velocity is constant in each time interval, which is a simplification, since in reality the velocity increases up to 90 to 120 seconds and then declines. The possible sources of error in determining the parameters of the mathematical function include (1) relatively high error of FPA assays (up to 10%); (2) small number of samples for each patient and missing measurements for some time intervals; and (3) small number of patients for each specific drug. Still, despite these limitations, the model developed described reality quite well as evidenced by very high correlation coefficients between FPA concentrations measured and calculated.

In addition to aspirin, other drugs used by us were



**Fig 3. Generation of thrombin ex vivo ( $\Delta$ ) before and ( $\blacktriangle$ ) 2 hours after ingestion of 500 mg aspirin (A, n = 17) or placebo (R, n = 5). Data represent means  $\pm$  SE of FPA concentrations in blood samples collected within the subsequent 30-second periods.**

**Table 3. Mean Values of Parameter  $\lambda$ , Characterizing the Time Course of Thrombinogenesis Ex Vivo**

Drug	n	Mean $\pm$ SE Before Drug	Mean $\pm$ SE After Drug	P*
Aspirin	16	0.509 $\pm$ 0.04	0.361 $\pm$ 0.04	.001
OKY-046	6	0.528 $\pm$ 0.08	0.403 $\pm$ 0.09	.176
Indomethacin	8	0.543 $\pm$ 0.05	0.539 $\pm$ 0.04	1.000
Ticlopidine 3 h	6	0.444 $\pm$ 0.05	0.448 $\pm$ 0.05	.755
Ticlopidine 5 d	7	0.508 $\pm$ 0.07	0.448 $\pm$ 0.06	.736
Placebo	5	0.560 $\pm$ 0.06	0.527 $\pm$ 0.07	.467

\*Wilcoxon test for paired samples.

deprived of any effects on thrombinogenesis. These drugs do not share a common mechanism of action on platelets. Indomethacin blocks cyclooxygenase, while OKY-046 is a highly specific inhibitor of thromboxane synthase. However, the clinical use of later drugs has been hampered by a substantial number of nonresponders. In addition to inhibiting thromboxane formation, OKY-046 reorients the cyclic endoperoxide metabolism toward other prostaglandins, such as PGD<sub>2</sub> and to PGI<sub>2</sub>. OKY-046 does not inhibit the formation of the proaggregatory cyclic endoperoxides, and its net action therefore is subject to a complex balance of pro- and anti-aggregatory prostaglandins.<sup>19,20</sup> This explains pronounced variability in response to the drug; such variability in bleeding time response was also present among subjects studied by us. The action of ticlopidine is less clear.<sup>8</sup> It does not interfere with arachidonic acid cascade. Ticlopidine inhibits ADP-mediated platelet aggregation and antagonizes the interaction of fibrinogen with its platelet receptor, the membrane glycoprotein IIb-IIIa. These effects only become evident after 3 to 5 days of ticlopidine administration, suggesting that it acts as a prodrug. However, in our experiments, even after 5 days of administration, and despite prolonging effectively bleeding time, ticlopidine did not affect thrombinogenesis.

The mechanism by which aspirin affects thrombinogenesis remains unknown. Inhibition of platelet activation through blockage of cyclooxygenase does not seem to be involved. In our study indomethacin, at a dose known to block platelet cyclooxygenase, prolonged bleeding time to the same extent as aspirin, but was deprived of any effects on thrombinogenesis. Interaction of aspirin with endothelial and subendothelial structures can be ruled out, since the phenomenon described was also observed in vitro. The possibility that aspirin acted by inducing structural changes in fibrinogen can be dismissed; 2 hours after aspirin administration, thrombin activity toward exogenous fibrin-

ogen was impaired. Furthermore, aspirin had no effect on thrombin time. It is also unlikely that aspirin could act by depressing synthesis of coagulation factors in the liver. Such a depression was observed only after massive doses of aspirin, amounting to 10 g/d orally.<sup>21</sup>

We suggest rather that aspirin impaired thrombinogenesis by acetylating prothrombin and/or platelet membranes. Aspirin is known to acetylate numerous biologic macromolecules, and the site of acetylation has been demonstrated to be the lysine residues of the N-terminal amino group. Already in 1974, Han and Ardlie<sup>22</sup> demonstrated that in vitro incubation of thrombin with very high concentrations of aspirin inhibited its aggregatory action on platelets. In a study by Cattaneo et al,<sup>23</sup> 20 mg/kg of aspirin, divided into three daily doses, produced a slight, but statistically significant, decrease of factor II biological activity on days 2 to 8 of administration. The plasma levels of other clotting factors, studied by biological methods, remained unchanged. It is not known whether a single 500-mg dose of aspirin could have a mild depressant effect on factor II biological activity. If that was the case, the importance of this inhibitory effect could be amplified during the exponential course of thrombin generation.

Another possibility would be acetylation of platelets membrane. Indeed, aspirin acetylates platelets membrane proteins and reduces membrane fluidity.<sup>24</sup> Changes in physical properties of the membranes might impair their catalytic power to assemble the prothrombinase complex. Release of platelet procoagulant factors could be hampered. Acetylation of guanosine triphosphate (GTP)-binding proteins or thrombin receptors could also affect adversely the process of thrombin formation, since platelet activation plays an important role in explosive generation of thrombin.<sup>25</sup>

A widely held notion is that protective action of aspirin is mediated by the inactivation of platelet cyclooxygenase. The results here presented point to another mechanism, complementary to platelet cyclooxygenase inactivation. Impairment of thrombin generation by aspirin might be of clinical relevance. In critical narrowing of an artery, building up of a clot would be prolonged and delayed because of late appearance of thrombin. In patients on chronic aspirin prophylaxis, depressed levels of plasma FPA have been observed recently.<sup>26</sup> This observation, explicable by the results of the present study, is consistent with lower levels of circulating thrombin activity. It is tempting to think that continuous dampening of thrombin formation by aspirin might be one of the mechanisms responsible for its prophylactic and therapeutic efficacy.

**Table 4. Mean Bleeding Time (s) Before and After the Drugs Studied**

Drug	n	Mean $\pm$ SE Before Drug	Mean $\pm$ SE After Drug	P*
Aspirin	22	357.5 $\pm$ 19.7	772.3 $\pm$ 98.9	.00005
OKY-046	6	439.6 $\pm$ 102.6	883.3 $\pm$ 318.8	.136
Indomethacin	9	336.4 $\pm$ 23.9	628.9 $\pm$ 109.7	.013
Ticlopidine 3 h	6	350.0 $\pm$ 19.3	440.0 $\pm$ 73.3	.295
Ticlopidine 5 d	7	345.7 $\pm$ 52.8	1641.4 $\pm$ 83.1	.000001
Placebo	5	348.1 $\pm$ 32.9	329.4 $\pm$ 36.6	.776

\*Paired Student's *t*-test.

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