INHIBITORY EFFECT OF TIARAMIDE ON AGGREGATION  
IN VIVO AND ON ELECTROPHORESIS OF RABBIT PLATELETS

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Abstract—Effects of tiaramide, aspirin and indomethacin were studied on rabbit platelet aggregation in vivo and on platelet electrophoretic mobility. When tiaramide (6 mg/kg), aspirin (30 mg/kg) or indomethacin (1.3 mg/kg) was injected into the ear vein of rabbit during 60 sec, tiaramide only inhibited ADP-induced aggregation, 20 min after the injection. All three drugs prevented collagen-induced aggregation 20 and 120 min after the injection. The inhibitory effects on the aggregation of tiaramide are presumably independent of prostaglandin synthesis, because malondialdehyde (a metabolite of PGG₂) production was not influenced. Tiaramide reduced cyclic AMP levels in platelets after 20 min incubation, despite the ability of this agent to inhibit platelet aggregation. Tiaramide, aspirin and indomethacin per se has no effect on platelet electrophoretic mobility, while tiaramide prevented the decrease in the mobility produced by ADP. Tiaramide and aspirin also depressed the decrease in the mobility produced by collagen.

Anti-inflammatory drugs such as aspirin and indomethacin have a marked inhibitory action on platelet aggregation (1), inhibit collagen-induced aggregation and the secondary wave of aggregation caused by ADP, in humans (1). Tiaramide, one of the non-steroidal anti-inflammatory drugs, reportedly inhibits the primary aggregation by ADP in rabbit platelets (2). Mechanisms which may be involved in the inhibition of platelet aggregation increase intracellular cyclic AMP levels by stimulating adenylate cyclase (3) or by inhibiting cyclic phosphodiesterase (4). Inhibition of the synthesis of prostaglandins such as aspirin-like drugs also occurs (5). Several specific antagonists such as AMP or adenosine for ADP inhibit platelet aggregation (6) and some tricyclic anti-depressants prevent the aggregation by their membrane stabilizing action (7).

On the other hand, the surface of blood platelets carries various types of charged groups and there is a net negative charge. Hampton and Mitchell reported a decrease in the negative charge, in the presence of ADP and collagen (8). As a result the platelets aggregate. Agents which have an acid group in their molecules may increase the platelet surface charge and decrease the aggregation (9). Therefore, it was of interest to study the effects of tiaramide, aspirin and indomethacin on the electrokinetic charge, per se, and influences on the changes of the electrophoretic mobilities induced by ADP and collagen, in platelets.

Tiaramide, a complex with hydrochloride is reportedly a potent inhibitor of platelet aggregation as produced by ADP and collagen in the optical density method and the Chandler loop method (10). We attempted to demonstrate the inhibitory effect of tiaramide on aggregation of rabbit platelets in vivo, as compared with effects of aspirin and indomethacin.
Also determined was the influence of tiaramide on malondialdehyde (MDA) production, on cyclic AMP, and platelet electrophoretic mobility.

MATERIALS AND METHODS

Rabbits of both sexes weighing 2.5 to 3.0 kg were used. Platelet-count ratio method (11) was used for the quantitative determination of circulating platelet aggregates in vivo. Blood (1.8 ml) was withdrawn from the ear vein of a rabbit with syringe containing 0.2 ml of 3.8% sodium citrate. The total mixture was separated into 4 equal samples. Two were preincubated for 3 min at 37°C, to which was added ADP (0.05 ml) and collagen (0.05 ml), respectively, followed by continuous stirring for 5 min. Then EDTA-formalin solution (pH 7.4) was added. A third aliquot was incubated with 0.9% NaCl, and then was added buffered EDTA solution (pH 7.4). Thereafter all three samples were prepared according to the method of Wu and Hoak (11). The samples were kept at room temperature for 15 min and then centrifuged at 700 rpm for 10 min to obtain platelet-rich plasma (PRP). The platelets were counted using a Coulter counter (Coulter Electronics, Inc.). The platelet-count ratio was calculated according to the formula:

Platelet-count ratio = \frac{\text{platelet-count in EDTA-formalin PRP}}{\text{platelet-count in EDTA PRP}}

Tiaramide (6 mg/kg), aspirin (30 mg/kg) or indomethacin (1.3 mg/kg, pH 6.8) were injected into the ear vein in one minute. Twenty min, 2 hr and 24 hr after the injection, the effects of the drugs on aggregation were examined using the Coulter counter.

Estimation of MDA production: After incubation of the PRP with the drugs for 20 min at 37°C, 77 mM EDTA-saline (0.05 ml) was added to the PRP and centrifuged. The sediment was suspended in 0.15 M phosphate buffered saline and treated according to the method of Stuart et al. (12).

Estimation of cyclic AMP: The platelet cyclic AMP level was determined by the binding protein method, according to Yajima and Ui (13). Cyclic AMP binding protein was obtained from rat liver. Rabbit PRP (6 x 10^5 platelets/mm^3), 0.8 ml, was incubated with 10^{-4} M of tiaramide, aspirin or indomethacin (0.1 ml) for 20 min at 37°C. Immediately the PRP was centrifuged at 3000 rpm for 3 min at 0°C and to the sediment which had been subjected to freezing and fusion, was added 0.8 ml of distilled water. The supernatant was treated according to the technique described elsewhere (12).

Electrophoresis: Platelets in concentrations of 10^4/mm^3 of plasma were used. Eight parts of PRP were incubated slowly with one part of anti-inflammatory drug for 20 min at 25°C, and then one part of ADP or collagen (25 µg/ml) was added. Electrophoretic mobility was measured using a cell electrophoresis microscopic apparatus developed by Sugiura Laboratory Inc. In each sample the rates of migration of 20 platelets were obtained at the stationary level in electric field with constant current of 2.0 mA at 25°C. The measurement of platelet migration was begun 5 min after ADP or collagen and finished within 15 min. The viscosity and specific resistance of the PRP were determined by means of an Ostwald's
The drugs used were: Adenosine diphosphate (ADP, Sigma), collagen (Hormone-Chemie München), ^4^H-cyclic AMP (The Radio Chemical Center), cyclic AMP (Seishin P.H. Co.), Malondialdehyde (MDA, Tokyo Kasei), tiaramide (Fujisawa Pharmaceutical Co. Ltd.), Aspirin (Torii Co. Ltd.) and Indomethacin (Sumitomo Chemical Co. Ltd.).

RESULTS

Effect of tiaramide on platelet-count ratio: Figure 1 shows that the platelet-count ratio decreased as the ADP or collagen concentration was increased. In a platelet-count technique, the ratio increased with increasing inhibition of platelet aggregation, but decreased with its acceleration. ADP (5 x 10^-6 M) induced more than 60% aggregation in whole blood, and this is almost the same as in PRP (2). The platelets in whole blood were more responsive to collagen than those in PRP. The former aggregated in the presence of 1 µg collagen /ml, but the latter did not respond sufficiently even to 10 µg/ml (2). Collagen in 2.5 µg/ml was used in this study. Aggregation by ADP was significantly inhibited (P<0.05)
20 min after tiaramide, but, at 2 hr the effect of the drug was not apparent (Fig. 2). Aspirin, 30 mg/kg, tended to inhibit the aggregation 2 hr after the injection. Collagen-induced aggregation was significantly inhibited by all drugs 20 min, 120 min and 24 hr after the injection (except the indomethacin at 24 hr) (Fig. 3).

*MDA production*: A large amount of MDA, a metabolite of prostaglandin endoperoxides, was formed by platelets from arachidonic acid (Fig. 4). Tiaramide, even in

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**Fig. 2.** Tiaramide (6 mg/kg) inhibited platelet aggregation induced by ADP (5 x 10^-6 M) at 20 min after i.v. administration. Values are the mean ± standard error of 6 rabbits. Significantly different from the control, *P<0.05. •: tiaramide (6 mg/kg), ■: aspirin (30 mg/kg), ▲: indomethacin (1.3 mg/kg).

**Fig. 3.** Tiaramide (6 mg/kg), aspirin (30 mg/kg) and indomethacin (1.3 mg/kg) inhibited platelet aggregation induced by collagen (2.5 μg/ml) 20 min, 120 min and 24 hr after i.v. administration. (except indomethacin at 24 hr). Values are the mean ± standard error of 6 rabbits. Significantly different from the control, *P<0.05, **P<0.01, ***P<0.001. •: tiaramide, ■: aspirin, ▲: indomethacin.
a high concentration had no effect on MDA production (Fig. 5), but aspirin and indomethacin markedly inhibited the production.

Intracellular cyclic AMP level in platelets: A general hypothesis has been proposed that some agents inhibit platelet aggregation by raising the cyclic AMP level in platelets. Since tiaramide has a significant inhibitory effect on ADP-induced aggregation, an experiment was performed to see whether it would affect the platelet cyclic AMP level. Tiaramide (10^-4 M) decreased the platelet cyclic AMP level to 73.6±3.06 % of the control (0.9 % NaCl, 92.1±9.15 p mole/4.8 x 10^8 platelets, P<0.001, n=7) during 20 min incubation at 37°C. Aspirin and indomethacin (10^-4 M) did not change the cyclic AMP level.

Platelet electrophoresis: All three drugs per se and saline had no effect on the electrophoresis of platelets.
phoretic mobility of platelets (Table 1-1). ADP (5 x 10^{-8} M) and collagen (25 µg/ml) reduced the mobility (Table 1-2, 3). Only tiaramide significantly restored the electrophoretic mobility reduced by ADP (Table 1-2). Tiaramide and aspirin restored the mobility reduced by collagen, but not indomethacin (Table 1-3).

Table 1. Effect of tiaramide, aspirin and indomethacin on platelet electrophoresis and on the decrease in mobility produced by ADP and collagen

| Drug (10^{-4} M) | 0.9% NaCl | ADP (5 x 10^{-6} M) | Collagen (25 µg/ml) |
|------------------|-----------|---------------------|--------------------|
|                  | n=10      | n=11                | n=10               |
| 0.9% NaCl        | 0.87±0.025| 0.76±0.019          | 0.80±0.009         |
| Tiaramide        | 0.87±0.021| 0.82±0.020**        | 0.83±0.006*        |
| Aspirin          | 0.87±0.024| 0.74±0.017          | 0.83±0.007*        |
| Indomethacin     | 0.86±0.019| 0.74±0.016          | 0.82±0.083         |

The value was expressed as the mean±standard error. Electrophoretic mobility was expressed as μ/sec/v/cm. PRP (10^4 platelets/mm³ of plasma) was incubated with the drug for 20 min at 25°C, to which was added ADP (5 x 10^{-6} M), collagen (25 µg/ml) or 0.9% NaCl. Asterisk indicates a significant prevention of the decrease in mobility produced by ADP or collagen (**: P<0.02, *P<0.05).

DISCUSSION

In a previous study using the turbidimetric method and the Chandler loop in vitro (2), tiaramide markedly inhibited ADP- or collagen-induced platelet aggregation. In the present in vivo study, the drug also inhibited ADP- or collagen-induced aggregation. The anti-inflammatory drugs were administered in the maximum dose used in clinical therapy. Aspirin tended to inhibit ADP-induced aggregation, because according to a calculation, the blood aspirin level was about 30 times of the (10^{-4} M) in vitro. The blood tiaramide level in vivo was 3 times 10^{-4} M in vitro and the indomethacin level was the same 10^{-4} M. Since tiaramide inhibited the aggregation produced by both ADP and collagen, it may be more potent than aspirin in relieving patients from some kinds of thrombosis, ischemic heart desease, and other cardiovascular diseases.

It seemed of interest to investigate the mechanism taking part in blocking the effect of tiaramide on platelet aggregation. Some reports demonstrated that the agents that increase the intracellular cyclic AMP levels in platelets inhibit the aggregation (3, 4). However, the present study seems to indicate that the inhibitory action of tiaramide is independent of the ability to increase the platelet cyclic AMP level. Furthermore, the mechanism of inhibitory effect of tiaramide on the aggregation might be different from those of aspirin and indomethacin. Collagen produces platelet aggregation by activating phospholipases to liberate arachidonic acid from platelet phospholipids (14). The arachidonic acid is then converted by a platelet cyclo-oxygenase into prostaglandin endoperoxides (PGG₂, PGH₂) (15), which are in turn transformed to a powerful platelet aggregating agent, thromboxane A₂ (16). And MDA is a metabolite of PGG₂ which is formed during platelet aggregation in response to collagen. Aspirin and indomethacin reduced the basal MDA level, since they are in-
hibitors of cyclo-oxygenase. Accordingly, the fact that tiaramide did not depress MDA production (Fig. 5) clearly suggests that the mechanism differs from that of aspirin-like drugs.

Zucker and Peterson suggested that collagen releases ADP and 5-HT by 2 mechanisms, only one of which is inhibited by aspirin (17). The other is presumably a prostaglandin-independent pathway, because even in the presence of a maximally effective concentration of aspirin, platelet release reaction was increased by increasing the concentration of collagen (17). In our experiment, aspirin and indomethacin inhibited collagen-induced aggregation and reduced the MDA level. These drugs, therefore, seem to prevent collagen-induced aggregation by inhibiting the production of prostaglandin endoperoxides. However, the prostaglandin-independent pathway is not excluded, because the platelet-count ratios after the administration of aspirin and indomethacin were considerably lower than 1.0, indicating that the inhibitory effects were not complete. Therefore, the possibility cannot be excluded that tiaramide inhibits the aggregation by a prostaglandin-independent pathway.

The aggregating agents decreased the electrophoretic mobility of platelets (7). Gröttum reported that increasing concentrations of dextran sulphate and heparin increased the electrophoretic mobility of platelets, and did not aggregate the platelets (9). And PGE\(_1\), a powerful inhibitor of platelet aggregation, prevents the decrease in mobility induced by ADP or noradrenaline (18). Tiaramide is an alkaline drug and not an acid like heparin, dextran sulphate and PGE\(_1\). Tiaramide restored considerably the electrophoretic mobility of platelets reduced by both ADP and collagen while indomethacin did not. These effects and the cyclic AMP decreasing effect of tiaramide suggest that the mechanism involved in the platelet aggregation preventing action of tiaramide differs from that of cyclo-oxygenase inhibiting drugs (indomethacin and aspirin). Nachman (19) reported that AMP, ATP and 2-chloro-adenosine, known inhibitors of platelet aggregation, blocked ADP membrane binding whereas cyclic AMP, adenosine and prostaglandin E\(_1\) did not. On the other hand, Boullin et al. (20) concluded that ADP induced platelet aggregation by binding to specific receptors probably located on the plasma membrane, and that PGE\(_1\) inhibited the aggregation by interfering with the ADP binding. Our experiments suggest that inhibition of decrease in negative charge of platelets produced by ADP and collagen by tiaramide may occur by interference with the ADP and collagen binding to receptors on the plasma membrane. Further study is necessary to clarify these problems.

Previous reports (8, 9) and the results of the present study suggest that a decrease in the platelet electrokinetic potential is essential for platelet aggregation and that inhibition of the decrease in mobility may be one explanation for the inhibition of the aggregation.

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