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IRREVERSIBLE INHIBITION OF THROMBOXANE (TX) A_2 SYNTHESIS BY Y-20811, A SELECTIVE TX SYNTHETASE INHIBITOR

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Abstract—As Y-20811, sodium (±)-4-[α-hydroxy-5-(1-imidazolyl)-2-methylbenzyl]-3,5-dimethylbenzoic acid, has been reported to inhibit serum thromboxane (TX) A_2 production with a long duration of action, its mechanism of action was investigated. When [^{3}H]Y-20811 (3 mg/kg) was administered orally to rats, the peak platelet concentration of Y-20811 was obtained 1 hr after the administration, and the $T_{\frac{1}{2}}$ was 43 hr. The peak plasma concentration of Y-20811 was also obtained 1 hr after administration, but the elimination of Y-20811 from plasma was faster ($T_{\frac{1}{2}}\alpha$ = 1.5 hr, $T_{\frac{1}{2}}\beta$ = 15 hr) than that observed in platelets. Serum TXA_2 (estimated as TXB_2) production was inhibited significantly from 1 to 72 hr after the oral administration of unlabeled Y-20811 (3 mg/kg), which temporally resembled the change of the platelet Y-20811 concentration. In platelet-rich plasma, [^{3}H]Y-20811 completely inhibited TXA_2 production at about 1500 pg/10^9 platelets, and the IC_{50} level was about 600 pg/10^9 platelets, which was similar to values obtained in *ex vivo* studies. In addition, inhibition of TXA_2 production by Y-20811 still remained after washing the drug-pretreated microsomes, whereas that of dazoxiben completely disappeared. A similar irreversible inhibition of TXA_2 production was observed with aspirin. These results suggest that Y-20811 may firmly combine with platelet TX synthetase and may irreversibly inhibit TXA_2 production.

Thromboxane (TX†) A_2 has been considered to be a major participant in the pathogenesis of ischemic circulatory disorders such as myocardial infarction and cerebral infarction; therefore, the drugs which inhibit TXA_2 production have been expected to be effective in the treatment of such disorders [1, 2]. As circulating platelets, in these situations, are exposed to several aggregating substances such as atherosclerotic plaques or subendotherial collagen fibers, it is important to inhibit platelet function for a prolonged period in order to prevent these disorders. Aspirin [3, 4] which inhibits TXA_2 production has been used to treat such disorders because of its long-duration of action. It has been reported to inhibit cyclooxygenase irreversibly by acetylating its active site [5].

Y-20811 [6], sodium (±)-4-[α-hydroxy-5-(imidazolyl)-2-methylbenzyl]-3,5-dimethylbenzoic acid, is a selective TX synthetase inhibitor. It has already been reported that its duration of action is long enough to be equal to the life span of platelets. This feature of Y-20811 led us to clarify the mode of action. In this paper, we have demonstrated that Y-20811 firmly combines with TX synthetase and inhibits TXA_2 production for a long time which is similar to the life span of platelets reported elsewhere [7], while it is eliminated quickly from plasma.

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† Abbreviations: TX, thromboxane; Y-20811, sodium (±) - 4-[α-hydroxy-5-(1-imidazolyl)-2-methylbenzyl] - 3,5-dimethylbenzoic acid; PG, prostaglandin; HHT, 12L-hydroxy-5,8,10-heptadecatrienoic acid; 12-HETE, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid; AA, arachidonic acid; PRP, platelet-rich plasma; PPP, platelet-poor plasma; SPB, standard platelet buffer; and RIA, radioimmunoassay.

MATERIALS AND METHODS

Materials. [^{3}H]Y-20811, unlabeled Y-20811, dazoxiben and aspirin were synthesized by The Laboratory of Yoshitomi Pharmaceutical Industries, Ltd. The labeled compound showed a specific radioactivity of 325.1 μCi/mg, and its radiochemical purity was 102.4%. These drugs were dissolved in dimethyl sulfoxide for *in vitro* experiments, and labeled or unlabeled Y-20811 was dissolved in 0.5% methylcellulose solution for oral administration. Arachidonic acid (AA) sodium salt, TXB_2, prostaglandin (PG) E_2, $PGF_2\alpha$, PGD_2, 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) were obtained from Funakoshi Yakuhin (Tokyo, Japan). [1-^{14}C]AA (58 mCi/mmol) was purchased from Amersham International (Amersham, U.K.).

Animals. Male Sprague–Dawley rats (Seiwa Laboratory Animals, Fukuoka, Japan) were used. Four animals a group were used for the determination of drug concentration. For TXA_2 production, 20 animals a group were used, since individual TXA_2 formation varied.

Changes of plasma or platelet ^3H levels. [^{3}H]Y-20811 (3 mg/kg) was administered orally by gastric intubation to rats weighing 200–300 g, and blood samples were obtained from their abdominal aortas using siliconized syringes containing 0.1 vol. of 4.5 mM EDTA solution under light ether anesthesia.

Platelet-rich plasma (PRP) was prepared from anticoagulated blood by centrifugation at 200 *g* for 10 min, and platelet-poor plasma (PPP) by centrifugation at 1400 *g* for 10 min. Two milliliters of PRP was centrifuged at 800 *g* for 10 min and the supernatant was discarded. The remaining platelet pellet was resuspended in an equal volume of standard platelet buffer (SPB; 3.2 mM KH_2PO_4, 4.8 mM Na_2HPO_4, 80 mM NaCl, 45 mM citric acid, 4.4 mM glucose, pH 6.5). This suspension was centrifuged at 750 *g* for 10 min at 4°, the supernatant was discarded, and the platelet pellet was resuspended in an equal volume of SPB. This operation was repeated three times and the pellet was finally resuspended in SPB (washed platelet suspension). Platelet counts were made with a platelet analyzer (Baker 810, Baker Diagnostics, PA, U.S.A.), and the platelet count of washed platelet suspension was adjusted to $10^6/\mu L$ with SPB. In a preliminary study, we confirmed that the loss of Y-20811 from platelets was extremely low during washing procedure.

The radioactivity of samples was measured with a liquid scintillation counter (Packard, model 460). Drug concentration was calculated based upon specific activity and radioactivity detected, and was expressed as Y-20811 concentration.

Ex vivo *serum TXA_2 production*. Blood samples of rats (weighing 400–500 g) who had received unlabeled Y-20811 (3 mg/kg) orally were obtained from the abdominal aorta under light ether anesthesia. The serum was prepared by incubating the blood at 37° for 60 min, followed by centrifugation (1400 *g*) at 4° for 10 min. As TXA_2 is extremely short-lived, its production was assayed by measuring the stable degradation product, TXB_2, by the radioimmunoassay (RIA) method. The RIA of TXB_2 was done on unextracted samples, according to the procedure described, using a [^{3}H] RIA kit (New England Nuclear, Boston, MA, U.S.A.). Using this method, we could detect 5–1000 pg/assay tube of TXB_2.

TXA_2 production and drug concentration in platelets in vitro. Blood samples were obtained using siliconized syringes containing 0.1 vol. of 3.8% sodium citrate solution from the abdominal aorta of rats, weighing 350–400 g, under light ether anesthesia. PRP was prepared by centrifuging the blood as mentioned above. PRP (2.5 mL) added with 25 μL of various concentrations of [^{3}H]Y-20811 (10 nM–10 μM) was incubated with shaking at 37° for 20 min. Three microliters of AA (final concentration; 0.5 mM) was added to a portion (0.3 mL) of incubated PRP and was incubated with stirring (1000 rpm) at 37° for 5 min. The reaction was stopped by adding 20 μL of 2 M citric acid, and the reaction mixture was centrifuged (1400 *g*) at 4° for 10 min. TXA_2 concentration in this supernatant was determined by the RIA method described above.

For the determination of the drug concentration, washed platelet suspensions were prepared from the remaining incubated PRP, and their ^{3}H concentrations were determined using the methods described above.

Irreversible inhibition of TXA_2 production. Human platelet microsomes obtained from Ran Biochemicals (Tel-Aviv, Israel) were used as a source of TX synthetase. For the assay, [1-^{14}C]AA was incubated with human platelet microsomes (0.5 mg protein) [8]. TXB_2 was chosen as a reaction product of TX synthetase, and was extracted into ethyl ether. The extract was evaporated to dryness. The residue was dissolved in a small amount of chloroform–methanol (2:1) and subjected to thin-layer chromatography in a mixture of chloroform–ethyl acetate–methanol–acetic acid–water (70:30:8:1:0.5). Authentic samples (AA, HHT, 12-HETE, PGD_2, PGE_2, TXB_2 and $PGF_2\alpha$) were also co-chromatographed, and visualized with iodine vapor. The radioactive products were located by a radiochromatogram scanner and quantitated by a standard liquid scintillation procedure.

To evaluate the reversibility of the inhibition of TXA_2 production, each drug was preincubated with human platelet microsomes, and diluted with 4 vol. buffer and centrifuged at 105,000 *g* for 1 hr. The pellet was resuspended in a reaction mixture containing [1-^{14}C]AA, and TXA_2 production was measured.

Statistical analysis. Statistical analysis was carried out using Student's *t*-test.

RESULTS

When [^{3}H]Y-20811 (3 mg/kg) was administered orally to rats, the maximum platelet concentration of Y-20811 (1450 ± 40 pg/10^9 platelets) was obtained 1 hr after the administration. The platelet concentration of Y-20811 declined slowly and its terminal half-life ($T_{\frac{1}{2}}$) was 43 hr. The maximum plasma concentration of Y-20811 (552 ± 29 ng/mL) was also obtained 1 hr after the administration. The plasma concentration of Y-20811 declined biexponentially ($T_{\frac{1}{2}}\alpha = 1.5$ hr, $T_{\frac{1}{2}}\beta = 15$ hr), which was faster than that observed in the platelets (Fig. 1).

Serum TXA_2 (estimated as TXB_2) production significantly declined from 1 to 72 hr after the oral administration of unlabeled Y-20811 (3 mg/kg), which temporally correlated with the change of the platelet Y-20811 concentration. TXA_2 production was inhibited by 94 and 45% at 1 and 48 hr, respectively, when the platelet Y-20811 concentrations were 1446 and 884 pg/10^9 platelets, respectively (Fig. 1).

As the platelet Y-20811 concentrations observed in *ex vivo* experiments were at extremely low levels, we studied *in vitro* experiments to determine whether these concentrations were effective to inhibit TX synthetase. Various concentrations (10 nM–10 μM) of [^{3}H]Y-20811 were incubated with PRP, and 20 min later TXA_2 production in PRP induced by AA (0.5 mM) and platelet ^{3}H concentrations in washed platelet suspensions were measured. TXA_2 (estimated as TXB_2) production in PRP was inhibited and the Y-20811 concentrations in the platelets increased in a concentration-dependent manner (Fig. 2a); the relation between the inhibition of TXA_2 production and platelet Y-20811 concentration was plotted (Fig. 2b). There was a positive correlation between them, and the TXA_2 production was inhibited by 50% at a concentration of about 600 pg/10^9 platelets and by 100% at a concentration of about 1500 pg/10^9 platelets. This concentration–

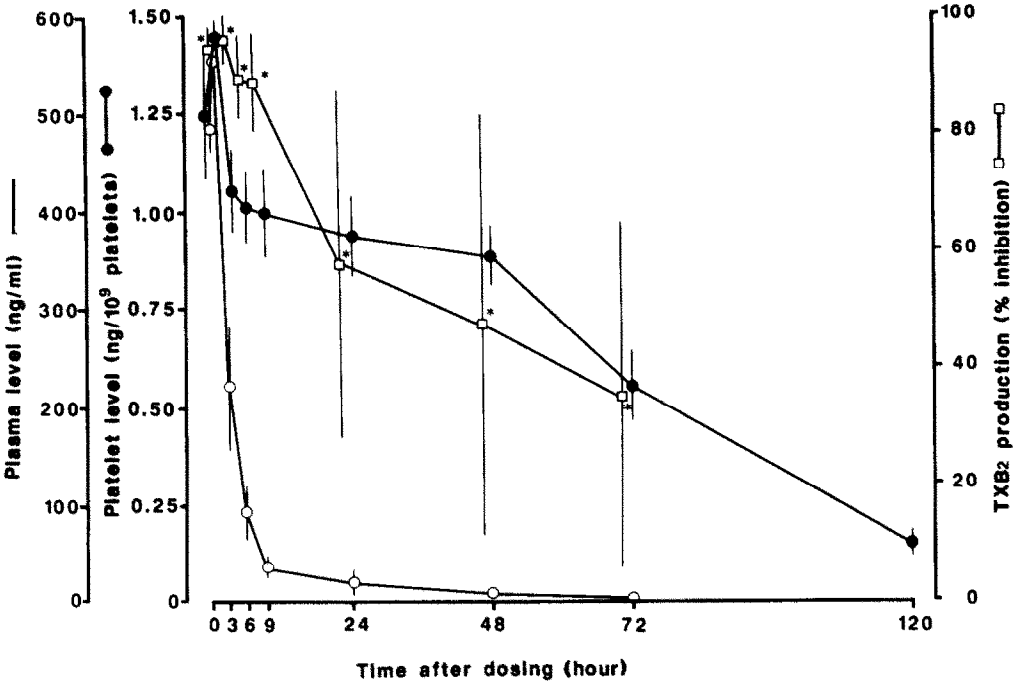

Fig. 1. Relationship between plasma or platelet Y-20811 levels and the inhibition of serum TXB_2 production after oral administration of labeled or unlabeled Y-20811 (3 mg/kg). To determine the Y-20811 concentration of both plasma and platelets, ^3H levels were measured. Each point is the mean ± SD (N = 4 for plasma or platelet, N = 20 for serum TXB_2 production). Key: * $P < 0.01$ vs non-treated group.

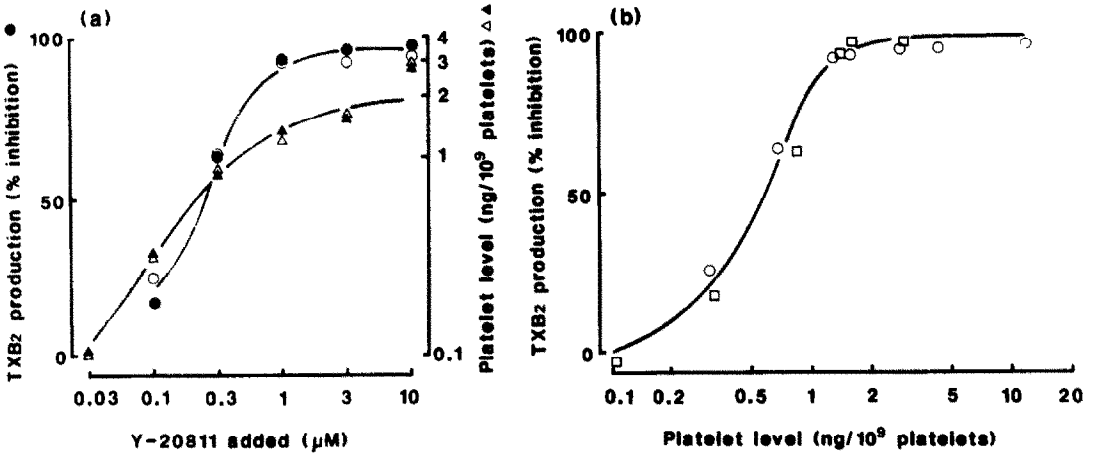

Fig. 2. Relationship between platelet Y-20811 levels and the inhibition of platelet TXA_2 (estimated as TXB_2) production *in vitro*. (a) Following the addition of [^3H]Y-20811 to PRP, platelet TXA_2 production (○, ●) was inhibited and platelet Y-20811 concentration (△, ▲) increased in a concentration-dependent manner. (b) There was a positive correlation between platelet TXA_2 production and platelet Y-20811 concentration. Results were obtained from two separate experiments.

response curve was similar to that observed in *ex vivo* experiments.

The reversibility of inhibition of TXA_2 production was examined by washing the human platelet microsomes pretreated with Y-20811, aspirin and dazoxiben. As shown in Fig. 3, the control platelet microsomes retained about 80% of their TXA_2 production activity after the washing treatment. The inhibitory effect of dazoxiben on TXA_2 production completely disappeared after washing, indicating

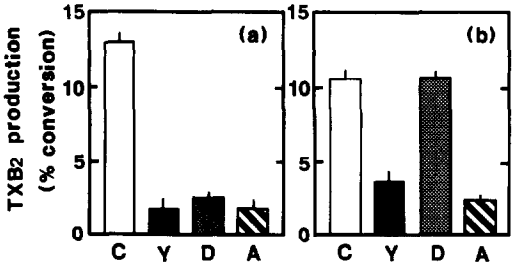

Fig. 3. Effect of washing treatment on the reversibility of TXA_2 production inhibition by Y-20811, dazoxiben and aspirin. TXA_2 production activity was measured in terms of the production of TXB_2 from [1-^{14}C]AA by human platelet microsomes. The results are the means ± SD of four assays. (a) Microsomes were preincubated with drugs for 3 min at 37° and subsequently incubated with AA for 10 min at 37°. (b) Microsomes were preincubated with drugs for 3 min at 37°, followed by dilution and centrifugation. Then the pellet was incubated with AA for 10 min as described above. Key: (C) control; (Y) Y-20811 (1 μM); (D) dazoxiben (1 μM); and (A) aspirin (10 mM).

that its inhibition was reversible. In contrast, the inhibition of TX production by Y-20811 decreased only slightly after the washing treatment. Aspirin, an irreversible inhibitor of cyclooxygenase, also retained its inhibitory effect on TXA_2 production.

DISCUSSION

Y-20811 [6] is a potent selective TXA_2 synthetase inhibitor which has been studied for clinical efficacy in prevention of both restenosis after percutaneous transluminal coronary angioplasty and reocclusion after percutaneous transluminal coronary recanalization. As the inhibitory effect of Y-20811 on TXA_2 production lasts for 7 days [9], once-daily medication has been adopted in clinical treatment. The usefulness of aspirin as an anti-thrombotic agent is derived from the property that its effects last for the life-span of the platelets because it inhibits cyclooxygenase by irreversibly acetylating its active site [5]. As the effect of Y-20811 on TXA_2 production lasted as long as that of aspirin, the inhibition of TX synthetase was considered to be an irreversible one.

In the present study, the inhibitory effect on serum TXA_2 production by Y-20811 lasted for 72 hr, which is similar to the half-life of platelets in rats [7]. The elimination of Y-20811 (estimated as ^{3}H concentration) from plasma, however, was fast ($T_{\frac{1}{2}}\alpha$ and $T_{\frac{1}{2}}\beta$ were 1.5 and 15 hr, respectively). Thus, it became clear that the long-duration of the action of Y-20811 on TXA_2 production did not depend on its plasma levels. Although there is a rough correlation between TXA_2 production and plasma drug levels during the decay phase (9–72 hr), plasma drug levels (< 40 ng/mL) between 9 and 72 hr were considered to be too low to inhibit platelet TXA_2 production, since the IC_{50} values of Y-20811 for *in vitro* platelet aggregation ranged between 9.8 and 17 μM (3.9 to 6.7 μg/mL) [6]. Additionally, it has been demonstrated in this paper that platelet TXA_2 production was inhibited at a concentration over 0.3 μM (120 ng/mL) (Fig. 2a). On the other hand, Y-20811 (estimated as ^{3}H concentration) was detected in platelets with a $T_{\frac{1}{2}}$ of 43 hr. This elimination curve was closely similar to the time course of the inhibition on TXA_2 production by Y-20811. These results suggest that Y-20811 owes its long-duration of action to its platelet levels.

The platelet concentration of Y-20811 was at extremely low levels (1446 ± 40 pg/10^9 platelets at 1 hr after the administration), so we studied the correlation between the inhibition of TXA_2 production and platelet Y-20811 concentrations *in vitro*. TXA_2 production was inhibited by 50% at a concentration of about 600 pg/10^9 platelets, and by 100% at a concentration of about 1500 pg/10^9 platelets. This correlation between platelet TXA_2 production and platelet Y-20811 concentration was very similar to that observed in *ex vivo* experiments. Thus, the inhibition of platelet TXA_2 production was effective at a low platelet concentration of Y-20811.

As the inhibition of TX production by Y-20811 appeared to be irreversible, we studied the binding of Y-20811 to TX synthetase. The inhibition of TXA_2 production by pretreatment with Y-20811 remained after the washing procedure. The inhibition by dazoxiben [8], a relatively short-acting TX synthetase inhibitor, disappeared after the same washing treatment, while that by aspirin [5], an irreversible inhibitor of cyclooxygenase, remained. These results suggest that the inhibition of TX synthetase by Y-20811 is irreversible. The irreversible inhibition of cyclooxygenase by aspirin has been reported to derive from the acetylation of the enzyme protein [5]. Y-20811 has no apparent component corresponding to the acetyl group in aspirin. But one characteristic component, different from other TX synthetase inhibitors [10, 11], is the hydroxy moiety. This hydroxy moiety is essential for the inhibition of TX synthetase (unpublished data). The position of this moiety appears to coincide with that of the hydroxy moiety in the hydrophobic chain of PGH_2, the substrate of TX synthetase, using the computer aided drug design system. Thus, it is possible that the hydroxy moiety of Y-20811 contributes to the irreversibility of TX synthetase inhibition, although other parts such as the imidazole and carboxyl also play an important role in the interaction with TX synthetase, as reported with other TX synthetase inhibitors.

In conclusion, the inhibition of TXA_2 production by Y-20811 is dependent on its platelet concentration. As the binding of Y-20811 with TX synthetase appears to be firm and irreversible, its inhibitory effect on TX synthetase lasts for the life-span of the platelets. These findings suggest that the compound may be useful in preventing thrombotic disorders in once-daily doses because of its long-duration of action.

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