Intravenous infusion of a selective inhibitor of thromboxane A₂ synthetase in man: influence on thromboxane B₂ and 6-keto-prostaglandin F₁α levels and platelet aggregation

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ABSTRACT The effect of the selective thromboxane A₂ synthetase inhibitor OKY-1581, a pyridine derivative [sodium (E)-3-(4-(3-pyridylmethyl)phenyl)-2-methyl-2-propenoate], on thromboxane B₂ and 6-keto-prostaglandin F₁α levels and platelet aggregation was studied in human volunteers. To clarify its effectiveness as an enzyme inhibitor, OKY-1581, at doses of 17, 83, 167, 417, 833, and 1667 µg/kg (n = 5 for each group), was injected intravenously, or was infused (10 µg/kg/min; n = 5) over 3 hr on 3 successive days. OKY-1580 (OKY-1581 free acid) was rapidly converted to its main β-oxidized product, OKY-1565, and its reduced form, OKY-1558. During the study, plasma thromboxane B₂ levels, inhibition of thromboxane B₂ production in serum, and inhibition of rabbit platelet thromboxane A₂ synthetase were monitored continuously. Twenty-five minutes after the injection of the above doses, plasma thromboxane B₂ levels decreased by 4 ± 7%, 40 ± 14%, 57 ± 7%, 68 ± 6%, 93 ± 5%, and 96 ± 5% (mean ± SD), respectively. Thromboxane B₂ production in serum was decreased by 2 ± 8%, 70 ± 10%, 75 ± 8%, 81 ± 10%, 95 ± 10%, and 96 ± 8%, respectively, and rabbit platelet thromboxane A₂ synthetase by 2 ± 7%, 52 ± 8%, 79 ± 10%, 80 ± 9%, 96 ± 8%, and 95 ± 7%. These parameters returned to the control levels 24 hr after the injection. During infusion of OKY-1581 at a rate of 10 µg/kg/min for 3 hr, plasma thromboxane B₂ levels decreased significantly, and inhibition of thromboxane B₂ production in serum and of rabbit platelet thromboxane A₂ synthetase was also significant. Intravenous infusion of this drug reduced platelet aggregation induced by arachidonate (2 mM) significantly. In serum of incubated whole blood, after the treatment with OKY-1581, serum 6-keto-prostaglandin F₁α production was increased significantly. OKY-1581 caused no untoward symptoms or changes in hemodynamic parameters or electrocardiographic or laboratory results, including those for bleeding time and coagulation. In cardiovascular diseases in which thromboxane A₂ may be involved in the pathogenesis, this selective inhibitor of thromboxane A₂ synthetase may become a useful drug because it inhibits thromboxane A₂ production and arachidonate-induced platelet aggregation.


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THROMBOXANE A₂ is produced in platelets from arachidonate and is a potent platelet aggregator and vasoconstrictor. An imbalance between thromboxane A₂ and prostacyclin, which is generated in the vascular endothelium, or excessive production of thromboxane A₂ is suspected of playing a role in the pathogenesis of thromboembolic and cardiovascular diseases. In view of such a potential pathologic role of thromboxane A₂, the selective inhibition of thromboxane A₂ synthetase, which would reduce thromboxane A₂ production in patients and might have the additional bene-
favorable effect of making more endoperoxides available for conversion to prostacyclin, is desirable. This potential benefit cannot be obtained with such antiplatelet drugs as aspirin, which primarily inhibits cyclooxygenase. Therefore, inhibition of thromboxane \( A_2 \) synthetase seems to be an attractive new approach to the control of thromboembolic disease. This report evaluates the effect of a selective inhibitor of thromboxane \( A_2 \) synthetase in human volunteers.

Materials and methods

Experimental subjects. Normal healthy men, 25 to 40 years old and weighing 50 to 60 kg, gave informed consent for this study. Before OKY-1581 was administered, each patient was screened for medical illness; a history and biochemical and hematologic profile were obtained and each patient underwent physical, chest x-ray, and electrocardiographic examinations. Subjects with any clinically relevant disease or laboratory test abnormality were excluded. Subjects were requested to abstain from all drugs and were admitted to Kyoto University Hospital the night before the study. They were fed fat-free diets during the study to minimize lipid interference with platelet aggregation studies; two 21-gauge butterfly needles, one for drug administration and the other for blood sampling, fitted with three-way stopcocks were inserted into the veins of both forearms 1 hr before drug administration to eliminate the need for frequent venipuncture. These systems were kept patent with 0.6 ml of heparinized saline (100 U/ml).

Drug. OKY-1581 was supplied by Ono Pharmaceutical Company (Osaka, Japan). Continuous infusion was delivered by means of an infusion pump (Nipro Company; Osaka, Japan). The diluent was normal saline. Drug solutions, prepared each day of administration, were stable for at least 24 hr at room temperature. The structure of OKY-1581, sodium (E)-3-[4-(3-pyridylmethyl)phenyl]-2-methyl-2-propenoate, is illustrated in figure 1. OKY-1581 was injected intravenously (\( \mu g/kg \)) or was infused for 3 hr on 3 successive days (10 \( \mu g/kg/min \)).

Clinical tests. Vital signs and electrocardiograms were recorded by nurses at frequent intervals. Biochemical and hematologic examinations were performed at both the initiation and termination of the study.

Platelet aggregation tests. At scheduled times during the study, platelet-rich plasma (PRP) was prepared from whole blood (3.15 ml) anticoagulated with 3.8% citrate (0.35 ml). Samples were centrifuged in plastic tubes successively at 300 and 2000 g for 10 min to collect, respectively, PRP and platelet-poor plasma (PPP). PRP was diluted with PPP as needed to give a final count of 300,000/mm\(^3\).

Aggregation was induced in 0.24 ml aliquots of PRP by the addition of 0.02 ml of arachidonate (2 mM). The light transmission of PRP and that of PPP were taken as 0 and 100%, respectively. The values (%) were determined at 5 min.

\[ \text{Sodium (E)-3-[4-(3-pyridylmethyl)-phenyl]-2-methyl propenoate} \]

\( \text{Mol. wt. 275.28} \)

FIGURE 1. Structure of OKY-1581.

**Determination of plasma thromboxane \( B_2 \) level.** Plasma thromboxane \( B_2 \) level was determined by radioimmunoassay, and the purification before radioimmunoassay was done with a reverse-phase column. Blood was drawn from an antecubital vein into tubes containing 1 mM of EDTA and 0.1 mM of indomethacin. Samples were centrifuged at 1500 g for 20 min, and plasma was frozen and kept at \(-80^\circ C\) until assay. Anti-thromboxane \( B_2 \) antiserum and \(^{3}H\)-thromboxane \( B_2 \) (100 to 150 Ci/mmol) were obtained from New England Nuclear Corporation. Authentic thromboxane \( B_2 \) was obtained from Ono Pharmaceutical Company.

After the preconditioning of a Sep-Pak column (Waters C-18 reverse-phase column) with 10 ml of distilled water and 20 ml of ethanol, 1.0 ml of EDTA-indomethacin-treated plasma acidified by 100 \( \mu l \) of 2N HCl was applied to the column. Step-wise elution was performed by the following procedures: washing with 5 ml of distilled water, washing with 5 ml of 15% ethanol, washing with 5 ml of petroleum ether, and final elution with 5 ml of ethyl acetate. The total recovery rate when \(^{3}H\)-thromboxane \( B_2 \) was used was 95%. The final eluant was centrifuge-evaporated to dryness at 50°C and reconstituted in 150 \( \mu l \) of 0.1M phosphate-buffered saline (0.9%), pH 7.4, with gelatin (0.1%); 100 \( \mu l \) of this solution was incubated with antiserum and tritiated thromboxane \( B_2 \). The final assay volume was 0.5 ml. The mixture was incubated for 16 hr at 4°C. Antibody-bound thromboxane \( B_2 \) was separated from the unbound compound with 0.5 ml of dextran-coated charcoal (mixture of 3.75 mg of dextran and 37.5 mg of charcoal per milliliter) by centrifugation at 1000 g for 10 min, and the amount of antibody-bound thromboxane \( B_2 \) in the supernatant was determined. Validation of this assay was by dilution and recovery studies. All assays had an 80% recovery. The sensitivity of the assay was 10 pg/ml plasma. The cross-reactivity of this antibody was as follows: thromboxane \( B_2 \) 100%, prostaglandin \( D_2 \) 10%, prostaglandin \( E_2 \) 0.2%, prostaglandin \( A_2 \) 0.2%, prostaglandin \( F_2 \) 0.2%, and 6-keto-prostaglandin \( F_1 \) 0.2%. When \(^{3}H\)-prostaglandin \( D_2 \) was used, 95% of prostaglandin \( D_2 \) added to plasma was found to be excluded by the above sample purification. The ability of the assay to detect known amounts of thromboxane \( B_2 \) is shown in figure 2. Plasma thromboxane \( B_2 \) concentrations were expressed in picograms per milliliter. In our laboratory, this method has a coefficient of variation of 5.3%. The normal value was 90 ± 25 pg/ml (\( n = 50 \)).

**Inhibition of thromboxane \( B_2 \) production in serum.**

\[ Y=0.80X+74.7 \]

\[ Y=0.80X \]

FIGURE 2. Radioimmunoassay of plasma thromboxane \( B_2 \). The horizontal axis represents the amount of thromboxane \( B_2 \) added to plasma and the vertical axis the thromboxane \( B_2 \) measured by radioimmunoassay. The solid line is the regression line (\( r = .96 \)), and the dotted line shows the regression line when a known amount of thromboxane \( B_2 \) was added to normal saline instead of to plasma (\( r = .98 \)).

CIRCULATION
rum thromboxane B₂ production levels were measured in serum derived from 1 ml of whole blood allowed to clot at 37°C for 1 hr in plain glass tubes (ng/3 × 10⁸ platelets).[4] The details of the radioimmunoassay were the same as those of the assay used for determination of plasma thromboxane B₂ levels. The effect of plasma containing OKY-1581 and its metabolites on the inhibition of formation of thromboxane B₂ in vitro was expressed as percentage inhibition. The normal value of serum thromboxane B₂ production levels was 68 ± 25 ng/3 × 10⁸ platelets (n = 50).

6-Keto-prostaglandin F₁α production in serum. Levels of serum 6-keto-prostaglandin F₁α production were obtained in serum derived from 1 ml of whole blood allowed to clot at 37°C for 1 hr in plain glass tubes (ng/ml). During purification on the reverse-phase column before radioimmunoassay, 5% ethanol was used instead of 15% ethanol in this assay. 6-Keto-prostaglandin F₁α antisera was purchased from Cappel Laboratories. [³H]-6-Keto-prostaglandin F₁α (120 to 180 Ci/mmol) was obtained from New England Nuclear Corporation. Authentic 6-keto-prostaglandin F₁α was supplied by Ono Pharmaceutical Company. The cross-reactivity of this antibody was a follows: 6-keto-prostaglandin F₁α 100%; prostaglandin A₁, A₂, B₁, DHF₁α, DHKE₂α, D₂, and thromboxane B₂ less than 0.01%; prostaglandin E₁ 0.1%; prostaglandin E₂ 1.2%; prostaglandin F₂α 1.9%; and prostaglandin F₂α 2.6%. The normal value was 0.5 ± 0.13 ng/ml (n = 20).

Inhibition of rabbit platelet thromboxane A₂ synthetase. [1-¹⁴C]-Arachidonic acid (40 to 60 mCi/mmole) was purchased from New England Nuclear Corporation. Sheep vesicular gland microsomes were obtained from Ran Biochemicals (Tel Aviv, Israel). [1-¹³C]-Prostaglandin H₂ was prepared by the method of Yoshimoto et al.[5] Fresh citrated rabbit blood was collected through a polyethylene tube placed in the carotid artery and centrifuged at 200 g for 10 min. The PRP was removed and centrifuged at 2000 g for 20 min. The pellets were suspended in cold 0.1M Tris HCl, pH 8.0 (4 × 10⁹/ml washed platelets). Plasma samples containing OKY-1581 and its metabolites were added to the washed rabbit platelets to measure inhibition. For the formation of thromboxane B₂, the washed platelets (4 × 10⁹) were incubated with 50 mM L-[¹⁴C]-prostaglandin H₂ (5 × 10⁶ cpm) at 24°C for 1 min in 0.1M potassium phosphate, pH 7.4 (0.1 ml). Termination of the reaction and extraction of the radioactive materials was performed by previously described methods.[6] Thin-layer chromatography was carried out in chloroform/ethyl acetate/methanol/acetic acid/water (70:30:8:1:0.5). The radioactive zones were located by autoradiography and quantitated by a standard liquid scintillation counting procedure. The effect on thromboxane B₂ generation (cpm) was expressed as percentage inhibition.

Determination of plasma concentration of OKY-1581 and its metabolites. An internal standard, OKY-1630, (E)-3-(4-(4-methyl-3-pyridylmethyl) phenyl)-2-methyl-2-propenoic acid (500 mg), was added to 1 ml of plasma and acidified by 1N HCl to pH 3.0. Samples were transferred to an Amberlite XAD-2 column. The eluent was evaporated to dryness by methanol. After the addition of 0.1N HCl and ethyl acetate (1/1, vol/vol), the extract was treated with diazomethane and evaporated to dryness. The methyl esters of OKY-1581 and its metabolites in methanol were analyzed by gas chromatography—mass spectrometry. A JEOV 20 KP gas chromatograph and JMSD-100 mass spectrometer (Nippon Denshi Company, Japan) were used. The column was 2 mm × 1.5 m inner diameter, of 3% OV-17, and operated at 235°C. The helium pressure was 1.6 kg/cm². The electron energy was 20 eV and the ion source temperature was 270°C.

Thromboxane A₂. Thromboxane A₂ was generated by incubating 1 μg of prostaglandin H₂ in aceton with 410 μg horse platelet microsomes suspended in 0.1 ml of 50 mM Tris HCl buffer at pH 7.5. All microsome incubations were for 2 min at 0°C. The entire 20 μl reaction mixture was applied to a cuvette for platelet aggregation. The composition of platelet aggregation for the analysis of interaction between OKY-1581 and thromboxane A₂ was as follows: 200 μl of PRP, 20 μl of thromboxane A₂, and 20 μl of OKY-1581 or vehicle.

Statistics. Values are presented as mean ± SD. Student-Newman-Keuls’ multiple comparison test with one-way analysis of variance was used. A p value less than .05 was considered to indicate significance.

Results

Figure 3 shows dose-response curves of OKY-1581 for inhibition of plasma thromboxane B₂, inhibition of thromboxane B₂ production in serum, and inhibition of rabbit thromboxane A₂ synthetase. Values were obtained 25 min after the injection of drug. These data show dose-related changes in these three parameters. OKY-1580, the free acid form of OKY-1581, was transformed in plasma into OKY-1558, 3-[4-(4-pyridylmethyl)phenyl]-2-methyl-propionic acid, by reduction, or into OKY-1565, 4-(3-pyridylmethyl) benzoic acid, by β-oxidation in plasma.

Figure 4 shows the metabolic fate of the three compounds after the injection of 417 μg/kg. Two hours after injection, OKY-1580 disappeared, and OKY-1565 appeared at 4.8 min and persisted for 24 hr. OKY-1558 was detectable for only 1 hr.

The effectiveness of OKY-1581 as a thromboxane A₂ synthetase inhibitor was tested with the following three parameters: plasma thromboxane B₂ levels (figure 5, A), inhibition of thromboxane B₂ production in serum (figure 5, B), and inhibition of rabbit platelet thromboxane A₂ synthetase (figure, 5 C). After the injection, peak effects on plasma thromboxane B₂ levels were obtained at 25 min. During the infusion of OKY-1581 at a rate of 10 μg/kg/min for 3 hr on 3 successive days (n = 5), plasma thromboxane B₂ levels decreased significantly (percentage of inhibition 2 hr after the start of the infusion: 82 ± 5%, 78 ± 4%, and 78 ± 2%), and thromboxane B₂ production in serum (91 ± 5%, 88 ± 6%, and 87 ± 5%) and rabbit platelet thromboxane A₂ synthetase (90 ± 10%, 89 ± 10%, and 88% ± 7%) were also significantly inhibited. Changes in platelet aggregation induced by arachidonate (2 mM) were as follows: 31.0 ± 28.0% vs 6.2 ± 5.1%, 42.6 ± 28.2% vs 1.7 ± 2.9%, and 39.4 ± 6.8% vs 1.8 ± 1.6%. In a placebo study, the vehicle (glycine buffer, pH 9.1) was given by a single injection or in a 3 hr infusion. Placebo had no effect on the three parameters.

During the administration of OKY-1581, arachidonate-induced platelet aggregation decreased significantly (table 1). However, when studied in vitro only,
OKY-1581 at final concentrations of more than 8.3 mg/ml suppressed the platelet aggregation induced by thromboxane A₂, which was generated by prostaglandin H₂ and platelet microsome thromboxane A₂ synthetase.

Figure 6 illustrates the effect of OKY-1581 on 6-keto-prostaglandin F₁α production in serum 1 hr after the treatment. OKY-1581 increased 6-keto-prostaglandin F₁α production in serum significantly.

There were no clinically relevant changes in heart rate or blood pressure, and no adverse symptoms or signs. Routine laboratory tests, including those for bleeding time, showed no important abnormalities.

**Discussion**

Thromboxane A₂, an extremely potent inducer of platelet aggregation and smooth muscle contraction, is produced by activated platelets. Excess formation of thromboxane A₂ is considered to play an important role in the pathophysiology of thromboembolic diseases, especially in the development of arteriosclerotic or cardiovascular diseases. To control or depress the formation of thromboxane A₂, aspirin and other non-steroidal anti-inflammatory drugs that react with cyclooxygenase may be used. However, the inhibition of cyclooxygenase activity concomitantly depresses the formation of prostacyclin. Thus, the selective inhibition of thromboxane A₂ synthetase, which catalyzes the synthesis of thromboxane A₂ from prostaglandin H₂, is considered to be an attractive new therapeutic approach. Thromboxane A₂ synthetase is inhibited by various pharmacologic agents including imidazole and its derivatives, pyridine and its derivatives, 9,11-azoprosta-5,13-dienoic acid, and 11α-carboxy-thromboxane A₂ analogue. We studied the effects of the selective inhibition of thromboxane A₂ synthesis on thromboxane A₂ formation and platelet aggregation, and tested the clinical safety of OKY-1581. Inhibition of thromboxane A₂ synthesis was determined by the following three methods: (1) Plasma thromboxane B₂ levels were determined by radioimmunoassay after purification by a reverse-phase column to eliminate substances that may nonspecifically bind to thromboxane B₂ antibody, such as albumin, neutral lipid, phospho-
l lipid, and other prostaglandin metabolites. (2) Thromboxane B2 generation in serum was inhibited by allowing whole blood to clot in response to endogenously formed thrombin. This method has sometimes been used to evaluate thromboxane B2 levels, since this compound, at nanogram concentrations, is easy to detect. However, this value shows only the ability to generate thromboxane B2 in platelets and does not represent plasma thromboxane B2 levels. (3) To confirm directly its action on platelet thromboxane A2 synthetase, we measured the effect of OKY-1581 on the conversion from prostaglandin H2 to thromboxane A2 using rabbit platelet thromboxane A2 synthetase. Administration of OKY-1581 lowered plasma thromboxane B2 levels and inhibited both thromboxane B2 production in serum and thromboxane A2 synthetase in rabbit platelets in a dose-related manner. The effect of OKY-1581 on plasma thromboxane B2 levels and its inhibition of thromboxane B2 production in serum and platelet aggregation were short-lived (figure 5 and table 1).

It is not known what changes occur in the metabolism of cyclic endoperoxides when thromboxane A2 formation is inhibited by OKY-1581. Theoretically, prostaglandin D2, E2, or I2 (prostacyclin) levels may increase. It has been reported that the reduction of thromboxane B2 formation is associated with a rise in the plasma level of 6-keto-prostaglandin F1α, a prosta-
TABLE 1
Effects of OKY-1581 on induction of platelet aggregation (%) by 2 mM arachidonate

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Values are mean ± SD.
^p < .05 vs control value. The light transmission of PRP and that of PPP were taken as 0 and 100%, respectively (n = 5, for each dose).

cyclin metabolite. However, the levels and even the existence of 6-keto-prostaglandin F₆₀ in plasma are now being questioned. Figure 6 shows that the selective inhibition of thromboxane B₂ formation concomitantly redirected platelet endoperoxides into 6-keto-prostaglandin F₆₀ production in serum. This increased production of serum 6-keto-prostaglandin F₆₀ could occur in monocytes. However, treatment with aspirin, a cyclooxygenase inhibitor, would inhibit both thromboxane B₂ formation and 6-keto-prostaglandin F₆₀ production in serum.

After injection, OKY-1581 is rapidly converted to OKY-1565 or 1558. The 50% inhibitory concentrations for these three substances in the purified thromboxane B₂ synthetase system are 6, 260, and 27 nM, respectively.

In our studies in vivo, arachidonate-induced platelet aggregation was inhibited by OKY-1581, although in vitro, OKY-1581 (8.3 mg/ml) antagonized thromboxane A₂-induced platelet aggregation. Thus, high concentrations of OKY-1581 may have a dual action: inhibition of thromboxane A₂ formation and antagonism of platelet aggregation caused by thromboxane A₂. This dual action of thromboxane A₂ synthetase has been reported previously. The mechanism of the inhibition of platelet aggregation is unknown; OKY-1581 may cause irreversible damage in platelets, but this antiaggregatory dose of OKY-1581 results in levels nowhere near those obtained in vivo.

In the treatment of patients in whom thromboxane A₂ might be involved in the pathophysiology of cardiovascular disease, the inhibition of thromboxane A₂ synthetase may become a useful method. However, in clinical use, we must be aware of the adverse effects that may arise with the long-term inhibition of thromboxane A₂ synthetase.

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References
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FIGURE 6. Effect of OKY-1581 on 6-keto-prostaglandin F₆₀ production in serum. (p < .001 vs control values). C = control; n = 5, each group.


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