The Effect of a Thromboxane Synthetase Inhibitor, OKY-046, On Urinary Excretion of Immunoactive Thromboxane B2 and 6-Keto-Prostaglandin F1α in Patients With Ischemic Cerebrovascular Disease

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SUMMARY Thromboxane synthetase activity is selectively inhibited by (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046). A single dose of 100 mg OKY-046 was orally administered to patients with ischemic cerebrovascular disease and healthy volunteers. Platelet aggregation and thromboxane B2 (TXB2) generation of intact and homogenized platelets induced by 1.0 mM sodium arachidonate were measured before and at 1, 4, 6, and 8 h after dosing. OKY-046 inhibited arachidonate-induced aggregation in platelet rich plasma from some, but not all, individuals, whereas platelet TXB2 generation was almost completely inhibited by a single dose of 100 mg OKY-046, in all of the patients and healthy volunteers. Endogenous TXA2 and prostacyclin (PGI2) biosynthesis were assessed by measurement of urinary immunoreactive TXB2 (i-TXB2) and 6-keto-PGF1α (i-6-keto-PGF1α) before and at 0–3, 3–6, 6–9 h after dosing. OKY-046 increased the urinary i-6-keto-PGF1α coincidently with a decrease of urinary i-TXB2, both in patients and healthy volunteers. These effects of a selective thromboxane synthetase inhibitor will improve a disturbed balance between TXA2 and PGI2, associated with the development of ischemic cerebrovascular disease.

THROMBOXANE A2 (TXA2) generated by platelets is not only a proaggregatory but also a potent vasoconstrictory agent. On the other hand, prostacyclin (PGI2) generated in blood vessels is a potent vasodilator and antiaggregatory agent. Aspirin has a marked effect on platelet function by inhibiting cyclooxygenase of platelets, whereas cyclooxygenase inhibitors such as aspirin coincidently result in a reduction of PGI2 generation in blood vessels. In view of the potential importance of these compounds in thrombogenic disorders such as ischemic cerebrovascular disease and myocardial infarction, several studies have been performed to find a dose of aspirin that would selectively block platelet cyclooxygenase. Studies with laboratory animals and humans have failed to dissociate the effects of single doses of aspirin on platelets and vascular cells. Therefore, selective inhibition of thromboxane synthetase is an attractive way to resolve the "aspirin dilemma."

Selective thromboxane synthetase inhibitors inhibit platelet aggregation and preserve the capacity to generate PGI2. Furthermore, they will lead to a redirection of prostaglandin endoperoxides from TXA2 production to PGI2 production. A selective thromboxane synthetase inhibitor, dazoxiben, has been reported to increase PGI2 biosynthesis coincidently with inhibiting TXA2 generation of platelets in healthy human volunteers.

The purpose of this investigation was to determine the effect of a selective thromboxane synthetase inhibitor, (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride monohydrate (OKY-046), on endogenous TXA2 and PGI2 biosynthesis in diseases with abnormal platelet-vascular homeostasis such as ischemic cerebrovascular disease. Since both TXA2 and PGI2 are rapidly inactivated to more stable substances, TXB2 and 6-keto-PGF1α respectively, endogenous TXA2 and PGI2 biosynthesis were assessed by measurement of the urinary excretion of immunoreactive TXB2 (i-TXB2) and 6-keto-PGF1α (i-6-keto-PGF1α).

Patients and Methods

Subjects and Samples

Five patients with ischemic cerebrovascular disease (4 male, 1 female; aged 54–63 yr; wt 48–65 Kg) and 2 healthy human volunteers (2 male; aged 30–32 yr; wt 62–67 Kg) participated in the study. All patients had a diagnosis of mild or moderate cerebral thrombotic infarction in a chronic stage, according to clinical history, neurological examination and computerized tomography. None of them had received drugs known to interfere with platelet function and prostaglandin metabolism. Informed consent was obtained from each subject prior to the initiation of the study in accordance with the Helsinki Declaration of 1975. The subjects fasted overnight and received 100 mg OKY-046. Urine was collected for i-TXB2 and i-6-keto-PGF1α determination for 3 h before dosing and for 0–3, 3–6, and 6–9 h postdosing, and frozen immediately at −20°C until assayed. It was cleared by centrifugation at 4°C preceding extraction. Blood was collected in sodium citrate solution (3.8%, 1 part + 9 parts of blood) for the measurement of platelet aggregation and
thromboxane generation before dosing and at 1, 4, 6, and 8 h postdosing.

Platelet Function Studies

Platelet rich plasma (PRP) was prepared by centrifugation of the citrated blood at 200 × g for 10 min at room temperature. The remaining sample was centrifuged further at 1400 × g for 30 min to yield platelet poor plasma (PPP). Platelets in PRP were counted with a phase contrast microscope and the platelet count in PRP was adjusted to 250,000/μl with PPP. Aggregation responses induced by 1 mM sodium arachidonate were measured at 37°C within 2 hrs of blood collection using an aggregometer (Rikadenki, Japan) to record percentage of change in light transmission of PRP (set at 0 %) relative to PPP (set at 100%). The maximal rate of aggregation was estimated from the steepest part of the curve. Homogenates of PRP were obtained by repeated (3 times) freezing in dry ice-acetone and thawing. The capacity of the intact and homogenised platelets for TXB₂ generation was determined by measuring the amount of TXB₂ that was produced when the intact and homogenised PRP was stirred with 1.0 mM sodium arachidonate at 37°C for 5 min. Two hundred μl of the incubated PRP was then immediately mixed with 800 μl of cold phosphate buffer, containing 10⁻⁴ M indomethacin, and frozen in dry ice-acetone. TXB₂ was subsequently measured by a radioimmunoassay technique and the results were expressed as ng/5 min/2.5 × 10⁷ platelets.

Urinary Excretion of i-TXB₂ and i-6-keto-PGF₁α

Urinary i-TXB₂ was measured by a modification of our previously reported radioimmunoassay method, using ethyl acetate extraction and a silica extraction column (Bond-Elut Si Extraction Column, 500 mg, Analytichem International). To 3 ml of urine, about 3,000 dpm of ³H-TXB₂ was added for recovery estimation. The samples were then acidified to pH 3 with 2 M citric acid and extracted twice with 3 ml ethyl acetate. The organic layer was collected and dried by evaporation under vacuum at 40°C. Separation of TXB₂ was performed according to the modified Jaffe’s method. The dry residue was reconstituted in 0.2 ml solvent 3 (benzene:ethyl acetate:methanol, 60:40:10) and 0.8 ml of solvent 1 (benzene:ethyl acetate, 60:40) and applied to a silica column that had been prewashed with 5 ml of solvent 4 (benzene:ethyl acetate:methanol, 60:40:20) and 1.5 ml of solvent 1. The column was eluted serially with 6 ml of solvent 1, 6 ml of solvent 2 (benzene:ethyl acetate:methanol, 60:40:2) and 16 ml of solvent 2. The last fraction was dried by evaporation under vacuum at 40°C and was reconstituted in the radioimmunoassay buffer. Although the cross-reactivity with 2,3-dinor-TXB₂ was 40%, this system completely isolated TXB₂ from 2,3-dinor-TXB₂. The overall recovery of ³H-TXB₂ ranged between 60–70%. Addition of known amounts of TXB₂ to urine yielded an excellent correlation of measured and predicted values (r = 0.999). Preliminary studies demonstrated that the concentration of TXB₂ was unaltered by urine volume, similar to the observations of others. For this reason, urinary i-TXB₂ was expressed as units of concentration (pg/ml).

Urinary i-6-keto-PGF₁α was measured by a modification of our previously reported radioimmunoassay method. Three ml of urine was extracted and applied to a silica column as described above for TXB₂. The column was eluted serially with 6 ml of solvent 1, 10 ml of solvent 2, and 2 ml of solvent 4. The last fraction was dried by evaporation and was reconstituted in the radioimmunoassay buffer. The overall recovery of ³H-6-keto-PGF₁α ranged between 70–80%. Addition of known amounts of 6-keto-PGF₁α to urine yielded an excellent correlation of measured and predicted values (r = 0.999). Urinary i-6-keto-PGF₁α was expressed as units of pg/mg creatinine.

Statistics

All results are reported as the mean ± SEM. Statistical analysis was assessed by paired Student’s t test.

Results

Platelet Aggregation and TXB₂ Generation

The effect of OKY-046 (100 mg) on the platelet aggregation and concomitant TXB₂ generation in 5 patients with ischemic cerebrovascular disease is shown in table 1. The irreversible platelet aggregation that always occurred when 1.0 mM sodium arachidonate was added to PRP was inhibited by OKY-046 in PRP from 4 of 5 patients, while OKY-046 had no effect on the aggregation in PRP from 1 of 5 patients. On the other hand, the peak inhibition of the concomitant TXB₂ generation did not differ among all of the patients.

The amount of TXB₂ generated by homogenised PRP before and after 5 patients with ischemic cerebrovascular disease ingested 100 mg OKY-046, is shown in figure 1. TXB₂ generation fell significantly (p < 0.001) from a predosing value of 23.7 ± 2.3 to 0.9 ± 0.3 at 1 h, 1.8 ± 0.6 at 4 h, 3.5 ± 1.2 at 6 h, and 5.4 ± 1.7 ng/5 min/2.5 × 10⁷ platelets at 8 h after administration of OKY-046. Both the peak inhibition and duration of inhibition of TXB₂ generation did not differ from those of two healthy volunteers.

Urinary Excretion of i-TXB₂ and i-6-keto-PGF₁α

The effect of OKY-046 on urinary i-TXB₂ and i-6-keto-PGF₁α in 5 patients with ischemic cerebrovascular disease is shown in figure 2. OKY-046 caused a statistically significant (p < 0.01) reduction of urinary i-TXB₂ concentrations at 0–3 and 3–6 h after dosing. Urinary i-TXB₂ averaged 132 ± 41 and 123 ± 21 pg/ml in the 0–3 and 3–6 h urines as compared with 231 ± 36 pg/ml before dosing. Although it was not statistically significant, there was a similar trend for the 6–9 h urines (129 ± 21 pg/ml; n = 4). OKY-046 resulted in a modest but significant increase (p < 0.05) in urinary excretion of i-6-keto-PGF₁α at 0–3 and 3–6 h after dosing. Urinary i-6-keto-PGF₁α rose from 260 ± 55 pg/mg creatinine before dosing to 390 ± 98 and 425 ± 79 pg/mg creatinine in the 0–3 and 3–6 h urines.
The average percent aggregation and concomitant TXB \(_2\) generation obtained for 1.0 mM sodium arachidonate before and after drug treatment are presented. *\(p < 0.01\), †\(p < 0.001\) relative to predosing values.

Though not statistically significant, there was a similar trend for the 6–9 h urines (425 ± 103 pg/mg creatinine; \(n = 4\)). Both the reduction of urinary i-TXB \(_2\) concentrations and the increment of urinary i-6-keto-PGF\(_{1\alpha}\) excretion did not differ from those of two healthy volunteers.

**Discussion**

The purpose of the present study has been to determine the effect of a selective thromboxane synthetase inhibitor on the metabolism of prostaglandin endoperoxides in patients with ischemic thrombotic diseases. To that end, it is necessary to investigate platelet TXB\(_2\) generation and the biosynthesis of TXA\(_2\) and PGI\(_2\).

We quantitated the effect of OKY-046 on platelet TXB\(_2\) generation *ex vivo*. Minno et al\(^{14}\) have reported that it is impossible to determine the exact recovery of platelet cyclooxygenase after administration of aspirin by employing a single aggregating agent or measuring serum TXB\(_2\) levels. We also observed that the amount of TXB\(_2\), generated by intact platelets at 6 or 8 h after administration of OKY-046, depended on the degree of aggregation responses induced by 1.0 mM sodium arachidonate. From these reasons, the recovery of platelet thromboxane synthetase activity after administration of OKY-046 was determined by adding 1 mM sodium arachidonate to the homogenised PRP. Our results indicate that a single dose of 100 mg OKY-046 inhibits platelet thromboxane synthetase activity by 99% during the first hour and by 76% after 8 h.

We determined the effect of OKY-046 on endogenous TXA\(_2\) and PGI\(_2\) biosynthesis by measuring urinary i-TXB\(_2\) and i-6-keto-PGF\(_{1\alpha}\). There are several unresolved issues about the origin of urinary TXB\(_2\) and 6-keto-PGF\(_{1\alpha}\). Urinary TXB\(_2\) and 6-keto-PGF\(_{1\alpha}\) may arise both from glomerular filtration of plasma\(^{15,16}\) and renal source.\(^{17,18}\) The increase in i-6-keto-PGF\(_{1\alpha}\) as measured might include cross-reacting metabolites of PGI\(_2\), such as 2,3-dinor-6-keto-PGF\(_{1\alpha}\), which is one of the major urinary metabolite of PGI\(_2\),\(^{9}\) because we could not separate 6-keto-PGF\(_{1\alpha}\) from 2,3-dinor-6-keto-PGF\(_{1\alpha}\). Therefore, the reduction of urinary i-TXB\(_2\) and the augmentation of urinary i-6-keto-PGF\(_{1\alpha}\) excretion may have resulted from changes in the plasma concentration and renal synthesis. Because we did not measure 6-keto-PGF\(_{1\alpha}\) and TXB\(_2\) in blood, the source of the products measured here must remain speculative.

Our present results indicate that 100 mg of OKY-046 decreases TXA\(_2\) biosynthesis and increases PGI\(_2\) biosynthesis. The increment of PGI\(_2\) may result from
endothelial "steal" of platelet prostaglandin endoperoxides and endoperoxides "redirection" in tissues that can synthesize both PG\(_I_2\) and TXA\(_2\), such as blood vessels, lung and kidney. Urinary i-TXB\(_2\), concentrations fell to only 50% of control values on 100 mg of OKY-046. When we consider that OKY-046 almost completely inhibited TXB\(_2\) generation by platelets \textit{ex vivo}, the most probable interpretation of these data is that extraplatelet sources less sensitive to OKY-046 contribute to urinary i-TXB\(_2\) excretion.\(^9\) The exact reasons remain to be cleared. The increase of PG\(_I_2\) biosynthesis by 100 mg of OKY-046 was certainly modest and such an increment is unlikely to result in plasma concentrations consistent with PG\(_I_2\) functioning as a circulating hormone \textit{in vivo}. However, it may inhibit platelet aggregation at local sites of interaction with endothelium. Although it has been reported that platelet aggregation induced by collagen was unaltered \textit{ex vivo} by a thromboxane synthetase inhibitor, dazoxiben,\(^8\) this increment of PG\(_I_2\) biosynthesis can be expected to increase the effect of OKY-046 on platelet aggregation \textit{in vivo}.

Our results indicate that a thromboxane synthetase inhibitor, OKY-046, increases PG\(_I_2\) biosynthesis co incidently with inhibition of TXA\(_2\) biosynthesis in patients with ischemic cerebrovascular disease.

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