Pharmacological Studies on the TXA₂ Synthetase Inhibitor (*E*)-3-[*p*-(1*H*-Imidazol-1-ylmethyl)Phenyl]-2-Propenoic Acid (OKY-046)

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Accepted April 10, 1986

Abstract—The effects of (*E*)-3-[*p*-(1*H*-imidazol-1-ylmethyl)phenyl]-2-propenoic acid (OKY-046) on thromboxane A₂ (TXA₂) synthetase in vitro and on experimental animal models of sudden death and cerebral infarction were studied. IC₅₀ values of OKY-046 for the TXA₂ synthetase of human, rabbit, dog and guinea pig washed platelets were 0.004, 0.004, 0.26 and 2.4 μ M, respectively. OKY-046 at concentrations up to 1 mM, however, did not inhibit prostacyclin (PGI₂) synthetase from bovine aorta microsomes or cyclooxygenase and PGE₂ isomerase from sheep seminal vesicle microsomes. Similarly, platelet 12-lipoxygenase was not affected by OKY-046. Evidence for a re-direction of arachidonate metabolism from thromboxane synthesis toward PGI₂ synthesis was obtained using rat peritoneal cells. Namely, OKY-046 increased PGI₂ production accompanied by an inhibition of TXA₂ production at a concentration of more than 1 μ M. OKY-046 at a dose of 0.1 mg/kg (i.v.) in dogs inhibited the aortic and mesenteric arterial contraction of rabbit induced by the addition of arachidonate to extracorporated blood of the dogs. OKY-046 at a dose of 0.3 mg/kg (i.v.) prevented the arachidonate-induced sudden death and also decreased the incidence of cerebral infarction induced by injection of arachidonate into the internal carotid artery in rabbits. Aspirin also decreased the incidence of cerebral infarction at a dose of 30 mg/kg (i.v.). These results suggest that OKY-046 may be valuable for the treatment of cerebrovascular and cardiovascular diseases associated with vasoconstriction and thrombosis due to TXA₂.

Thromboxane A₂ (TXA₂), which was discovered by Hamberg et al. in 1975 (1), is a highly unstable but biologically active substance and was shown to be biosynthesized from prostaglandin endoperoxides. TXA₂ is mainly produced by platelets and induces not only a potent platelet aggregation, but also blood vessel contraction. TXA₂ synthetase is localized in the microsomal fractions of various cells, and this enzyme has been partially purified from microsomes of blood platelet (2, 3) and lung (4, 5). Recently, Ullrich and Haurando (6) purified TXA₂ synthetase in human blood

platelet by affinity chromatography and demonstrated this enzyme to be identical with a cytochrome P-450. Prostacyclin (PGI₂) was discovered by Moncada et al. in 1976 (7) and exhibits quite opposite physiological activities to TXA₂. Recently, it has been suggested that the balance of production of TXA₂ and PGI₂ is important for the maintenance of homeostasis in the cardiovascular system and an imbalance toward the overproduction of TXA₂ leads to various diseases such as angina (8) and myocardial infarction (9). Accordingly, selective inhibitors of TXA₂ synthetase (10–13) or specific antagonists

Fig. 1. Chemical structure of OKY-046.

of TXA₂ (14, 15) have been extensively investigated. Naito et al. (16) have already reported that OKY-046 (Fig. 1) inhibits platelet aggregation and production of TXA₂ during aggregation of rabbit platelets *in vitro*. We have examined the effect of the TXA₂ synthetase inhibitor OKY-046 on enzymes of the arachidonic acid cascade, on arachidonic acid metabolism in platelets of various animals and on experimental animal models of arachidonate-induced sudden death and cerebral infarction.

Materials and Methods

Animals

Male Sprague-Dawley rats (400–500 g), male Hartley guinea pigs (450–500 g), male and female mongrel dogs (10–13 kg) and male Japanese white rabbits (2–2.5 kg) were used.

Reagents

OKY-046 (sodium salt or hydrochloride salt) was synthesized in our laboratory. PGE₂, PGF_{2α}, PGD₂, TXB₂ and 6-keto-PGF_{1α} were synthesized by the Ono Pharmaceutical Co., Ltd. [1-¹⁴C] arachidonic acid (47 mCi/mmol) was purchased from New England Nuclear (U.S.A.). Sheep seminal vesicle microsomes were purchased from Ran Biochemicals (Israel). [1-¹⁴C]PGH₂ was prepared from [1-¹⁴C] arachidonic acid using sheep seminal vesicle microsomes by the method of Yoshimoto et al. (2). 15-HETE was prepared by incubating arachidonic acid with soybean lipoxygenase followed by reduction with sodium borohydride. Other materials were obtained from the following sources: atropine sulfate (Takeda Chemical Ind., Ltd., Japan), methysergide bimalate (Sandoz A.G., Switzerland), phenoxybenzamine hydrochloride (Tokyo Kasei Kogyo Co., Ltd., Japan), propranolol hydrochloride (Wako Pure Chemical Ind., Ltd., Japan), mepyramine maleate (May & Baker Ltd., England), hemoglobin, glutathione, L-tryptophan,

sodium arachidonate, soybean lipoxygenase, indomethacin (Sigma Chemical Company, U.S.A.) and aspirin (Venopirin® Green Cross Corp., Japan).

Preparation of enzymes

TXA₂ synthetase of blood platelets was prepared as follows: Human, rabbit and guinea pig blood was collected in sodium citrate solution (1 volume of 3.8% sodium citrate+9 volume of blood). Dog blood was collected in the presence of heparin (3 U/ml). In the case of guinea pigs and dogs, the collection of blood was carried out under anesthesia induced by ether and sodium pentobarbital, respectively. The collected blood was centrifuged at 300×g for 10 min. The platelet-rich plasma was obtained and re-centrifuged at 1,800×g for 10 min. The platelets obtained were washed twice with saline containing 10 mM EDTA·2Na. The washed platelets were suspended in 0.01 M phosphate buffer (pH 7.4) and homogenized.

TXA₂ synthetase of rabbit and guinea pig lung and PGI₂ synthetase of bovine aorta were prepared as follows: Each tissue or organ was homogenized in three or four volumes of 0.01 M phosphate buffer (pH 7.4) and centrifuged at 10,000×g for 30 min. The supernatant was centrifuged at 100,000×g for 60 min, and the microsomal fraction was homogenized after suspending in buffer of the same composition.

Preparation of rat peritoneal cells

The peritoneal cavity of rats was washed with Tyrode-heparin (5 U/ml) solution, and the peritoneal cells were collected by centrifuging the washed solution at 450×g for 5 min. The contaminating erythrocytes in the collected peritoneal cells were removed by decreasing the ion strength. The peritoneal cells were suspended in Tyrode's solution (10⁷ cells/ml).

Enzyme assays

TXA₂ synthetase and PGI₂ synthetase: Five nmol of [1-¹⁴C]PGH₂ (5×10⁴ cpm) and platelet homogenate (TXA₂ synthetase) or bovine aorta microsomes (PGI₂ synthetase) were incubated in 100 μl of 0.1 M phosphate buffer (pH 7.4) at 24°C for 2 min, and the formation of TXB₂ (stable metabolite of TXA₂) or 6-keto-PGF_{1α} (stable metabolite of PGI₂) was measured, respectively. The

yields of reaction products were estimated according to the methods of Miyamoto et al. (17) for TXB₂ and Watanabe et al. (18) for 6-keto-PGF_{1α}. The conversion rates of PGH₂ to TXB₂ or 6-keto-PGF_{1α} were estimated as TXA₂ synthetase or PGI₂ synthetase activity, respectively.

Cyclooxygenase and PGE₂ isomerase:

Five nmol of [1-¹⁴C]arachidonic acid (1×10^5 cpm) and 0.1 mg sheep vesicular gland microsomes were incubated at 24°C for 2 min in the presence of 0.2 nmol hemoglobin, 0.5 nmol L-tryptophan and 0.5 nmol glutathione in 100 μ l of 0.1 M phosphate buffer (pH 7.4). Termination of the reaction and extraction of the products were carried out using the method of Miyamoto et al. (17). Thin layer chromatography (TLC) was carried out using the solvent system of ethylether/methanol/1 M acetic acid (9:2:0.1), and the amounts of arachidonic acid and PGE₂ separated were estimated by radioactivity. The consumption rate of arachidonic acid was estimated as cyclooxygenase activity, and the ratio of the formed PGE₂ to the consumption of arachidonic acid was estimated as PGE₂ isomerase. In order to determine the degree of inhibition, OKY-046 was preincubated at 24°C for 2 min with each enzyme.

Arachidonic acid metabolism in platelets and rat peritoneal cells

Five nmol of [1-¹⁴C]arachidonic acid (10^5 cpm) and homogenates of washed platelets (2×10^8 cells) were incubated at 24°C for 2 min in the presence of 0.2 nmol hemoglobin and 0.5 nmol L-tryptophan in 100 μ l of 0.1 M phosphate buffer (pH 7.4). The procedures for the estimation of the amount of residual arachidonic acid and the amounts of TXB₂ and 12-HETE formed were the same as those for the TXA₂ synthetase assay. 15-HETE was used as a standard compound for TLC instead of 12-HETE because the mobility of both compounds on TLC or HPLC were quite similar (19, 20).

In the case of rat peritoneal cells, 1 nmol of [1-¹⁴C]arachidonic acid (10^5 cpm) and peritoneal cells ($10^6/100$ μ l) were incubated at 24°C for 2 min. The procedures for the estimation of the amounts of various prostanoids formed were the same as those

for the PGI₂ synthetase assay.

The effect of OKY-046 was studied by preincubation with platelets or peritoneal cells for 2 min at 24°C prior to the addition of arachidonic acid.

Effect on arachidonate-induced arterial contraction

This experiment was carried out using the apparatus as shown in Fig. 2 according to the method of Vane (21). Adult mongrel dogs were anesthetized with barbital sodium (300 mg/kg, i.p.) and then heparinized (1,000 U/kg, i.v.). The rabbit thoracic aorta and mesenteric artery were isolated to prepare helical specimens, and they were superfused with dog blood pumped from the common carotid artery at a rate of 5 ml/min and then returned to the femoral vein. A solution containing 5×10^{-7} g/ml atropine, 5×10^{-7} g/ml mepyramine, 5×10^{-7} g/ml phenoxybenzamine, 10^{-7} g/ml methysergide and 10^{-6} g/ml propranolol was added continuously to assay tissues at the rate of 0.1 ml/min throughout the experiment. A hundred μ l of sodium arachidonate solution (1 mg/ml) dissolved in 0.05 M phosphate buffer (pH 7.4) was dripped into the blood just above the rabbit aorta specimen. The arachidonate-induced arterial contractions were recorded by a recticorder (Nihon Kohden

Fig. 2. Experimental apparatus in dog blood superfusion method.

RM-85) through an isometric transducer. The drug was injected into the femoral vein, and the arterial contraction was measured at ten-minute intervals. Statistic significance was evaluated by Student's *t*-test.

Sudden death

Sodium arachidonate dissolved in 0.05 M phosphate buffer (pH 7.4) was injected into the auricular vein of rabbits at a dose of 4 mg/kg to induce sudden death. The drug was injected intravenously 2 min prior to the injection of sodium arachidonate.

Cerebral infarction

Unanesthetized male rabbits were restrained in a supine position. A polyethylene tube was inserted retrogradely to the left external carotid artery. Sodium arachidonate solution (10 mg/ml) dissolved in M/15 phosphate buffer (pH 7.4) was infused at a dose of 1 mg/body/min for 30 min into the internal carotid artery without obstructing the blood flow. Twenty-four hours later, animals were sacrificed, and their brains were removed and fixed with 10% formaldehyde-saline. OKY-046 or aspirin was administered intravenously from the auricular vein 2 min or 10 min, respectively, before the initiation of sodium arachidonate infusion. The effect of the drugs was evaluated by calculating the area of infarction in the left hemisphere. The results were analyzed by the χ^2 test.

Results

Effect of OKY-046 on TXA₂ synthetase and other enzymes of the arachidonic acid cascade: OKY-046 inhibited the various TXA₂ synthetases in a dose-dependent manner as described in Fig. 3. The concentration of OKY-046 which gave 50% inhibition (IC₅₀ value) of TXA₂ synthetases from human platelets, rabbit platelets and rabbit lung were the same, 0.004 μ M. IC₅₀ values for dog platelets, guinea pig platelets and guinea pig lung enzymes were 0.26, 2.4 and 3.4 μ M, respectively. On the other hand, OKY-046 did not inhibit PGI₂ synthetase from bovine aorta, cyclooxygenase or PGE₂ isomerase from sheep seminal vesicle microsomes, even at a concentration of 1 mM.

Effect of OKY-046 on the metabolism of arachidonic acid in platelets: OKY-046

Fig. 3. Effect of OKY-046 on various TXA₂ synthetases and other enzymes. OKY-046 was pre-incubated with enzymes at 24°C for 2 min. Then, the substrate 5 nmol [1-¹⁴C]PGH₂ or [1-¹⁴C]arachidonic acid was added and incubated for 2 min. The reaction products were submitted to thin-layer chromatography, and autoradiography was performed. The corresponding fraction was scraped and radioactivity was measured by a scintillation counter. A: rabbit platelet TX synthetase, B: human platelet TX synthetase, C: rabbit lung TX synthetase, D: dog platelet TX synthetase, E: guinea pig platelet TX synthetase, F: guinea pig lung TX synthetase, G: PGI₂ synthetase, H: cyclooxygenase, I: PGE₂ isomerase.

inhibited the formation of TXB₂ by human, dog and rabbit platelets at concentrations greater than 0.1 μ M (Fig. 4). In guinea pig platelets, more than 10 μ M of OKY-046 was required to inhibit the formation of TXB₂.

In addition, OKY-046 did not affect the formation of 12-HETE or the consumption of arachidonic acid in platelets from any of the four species. Thus, OKY-046 does not inhibit the 12-lipoxygenase or cyclooxygenase in platelets of these four animals.

Effect of OKY-046 on the metabolism of arachidonic acid in rat peritoneal cells: As depicted in Fig. 5, OKY-046 inhibited the production of TXB₂ at a concentration of more than 1 μ M, but at the same time, it increased the production of 6-keto-PGF_{1 α} in a dose-dependent manner. A small increase of other prostanoids such as PGE₂, PGF_{2 α} and PGD₂ was observed.

Effect of OKY-046 on arachidonate-induced arterial contraction: OKY-046 significantly inhibited the arachidonate-induced contraction of rabbit thoracic aorta and

Fig. 4. Effect of OKY-046 on arachidonate metabolism in platelets. OKY-046 was preincubated with homogenates of washed platelets (2×10^8 cells) at 24°C for 2 min. Then 5 nmol [$1\text{-}^{14}\text{C}$]arachidonic acid was added and incubated for 2 min. The metabolites of arachidonic acid were extracted and determined in the same way as described in the Fig. 3 legend. $\bullet$: human, $\circ$: dog, $\triangle$: guinea pig, $\blacktriangle$: rabbit, —: arachidonic acid (the amount of consumption), - - - : 12-HETE (the amount formed), : TXB₂ (the amount formed).

Fig. 5. Effect of OKY-046 on arachidonate metabolism in rat peritoneal cells. OKY-046 was preincubated with rat peritoneal cells suspended in Tyrode's solution at 24°C for 2 min. Then, 1 nmol [$1\text{-}^{14}\text{C}$]arachidonic acid was added and incubated for 2 min. The metabolites of arachidonic acid were extracted and determined in the same way as described in the Fig. 3 legend. Production rates were expressed as the percentage to the amount of [$1\text{-}^{14}\text{C}$]arachidonic acid used.

mesenteric artery superfused by blood from dog common carotid artery at a dose of 0.1 mg/kg. The degree of inhibition was almost identical in both specimens (Fig. 6). Maximum inhibition of about 40–50% was observed 3–10 min after injection of OKY-046, and the

inhibitory effect lasted for 20–30 min.

Effect of OKY-046 on sudden death: Following intravenous injection of sodium arachidonate (4 mg/kg), all the rabbits in the control group died within 2–3 min. OKY-046 administered 2 min before the injection of sodium arachidonate provided protection against sudden death at a dose of more than 0.3 mg/kg (Table 1).

Effect of OKY-046 on cerebral infarction: Cerebral infarction in rabbits was accomplished by the continuous infusion of sodium arachidonate into the internal carotid artery. The rate of incidence and the degree of cerebral infarction are shown in Fig. 7. In Fig. 8, photographs of the brains in each group are shown. OKY-046 significantly inhibited the incidence of cerebral infarction at doses of 0.3 and 1.0 mg/kg. Aspirin provided a protective effect at a dose of 30 mg/kg.

Discussion

OKY-046, an imidazole derivative, had a potent inhibitory effect on TXA₂ production from endoperoxide PGH₂ by the TXA₂ synthetase of human and rabbit platelet preparations. However, the inhibitory potencies against dog and guinea pig platelet TXA₂ synthetase were lower, indicating species differences.

OKY-046 did not inhibit cyclooxygenase,

Fig. 6. Effect of OKY-046 on arterial contraction in the dog superfusion model. The helical specimens of rabbit thoracic aorta and mesenteric artery were superfused with heparinized blood from a dog pumped from the common carotid artery and returned to the femoral vein. In the presence of various blockers, 100 μ l of sodium arachidonate (10 mg/ml) was dripped in the blood just above the upper tissue to induce arterial contraction. The degree of contraction was expressed as a percentage of the resting value. Each point shows the mean $\pm$ S.E. of 5–6 experiments. *, **: significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively. $\circ$: control, $\blacktriangle$: 0.01 mg/kg, $\bullet$: 0.1 mg/kg.

Fig. 7. Effect of OKY-046 and aspirin on cerebral infarction in rabbits. Cerebral infarction was accomplished by the injection of sodium arachidonate into the internal carotid artery at a dose of 1 mg/body/min for 30 min. OKY-046 or aspirin was administered intravenously 2 or 10 min, respectively, prior to injection of sodium arachidonate. Twenty-four hours later, rabbits were sacrificed and the brains were isolated. The degree of incidence of cerebral infarction was observed. The grades +, ++ and +++ indicate a partial, a half and whole infarction of the left hemisphere, respectively.

Table 1. Protective effect of OKY-046 on arachidonate-induced sudden death in rabbits

	Dose (mg/kg)	No. of survivals/ No. of test animals	Survival (%)
Control		0/10	0
OKY-046	0.3	2/5	40
	1.0	4/6	67

Drug was administered intravenously 2 min before the injection of 4 mg/kg sodium arachidonate, i.v.

Fig. 8. Photograph of rabbit brains from each group. Five specimens of each group were selected in the order of severity.

PGI₂ synthetase or PGE₂ isomerase at concentrations as high as 1 mM. Moreover, as described in Fig. 3, OKY-046 did not affect platelet 12-lipoxygenase. These results indicate that this compound selectively inhibits TXA₂ synthetase in the arachidonic acid cascade.

In rat peritoneal cells, OKY-046 modified

the products formed from arachidonate such that the production of TXB₂ was reduced, but the production of PGI₂ was increased. Similar results were reported by Kitamura et al. (22) for OKY-046 in perfused guinea pig lung and by Randall et al. (13) for UK-37248, another TXA₂ synthetase inhibitor, in perfused rabbit lung. These effects could be explained

as follows: The endoperoxide PGH_2 which accumulates as a result of selective inhibition of TXA_2 production might be used as a substrate for PGI_2 synthetase.

OKY-046 significantly inhibited the arachidonate-induced arterial contraction of isolated aorta specimens superfused by dog blood. Since arachidonate-induced arterial contraction in the presence of the various antagonists could be caused by TXA_2 formed in dog blood, the suppression of arterial contraction by OKY-046 might be due to the inhibition of TXA_2 synthesis in dog blood platelets.

OKY-046 protected rabbits against sudden death induced by injection of sodium arachidonate. Lefer et al. (23) reported that UK-37248 demonstrated a protective effect on arachidonate-induced sudden death accompanied by suppression of the elevation of TXB_2 level in plasma, and that TXA_2 formation caused the sudden death. Therefore, it could be argued that the inhibitory effect of OKY-046 was due to the inhibition of TXA_2 formation in rabbits.

In the cerebral infarction model, OKY-046 showed a potent suppressive effect on the incidence of infarction, and this effect of OKY-046 was 30–100 times more potent than aspirin. It seems that the formation of a platelet thrombus might lead to the occurrence of cerebral infarction in this model. It has been reported by Naito et al. (16) that OKY-046 showed a more potent inhibitory effect on rabbit platelet aggregation and on the production of TXA_2 during platelet aggregation than aspirin. It seems that the difference in the effects of both compounds on platelet function in vitro might be reflected in the experiment of cerebral infarction in vivo.

In conclusion, OKY-046 potently inhibited TXA_2 synthetase without affecting other enzymes related to the arachidonic acid cascade, and it exhibited a suppressive effect on the vascular contraction and the generation of the thrombosis and cerebral infarction through an inhibition of TXA_2 production. From these results, OKY-046 may be a valuable drug for the treatment of cerebrovascular and cardiovascular diseases associated with vasoconstriction and thrombosis due to TXA_2 .

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