EFFECT OF OKY-046, A THROMBOXANE A₂ SYNTHETASE INHIBITOR, ON ARACHIDONATE-INDUCED PLATELET AGGREGATION: POSSIBLE ROLE OF "PROSTAGLANDIN H₂ STEAL" MECHANISM

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To clarify the mode of action of a selective thromboxane A₂ (TXA₂) blockade in platelet reactivity, we examined the effect of (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride (OKY-046), a potent TXA₂ synthetase inhibitor, on human platelet aggregation induced by arachidonic acid (1 mM) in the absence and presence of aspirin-treated aortic microsomes containing prostacyclin (PGI₂) synthetase activity *ex vivo*. The production of TXA₂ and PGI₂ in platelet rich plasma was determined by the amounts of their stable catabolites, TXB₂ and 6-keto-PGF_{1α} respectively, measured by radioimmunoassay. In the absence of aortic microsomes, OKY-046 (> 10⁻⁵ M) produced more than 90% inhibition of TXA₂ production, whereas platelet aggregation was less inhibited, about 40% inhibition over control, by OKY-046 in that concentration. In the presence of aortic microsomes, the inhibitory effect of OKY-046 on platelet aggregation was markedly augmented in a dose-dependent manner in proportion to the increment of PGI₂ production, which paralleled the OKY-046-induced inhibition of TXA₂. These results suggest that a selective TXA₂ blockade produces effects on platelet aggregation mainly in dual fashion in the presence of PGI₂ synthetase: one is due to mere inhibition of TXA₂ synthetase and the other is due to the enhancement of PGI₂ production probably involving "prostaglandin H₂ (PGH₂) steal" mechanism, in which PGH₂ accumulated in platelets is partly converted to a substrate of PGI₂ synthetase in aortic microsomes to produce PGI₂.

THROMBOXANE A₂ (TXA₂) is a main product of arachidonic acid metabolism in platelets and is the most potent inducer of platelet aggregation.¹ Formation of this substance is increased under several conditions characterized by a

thrombotic diathesis.²⁻⁴ Therefore, drugs that inhibit TXA₂ production, especially selective inhibitors of this substance, have recently received much attention.⁵

We previously found that a TXA₂ synthetase inhibitor is effective in a partially constricted canine coronary artery in abolishing cyclic reduction of coronary flow (CRCF),⁶ which is known to originate from intravascular platelet aggregation.^{6,7} When this inhibitor was intravenously injected during CRCF, CRCF was ef-

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Fig.1. Typical time courses of platelet aggregation in a case, in which the effect of a TXA_2 synthetase inhibitor, OKY-046, on platelet aggregation was markedly potentiated in the presence of aortic microsomes containing PGI_2 synthetase activity. OKY-046 and aortic microsomes were pre-incubated with platelet rich plasma for 1 min at room temperature before induction of aggregation. Platelet aggregation nearly plateaued at 5 min after the addition of arachidonic acid. The amount of aortic microsomes used in this study was 0.1 mg/ml, which did not affect arachidonic acid-induced platelet aggregation *per se*.

fectively suppressed accompanying marked reduction of thromboxane B_2 (TXB_2 , a stable catabolite of TXA_2) levels and instantaneous elevation of 6-keto-prostaglandin $\text{F}_{1\alpha}$ (6-keto- $\text{PGF}_{1\alpha}$, a stable catabolite of prostacyclin) levels in the coronary sinus. These findings suggest that the antithrombotic effect of a selective TXA_2 synthetase inhibitor could be derived from the reduction of platelet TXA_2 production and the augmentation of vascular prostacyclin (PGI_2) production. Vascular microsomes are reported to utilize prostaglandin H_2 (PGH_2), a common precursor of TXA_2 and PGI_2 , released from platelets to produce PGI_2 when platelet TXA_2 synthetase is inhibited^{8,9}. These interactions between platelet and vascular wall *in vitro* let us speculate that a TXA_2 synthetase inhibitor can

potently suppress platelet aggregation *in vivo* through such a mechanism termed “ PGH_2 steal”.

The present study was designed to examine whether “ PGH_2 steal” mechanism can operate on human platelet rich plasma and to clarify how such a mechanism contributes to the anti-aggregating effect of a TXA_2 synthetase inhibitor.

METHODS

Preparation of Platelet Rich Plasma

Blood was collected by clean venipuncture from 20 human subjects, who denied having taking medication containing aspirin for two weeks previously, into one-tenth volume of 3.8% trisodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation at 600 rpm for 10 min at room temperature. The upper turbid layer of PRP was transferred to a polystyrene tube and the residual blood was centrifuged at 3000 rpm for 15 min to obtain platelet poor plasma (PPP). The platelet count in the PRP was determined using a Coulter Counter and the PRP was diluted to a count of $2.5 \times 10^5/\mu\text{l}$ using autologous PPP and was used within 2 hours of preparation.

Preparation of Aortic Microsomes

Bovine aortas were obtained from a local abattoir and the adventitia was stripped off and discarded. For preparation of the microsomes, the aortic wall was crushed into a fine powder, resuspended (1:4, W:V) in 50 mM Tris buffer (pH 7.5) with 1g aspirin and homogenized at high-speed in a “Polytron” homogenizer. Microsomal fractions were prepared according to the method previously described by Gryglewski et al.¹⁰ and contained 51% protein as determined by the method of Lowry et al.¹¹ They were resuspended in deionized water and lyophilized. These aortic microsomes were employed as the source of PGI_2 synthetase^{9,10}.

Platelet Aggregation Studies

Platelet aggregation in fresh PRP was monitored in a model RAM Multichannel Platelet Aggregometer (Rikadenki, Japan) with stirring at 1,100 rpm using the method of Born.¹² Platelets were pre-incubated with a potent TXA_2 synthetase inhibitor, (E)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid hydrochloride (OKY-046, 10^{-8} to 10^{-4} M)¹³ and aortic microsomes diluted with 50 mM Tris buffer (pH 7.5) for 1 min at room temperature before the addition of 25 μl

Fig. 2. Effect of OKY-046 in the different concentrations on platelet aggregation in the absence (open circles) and presence (closed circles) of aortic microsomes. The inhibition of platelet aggregation in the presence of more than 10^{-6} M OKY-046 was markedly enhanced when aortic microsomes were present. Platelet aggregation was expressed as % aggregation over control.

arachidonic acid (1 mM). There was no difference in the inhibitory effect of OKY-046 on TXB_2 production for more than 1 min of pre-incubation time. The final volume of platelet suspension was always 300 μl , containing 6.25×10^7 platelets and 8 mM Tris buffer. Platelet aggregation was expressed as % aggregation over control (in the absence of OKY-046 and aortic microsomes) at 5 min after addition of arachidonic acid. In this study, the amount of aortic microsomes employed was 0.1 mg protein/ml, which did not affect arachidonic acid-induced platelet aggregation *per se*. Arachidonic acid (sodium salt) was obtained from the Sigma Chemical Co.; OKY-046 was supplied by Ono Pharmaceutical Co. and Kissei Pharmaceutical Co., Japan.

TXB₂ and 6-keto-PGF₁ α Production

At the end of aggregation study, indomethacin (0.1 mM) and ethylenediamine tetraacetic acid (1 mM) were added to the PRP samples to avoid the secondary production of TXA_2 and PGI_2 . They were stored at -20°C until assay. To examine the time courses of these production, platelet aggregation was stopped by adding these substances at the specified time intervals. The

PRP samples were carefully thawed and were diluted with saline and the production of immunoreactive TXB_2 and 6-keto-PGF₁ α in the diluted samples were measured using a specific radioimmunoassay for TXB_2 and 6-keto-PGF₁ α according to the method previously described^{2,6}

RESULTS

Effect of OKY-046 on Arachidonic Acid-Induced Platelet Aggregation

Figure 1 shows the typical time courses of platelet aggregation induced by arachidonic acid in a representative case. 10^{-5} M OKY-046 exhibited only a slight inhibition of platelet aggregation, while in the presence of aortic microsomes, this agent markedly inhibited platelet aggregation and further exhibited disaggregating effect. To investigate the anti-aggregating effect of OKY-046 *ex vivo*, platelet aggregation was examined at 5 min after adding arachidonic acid in the presence of OKY-046 (10^{-8} to 10^{-4} M) in the PRP samples obtained from 20 cases (Fig. 2). In the absence of aortic microsomes, platelet aggregation was little inhibited at more than 10^{-6} M OKY-046 and about 40% inhibition was

Fig.3. Typical time courses of TXB₂ (left panel) and 6-keto-PGF_{1α} (right panel) production in the platelet rich plasma after the induction of platelet aggregation by arachidonic acid (1 mM) in the absence and presence of OKY-046 and aortic microsomes. Compared with control (open circles), 10⁻⁵ M OKY-046 (closed circles) exhibited the significant inhibition of TXB₂ production but no 6-keto-PGF_{1α} production. However, 6-keto-PGF_{1α} production was markedly augmented accompanying the inhibition of TXB₂ production in the presence of 10⁻⁵ M OKY-046 with the aid of aortic microsomes (triangles).

Fig.4. Effect of OKY-046 on TXB₂ (left panel) and 6-keto-PGF_{1α} (right panel) production in the platelet rich plasma for 5 min after arachidonic acid (1 mM)-induced aggregation in the absence (open circles) and presence (closed circles) of aortic microsomes. Though there was no difference in TXB₂ production under these conditions, 6-keto-PGF_{1α} production was significantly augmented at the concentrations of more than 10⁻⁶ M OKY-046 in the presence of aortic microsomes.

attained at 10⁻⁴ M OKY-046. In the presence of aortic microsomes, the inhibition was markedly potentiated, so that even 10⁻⁶ M OKY-046 gave

45% inhibition with a maximal inhibition of 82% above 10⁻⁵ M (Fig. 2). These findings indicated that anti-aggregating agent, possibly PGI₂, was

Fig. 5. Correlation between the augmented inhibition of platelet aggregation and the amount of 6-keto-PGF $_{1\alpha}$ both produced by OKY-046 in platelet rich plasma coexisted with aortic microsomes. The open circles represent the mean values of platelet aggregation inhibited and 6-keto-PGF $_{1\alpha}$ produced both which were subtracted their values in the absence of aortic microsomes from those in the presence of aortic microsomes in each concentration of OKY-046.

released into the reaction medium with the aid of aortic microsomes after the addition of arachidonic acid to the PRP samples.

Effect of OKY-046 on the Production of TXA $_2$ and PGI $_2$

Figure 3 shows the typical time courses of TXA $_2$ and PGI $_2$ production, estimated by the amounts of TXB $_2$ and 6-keto-PGF $_{1\alpha}$ released respectively, in a PRP sample during arachidonic acid-induced platelet aggregation. In the absence of OKY-046 and aortic microsomes (control), TXA $_2$ was precipitously produced and its amount plateaued at 5 min after the addition of arachidonic acid. When aliquotes of PRP were pre-incubated with 10^{-5} M OKY-046, the arachidonic acid-induced TXA $_2$ production was markedly inhibited (Fig. 3, left panel). The extent of the inhibitory action of OKY-046 was virtually similar, both in the absence and presence of aortic microsomes. In contrast, in the presence of aortic microsomes, addition of arachidonic acid provoked marked production of PGI $_2$ in PRP pre-incubated with 10^{-5} M OKY-046, while PGI $_2$ production was not detected in control PRP (Fig. 3, right panel). Since TXA $_2$ and PGI $_2$ production nearly plateaued at 5 min after the

addition of arachidonic acid, we examined the effect of OKY-046 in the different concentrations on these production at 5 min of aggregation in the absence and presence of aortic microsomes (Fig. 4). There was virtually no difference in TXA $_2$ production under these conditions: 10^{-6} M OKY-046 exhibited about 50% inhibition of TXA $_2$ production, and more than 90% inhibition was attained at 10^{-5} M. It was also found that PGI $_2$ production, which was only seen in the presence of aortic microsomes, was augmented along with the increase in the concentration of OKY-046, in that PGI $_2$ production was markedly potentiated at more than 10^{-6} M OKY-046 which was enough to inhibit TXA $_2$ production significantly, reaching the maximal production of about 26 ng/5 min/ml at 10^{-4} M OKY-046 (Fig. 4).

Correlation between the Effects of OKY-046 on Platelet Aggregation and Prostanoid Production

Since the potentiated inhibition of platelet aggregation by OKY-046 in the presence of aortic microsomes was assumed to be related to enhanced production of PGI $_2$ in addition to the inhibition of TXA $_2$ production, we examined the correlation between the augmented inhibition of

Fig. 6. Potentiation of the inhibitory effect of OKY-046 on platelet aggregation in the presence of aortic microsomes. The open circles represent the effect which was derived by only the inhibition of TXA_2 synthesis in the absence of aortic microsomes, while closed circles represent the effect which was derived by the complex system of platelets and aortic microsomes. Shaded area represents the additional inhibitory actions of OKY-046 on platelet aggregation due to PGI_2 production.

platelet aggregation and PGI_2 , estimated by the amount of 6-keto- $\text{PGF}_{1\alpha}$, both produced by OKY-046 in PRP including aortic microsomes (Fig. 5). In proportion as PGI_2 production was augmented along with the increase in the concentration of OKY-046, the additional inhibition of platelet aggregation, that is, increment in the inhibitory effect of OKY-046 on platelet aggregation caused by the presence of aortic microsomes, was linearly increased. Figure 6 shows the inhibitory effects of OKY-046 on platelet aggregation and TXA_2 production both in the absence and presence of aortic microsomes. In the absence of aortic microsomes, OKY-046 caused the reduction of TXA_2 production in a dose-dependent manner, whereas platelet aggregation was less inhibited. In the presence of aortic microsomes, however, the inhibitory effect of OKY-046 on TXA_2 production paralleled its effect on the inhibition of platelet aggregation. The augmented inhibitory effect on platelet aggregation was almost twice as much in each concentration of OKY-046 as that in the absence of aortic microsomes.

DISCUSSION

The inhibitory effect of OKY-046 on arachidonic acid-induced platelet aggregation was markedly enhanced associated with augmented PGI_2 production when platelets coexist with aortic microsomes *ex vivo*. Human platelet aggregation, induced by arachidonic acid, was inhibited only by 40%, at most, under mere TXA_2 synthesis inhibition, possibly due to the augmented production of endoperoxide PGH_2 , a proaggregatory precursor of TXA_2 . However, the inhibitory effect of OKY-046 on platelet aggregation in the presence of aortic microsomes containing PGI_2 synthetase was markedly augmented in a dose-dependent manner in proportion to the increment of PGI_2 production. It was also noted that PGI_2 production in PRP paralleled the OKY-046-induced inhibition of TXA_2 . These findings indicate that a TXA_2 synthetase inhibitor potentiates its effect on the inhibition of platelet aggregation *ex vivo* associated with augmented PGI_2 production in the presence of PGI_2 synthetase, which can utilize PGH_2 released from platelets to produce PGI_2 .

In platelets, the dominant route of transformation of proaggregatory endoperoxide PGH_2 is to the unstable substance TXA_2 which is a more potent aggregating agent¹. Many studies have found to report the increase in some prostaglandins such as prostaglandin D_2 ,¹⁴ E_2 and $\text{F}_{2\alpha}$ ¹⁵⁻¹⁷ when this step is selectively inhibited by TXA_2 synthetase inhibitors. It has been also postulated that PGH_2 accumulated in platelets by these agents is partly converted to a substrate of PGI_2 synthetase in vascular endothelium to produce PGI_2 ¹⁸⁻²⁰ and the amounts of PGH_2 stolen from platelets to vascular endothelium should increase with TXA_2 synthetase inhibition ("PGH₂ steal" mechanism)^{8,9}. However, it has been undefined whether such a mechanism between platelets and vascular wall could contribute to anti-platelet action of these agents.

The present study examining the effect of OKY-046 on platelet aggregation of human PRP coincubated with aortic microsomes, revealed that a TXA_2 synthetase inhibitor more effectively inhibited platelet aggregation in proportion to augmented amounts of PGI_2 production through this mechanism, though the amounts of PGH_2 stolen from platelets to aortic microsomes to produce PGI_2 were small (less than 5% of the decrement of TXA_2). In accordance with these findings, it is feasible that PGI_2 , but not other prostaglandins, produced when TXA_2 synthesis is selectively blocked in the presence of PGI_2 synthetase, plays an important role in the augmented inhibition of platelet aggregation. In other words, a TXA_2 synthetase inhibitor produces mainly the dual actions on the platelet aggregation in the presence of PGI_2 synthetase; one is due to mere inhibition of proaggregatory TXA_2 and the other is due to the augmented production of antiaggregatory PGI_2 deriving from "PGH₂ steal" mechanism.

Though it is difficult to extrapolate results obtained from the investigation of platelets *ex vivo* to the *in vivo* situation, it would seem that inhibition of TXA_2 synthetase affects platelet behavior in local site more dramatically *in vivo* than does *ex vivo* as long as the endothelium has intact activity of PGI_2 synthetase. These contentions may be supported by our previous data⁶ in that a TXA_2 synthetase inhibitor actively suppressed intracoronary platelet aggregation in an experimental model with increased PGI_2 levels in the coronary venous blood. Atheromatous lesions contain relatively high concentrations of

lipid peroxides,²¹ one of which is 15-hydroperoxy arachidonic acid that greatly inhibits PGI_2 synthetase.¹⁰ Formation of PGI_2 could be of great physiological importance in the maintenance of the integrity of the vessel walls.²² In the pathophysiological conditions such as coronary artery disease, the balance between TXA_2 and PGI_2 generating systems is considered to shift towards the TXA_2 pathway,^{3,23} leading to irreversible platelet aggregation and vasoconstriction. The effect of a selective TXA_2 synthetase inhibitor on the clinical course in patients with coronary artery disease would depend considerably on the extent of vascular PGI_2 production. It is not known, however, whether "PGH₂ steal" mechanism, such as that observed under experimental conditions, actually takes place in the disordered coronary circulation in diseased patients when they are treated with TXA_2 synthetase inhibitors.

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