Effects of a Thromboxane A2-Receptor Antagonist, a Thromboxane Synthetase Inhibitor and Aspirin on Prostaglandin I2 Production in Endothelium-Intact and -Injured Aorta of Guinea Pigs

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ABSTRACT—We examined the effects of KW-3635, a thromboxane (TX) A2-receptor antagonist, and OKY-046, a TX synthetase inhibitor, on the prostaglandin (PG) I2 production in endothelium-intact and -injured guinea pig aorta and compared them with those of aspirin. In the endothelium-intact aorta, both the low (3 mg/kg) and the high (100 mg/kg) dose of aspirin similarly reduced the PGI2 production, as measured ex vivo 1 hr after the injury. In contrast, neither KW-3635 (10 mg/kg) nor OKY-046 (30 mg/kg) inhibited the PGI2 production. The endothelial injury, induced by balloon catheterization, caused a reduction of PGI2 production in the aorta and decline of plasma PGI2/TXA2 ratio. In the endothelium-injured animals, the high dose of aspirin further reduced the PGI2 production in the aorta, whereas KW-3635 and OKY-046 did not affect it. KW-3635 and OKY-046 also ameliorated the reduced ratio of PGI2/TXA2 in the plasma. The present results demonstrate that aspirin, but not KW-3635 or OKY-046, reduces the PGI2 production in the aorta either in the endothelium-intact or -injured state. It is thus suggested that the TXA2-receptor antagonist and the TX synthetase inhibitor have some advantages over aspirin when used for the prevention of acute thrombosis after percutaneous transluminal angioplasty.

Keywords: Angioplasty transluminal, Prostaglandin I2, Aspirin, Thromboxane receptor antagonist, Thromboxane synthetase inhibitor

Percutaneous transluminal angioplasty (PTA) has recently been the prevailing treatment for occlusive atherosclerotic disease in humans. Although the primary success rate for PTA reaches 90%, there is a considerable rate of restenosis, up to approx. 30% (1-4). The platelet adhesion early after angioplasty and intimal hyperplasia are supposed to be responsible for abrupt reocclusion and restenosis, respectively. After angioplasty, platelets adhere to the exposed subendothelial connective tissues or media in only a few minutes (5, 6). Adherent platelets then discharge not only various granular components to enhance the aggregatory response but also a variety of platelet-derived growth factors, which increase the migratory or the mitogenic activity of smooth muscle cells (7, 8). Therefore, early platelet aggregation appears to play a pivotal role in the occurrence of postangioplasty thrombotic occlusion and restenosis (9). One of the important factors involved in the process may be the locally reduced production of prostaglandin (PG) I2, which is a potent inhibitor of platelet aggregation (10). In fact, destruction or injury of the endothelium by balloon angioplasty is known to cause the reduction of PGI2 synthesis in the vessel (11, 12).

To prevent thrombosis after PTA, medication with various drugs including antiplatelet agents has been advocated (13). Aspirin, a cyclooxygenase inhibitor, is a potent inhibitor of platelet aggregation and is frequently prescribed for patients who underwent PTA so as to prevent abrupt reocclusion. The activity of aspirin is mainly based on its irreversible inhibition of platelet cyclooxygenase, resulting in the prevention of synthesis of the proaggregating thromboxane (TX) A2. However, the antiaggregatory effect of aspirin may be offset by the concurrent inhibition of vascular cyclooxygenase, leading to the decrease in the PGI2 producing ability.

As the platelets from guinea pigs are quite similar to those from humans with regard to the response to agonists and antagonists of the eicosanoids (14), it would
be preferable to use guinea pigs to study a response that is dependent on platelets. However, so far, guinea pigs have rarely been used as an experimental PTA model (15). The purposes of the present study using guinea pigs were: 1) to determine whether PGI₂ production after experimental balloon angioplasty is lowered or not and 2) to delineate the effects of aspirin, KW-3635, a TXA₂-receptor antagonist (16) and OKY-046, a TX synthetase inhibitor (17), on the aortic PGI₂ production in either the normal stare or in the injury state.

Fig. 1. Observation of the longitudinal segment of balloon catheter-injured guinea pig aorta by light microscopy. A: Injury of aortic intima with loss of endothelium and thrombotic formation are apparent. Direct magnification, × 10. B: Higher magnification of the area indicated by the arrow in Panel A. Intrusions of inflammatory cells into the thrombus are observed. Direct magnification, × 50.

MATERIALS AND METHODS

Male Hartley guinea pigs weighing 400–550 g (Japan Shizuoka Laboratory Animal Center, Inc., Hamamatsu) were used. Animals were randomly assigned to 2 groups, the endothelium intact group and the endothelium injured one. Each group consisted of the following 5 subgroups, each including 6 animals: 1: vehicle-treated, 2: aspirin (3 mg/kg)-treated, 3: aspirin (100 mg/kg)-treated, 4: KW-3635 (10 mg/kg)-treated, and 5: OKY-046 (30 mg/kg)-treated. In addition to the above groups, a sham-
operated group was set up. The doses of aspirin were determined according to the previous report (18): we used a low dose, 3 mg/kg, that did not inhibit sodium arachidonate (100 μM)-induced ex vivo platelet aggregation in guinea pigs 2 hr after administration and a high dose, 100 mg/kg, that completely inhibits the aggregation. The dose of KW-3635 examined, 10 mg/kg, was the minimum effective dose to inhibit the aggregation (18). The dose of OKY-046 was determined by a preliminary study examining the TXB₂ concentration in the serum. At the dose of 30 mg/kg, it almost completely prevented the elevation of TXB₂, which was elicited by intravenous injection of sodium arachidonate 2 hr after the drug treatment. The drug or the vehicle was administered 1 hr before the aortic endothelial injury, as described below; i.e., 2 hr before the withdrawal of blood and sacrifice of the animal. Several guinea pigs, with endothelium intact or injured aorta, were used for histological studies.

Endothelial injury

Endothelial injury was produced with a slight modification of the method described by McCaffrey et al. (19). In brief, 1 hr after the drug administration, the guinea pig was anesthetized with pentobarbital sodium (35 mg/kg, i.p.). The left carotid artery was exposed and a 2F Fogarty balloon catheter (Baxter Healthcare Corporation,
Irvine, CA, USA) was inserted 10 cm to the level of just proximal to the abdominal bifurcation. The balloon was then inflated with an inflator (Boston Scientific Corporation, Watertown, MA, USA) at 1.5 atm. The balloon was gently withdrawn to the level of the aortic arch and then deflated. This procedure was repeated 4 times. Sham-operated animals were subjected to the same procedure except the balloon was not inflated.

**Collecting blood samples**

In the endothelium-injured animal (under anesthesia), the blood was collected from the abdominal aorta 1 hr after the balloon catheterization (i.e., 2 hr after the drug administration). The endothelium-intact animal was anesthetized with sodium pentobarbital (35 mg/kg, i.p.) 2 hr after the drug administration, and then the blood was collected from the abdominal aorta. The animals were sacrificed by the massive blood withdrawal. The collected blood was mixed with 1/10 volume of 77 mM EDTA • 2Na containing 10−4 M indomethacin and thereafter centrifuged at 3000 rpm for 10 min. The obtained plasma was stored at −35°C until analyzed.

**Histological examination**

One hour after the balloon catheterization, the thoracic aorta was removed, washed thoroughly with saline and fixed in 10% neutral buffered formaldehyde. The aorta was dissected longitudinally and embedded in paraffin in a conventional manner. Each section was stained with hematoxylin and eosin, and it was examined histologically by light microscopy. The presence or absence of endothelial cells and mural thrombus and the extent of intimal thickening were examined in a “blind” manner.

**PGI₂ production in aorta**

After collecting the blood sample, the thoracic aorta from aortic arch to diaphragm was carefully removed and the surrounding connective tissues were freed and discarded. The aorta was cut into 15-mm ring segments and pre-incubated for 15 min in 3 ml of physiologically balanced salt solution of the following composition (20): 124 mM NaCl, 5 mM KH₂PO₄, 1.25 mM MgSO₄, 1.45 mM CaCl₂, 1.25 mM KH₂PO₄, 25 mM Heps and 8 mM glucose. The solution was maintained at 37°C and was oxygenated with a gas mixture of 95% O₂ and 5% CO₂. After a 15-min incubation, the incubation medium was exchanged, and the pre-incubation was continued for another 1 hr, during which time the production of PGI₂ reached a steady-state. Thereafter, the medium was exchanged and incubation was continued for another 30 min. This medium was stored at −35°C until measured. A 100-μl aliquot of the incubation medium was used for radioimmunoassay (RIA).

**RIAl analysis**

PGI₂ and TXA₂ were measured as 6-keto-PGF₁α and TXB₂, the stable hydrolyzed products of PGI₂ and TXA₂, respectively, as described by Maclouf (21) and Fitzpatrick (22). The concentration of 6-keto-PGF₁α in the incubation medium and the concentrations of 6-keto-PGF₁α and TXB₂ in the plasma were determined by RIA. The working buffer used was 0.1 M potassium phosphate buffer, pH 7.4, containing 0.9% (w/v) NaCl, 0.01% (w/v) Na₂SO₄ and 0.1% (w/v) gelatin. Antiserum for 6-keto-PGF₁α or TXB₂ was diluted 10,000 or 30,000 times, respectively, with the working buffer. [³H]6-Keto-PGF₁α or [³H]TXB₂ (New England Nuclear, Boston, MA, USA) (0.1 ml), 0.1 ml of anti-6-keto-PGF₁α antiserum or anti-TXB₂ antiserum, respectively, 0.2 ml of the working buffer and 0.1 ml of the sample were mixed. After incubation for 16 hr at 4°C, 1 ml of 0.5% charcoal/0.05% dextran T70 suspension in the working buffer was added to the assay mixture. The mixture was allowed to stand for 15 min and then centrifuged at 1,000 x g for 10 min at 4°C. The radioactivity in the supernatant was counted with a liquid scintillation counter (Model 14530; Packard, Meriden, CT, USA). The cross-reactivity of anti-6-keto-PGF₁α antiserum or anti-TXB₂ antiserum with other prostaglandins was less than 2% or 1%, respectively. Unlabeled 6-keto-PGF₁α and TXB₂ were purchased from Funakoshi Co., Ltd. (Tokyo).

**Drug treatment**

Aspirin (Nacalai Tesque Inc., Kyoto), KW-3635 and OKY-046 were orally administered 2 hr before sacrifice. KW-3635 (sodium (E)-11-[2-(5,6-dimethyl-1-benzimidazolyl)ethylidene]-6,11-dihydrodibenz[b,e]oxepine-2-carboxylate monohydrate) and OKY-046 were synthesized in our laboratories. All the drugs were suspended in 0.3% sodium carboxymethylcellulose (CMC) at an appropriate concentration so as to make the administration volume 5 ml suspension per kg body weight. Guinea pigs that were administered only 0.3% CMC served as the control.

**Statistical analyses**

Results are expressed as means ± standard errors of 6 experiments. Differences between two groups were analyzed by Student’s t-test or the Aspin-Welch test. P values less than 0.05 were considered to be statistically significant.

**RESULTS**

The balloon angioplasty caused significant endothelial damage in the aorta. Formation of fibrinous thrombi over abraded intimal areas, directly on the denuded lamina elastica interna, was observed in patches (Fig. 1A). In the
thrombus, some neutrophils, in addition to platelets, were present (Fig. 1B). Intimal thickening was not evident 1 hr after the balloon catheterization.

PGI₂ production, measured as 6-keto-PGF₁₀⁻, in either the endothelium-intact or the endothelium-injured aorta, is shown in Fig. 2. The 6-keto-PGF₁₀⁻ release from the aortas of vehicle-treated endothelium-intact (vehicle-intact) animals was not different from that of sham-operated animals (26.2±2.7 and 29.3±3.8 ng/100 mg dry weight/30 min, respectively). Aspirin at doses of 3 and 100 mg/kg significantly reduced the 6-keto-PGF₁₀⁻ release from the aortas of endothelium-intact animals. Neither KW-3635 (10 mg/kg) nor OKY-046 (30 mg/kg) caused any effect on the 6-keto-PGF₁₀⁻ release in endothelium-intact animals. After the endothelial injury by balloon catheterization, the 6-keto-PGF₁₀⁻ release from aortas was attenuated as compared with that in the vehicle-intact animals. Aspirin at a dose of 100 mg/kg significantly reduced the 6-keto-PGF₁₀⁻ release in endothelium-injured animals. KW-3635 (10 mg/kg) or OKY-046 (30 mg/kg) did not further reduce the 6-keto-PGF₁₀⁻ release, but rather tended to ameliorate the attenuation.

The effects on the plasma concentration of 6-keto-PGF₁₀⁻ are summarized in Fig. 3. In endothelium-intact animals, the plasma concentration of 6-keto-PGF₁₀⁻ was significantly reduced by the treatment with aspirin (3 and 100 mg/kg). KW-3635 (10 mg/kg) did not affect the plasma concentration of 6-keto-PGF₁₀⁻. OKY-046 (30 mg/kg) significantly reduced it, although the reason for this is not clear. Following endothelial injury, the plasma concentration of 6-keto-PGF₁₀⁻ fell significantly as compared with that of the vehicle-intact animals. In the aspirin (3 and 100 mg/kg)-treated groups, the plasma concentration of 6-keto-PGF₁₀⁻ remained low, whereas in the KW-3635 (10 mg/kg)- and OKY-046 (30 mg/kg)-treated groups, it recovered to almost the same level as that in the vehicle-intact animals. The effects on the plasma concentration of TXB₂ are summarized in Fig. 4. In both endothelium-intact and endothelium-injured animals, plasma TXB₂ tended to be reduced by the treatment with aspirin (3, 100 mg/kg) and OKY-046 (30 mg/kg), presumably because of the inhibition of cyclooxygenase and TX synthetase, respectively.

The effects on the ratio of 6-keto-PGF₁₀⁻ to TXB₂ in the plasma are shown in Fig. 5. There was no significant difference in the ratio between the sham-operated animals (1.74±0.44) and the vehicle-intact animals (1.74±0.19). In endothelium-intact animals, aspirin at doses of 3 and 100 mg/kg significantly reduced the ratio as compared with that of the vehicle-intact animals. Neither KW-3635 (10 mg/kg) nor OKY-046 (30 mg/kg) significantly affected the ratio. In the vehicle-treated endothelium-injured (vehicle-injured) animals, the ratio significantly declined, as compared with that of the corresponding endothelium-intact animals, owing to the decrease in 6-keto-PGF₁₀⁻ and the increase in TXB₂. In the endothelium-injured

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**Fig. 2.** Production of 6-keto-PGF₁₀⁻ in the guinea pig aorta. The dissected segment of guinea pig thoracic aorta was incubated in physiologically balanced salt solution. Released 6-keto-PGF₁₀⁻ was measured by radioimmunoassay as an index of PGI₂ production in the aorta. A: Endothelium-intact animals. B: Endothelium-injured animals. Each bar indicates the mean±S.E. of 6 experiments. *P<0.05, ***P<0.001; significantly different from the corresponding vehicle-treated animals by Student's t-test or the Aspin-Welch test.
animals, the low dose (3 mg/kg) aspirin, KW-3635 (10 mg/kg) and OKY-046 (30 mg/kg) significantly increased the ratio as compared with that of the vehicle-injured animals.

DISCUSSION

The present histological observation revealed the focal presence of platelet deposition and mural thrombus on the subendothelium of the endothelium-injured aorta.
This early platelet deposition and thrombus formation were also observed in the previous experimental studies (5, 7, 15). As the vascular production of PGI₂ is negatively correlated with the platelet deposition (5, 15), the reduced production of PGI₂ seems to, at least partly, be responsible for the platelet deposition on the subendothelium of the injured aorta. In fact, the present results demonstrated that in guinea pigs, like in other species, vascular production of PGI₂ decreased following balloon catheterization. This observation is in accordance with the previous one by Tschopp and Baumgartner (15) who showed the same decline of PGI₂ production in guinea pigs by using the bioassay system. It is thus reasonable to assume that the reduced production of PGI₂ is involved in the platelet deposition and the formation of thrombus in the endothelium-injured aorta.

In the endothelium-intact aorta, aspirin not only at 100 mg/kg, but even at 3 mg/kg reduced the production of PGI₂. Aspirin at 3 mg/kg, does not inhibit the ex vivo platelet aggregation induced by either sodium arachidonate (100 μM) or collagen (3 μg/ml) (18). Thus, the present result implies that aspirin even at the low dose, which does not affect platelet aggregation, could attenuate the ability of the endothelium to produce PGI₂. Indeed, the same phenomenon was also observed in normal volunteers and patients with atherosclerosis (23, 24). Cumulative administration of a low dose (3 mg/kg) aspirin significantly attenuated the production of PGI₂ by the cephalic (23) and the saphenous vein (24). In the present study, neither KW-3635 nor OKY-046, in contrast to aspirin, exerted any influence on the ability of aortas to produce PGI₂ in endothelium-intact animals. The present observation suggest that even the single low dose of aspirin might attenuate the ability of vessels to produce PGI₂.

In the endothelium-injured aorta, the high dose (100 mg/kg) of aspirin further reduced the PGI₂ production, while the low dose (3 mg/kg) of this drug did not affect it. On the other hand, the aortic PGI₂ production in KW-3635 (10 mg/kg)- or OKY-046 (30 mg/kg)-treated animals was not reduced as compared with that of the vehicle-injured animals. These results suggest that KW-3635 and OKY-046 may be more desirable drugs than the high dose aspirin since it reduced the production of PGI₂ as well as TXA₂. Furthermore, aspirin also has some drawbacks such as the well-known gastrointestinal adverse effect (25) and the sex difference in the response to the drug (26). In addition, the PGI₂ production by atheromatous vessels might be more easily inhibited by aspirin (27). Considering these observations, the TXA₂-receptor antagonist and the TX synthetase inhibitor may have some advantage over aspirin for the treatment of vascular thrombotic disorders.

A significant portion of vascular PGI₂ production originates from endothelial cells (12). In the present study, indeed, when the endothelium was injured by balloon catheterization, the PGI₂ production in the endothelium-denuded aorta declined significantly, and platelet deposition and thrombus formation occurred simultaneously. Concomitantly with the reduced PGI₂ production in the aorta, the plasma concentration of 6-keto-PGF₁α, was significantly reduced while that of TXB₂ tended to be increased, resulting in a significant fall in the ratio of 6-
Experimental models in animals (28–31). Inhibitory effects on thrombus formation more potently than aspirin in several exotherms have been shown to exert inhibitory effects on thrombus formation more potently than aspirin in several exotherms. OKY-046 also significantly recovered the ratio. Increased production of PGI2 from PGH2 by the inhibition of thromboxane synthetase (i.e., steal phenomenon) might have been highlighted when platelets were activated.

TXA2-receptor antagonists and TX synthetase inhibitors have been shown to exert inhibitory effects on thrombus formation more potently than aspirin in several experimental models in animals (28–31). Inhibitory effects of aspirin against thrombus formation vary according to the dose used (32, 33). Generally speaking, the antithrombotic action of aspirin is observed in its lower doses. Thus, aspirin therapy requires careful deliberation. Further studies are required to determine whether or not the TXA2-receptor antagonist or the TX synthetase inhibitor is more efficacious as an antithrombotic drug than aspirin, especially in the clinical setting.

In the present study, a TXA2-receptor antagonist and a TX synthetase inhibitor produced similar effects on the aortic PGI2 production; however, these two types of antiplatelet agents have different modes of action. The antithrombotic effect of the TX synthetase inhibitor seems at least partly be mediated via the increased production of PGI2 (34). On the other hand, the TXA2-receptor antagonist, but not the TX synthetase inhibitor, antagonizes TXA2 and PGH2, both of which exhibit a potent proaggregatory action (35, 36). Recently, the combined treatment with a TXA2 receptor antagonist and a TX synthetase inhibitor has been recommended (37, 38). The combined treatment seems to be theoretically preferable, since it can lead to the inhibition of PGH2 as well as TXA2 and, moreover, to locally increased PGI2 at the site of thrombus.

In summary, we demonstrated the reduction of PGI2 production in the balloon catheterized aorta of the guinea pig. In the endothelium-intact aorta, both the low (3 mg/kg) and the high (100 mg/kg) doses of aspirin, but not KW-3635 or OKY-046, reduced the PGI2 production. In the endothelium-injured aorta, the high dose of aspirin further reduced the PGI2 production, whereas the low dose of aspirin, KW-3635 and OKY-046 did not further suppress the PGI2 production. These results suggest that the TXA2-receptor antagonist and the TX synthetase inhibitor have some advantages over aspirin when used for the prevention of acute thrombosis after PTA.

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