The Novel Thromboxane A₂ Receptor Antagonist KW-3635 Abolishes the Cyclic Flow Reduction in the Canine Carotid Artery

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We tested the hypothesis that the abolition of the cyclic flow reduction (CFR) in the canine carotid artery is related to inhibition of *ex vivo* platelet aggregation following administration of KW-3635, a thromboxane A₂ receptor antagonist, or aspirin. The CFR was induced in the carotid artery of anesthetized dogs by mechanical injury and narrowing of the artery. After induction of CFR, KW-3635 or aspirin was administered every 30 min at doses of 0.1, 0.3, 1 and 3 mg/kg (i.v.). The *ex vivo* platelet aggregation, induced by sodium arachidonate and collagen, was also examined before and 15 min after each administration. KW-3635 and aspirin, at doses of 1 mg/kg i.v. and above, inhibited CFR and *ex vivo* platelet aggregation. These results suggest that CFR in the canine carotid artery is platelet-dependent.

Keywords thromboxane A₂; carotid thrombosis; KW-3635; aspirin

Thrombi of carotid, vertebral or basilar origin are the main sources of emboli that cause transient ischemic attacks (TIAs).¹⁾ Fibrous thrombi from infarct-related arteries are evident as emboli in retinal arteries.²⁾ The cumulative incidence of cerebral infarction in the 5 year period after the initial TIA is about 30%.^{3–5)} Thus, the prevention of thrombosis or atherosclerosis in these extracranial arteries is very important for the treatment of TIAs, which frequently lead to cerebral infarction. In fact, several antiplatelet agents (aspirin and ticlopidine) have been shown to reduce the incidence of stroke,^{6–9)} suggesting that the emboli responsible for TIAs come at least partly from platelet aggregates.

Severe stenosis of a coronary or carotid artery with endothelial injury induces a cycle of decline and abrupt restoration of the blood flow, termed cyclic flow reduction (CFR). It has been proposed that this phenomenon involves the formation and dislodgement of a platelet-mediated thrombus at the stenotic site, because the CFRs can be abolished by a variety of antiplatelet agents including aspirin,^{10,11)} thromboxane (TX) receptor antagonists,^{12,13)} TX synthetase inhibitors,^{13,14)} an inhibitor of ADP-induced platelet aggregation¹⁵⁾ and a fibrinogen receptor antagonist.¹¹⁾ In a model of carotid CFR, the dislodged platelet-fibrin thrombi are carried away by the circulation to peripheral regions as emboli. Since this phenomenon is supposed to resemble the nature of TIAs, a model of carotid CFR can serve as an experimental model of TIAs.

KW-3635 (sodium (*E*)-11-[2-(5,6-dimethyl-1-benzimidazolyl)-ethylidene]-6,11-dihydrodibenz[*b,e*]oxepine-2-carboxylate monohydrate) is a potent and selective TXA₂ receptor antagonist, which has been shown to inhibit the receptor binding of TXA₂ agonists and antagonists,¹⁶⁾ TXA₂-mediated platelet aggregation¹⁷⁾ and the constriction of various smooth muscle preparations in response to TXA₂.¹⁸⁾ Moreover, KW-3635 exhibits antithrombotic activity, as shown by its prevention of vascular reocclusion after thrombolysis with tissue-type plasminogen activator.¹⁹⁾

Although many agents have been evaluated in coronary CFR models, few studies have been carried out using carotid CFR models. Additionally, while a variety of antiplatelet agents have been shown to be effective in abolishing CFRs, the close relationship between this effect on CFRs and the antiplatelet effect has rarely been examined.²⁰⁾ The aims of the present study were 1) to evaluate the effect of KW-3635, a new TXA₂ receptor antagonist, on a canine model of carotid artery CFR in comparison with that of aspirin and 2) to test the hypothesis that the suppressing effects of these antiplatelet agents on the carotid CFR are based on their activity to inhibit platelet aggregation.

MATERIALS AND METHODS

All procedures used in this study were conducted according to the principles of the Experimental Animal Ethics Committee of Kyowa Hakko Kogyo Co., Ltd. Animals were kept in air-conditioned rooms and treated carefully until euthanasia was carried out.

Adult mongrel dogs of either sex weighing 6.2–14.4 kg were used. Animals were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated and ventilated with room air. Both the right femoral artery and vein were exposed and catheters were inserted to monitor blood pressure (AP-621G, Nihon Kohden, Tokyo, Japan) and for administration of the test compound, respectively. The catheter inserted into the femoral artery was also used to withdraw blood samples. A 5–6 cm segment of the left common carotid artery was isolated and an electromagnetic flow probe was placed at the proximal end of this section for monitoring carotid blood flow (MEV-3100, Nihon Kohden). All parameters were monitored on a recorder (WS-681G, Nihon Kohden).

The induction of the carotid CFR was carried out by a modification of the procedure described by Uchida and Murao.²¹⁾ A 1–2 cm segment of the exposed common carotid artery was de-endothelialized by gently squeezing the artery with a pair of forceps. Thereafter, an external

Fig. 1. Experimental Protocol of the Present Study

Arrows indicate the administration of either vehicle, KW-3635 or aspirin. At the point indicated as ●, blood samples were collected for *ex vivo* platelet aggregation.

plastic constrictor (MT Giken, Choufu, Japan) of suitable size was placed around the artery to reduce the carotid blood flow to approximately 20–40% of the basal value. Shortly after this, a typical pattern of CFR appeared, characterized by a cycle of gradual declines in blood flow to almost zero, followed by spontaneous restoration of the flow.

Figure 1 shows the experimental protocol of the present study. After the appearance of CFR, all animals were kept stabilized for at least 60 min to determine their baseline parameters. Then, they were divided into 3 groups: vehicle-treated, KW-3635-treated and aspirin-treated groups. In the case of the KW-3635 ($n=6$)- and aspirin-treated groups ($n=6$), KW-3635 or aspirin at a dose of 0.1 mg/kg was administered intravenously (2 mg/ml) within 10 s. Thereafter, at 30 min intervals, each agent was administered at doses of 0.3, 1, and 3 mg/kg. In the vehicle-treated group ($n=6$), the protocol was similar to that for the other two groups except that the vehicle alone (5% glucose solution, pH 9.0) was used.

The frequency of CFR was monitored and the value per 30 min was calculated. The amplitude was defined as the difference between the peak and nadir blood flows of each cycle. The mean amplitude during the pretreatment period or the treatment period (30 min) was calculated.

For *ex vivo* platelet aggregation, blood samples were obtained before the first administration of the agent or vehicle and then 15 min after each treatment. Blood was withdrawn from the artery and mixed with 1/10 its volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation of this mixture at $200 \times g$ for 5 min at room temperature. The sample was again centrifuged at $1000 \times g$ for 10 min to obtain platelet-poor plasma (PPP). Platelet aggregation was determined by measuring the optical density of PRP using an aggregometer (TE500, Erma Optical Works, Tokyo, Japan). When collagen (Hormon-Chemie, München, Germany) was used as an inducer of platelet aggregation, the submaximal concentration that was needed for aggregation was determined using the PRP obtained before drug treatment, and the suitable concentration of collagen was selected as either 9, 12, 20 or 30 $\mu\text{g/ml}$ (final concentration). PRP was prewarmed for 3 min at 1100 rpm and collagen was added to the PRP. When U-46619 (9,11-dideoxy-9 α ,11 α -epoxymethano-prostaglandin $F_{2\alpha}$) (Cayman Chemical, Ann Arbor, MI, U.S.A.) and sodium arachidonate (Sigma Chemical, St. Louis, MO, U.S.A.) were used for induction, epinephrine (10 μM) (Bosmin®, Daiichi Seiyaku, Tokyo, Japan) was added to the PRP which had been prewarmed for 3 min at 1100 rpm

and then, 1 min afterwards, U-46619 (3 μM) or sodium arachidonate (150 μM) was added to the PRP. In either case, the change in light transmission was monitored until the aggregation reached a plateau.

KW-3635, synthesized in our laboratories, was dissolved in 5% glucose (adjusted to pH 9.0 by 0.1 N KOH) immediately before use. Aspirin was purchased from Nacalai Tesque (Kyoto, Japan) and dissolved in 5% glucose solution (adjusted to pH 9.0 by 6 N NaOH) immediately before use.

All data were expressed as means $\pm$ standard errors (S.E.). For the analysis of the degree of reduction in carotid blood flow before drug treatment, the non-parametric Kruskal–Wallis test followed by a Tukey-type multiple comparison test was used. For the analysis of the other data, a non-parametric Friedman test followed by the Sign–Wilcoxon test was used. p values less than 0.05 were considered statistically significant.

RESULTS

Before drug treatment, stenosis of the common carotid artery in the vehicle-, KW-3635- and aspirin-treated groups reduced the carotid blood flow to $23.3 \pm 4.4\%$, $40.3 \pm 8.9\%$ and $36.2 \pm 5.9\%$, respectively. The differences in these were not statistically significant.

A typical recording of CFR in the vehicle-treated group is shown in Fig. 2A. In this group, once CFR was induced, it persisted for at least 3 h. The mean frequency of CFR ranged from 3.3 to 4.0 per 30 min and was unaffected by the administration of the vehicle (Table I). The amplitude did not change throughout the experiment in the vehicle treated group, either, ranging from 20.8 to 24.1 ml/min/cycle. Figure 2B shows a representative recording of CFR in the KW-3635-treated group. Administration of 0.1 mg/kg KW-3635 did not produce any effect on CFR, while 0.3 mg/kg of KW-3635 tended to reduce the amplitude. KW-3635 at a dose of 1 mg/kg markedly reduced the frequency, and finally, KW-3635 abolished CFR at a dose of 3 mg/kg (Table I). A typical recording of CFR in the aspirin-treated group is shown in Fig. 2C. The same pattern was observed as for KW-3635 and aspirin, which abolished the CFR at a dose of 1 mg/kg (Table I).

The percentage aggregation induced by 3 μM U-46619, 150 μM sodium arachidonate and collagen (9–30 $\mu\text{g/ml}$) during the course of the experiment is shown in Fig. 3. Administration of the equivalent volume of vehicle did not affect the platelet aggregation induced by any of the agonists tested. KW-3635 inhibited *ex vivo* platelet aggregation induced by U-46619 (3 μM) at a dose of 1 mg/kg. It also markedly inhibited the aggregation induced by sodium arachidonate (150 μM) and collagen (9–30 $\mu\text{g/ml}$). Aspirin, at a dose of 1 mg/kg, significantly inhibited the aggregation induced by sodium arachidonate and collagen (Fig. 3), but not that induced by U-46619 (3 μM) (unpublished observation).

DISCUSSION

It has been proposed that CFR, defined as the periodic

Fig. 2

TABLE I. Mean Frequency and Amplitude of Carotid Cyclic Flow Reduction (CFR)

	Pre	0.1 mg/kg	0.3 mg/kg	1 mg/kg	3 mg/kg
Vehicle					
Frequency (/30 min)	3.5 ± 0.5	4.0 ± 0.4	3.3 ± 0.3	3.3 ± 0.4	3.8 ± 0.3
Amplitude (ml/min/cycle)	24.1 ± 2.7	21.8 ± 2.7	23.2 ± 3.6	22.3 ± 3.1	20.8 ± 3.4
KW-3635					
Frequency (/30 min)	2.7 ± 0.2	3.0 ± 0.3	2.3 ± 0.3	0.7 ± 0.3 ^{a)}	0.5 ± 0.3 ^{a)}
Amplitude (ml/min/cycle)	29.4 ± 5.5	21.7 ± 5.0 ^{a)}	23.8 ± 6.3	9.9 ± 4.8 ^{a)}	7.6 ± 5.4 ^{a)}
Aspirin					
Frequency (/30 min)	3.3 ± 0.3	3.8 ± 0.3	3.5 ± 0.4	1.3 ± 0.4 ^{a)}	0.2 ± 0.2 ^{a)}
Amplitude (ml/min/cycle)	22.1 ± 2.0	18.7 ± 1.5	18.8 ± 2.8	18.3 ± 4.7	0.0 ± 0.0 ^{a)}

Data are expressed as means ± S.E. of 6 experiments. ^{a)} $p < 0.05$; significantly different from the corresponding pre-administration value (Sign-Wilcoxon test).

formation and dislodgement of thrombi at the site of a stenosed artery, is mainly mediated by TXA_2 .^{12,14)} The increment in plasma TXA_2 , measured as TXB_2 , is observed at the distal end of a stenosed coronary artery during the appearance of CFR.¹⁴⁾ Furthermore, previous studies have demonstrated that CFR occurs, not by vasospasm of the stenosed artery, but by aggregated platelets in the narrowed lumen.²²⁾ Thus, it was of interest to determine whether or not agents inhibiting TXA_2 -mediated platelet aggregation would abolish CFR and suppress platelet function at the same dose. Although there have been several reports of the relationship between the inhibitory effects of CFR and antiplatelet activity,²³⁻²⁵⁾ the

inhibitory effects of the agents on *ex vivo* platelet aggregation were usually tested only at one dose, that producing complete inhibition of CFR. In contrast, the present study, the antiplatelet effects were tested at different doses given one after the other.

The present results demonstrate that both KW-3635 and aspirin effectively abolish CFR almost at the same dose that inhibits *ex vivo* collagen- and sodium arachidonate-induced platelet aggregation, which is supposed to be mediated by TXA_2 . KW-3635 also inhibits U-46619-induced platelet aggregation *ex vivo* at a dose of 1 mg/kg, which is the minimum effective dose for abolishing CFR. The inhibitory effect of KW-3635 on collagen-induced

Fig. 2. Representative Recordings of Carotid Blood Flow (A) vehicle, (B) KW-3635, (C) aspirin.

platelet aggregation was not so marked as that on U-46619-induced aggregation, perhaps because collagen-induced platelet aggregation is not entirely mediated by TXA_2 . The present results are well consistent with previous studies showing that TXA_2 is one of the most important mediators of the onset and persistence of CFR.¹²⁻¹⁴ KW-3635 possibly antagonizes TXA_2 by blocking its receptors while aspirin suppresses TXA_2 production by inhibiting cyclooxygenase. Thus, it is quite reasonable to assume that both agents, by inhibiting TXA_2 -dependent platelet aggregation, were able to abolish CFR and inhibit platelet aggregation at the same dose.

Several investigators have reported that CFR becomes more resistant to cyclooxygenase inhibitors as the stenosis becomes severer.^{11,26} In fact, Willette *et al.*¹¹ reported that, in a canine carotid artery CFR model, treatment with aspirin at 5 mg/kg i.v. was ineffective in 6 out of 9 dogs. We speculate that, under such circumstances when cyclooxygenase inhibitors are unable to abolish CFR, the degree of the stenosis must be too severe. Actually, Willette *et al.*¹¹ and Aiken *et al.*²⁶ mechanically disrupted the thrombus to restore the reduced blood flow, perhaps because it did not recover spontaneously. In the present study, all the dogs showed a spontaneous decline and recovery of carotid blood flow, suggesting that the degree of stenosis in our model was mild compared with

Fig. 3. Effects of KW-3635 and Aspirin of *ex Vivo* Platelet Aggregation (A) U-46619 (3 μM) (pre-stimulated with 10 μM epinephrine), (B) sodium arachidonate (150 μM) (pre-stimulated with 10 μM epinephrine), (C) collagen (9–30 $\mu\text{g/ml}$). —○—, vehicle-treated ($n=6$); —●—, KW-3635-treated ($n=6$); —□—, aspirin-treated ($n=6$). *a*) $p < 0.05$; significantly different from the corresponding pre-treatment value (Sign-Wilcoxon test).

that studied by Willette *et al.*¹¹ and Aiken *et al.*²⁶

In conclusion, aspirin and the TXA_2 receptor antagonist KW-3635 both exhibit antithrombotic and antiplatelet activities at the same dose in a canine model of carotid CFR. These results suggest that the formation and abolition of CFR in the present study depends on platelet aggregation. Administration of a TXA_2 receptor antagonist may be effective in the prophylaxis and treatment of TIAs.

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