Antiplatelet activities of FK409, a new spontaneous NO releaser

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1 We reported that (±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (FK409) released nitric oxide (NO) spontaneously with a chemiluminescence analyzer. The aim of this study was to compare antiplatelet activities of FK409, a new NO releaser, with those of isosorbide dinitrate (ISDN) *in vivo* and *in vitro*. In order to elucidate the differences in antiplatelet activities between FK409 and ISDN, we compared their modes of action.

2 In *in vitro* experiments, FK409 had a more potent inhibitory effect on rat platelet aggregation induced by adenosine 5'-diphosphate (2.0 µM) than ISDN (IC₅₀ = 4.32 ± 0.95 µM and >100 µM respectively).

3 In the rat extracorporeal shunt model (*in vivo* experiments), FK409 suppressed thrombus formation dose-dependently from 0.32 mg kg⁻¹, p.o. and showed the maximum inhibition (52% inhibition vs. vehicle treatment) at 10 mg kg⁻¹, p.o., while ISDN showed no inhibition at 10 mg kg⁻¹ and only 17% inhibition at 32 mg kg⁻¹, p.o.

4 FK409 could generate nitrite, which is an oxidative product of NO, much faster than ISDN in phosphate buffer solution and rat plasma during 60-min incubation at 37°C.

5 These data show that FK409 has more potent antiplatelet effects than ISDN, by acting through spontaneously released NO.

Keywords: FK409; isosorbide dinitrate; nitric oxide; antiplatelet activity

Introduction

(±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (FK409), the chemical structure of which is shown in Figure 1, is a structurally unique vasodilator discovered from the fermentation products of *Streptomyces griseosporus* (Hino *et al.*, 1989). FK409 produced a potent vasorelaxation mediated by the elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in dog isolated coronary artery (Yamada *et al.*, 1991) and rabbit aorta (Sibata *et al.*, 1991). In addition, the vasorelaxation and the elevation of cyclic GMP were inhibited by methylene blue, an inhibitor of soluble guanylate cyclase. Owing to these facts, although FK409 has no O-NO₂ functional group (unlike organic nitrates) it seems to have a mode of action similar to that of organic nitrates reported by Katsuki *et al.* (1977) and Schultz *et al.* (1977). Previously, it has been reported that organic nitrates showed various pharmacological actions via nitric oxide (NO) (Feelish & Noack, 1987a,b), which is generally accepted as EDRF (Palmer *et al.*, 1987). Recently, we reported that FK409 decomposed and released NO spontaneously in solution (Kita *et al.*, 1994).

It has been shown that the antiplatelet effect of organic nitrates acting via NO (Mellion *et al.*, 1981; Radomski *et al.*, 1987) played an important role in the prevention of acute coronary syndromes such as unstable angina pectoris and acute myocardial infarction (Hjemdahl-Monsen *et al.*, 1986; Fuster *et al.*, 1988). Thus, the antiplatelet action could be an important aspect in the treatment of patients with ischaemic cardiovascular diseases.

The purpose of this study was to evaluate the antiplatelet effects of FK409 in rats; we compared the *in vitro* and *in vivo* effects of FK409 with those of isosorbide dinitrate (ISDN) (Figure 1), which is an organic nitrate and the most popular orally active drug for ischaemic cardiovascular diseases. Additionally we related the differences in the biological potencies of FK409 and ISDN to those of their modes of actions.

Methods

Animals

Sprague-Dawley (SD) rats were purchased from Nihon SLC Co. (Sizuoka, Japan). In *in vivo* experiments, rats were fasted for 24 h and given each drug orally, suspended in 0.5% methylcellulose, in a volume of 5 ml kg⁻¹.

In vitro platelet aggregation

Male SD rats weighing 240–325 g, were anaesthetized with diethyl ether and blood was collected from the abdominal aorta into plastic vessels containing 2.2% trisodium citrate (10⁻¹ volume). Platelet-rich plasma was obtained from the supernatant fraction after centrifugation of blood at 200 g for 10 min. The effect of FK409 or ISDN on platelet aggregation was determined by Born's turbidimetric method (Born & Cross, 1968) using an aggregometer (Hema Tracer 801, Niko Bioscience Co., Tokyo, Japan). To 225 µl of platelet-rich plasma in a cuvette, 25 µl of each drug dissolved in Tris-acetate (25 mM) and NaCl (120 mM) buffer adjusted to pH 7.4 or vehicle was added and incubated at 37°C for 2 min. After the incubation, platelet aggregation was induced by the addition of 5 µl of adenosine 5'-diphosphate (ADP; final concentration, 2.0 µM). In order to evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve for 7 min after the addition of ADP. The effects of each drug were expressed as % inhibition of ADP-induced platelet aggregation compared with vehicle treatment. The IC₅₀ value is the drug concentration required to produce 50% inhibition; this was calculated logarithmically for platelet-rich plasma from each rat.

Extracorporeal shunt model

The procedure was performed as described previously (Umetsu & Sanai, 1978). Briefly, male SD rats weighing 325–405 g were anaesthetized with sodium pentobarbitone

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Figure 1 Chemical structures of FK409 and ISDN.

(50 mg kg⁻¹, i.p.). A silk thread (5 cm; Tire No. 50, Fujix Co., Osaka, Japan) was placed in the polyethylene tubing (6 cm; Hibiki Size 7, Kunii Co., Tokyo, Japan). Both ends of this tubing were connected to other polyethylene tubing (10 cm; Hibiki Size 4, Kunii Co.). The extracorporeal shunt filled with heparin solution (50 iu ml⁻¹) was introduced between the right carotid artery and the left jugular vein and blood circulation was then established. After 20 min, the blood flow was stopped at the artery side with a pinch cock. Then, the middle tubing was taken away and the thread coated with thrombus was carefully pulled out of the tubing. Immediately, the wet weight of the thrombus was measured. Another new polyethylene tubing containing a silk thread and filled with the heparin solution was inserted between the arterial and venous tubing ends. Blood circulation was re-established by opening the pinch cock. After 20 min, the wet weight of the silk thread was again measured for the second time. Then, the third blood circulation was performed. Thrombus weight was determined by subtracting the weight of a silk thread immersed in heparinized blood from that of the thread coated with thrombus. The total thrombus weight obtained by three blood circulations was taken as a net thrombus weight in one experiment. FK409, ISDN or vehicle was given orally 60 min before the first blood circulation. The effects of each drug were expressed as % inhibition of thrombus formation compared with vehicle treatment.

Nitrite analysis

In the determination of nitrite concentrations in sodium phosphate buffer (0.1 M; PB), FK409 and ISDN were dissolved in PB at 1.5 mM and immediately incubated at 37°C. For the determination of nitrite concentrations in plasma, blood from male SD rats weighing 325–375 g, which had been anaesthetized with ethylether, was collected from the abdominal aorta into plastic vessels containing 4% tripotassium EDTA (40⁻¹ volume). Plasma was obtained from the supernatant fraction after centrifugation of blood at 1000 g for 15 min. FK409 and ISDN were dissolved in dimethyl sulphoxide at 75 mM and 0.05 ml was added to 2.45 ml of plasma (final drug concentration: 1.5 mM) and immediately incubated at 37°C. Prior to incubation and at various time intervals, the concentrations of nitrite were determined by diazotization. To 0.05 ml of PB or plasma containing drug, 3.95 ml of HCl (0.05 N for plasma and 0.5 N for PB), 0.5 ml of 0.2% sulfanilic acid and 0.5 ml of 0.1% N-(naphthyl)-ethylenediamine was added subsequently (Bell *et al.* 1963). Absorbance of a purple dye at 548 nm was measured with a spectrophotometer (UV-2200, Shimadzu Co., Kyoto, Japan). For the standard curve, sodium nitrite was used under the same experimental conditions.

Drugs

FK409 and ISDN were synthesized by Fujisawa Pharmaceutical Co. (Osaka, Japan). Sodium citrate, ADP and

Figure 2 Inhibitory effects of FK409 (●) and ISDN (▲) on platelet aggregation induced by ADP (2.5 μM) in rat platelet-rich plasma. The effects of each drug are expressed as % inhibition of ADP-induced platelet aggregation compared with vehicle treatment. Each value represents the mean + s.e.mean for five experiments.

tripotassium EDTA were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sulfanilic acid and N-(naphthyl)-ethylenediamine were purchased from Nacalai Tesque Co. (Kyoto, Japan).

Results

Effects on in vitro platelet aggregation

Figure 2 shows the inhibitory effects of FK409 and ISDN on ADP-induced platelet aggregation in rat platelet-rich plasma. FK409 (0.32–32 μM) dose-dependently suppressed this aggregation. The inhibition value of FK409 at 0.32–32 μM did not reach 100% and was 64% at 32 μM. The IC₅₀ value of FK409 was 4.32 ± 0.95 μM. This value is larger than the IC₅₀ value (0.75 μM) which we reported in the human platelet-rich plasma test (Kita *et al.*, 1994). ISDN showed only a 10% inhibition of platelet aggregation at 100 μM.

Effects on thrombus formation in the extracorporeal shunt model

To examine *in vivo* antiplatelet action of FK409 and ISDN, we evaluated these drugs in the rat extracorporeal shunt model. Figure 3 shows the effects of FK409 and ISDN on thrombus formation in this model. FK409 (0.32–32 mg kg⁻¹, p.o.) dose-dependently inhibited thrombus formation. FK409 showed maximum inhibition (52%) at 10 mg kg⁻¹, p.o., while

Figure 3 Inhibitory effects of FK409 (●) and ISDN (▲) on thrombus formation in the rat extracorporeal shunt model. The effects of each drug are expressed as % inhibition of thrombus formation compared with vehicle treatment. Each value represents the mean + s.e.mean for six experiments.

ISDN showed no inhibitory effects at 10 mg kg⁻¹, p.o. and produced only 17% inhibition at 32 mg kg⁻¹, p.o.

Nitrite generation in PB and rat plasma

As shown in Figure 4a, FK409 spontaneously generated nitrite which is an oxidative product of NO, while ISDN did not. The concentration of nitrite generated from FK409 was 0.239 ± 0.002 mM after 60-min incubation. The first-order generation of nitrite was observed for FK409, the rate constant being 0.0109 ± 0.0001 min⁻¹ ($r = 0.972 \pm 0.010$).

As shown in Figure 4b, ISDN could also generate nitrite time-dependently in rat plasma in contrast to PB. But the nitrite generation from ISDN was much slower than that from FK409 and did not occur in the first order. The concentrations of nitrite generated from FK409 and ISDN were 0.211 ± 0.005 and 0.0069 ± 0.002 mM after 60-min incubation, respectively. The first-order generation of nitrite was observed for FK409, the rate constant being 0.0875 ± 0.0023 min⁻¹ ($r = 0.997 \pm 0.001$).

Discussion

Organic nitrates such as nitroglycerin (Hampton *et al.*, 1967; Kowey *et al.*, 1981) and ISDN (DeCaterina *et al.*, 1984) have been shown to inhibit platelet aggregation in *in vitro* and *in vivo* experiments. However, in our experiments, ISDN showed very weak inhibitory effects on *in vitro* platelet aggregation and *in vivo* thrombus formation in rats. In comparison with ISDN, FK409 had much more potent antiplatelet effects in both *in vitro* and *in vivo* experiments. However, in both experiments, FK409 inhibited platelet function dose-dependently but could not cause complete inhibition even at maximum doses. In *in vitro* experiments, the inhibition induced by FK409 at 32 μ M was almost maximal and was only 64%. In *in vivo* experiments, the inhibitory effect of FK409 on thrombus formation reached a maximum at 10 mg kg⁻¹, p.o. and the inhibition was only 52% at this dose. Interestingly, in contrast with these results in rats, FK409 showed almost complete inhibition (97% inhibition) of platelet aggregation *in vitro* in man at 32 μ M and the slope of the concentration-inhibition curve was steeper (Kita *et al.*, 1994). It is not clear why FK409 could not inhibit platelet function completely, in both *in vitro* and *in vivo* experiments in rats, as it did in the human *in vitro* experiments.

Figure 4 Time-dependent generation of nitrite from FK409 (●) and ISDN (▲) in (a) phosphate buffer solution (pH 7.4) and (b) rat plasma at 37°C. The initial concentration of each drug was 1.5 mM. Each value represents the mean + s.e.mean for three experiments.

Recently, we reported that FK409, in contrast with ISDN, released NO spontaneously with a chemiluminescence analyzer (Kita *et al.*, 1994) and in this paper we have shown that FK409 spontaneously generated nitrite, which is an oxidative product, in PB solution and rat plasma. Taking these data into account the antiplatelet activity of FK409 in our experiments is probably mediated by NO released spontaneously from the compound.

In *in vitro* and *in vivo* experiments, ISDN showed weak inhibition of platelet function at the highest doses (10% inhibition at 100 μ M and 17% inhibition at 32 mg kg⁻¹, p.o. respectively). In order to explain the low efficacy of ISDN compared with FK409, we determined the concentrations of nitrite generated from ISDN and FK409 in rat plasma. In contrast with the result in PB, ISDN was able to generate nitrite in rat plasma. Feelish & Noack (1987a,b) reported that organic nitrates such as ISDN showed pharmacological actions via NO released from the compounds. In these reports, the sulphhydryl group of cysteine was required to biotransform organic nitrates to NO. Recently, it was reported that albumin in plasma catalyzed the denitrication of nitroglycerin in a reaction proceeding by way of its sulphhydryl moiety (Chong & Fung., 1990). From these facts, it was considered that ISDN weakly inhibited platelet function in platelet-rich plasma (*in vitro*) and blood (*in vivo*) at the highest doses through being biotransformed to NO by sulphhydryl moieties such as cysteine contained in serum proteins. The amount of nitrite generated from ISDN during 60-min incubation, however, was much less than that from FK409. These data reflected well the differences in antiplatelet activities between FK409 and ISDN.

In conclusion, we have shown that FK409 has more potent antiplatelet activity than ISDN *in vitro* and *in vivo*. These

beneficial effects of FK409 are mediated by spontaneously released NO, which is considered to be important for the prevention of acute coronary syndromes such as unstable angina pectoris and acute myocardial infarction in patients

(Hjemdahl-Monsen *et al.*, 1986; Fuster *et al.*, 1988). The preventive effects of FK409 in experimental animal models with acute coronary syndromes will be evaluated in future work.

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(Received February 25, 1994

Revised May 3, 1994

Accepted June 1, 1994)