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FR144420, a novel, slow, nitric oxide-releasing agent

Yasuhiro Kita [a,*], Kazumi Ohkubo [a], Yoshimi Hirasawa [a], Yoshiki Katayama [b], Mitsuko Ohno [a], Shigetaka Nishino [a], Masayuki Kato [a], Keizo Yoshida [a]

[a] *New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-chome, Kashima, Yodogawa-ku, Osaka 532, Japan*
[b] *Dojindo Laboratories, Kumamoto Techno Research Park, Tabaru 2025-5 Mashiki-machi, Kamimashiki-gun, Kumamoto 861-22, Japan*

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Abstract

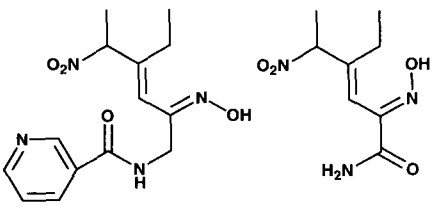
We report that (±)-(*E*)-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexeneamide (FK409) decomposes and releases nitric oxide (NO) spontaneously in solution. (±)-*N*-[(*E*)-4-Ethyl-3-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl]-3-pyridinecarboxamide (FR144420) was synthesized with the aim of discovering a compound with longer duration of effects in vivo, compared with FK409. FR144420, like FK409, released NO spontaneously in solution, but the amount of NO released from FR144420 during a 5-min incubation was half the amount from FK409. In addition, FR144420 spontaneously decomposed and generated nitrite, which is an oxidative metabolite of NO, at half the rate of FK409. In a vasorelaxant study with isolated rat aorta, FR144420 had a weaker potency than FK409 (EC_{50} = 54 and 8.1 nM, respectively). In in vivo studies, FR144420 decreased mean blood pressure immediately after intravenous and oral administration to conscious rats. The maximum hypotensive effects of FR144420 were less than those of FK409. However, the durations of FR144420-induced (i.v. and p.o.) hypotensive effects were longer than those of FK409-induced effects. In conclusion, FR144420 is more stable and releases NO more slowly in solution than does FK409. In in vivo experiments, FR144420 showed a longer duration of effects than FK409. FR144420 may be very useful for investigating the in vivo actions of NO.

Keywords: FR144420; FK409; Nitric oxide (NO)

1. Introduction

(±)-(*E*)-Ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexeneamide (FK409) (Fig. 1) is a structurally unique compound, which has been discovered from the fermentation products of *Streptomyces griseosporeus*, with vasorelaxant and antiplatelet activities (Hino et al., 1989). Recently we have reported that these vasorelaxant and antiplatelet effects are due to NO released spontaneously from FK409 (Kita et al., 1994a).

In in vivo experiments, FK409 shows beneficial effects in the treatment of ischemic cardiovascular diseases. FK409 has shown antianginal effects in dog (Isono et al., 1993) and rat coronary ischemic models (Kita et al., 1994b). In addition, FK409 suppresses myocardial infarction following occlusion and reperfusion in a rat coronay artery (Kita et al., 1994c), and inhibits thrombus formation in a rat extracorporeal model (Kita et al., 1994d). FK409, however, is very unstable and releases NO rapidly in plasma (Kita et al., 1994c), so the compound may have limited clinical use. A compound with prolonged NO-releasing ability could probably be useful for investigating the in vivo actions of NO. Recently, we have discovered a derivative, ((±)-*N*-[(*E*)-4-ethyl-3-[(*Z*)-hydroxyimino]-5-nitro-3-



FR144420 FK409

Fig. 1. Chemical structures of FR144420 and FK409.

* Corresponding author. Department of Pharmacology, New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-chome, Kashima, Yodogawa-ku, Osaka 532, Japan. Tel. +81-6-390-1144, fax +81-6-304-1192.

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hexen-1-yl]-3-pyridinecarboxamide; FR144420) (Fig. 1), of FK409, which has a longer duration of activity.

In the present study, we evaluated FR144420 and FK409 focussing on the NO-releasing rate in vitro and the duration of the hypotensive effects in vivo. In in vitro experiments, the decomposition rate and NO-releasing rate of FR144420 were compared with those of FK409. In addition, the vasorelaxant effects of both drugs in isolated rat aorta were also compared. In in vivo experiments, we evaluated the hypotensive effects of FR144420 and FK409 and compared the durations of the effects of both drugs. Finally we related the results of in vitro experiments to those of in vivo experiments.

2. Materials and methods

2.1. Determination of the concentration of NO released

FR144420 or FK409 was dissolved in dimethyl sulfoxide at a concentration of 25 mM. From each drug solution, 0.1 ml was added to 4.9 ml of sodium phosphate buffer (PB) solution (0.1 M; pH 7.4) containing 1.0 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) and the mixture was incubated at 37°C. After 5 min, aliquots were dispersed into a quartz flat cell (inner size: $60 \times 10 \times 0.31$ mm) and the concentration of released NO was determined by electron spin resonance (ESR) spectroscopy (JES FR-80, JEOL, Tokyo, Japan). Since C-PTIO reacts with NO in a molar ratio of 1:1 (Akaike et al., 1993), the NO concentration in PB solution was calculated from the decrease in the peak height of the ESR signal of C-PTIO in the lowest magnetic field. The conditions for ESR measurement were as follows: modulation frequency, 100 kHz; modulation amplitude, 0.25 mT; scanning field, 335.1 ± 5.0 mT; response time, 2 min; microwave power, 5 mW; microwave frequency, 9.422 GHz.

2.2. Determination of the concentrations of each drug and nitrite

FR144420 or FK409 was dissolved in dimethyl sulfoxide at a concentration of 25 mM. From each drug solution, 0.1 ml was added to 4.9 ml of PB solution and the mixture was incubated at 37°C. Prior to incubation and at various time intervals, aliquots were taken for the determination of the concentrations of drug and nitrite, respectively. For the determination of the concentration of each drug, 0.4 ml of a drug solution was added to 0.05 ml of 0.1 N HCl to stop decomposition and 0.01 ml of the mixture was injected into a high performance liquid chromatography system (LC-9A, Shimazu, Kyoto, Japan). The high performance liquid chromatography system consisted of a mobile phase of acetonitrile and distilled water (27:73) pumped through a CAPCEL-PAK C18 column (SG-120, Kanebo, Tokyo, Japan) with a column size of 4.6×150 mm at a flow rate of 1.0 ml/min. Both drugs were detected at 254 nm. The concentration of nitrite was determined by diazotization. To 0.05 ml of each drug solution, 3.95 ml of 0.5 N HCl, 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, Shimazu). For the standard curve, sodium nitrite was used under the same experimental conditions.

2.3. In vitro vasorelaxant study

Male Sprague-Dawley rats, weighing 290–340 g, were killed by stunning and exsanguination. The thoracic aorta was removed and cut into helical strips after removal of excess fat and connective tissues. The strip was mounted vertically in an organ bath containing 25 ml of Tyrode solution of the following composition: 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.4 mM NaH_2PO_4 and 5.6 mM dextrose. Isometric tension was measured with a force-displacement transducer (UL-10GR, Shinkoh, Tokyo, Japan) connected to an amplifier (AP-601G, Nihon Kohden, Tokyo, Japan) and was recorded with a polygraph (Recti-Horiz-8K, Sanei, Tokyo, Japan). The organ bath solution was maintained at 37°C and bubbled with a 95% O_2 and 5% CO_2 gas mixture. After the resting tension was adjusted to 0.5 g, the strip was contracted with 0.25 ml of (–)-norepinephrine (final concentration: 32 nM) 10 min after the addition of 0.25 ml of L-ascorbic acid (final concentration: 57 μM). Each drug, dissolved in dimethyl sulfoxide, was added to the organ bath cumulatively. The cumulative addition of DMSO as vehicle had no effect on the contraction. Finally, 0.25 ml of papaverine (final concentration: 100 μM) was added to the organ bath to obtain the maximum relaxation.

2.4. In vivo tests

2.4.1. Animals and drug administration

In i.v. experiments, male Sprague-Dawley rats, without being fasted, were given intravenously each drug dissolved in a mixture of polyethylene glycol, ethanol and distilled water (1:1:2), or vehicle only in a volume of 0.5 ml/kg. In p.o. experiments, male Sprague-Dawley rats were fasted for 24 h. The rats were given each drug orally, suspended with 0.5% methylcellulose, or vehicle only in a volume of 5 ml/kg.

2.4.2. Measurement of mean blood pressure in conscious rats

After male Sprague-Dawley rats, weighing 290–355 g (in i.v. experiments) and 245–325 g (in p.o. experiments), were anesthetized with diethyl ether, a polyethylene cannula filled with heparin solution was inserted into the left femoral artery to measure mean blood pressure. Another polyethylene cannula filled with saline was inserted into the left femoral vein to administer the drug intravenously. Mean blood pressure was measured with a pressure transducer (TP-400T, Nihon Kohden) connected to an amplifier and was recorded on a polygraph. Each drug or vehicle was administered intravenously or orally 2 h after cannulation. Changes in mean blood pressure were expressed as percentages of the pre-administration value.

2.5. Materials

FR144420 and FK409 were synthesized by Fujisawa Pharmaceutical Co. (Osaka, Japan). Carboxy PTIO was synthesized by Dojindo Laboratories (Kumamoto, Japan). Sulfanilic acid, *N*-(-naphthyl)-ethylenediamine, sodium nitrite, L-ascorbic acid and papaverine hydrochloride were purchased from Nacalai Tesque Co. (Kyoto, Japan). (−)-Norepinephrine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.6. Statistical analysis and data analysis

The data are presented as the means ± S.E.M. for the number of experiments indicated. For multiple comparisons, the data were analyzed using one-way analysis of variance followed by Dunnett's test. The EC_{50} value expressed the drug concentration required to produce 50% of the papaverine-induced (100 μM) relaxation in isolated rat aorta. The EC_{50} value was logistically computed by regression analysis.

3. Results

3.1. The concentration of NO released

C-PTIO reacts with NO but not with nitrite or nitrate, which are oxidative metabolites of NO (data not shown). C-PTIO immediately reacted with NO released from FR144420 and FK409. As shown in Fig. 2, the concentrations of NO released from FR144420 (0.5 mM) and FK409 (0.5 mM) during 5-min incubation in PB solution were 0.050 ± 0.015 and 0.110 ± 0.006 mM, respectively. The amount of NO released from FR144420 was about half the amount from FK409.

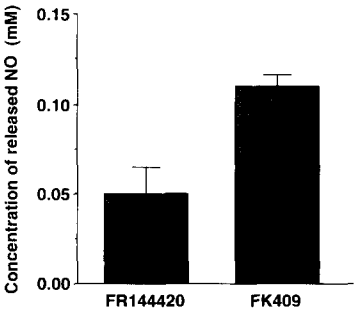

Fig. 2. The concentration of NO released from FR144420 and FK409 during 5-min incubation in PB solution at 37°C. The initial concentration of each drug was 0.5 mM. Each value represents the mean ± S.E.M. for three experiments.

3.2. Stability of each drug in solution

The decomposition curves of FR144420 and FK409 are shown in Fig. 3A. Although both compounds are stable as solids, decomposition occurred immediately when they were dissolved in PB and incubated at 37°C. First-order decomposition was observed for FR144420 and FK409, the rate constants and the correlation coefficients being 0.0087 1/min and 1.000 for FR144420, and 0.0187 1/min and 1.000 for FK409, respectively. FR144420 is about twice as stable as FK409.

As shown in Fig. 3B, FR1444420 and FK409 generated nitrite, an oxidative metabolite of NO, spontaneously and time dependently. First-order generation

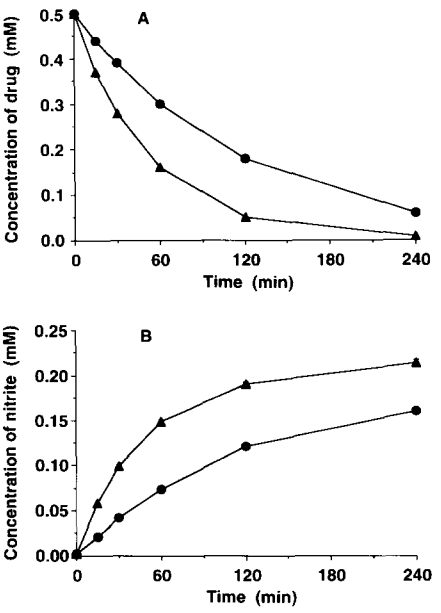

Fig. 3. Stability of FR144420 (●) and FK409 (▲) in PB solution at 37°C. (A) Time-dependent decomposition of FR144420 and FK409, and (B) time-dependent generation of nitrite from FR144420 and FK409. The initial concentration of each drug was 0.5 mM. Each value represents the mean ± S.E.M. for three experiments in the study of nitrite generation.

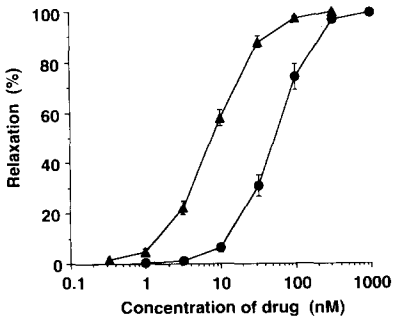

Fig. 4. The vasorelaxant effects of FR144420 (●) and FK409 (▲) on norepinephrine-induced (32 nM) contraction in isolated rat aorta. Relaxation with papaverine (100 μM) was expressed as 100%. Each value represents the mean ± S.E.M. for five experiments.

of nitrite was observed for FR144420 and FK409, the rate constants and the correlation coefficients for the ln([nitrite]$_\infty$ – [nitrite]) versus time plots being 0.0081 ± 0.0002 l/min and 0.999 ± 0.000 for FR144420, and 0.0159 ± 0.0005 l/min and 0.999 ± 0.001 for FK409, respectively. The nitrite-generating rate of FR144420 was about half the rate of FK409.

3.3. In vitro vasorelaxation

In isolated rat aorta contracted with norepinephrine, cumulative addition of FR144420 (1.0–1000 nM) or FK409 (0.32–320 nM) induced a concentration-dependent relaxation and the maximum relaxation produced by each drug was about 100%. Concentration-relaxation curves for both drugs are presented in Fig. 4. The EC_{50} values of FR144420 and FK409 were 54 ± 6 nM and 8.1 ± 0.8 nM, respectively. Thus, the vasorelaxant potency of FR144420 is weaker than that of FK409. The EC_{50} values of FK409 in the present study were greater than the value (1.0 nM) previously reported by us (Kita et al., 1994a). Although FK409 was dissolved in distilled water initially in the previous study, the compound was dissolved in dimethyl sulfoxide in the present study. The difference in the EC_{50} values of FK409 may have been due to the difference in the solvents for FK409.

3.4. Effects on mean blood pressure

Figs. 5 and 6 present the effects of drugs given intravenously and orally on mean blood pressure in conscious rats, respectively. Mean blood pressure values before intravenous administration of vehicle, FR144420 (1.0 and 3.2 mg/kg) and FK409 (1.0 and 3.2 mg/kg) were 120 ± 4, 121 ± 4, 124 ± 3, 122 ± 2 and 119 ± 4 mm Hg. The values before oral administration of vehicle, FR144420 (1.0 and 3.2 mg/kg) and FK409 (1.0 and 3.2 mg/kg) were 115 ± 2, 121 ± 3, 118 ± 1, 115 ± 2 and 126 ± 4 mm Hg. The pre-administration values were not significantly different among all groups

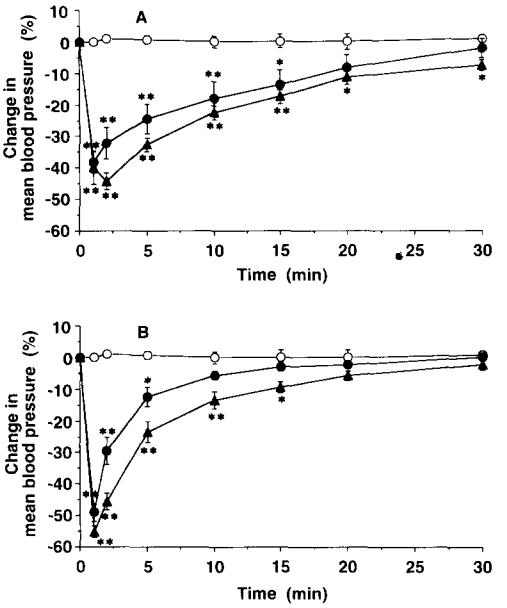

Fig. 5. Time course of the effects of intravenously administrered (A) FR144420 and (B) FK409 (○: vehicle, ●: 1.0 mg/kg, ▲: 3.2 mg/kg) on mean blood pressure. Changes in mean blood pressure were expressed as percentages of the pre-administration value. Each value represents the mean ± S.E.M. for five experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated group.

in both experiments. The mean blood pressure decreased immediately after intravenous and oral administration of both drugs. The maximum hypotensive effects of FR144420 were less than those of FK409 at

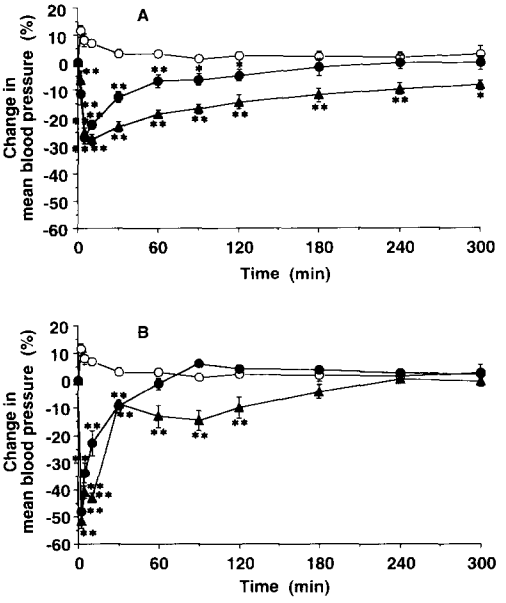

Fig. 6. Time course of the effects of orally administrered (A) FR144420 and (B) FK409 (○: vehicle, ●: 10 mg/kg, ▲: 32 mg/kg) on mean blood pressure. Changes in mean blood pressure were expressed as percentages of the pre-administration value. Each value represents the mean ± S.E.M. for five experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle-treated group.

the same doses in both experiments. However, the FR144420-induced hypotensive effects lasted longer than the FK409-induced effects. In the i.v. experiment, significant decreases in mean blood pressure lasted 15 and 30 min after the administration of 1.0 and 3.2 mg/kg of FR144420, respectively, whereas they lasted 5 and 15 min after the administration of the same doses of FK409, respectively. In the p.o. experiment, significant decreases in mean blood pressure lasted 120 and 300 min after the administration of 1.0 and 3.2 mg/kg of FR144420, respectively, whereas they lasted 30 and 120 min after the administration of the same doses of FK409, respectively.

4. Discussion

FK409 decomposes and releases NO immediately when the compound is dissolved in PB solution (Kita et al., 1994a). FK409 is so unstable and releases NO so rapidly that the biological activity in vivo does not last for a long period. FR144420, a derivative of FK409, has been discovered to be a compound with slow NO-releasing activity in solution. In the present studies, FR144420 also decomposed and released NO spontaneously in PB solution. The amount of NO generated from FR144420 during a 5-min incubation and the decomposition rate and the nitrite-generating rate of FR144420 were half those for FK409, as expected. These data were reflected in the results of an in vitro vasorelaxant study. The potency of FR1444420 in the study was weaker than that of FK409.

As described above, slower decomposition of FR144420 led to slower NO release. These facts show that the NO-releasing rates of FR144420 and FK409 depend on the decomposition rates of the compounds. In a stability study with high performance liquid chromatography, we observed the first step of the decomposition pathway of FR144420 and FK409. The first step of the decomposition of FR144420 and FK409 is probably a rate-limiting stage in the NO-releasing pathway of the compounds. Recently, we have clarified, using nuclear magnetic resonance spectroscopy, that the first step of the decomposition (NO-releasing) pathway is the subtraction reaction of the hydrogen bound at the α-carbon of the nitro moiety (data not shown).

We examined whether slower NO release from FR144420, compared with that from FK409, in in vitro experiments would result in a longer duration of activity of FR144420 in in vivo experiments. FR144420 significantly decreased mean blood pressure for a longer duration after both i.v. and p.o. administration in rats, compared with FK409. These results are probably due to better stability of FR144420. In addition, the maximum hypotensive effects of FR144420 were less than those of FK409 at the same doses in both in vivo experiments. These weaker responses to FR144420 reflected the weaker potency of the compound in the in vitro vasorelaxant study. Taking these data into account, it is considered that the differences in the in vitro and in vivo responses are due to differences in the rates at which these compounds decompose and release NO. In addition, although we have reported that oral absorption of FK409 is rapid (Kita et al., 1994a), the absorption of FR144420 also seems to be rapid, judging from the quick hypotensive responses in the present p.o. experiments of FR144420 and FK409.

The continuous administration of organic nitrates such as glyceryl trinitrate and isosorbide dinitrate leads to the development of tolerance as seen from the reduced vasorelaxant effects of the compounds (Zelis and Hason, 1975; Thadani et al., 1980). It is widely accepted that nitrate tolerance is produced by depletion of reduced sulfhydryl groups in the body during continuous nitrate exposure, leading to reduced biotransformation of organic nitrates to NO and to diminished vasodilation (Ignarro et al., 1981). On the other hand, FK409 can release NO spontaneously in contrast to organic nitrates, so continuous FK409 exposure would not be expected to lead to depletion of reduced sulfhydryl groups. This prediction is supported by the observation that tolerance to the hypotensive effect of FK409 is less than that to the effect of glyceryl trinitrate following continuous administration of either drug in rats (Isono et al., 1994). FR144420 spontaneously released NO. Therefore, FR144420, like FK409, is also expected to lead to less tolerance in vivo.

In conclusion, FR144420 is more stable than FK409 in solution and this results in a slower and more prolonged NO release in in vitro experiments. In addition, FR144420 has shown a longer duration of hypotensive effects than FK409 in in vivo experiments. Therefore FR144420 may be very useful for investigating the in vivo actions of NO.

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