Acceleration of Nitric Oxide (NO) Release from FK409, a Spontaneous NO Releaser, in the Presence of Sulfhydryl-bearing Compounds

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Purpose: Recently, we have reported that FK409 spontaneously releases nitric oxide (NO) in solution. In the present study, the influence of L-cysteine (Cys) and glutathione (GSH), which are typical sulfhydryl group-bearing compounds, on NO release from FK409 and biological action of FK409 was examined.

Methods. We evaluated the effects of Cys and GSH on NO release from FK409 by nitrite analysis or detection with a chemiiluminesence analyzer. In a biological study, the influence of Cys on inhibition of rat platelet aggregation of FK409 was investigated. In addition, the above mentioned characteristics of FK409 were compared with those of isosorbide dinitrate (ISDN).

Results. FK409 decomposed spontaneously with generation of nitrite in solution. Both Cys and GSH accelerated decomposition of FK409 and nitrite generation from FK409 in a concentration-dependent manner. When the NO levels in the headspace of FK409 solutions (0.5 mM) reached equilibrium with and without 25 mM Cys, the constant rate for NO release from FK409 in the presence of Cys was 13 times larger than that in the absence of Cys. In biological study, FK409 (100 μM) showed 56 and 90% inhibition of rat platelet aggregation in the absence and presence of 10 mM Cys, respectively, whereas ISDN (100 μM) showed 10 and 23% inhibition, respectively.

Conclusions. Decomposition of FK409 with generation of NO is spontaneous, and is accelerated in the presence of sulfhydryl group-bearing compounds, thereby potentiating the biological action of FK409.

KEY WORDS: FK409; isosorbide dinitrate; nitric oxide; L-cysteine; antiplatelet activity.

INTRODUCTION

(±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (FK409), whose chemical structure is shown in Figure 1, has been reported to be a structurally unique compound discovered from the fermentation products of *Streptomyces griseosporeus* (1) and shows vasorelaxant and antiplatelet effects *in vitro* (1) and *in vivo* (2). FK409 produces a potent vasorelaxation mediated by the elevation of cyclic guanosine-5′-monophosphate (cGMP) in isolated dog coronary artery (3). Thus, FK409 appears to have a mode of action similar to that of organic nitrates. Recently, we reported that a decrease in mean blood pressure by FK409 closely relates to an increase in plasma cGMP level in rat *in vivo* experiments (2). In addition, we have also shown that FK409 spontaneously decomposes and releases NO in solution (2). These facts indicate that biological actions of FK409 are induced through an increase in cGMP *via* NO released from FK409.

Biotransformation of organic nitrates such as glyceryl trinitrate (GTN) and isosorbide dinitrate (ISDN) to NO is required for their vasorelaxant and antiplatelet effects. Glutathione-S-transferase or cytochrome P_{450} in the liver and kidney is involved in the biotransformation of GTN to NO (4,5). Recently, it has been reported that a microsomal protein (approximately 200 kDa) found in vascular smooth muscle cells, which is neither glutathione-S-transferase nor cytochrome P_{450}, biotransforms some organic nitrates to NO (6,7). In addition, NO can be released from organic nitrates by a non-enzymatic mechanism involving the interaction with the sulfhydryl groups of some thiol compounds such as L-cysteine (Cys) (8).

Although sulfhydryl groups exist in high concentrations in the human body as thiol compounds, such as free Cys and glutathione (GSH) molecules or Cys residues in many polypeptides, it remains unclear whether decomposition and NO release in FK409 are affected by thiol compounds. It is important to obtain information on the effects of thiol compounds on NO release from FK409 in order to understand the influence of sulfhydryl groups on metabolism and biological activities of FK409 *in vivo*. In the present study, we evaluated the effects of Cys and GSH, which are typical sulfhydryl-bearing compounds, on NO release from FK409 and on the antiplatelet action of FK409, in a biological study. In addition, these characteristics of FK409 were compared with those of ISDN (Figure 1), which is an organic nitrate with two $O-NO_2$ groups in the structure, since we have previously compared many biological actions of FK409 with those of ISDN (2).

MATERIALS AND METHODS

Materials

FK409 and ISDN were prepared at Fujisawa Pharmaceutical Co. Ltd., (Osaka, Japan). L-Cysteine, L-serine, sulfanilic acid, N-(1-naphthyl)-ethylenediamine and sodium nitrite were purchased from Nacalai Tesque (Kyoto, Japan). Glutathione, sodium citrate and adenosine-5′-diphosphate (ADP) were purchased from Sigma Chemical (St. Louis, Missouri).

Animals

Male Sprague-Dawley rats (Nihon SLC, Shizuoka, Japan), weighing 345–385 g and 285–340 g, were used to obtain plasma for nitrite generation study and platelet aggregation study, respectively.

Determination of Drug Concentration

FK409 or ISDN was dissolved at a concentration of 1.5 mM in 0.1 M sodium phosphate buffer (PB) solution at pH 7.4 (pH of the solution was adjusted to 7.4 by sodium hy-

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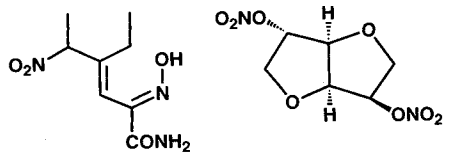

FK409 ISDN

Fig. 1. Chemical structures of FK409 and ISDN.

droxide solution, if necessary) with or without various amounts of Cys, GSH, and Ser. The samples were immediately incubated at 37°C. Prior to incubation and after 15 min, 0.5 ml of the solution was added to 0.5 ml of 1 % trifluoroacetic acid (TFA) to stop decomposition and 5 μl of the mixture was injected into a HPLC system, consisting of a SPD-2A variable-wavelength detector (Shimadzu, Kyoto, Japan) or a L-4000 UV detector (Hitachi, Tokyo, Japan) operating at 230 nm; a LC-9A pump (Shimadzu) or a L-6000 pump (Hitachi); a SIL-9A auto injector (Shimadzu) and a C-R5A integrator (Shimadzu) for peak processing. The HPLC conditions were as follows: mobile phase, distilled water and acetonitrile (75:25 for FK409 and 55:45 for ISDN) containing 0.05 % TFA solution; column, YMC R-ODS-1 5-ST (4.6 i.d. × 150 mm, YMC, Kyoto, Japan); flow rate, 0.9 ml/min for FK409 and 1.1 ml/min for ISDN.

Determination of Nitrite Concentration

In the study of nitrite generation in PB solution, FK409 or ISDN was dissolved at a concentration of 1.5 mM in 0.1 M PB solution at pH 7.4 (pH of the solution was adjusted to 7.4 by sodium hydroxide solution, if necessary) with or without various amounts of Cys, GSH, and Ser. The samples were immediately incubated at 37°C. The concentration of nitrite was determined by diazotization. To 0.05 ml of a drug solution prior to incubation, and after 15 min, 3.95 ml of 0.05 N HCl, 0.5 ml of 0.2% sulfanilic acid and 0.5 ml of 0.1% N-(1-naphthyl)-ethylenediamine were added subsequently. Absorbance of a purple dye at 548 nm was measured with a spectrophotometer (UV-1200, Shimadzu). For the standard curve, sodium nitrite was used under the same experimental conditions.

In the study of nitrite generation in plasma, FK409 or ISDN was dissolved at a concentration of 100 μM in 2 ml of plasma with or without 10 mM Cys and immediately incubated at 37°C. Plasma was obtained from the supernatant fraction after centrifugation (1000 × g for 10 min) of blood collected from the rat abdominal aorta into plastic vessels containing 4% tripotassium ethylenediamine tetraacetate (1/40 volume). To 0.2 ml of a drug solution, 3.8 ml of 0.01 N HCl, 0.5 ml of 0.2% sulfanilic acid and 0.5 ml of 0.1% N-(1-naphthyl)-ethylenediamine were subsequently added at 5 min after incubation of each drug. Absorbance of a purple dye at 548 nm was measured with a spectrophotometer (UV-2200, Shimadzu). For the standard curve, sodium nitrite was used under the same experimental conditions.

Determination of the NO Level in the Headspace of Drug Solution

The NO level in the headspace of FK409 solution was determined with a redox chemiluminescence analyzer (FES-450, Scholar Tec, Osaka, Japan) (9). FK409 was dissolved at a concentration of 0.5 mM in 20 ml of 0.1 M PB solution (pH 7.4) with or without 25 mM Cys, pre-flushed with N_2 gas in plastic vessels. The drug solution was incubated at 37°C and flushed with N_2 gas. N_2 gas was drawn through one needle into the drug solution and the headspace gas was then directed via a vacuum through a second needle directly into the NO analyzer and reacted with O_3 gas. The flow rate of total gas was 2.6 l/min (sample gas: 0.8 l/min, O_3 gas: 1.8 l/min) at one atmosphere. The NO level was measured with a time resolution of 150 ms per point. For the standard curve of NO, NO gas was injected into the NO analyzer, directly.

Platelet Aggregation Study

Blood from rats, which were anaesthetized with diethyl ether, was collected from abdominal aorta into plastic vessels containing 2.2% trisodium citrate (1/10 volume). Platelet-rich plasma (PRP) was obtained from the upper layer after centrifugation of blood at 200 × g for 10 min. The effects of FK409 or ISDN on platelet aggregation were determined by Born's turbidimetric method (10) using an aggregometer (Hema Tracer 801, Niko Bioscience, Tokyo, Japan). To 220 μl of PRP in the cuvette, 12.5 μl of 200 mM Cys dissolved in 25 mM Tris-acetate and 120 mM NaCl buffer (pH 7.4) or vehicle and 12.5 μl of drug dissolved in the buffer or vehicle were added and incubated at 37°C for 5 min. After incubation, platelet aggregation was induced by the addition of 5 μl of 100 μM ADP. 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.

Data Analysis

Data were presented as the means ± S.E.M. for the number of experiments as indicated. Student's t-test was used to determine significance of differences between the groups with and without Cys.

RESULTS

Stability of Drug in PB Solution

Decomposition of FK409 and ISDN was studied as a function of concentrations of Cys, GSH and Ser (Figure 2). After 15 min of incubation, 28% of FK409 spontaneously decomposed. Both Cys and GSH obviously accelerated decomposition of FK409 in a concentration-dependent fashion, however, it was not the case with Ser. In the presence of 25 mM Cys or GSH, the concentration of decomposed FK409 was approximately 2.5 times larger than that in the absence of them. On the other hand, ISDN did not decompose spontaneously. However, its decomposition occured only in the presence of Cys or GSH.

Nitrite Generation in PB Solution

Nitrite generation from FK409 and ISDN was studied as a function of concentrations of Cys, GSH and Ser (Figure 3). FK409 spontaneously generated nitrite, which is the oxida-

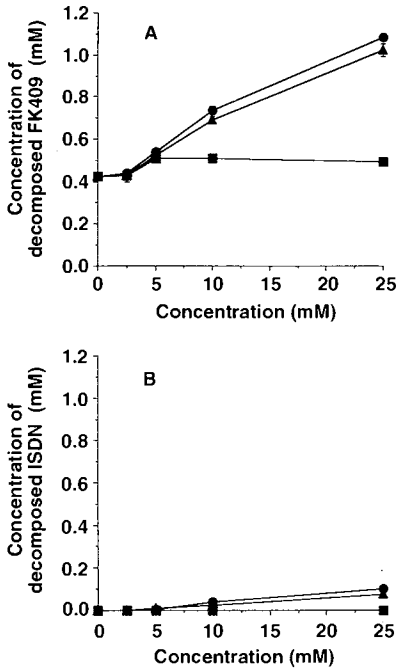

Fig. 2. Decomposition of (A) FK409 and (B) ISDN in PB solution as a function of concentrations of Cys (●), GSH (▲) and Ser (■) at 37°C. The initial concentration of each drug was 1.5 mM. The concentration of each drug decomposed was determined 15 min after incubation. Each value represents the mean ± S.E.M. for three experiments.

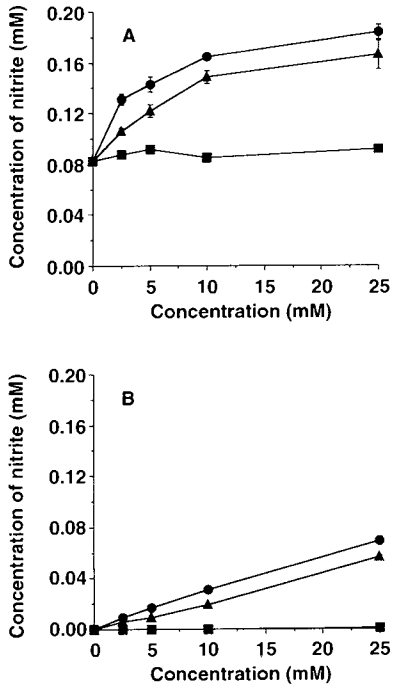

Fig. 3. Nitrite generation from (A) FK409 and (B) ISDN in PB solution as a function of concentrations of Cys (●), GSH (▲) and Ser (■) at 37°C. The initial concentration of each drug was 1.5 mM. The concentration of nitrite generated was determined 15 min after incubation of each drug. Each value represents the mean ± S.E.M. for three experiments.

tive product of NO, 15 min after incubation. Both Cys and GSH enhanced nitrite generation in a concentration-dependent fashion, however, Ser had no effect. The concentration of nitrite generated with 25 mM Cys or GSH was 2 times larger than that without them after 15 min of incubation. On the other hand, ISDN generated nitrite only in the presence of Cys or GSH.

NO Release from Drug Solution

The NO levels in the headspace of FK409 solutions are shown in Figure 4. The NO level in the headspace began to increase immediately when FK409 was dissolved in PB with or without 25 mM Cys and incubated at 37°C. In the absence of Cys, the NO level in the headspace reached equilibrium at a level of 0.1 ppm after 8 min of incubation and remained at this level for more than 5 min. The constant rate for NO release into the headspace was then calculated to be 10.6 nmol/min. In the presence of Cys, the NO level in the headspace reached the maximal level (1.3 ppm) after 2 min of incubation and remained at this level for 6 min. The constant rate for NO release into the headspace was then calculated to be 138.3 nmol/min. The constant rate for NO release in the presence of Cys was 13 times larger than that in the absence of Cys.

Nitrite Generation in Plasma

The concentrations of nitrite generated from FK409 and ISDN for 5 min of incubation at 37°C in plasma are shown in Figure 5A. FK409 generated nitrite in plasma with and with-

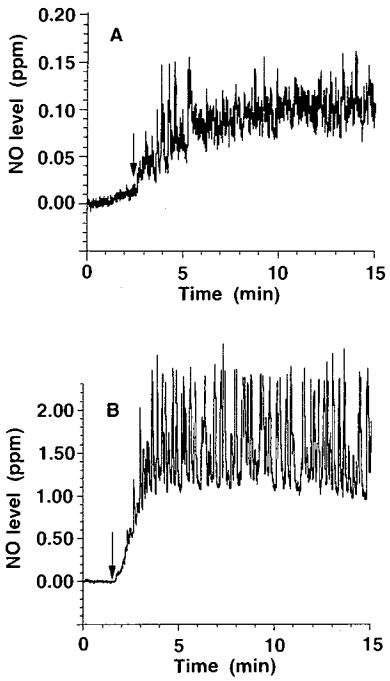

Fig. 4. Time-dependent NO generation from FK409 into the headspace of PB solution (A) without and (B) with 25 mM Cys at 37°C. The initial concentration of each drug was 0.5 mM. The time resolution was 150 ms per point. At the time pointed by (↓), FK409 was added to PB solution pre-flushed with N_2 gas.

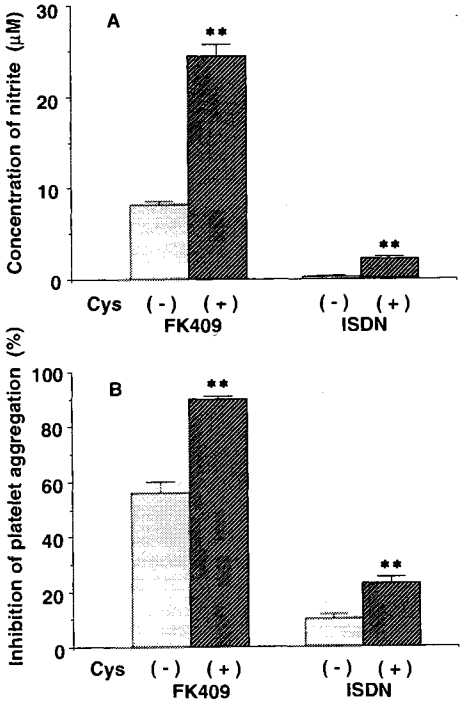

Fig. 5. (A) Nitrite generation from FK409 and ISDN in plasma with and without 10 mM Cys at 37°C. The initial concentration of each drug was 100 μM. The nitrite concentration was determined 5 min after incubation of each drug at 37°C. Each value represents the mean ± S.E.M. for three experiments. $^{**}P < 0.01$ compared with the group without Cys. (B) The inhibitory effects of FK409 (100 μM) and ISDN (100 μM) on ADP-induced platelet aggregation in rat PRP with and without 10 mM Cys. ADP was added 5 min after incubation of each drug or vehicle at 37°C. The effects of each drug were expressed as % inhibition of ADP-induced platelet aggregation compared with vehicle treatment. Each value represents the mean ± S.E.M. for five experiments. $^{**}P < 0.01$ compared with the group without Cys.

out 10 mM Cys. The concentration of nitrite generated for 5 min of incubation in the presence of Cys was significantly larger than that in the absence of Cys (0.0244 ± 0.0013 and 0.0082 ± 0.0003 mM, respectively). The concentration of nitrite generated from ISDN for 5 min of incubation in plasma with 10 mM Cys was also significantly larger than that in plasma without Cys (0.0021 ± 0.0002 and 0.0002 ± 0.0002 mM, respectively).

Antiplatelet Effects

Figure 5B shows the inhibitory effects of FK409 and ISDN on ADP-induced platelet aggregation in rat PRP. Each drug (100 μM) was pre-incubated for 5 min in PRP with or without 10 mM Cys. FK409 showed 56 ± 4% and 90 ± 1% inhibition of platelet aggregation in the absence and presence of Cys, respectively. These inhibitory effects were significantly different. ISDN also showed more potent inhibitory effect in the presence of Cys than that in the absence of Cys (23 ± 2 and 10 ± 2% inhibition, respectively).

DISCUSSION

Sulfhydryl groups exist in high concentrations in the human body as thiol compounds, including free Cys and GSH molecules, or Cys residues in many polypeptides. Therefore, it is important to obtain information on the effects of thiol compounds on NO release from FK409 in order to understand the influences of sulfhydryl groups on metabolism and biological activities of FK409 *in vivo*. In this paper, we studied the influence of thiol compounds such as Cys and GSH on NO release from FK409 and biological activity of FK409. The experiments reported here were designed to be suited to the concentrations of sulfhydryl groups in human blood (10–20 mM) as reported by Ellman (11).

Decomposition and generation of nitrite, which is the oxidative product of NO, in FK409 were enhanced in the presence of Cys or GSH in a concentration-dependent manner. But, Ser which is an amino acid containing no sulfhydryl moiety had no effect on decomposition and nitrite generation in FK409. On the other hand, ISDN did not decompose and generate nitrite in solution without Cys or GSH, in contrast to FK409. These results showed that the NO-releasing mechanism of FK409 appeared to be different from that of ISDN.

In addition, we confirmed that NO release from FK409 was accelerated by Cys with a chemiluminescence analyzer. The difference in the enhancement of the rate between NO generation detected with a chemiluminescence analyzer (13 times) and nitrite generation from FK409 (2 times) in the presence of 25 mM Cys, is based on the difference of each detection method for NO and nitrite generated from FK409. Thus, the time course of concentrations of nitrite generated from FK409 represents the rate of nitrite generation. On the other hand, the rate detected by the chemiluminescence method does not indicate the rate of NO generation from FK409 but the quantity of NO expelled by N_2 gas from the sample solution.

We have shown that FK409 has more potent antiplatelet activity than ISDN in human PRP and this activity of FK409 depends on spontaneous NO release (2). The antiplatelet effect, which was potentiated by Cys, of FK409 (100 μM) was more potent than that of ISDN (100 μM) in rat PRP. In order to explain the differences in potency of FK409 and ISDN, we determined the concentration of nitrite generated from the compounds in plasma. As seen in Figure 5A, nitrite generation from FK409 and ISDN was enhanced in the presence of Cys, and the concentration of nitrite generated from FK409 was larger than that from ISDN. These results indicate that potentiation of antiplatelet effects of FK409 and ISDN in the presence of thiol compounds is attributable to enhancement of NO generation from FK409 and ISDN by sulfhydryl groups on their molecules. Antiplatelet effects of FK409 and ISDN closely correlated with the concentrations of nitrite generated from the compounds in rat plasma. When the correlation was fitted with a sigmoidal curve as follows;

$$y = -100/\{1 + (x/IC_{50})^a\} + 100$$

where y was the % inhibition of platelet aggregation; x, the concentration of nitrite generated for 5 min; IC_{50}, the concentration of nitrite required to produce 50% inhibition of platelet aggregation; a, a "slope factor" that determined the steepness of the curve, the IC_{50} value, a value, and correlation coefficient (r) were calculated to be 5.90 μM, 1.22 and 0.987, respectively.

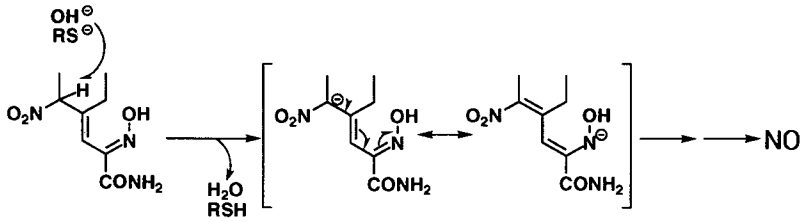

Fig. 6. Proposed scheme of NO release from FK409.

It has been reported that the nucleophilic attack by thiolate anion on the nitrogen atom of $O-NO_2$ group of GTN is involved in the NO-releasing pathway of GTN (12). Since FK409 has no $O-NO_2$ group, unlike organic nitrates, the NO-releasing pathway of FK409 appears to be different from that of ISDN. Recently, we suggested that the essential step of NO release from FK409 is the deprotonation reaction of the α-hydrogen atom of the nitro moiety, by ^{1}H-NMR analysis (13). We considered that thiolate anion affected the first step of FK409 degradation, and acted as a base, like hydroxyl ions, as shown in Figure 6. Thus, degradation and NO release in FK409 were accelerated in the presence of sulfhydryl-bearing compounds.

The continuous administration of organic nitrates such as ISDN and GTN leads to the development of tolerance as seen in the reduced vasorelaxant effects of ISDN and GTN and cross tolerance between ISDN and GTN (14). It is most widely accepted that nitrate tolerance is produced by depletion of reduced sulfhydryl group in the body during continuous nitrate exposure, leading to reduced biotransformation of organic nitrates to NO and to diminished vasodilation (15). The nucleophilic attack by thiolate anion is probably a trigger for this depletion of reduced sulfhydryl groups. This contrasts with the mechanism involved in the NO-releasing pathway of FK409, where the thiolate anion may be acting as a base, as hypothesized above. In this case, continuous FK409 exposure would not be expected to lead to depletion of reduced sulfhydryl groups. This prediction is supported by the observation of less tolerance to the vasorelaxant effect of FK409 than that of GTN in isolated dog coronary arteries following continuous exposure to each drug (3).

In conclusion, FK409 decomposes and releases NO spontaneously, in contrast to ISDN. In addition, the decomposition and NO release in FK409 are accelerated in the presence of sulfhydryl group-bearing compounds, in a similar manner to ISDN. These results indicate that sulfhydryl-containing compounds may potentiate the biological action of FK409.

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