Nitric Oxide (NO)-releasing Pathway of FK409 in the Presence of Sulfhydryl-bearing Compounds

Shinichi Fukuyama,[1,3] Toshio Azuma,[1] Yoshimi Hirasawa,[2] Noritugu Morokoshi,[1] Toshihiro Akama,[1] Shigetaka Koda,[1] and Yasuhiro Kita[2]

Received February 25, 1996; accepted April 20, 1996

Purpose. We have recently reported that degradation of FK409 with generation of NO is spontaneous and is accelerated in the presence of sulfhydryl-bearing compounds, such as L-cysteine (Cys) and glutathione (GSH). The purpose of the present study is to investigate the NO-releasing pathway of FK409 in the presence of sulfhydryl-bearing compounds.

Methods. The degradation process of FK409 in the presence of Cys or GSH was investigated by means of ^{1}H-nuclear magnetic resonance (NMR) spectroscopy and high-performance liquid chromatography (HPLC).

Results. The degradation of FK409 in the presence of Cys was dependent on concentration of Cys, and showed pH-dependency, accelerating with an increase in pH. The ^{1}H-NMR spectra of FK409 with Cys suggested that time-dependent elimination of the hydrogen atom at the α-position of the nitro moiety (5-position) was accelerated by Cys in weakly alkaline solution. Cys and GSH were transformed readily, concomitant with FK409 degradation, to give their oxidized forms and probably S-nitrosothiols.

Conclusion. The effect of sulfhydryl-bearing compounds on FK409 degradation is due to the acceleration of deprotonation of the hydrogen atom at the 5-position by thiolate anion as well as hydroxyl ion. Sulfhydryl-bearing compounds reacted with the released NO resulting in formation of disulfides *via* intermediate S-nitrosothiols.

KEY WORDS: FK409; nitric oxide; L-cysteine; glutathione.

INTRODUCTION

FK409, (±)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (Figure 1), shows both potent vasorelaxant and anti-platelet activities (1), which occurs *via* cGMP elevation (2) like those of organic nitrates such as isosorbite dinitrate (ISDN) and glyceryl trinitrate (GTN) (3). However, FK409 decomposes and subsequently releases NO spontaneously in solution (4), in contrast to organic nitrates. Furthermore, in the preceding paper, we shows that NO release from FK409, following its degradation, is accelerated in the presence of sulfhydryl group-bearing compounds such as L-cysteine (Cys) and glutathione (GSH) (5) like that of ISDN (6).

Nucleophilic attack by thiolate anion on the nitrogen atom of the O-NO_2 group is involved in the NO-releasing pathway of organic nitrates (7). However, FK409 has no O-NO_2 group, unlike organic nitrates, so the NO-releasing pathway of FK409 in the presence of sulfhydryl-bearing compounds is presumably different from that of organic nitrates. Recently, we showed that the essential step for FK409 degradation followed by spontaneous NO release is the deprotonation reaction of the α-hydrogen atom of the nitro moiety (at the 5-position), by means of ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (8). Thus, the thiolate anion derived from thiol compounds is expected to be responsible for the removal of the α-hydrogen atom. Sulfhydryl groups exist in high concentration *in vivo* as thiol compounds, including free Cys and GSH molecules, or Cys residues in many polypeptides. For example, Ellman reported that the concentration of sulfhydryl groups in human blood was 10–20 mM (9). We have already reported that sulfhydryl-containing compounds potentiated the biological action of FK409 *via* spontaneous NO release from the compound (5,10). However, we have as yet very little information as to how sulfhydryl-bearing compounds are involved in FK409 degradation.

In the present study, we investigated the NO-releasing pathway of FK409 in the presence of sulfhydryl-bearing compounds by several physicochemical techniques. We also proposed a possible scheme for NO release from FK409 in the presence of sulfhydryl-bearing compounds.

MATERIALS AND METHODS

Materials

FK409 was synthesized at Fujisawa Pharmaceutical Co. Ltd,. (Osaka, Japan). Cys was obtained from Nacalai Tesque (Kyoto, Japan). GSH and its oxidized form (GSSG) were purchased from Sigma Chemical (St. Louis, MO).

Determination of Concentrations of FK409 and GSSG by Means of High-performance Liquid Chromatography (HPLC)

FK409 was dissolved at a concentration of 1.5 mM in 0.1 M sodium phosphate buffer solution (PB) at various pH values with or without 10mM Cys. The samples were immediately incubated at 37°C. At appropriate time intervals, 1 ml of the solution was added to 1 ml of 1% trifluoroacetic acid (TFA) solution to stop the degradation and 5 μl of the mixture was injected into a HPLC system, consisting of a SPD-2A variable-wavelength detector (Shimadzu, Kyoto, Japan) operating at 230 nm; a LC-9A pump (Shimadzu); a SIL-9A (Shimadzu) auto injector and a C-R5A integrator (Shimadzu) for peak processing. The HPLC conditions were as follows: mobile phase, a mixture of distilled water and acetonitrile (3:1, v/v) containing 0.05% TFA solution; column, YMC Pack ODS-AM (4.6 i.d. × 150 mm, YMC, Kyoto, Japan); flow rate, 1.0 ml/min.

In order to investigate the breakdown process of sulfhydryl-bearing compounds in the presence of FK409, we attempted to determine breakdown products of GSH which could be detected readily by reverse-phase HPLC. FK409 was dissolved at a concentration of 1.5 mM in 0.1 M PB at pH 7.4 with or without 10 mM GSH. The samples were immediately incubated at 37°C. At appropriate time intervals, 1 ml of the solution was added to 3 ml of 0.7% TFA solution to stop further degradation, and 10 μl of the mixture was injected into a LC-

[1] Analytical Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6, 2-chome, Kashima Yodogawa-ku, Osaka 532, Japan.
[2] New Drug Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 1-6,2-chrome, Kashima Yodogawa-Ku, Osaka 532, Japan.
[3] To whom correspondence should be addressed.

0724-8741/96/0800-1238$09.50/0

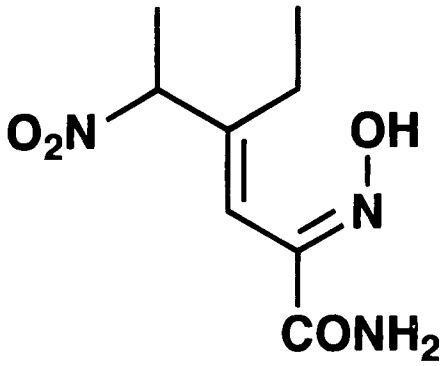


Fig. 1. Chemical structure of FK409

10A system (Shimadzu). The HPLC conditions were as follows: detection wavelength, 230 nm; mobile phase, a mixture of distilled water and acetonitrile (30:1, v/v) containing 0.05% TFA solution; column, YMC Pack ODS-AM (4.6 i.d. × 150 mm); flow rate, 0.9 ml/min.

^{1}H-NMR Spectroscopy Study

FK409 was dissolved in dimethylsulfoxide (DMSO)-d_6 at a concentration of 30 mM. An aliquot (0.1 ml) of the solution was added to 1.9 ml of 0.1 M PB (dissolved in D_2O) at pD 8.1 with or without 10.5 mM Cys. At appropriate time intervals, ^{1}H-NMR spectra were recorded with a model 500AMX (500 MHz, Bruker, Karlsruhe, Germany) at 37°C. 3-(Trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP) was used as an internal standard of the chemical shift.

RESULTS

The pH-Dependency of FK409 Degradation in the Presence of Cys

Degradation-profile of FK409 was studied as a function of pH with or without Cys (Figure 2). Pseudo-first order kinetics were observed at all pH values, the degradation rate constants at each pH value being obtained from linear least-squares regression of semilogarithmic first-order plots. Degradation rate constants with Cys were essentially equal to those without Cys below pH 5.4, whereas the rate constants with Cys are larger than those without Cys above pH 5.4. Cys obviously accelerated FK409 degradation in a pH-dependent fashion. In the presence of 10 mM Cys at pH 8.2, the degradation rate constant was approximately 4 times larger than that in the absence of Cys.

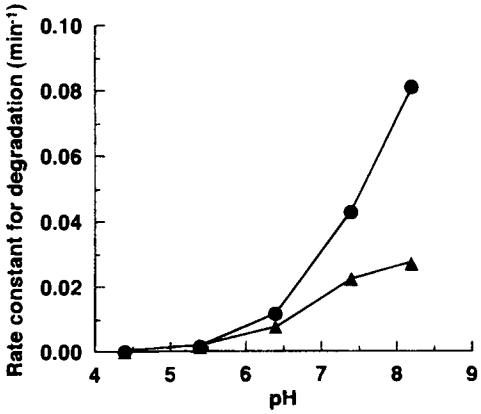


Fig. 2. The pH-dependency of FK409 degradation in 0.1M PB with (●) or without (▲) 10 mM Cys at 37°C. The initial concentration of FK409 was 1.5 mM.

FK409 Degradation Estimated by ^{1}H-NMR Spectroscopy

The time-dependent changes of ^{1}H-NMR spectra for FK409 in PB with and without 10 mM Cys were studied. The ^{1}H-NMR spectra of FK409 in 0.1 M PB containing 5% (v/v) DMSO-d_6 (at pD 8.1) without Cys are shown in Figure 3. The signals of FK409 were assigned as follows: triplet at 0.99 ppm [3H, (a)] and quartet at 2.14 ppm [2H, (c)] from $CH_3CH_2^-$; doublet at 1.75 ppm [3H, (b)] and quartet at 5.45 ppm [1H, (d)] from $CH_3CH(NO_2)-$; singlet at 6.24 ppm [1H, (e)] from $-CH = C<$. After 7-min standing, signals corresponding to the degradation products of FK409 have appeared, with the elimination of the signals of FK409. When FK409 degraded, the signals, except for that at 5.45 ppm (d), shifted as shown in Figure 3; (a)→(a'), (b)→(b'), (c)→(c'), and (e)→(e'). The signal at 5.45 ppm (d) had gradually decreased concomitantly with FK409 degradation. The elimination of the hydrogen atom at the 5-position suggested the contribution of hydroxyl ion in the degradation process of FK409 as reported previously (8).

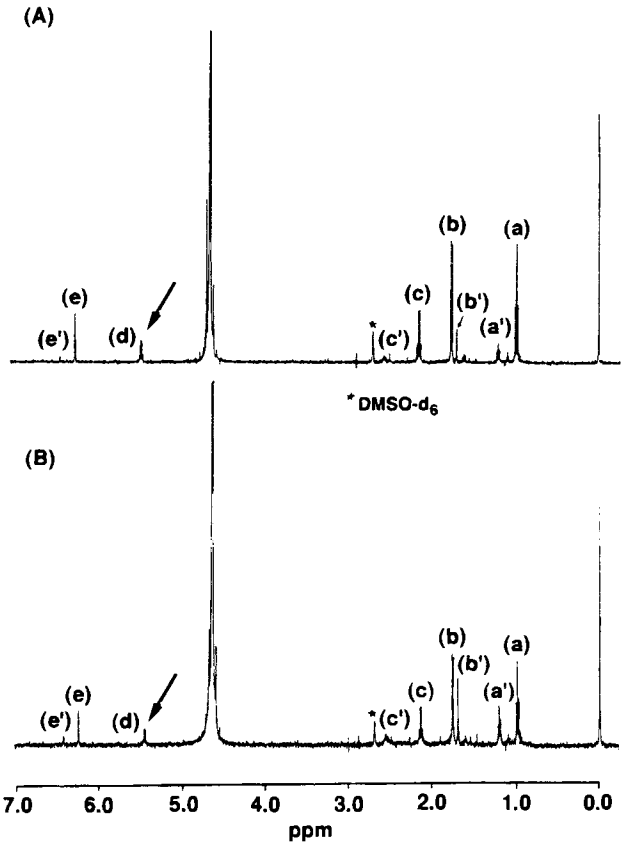


Fig. 3. Time-dependent ^{1}H-NMR spectral change of FK409 in 0.1 M PB (pD 8.1) containing 5% (v/v) DMSO-d_6 at 37°C without Cys. (A) and (B) indicate NMR spectra after 7- and 20-min standing, respectively. The initial concentration of FK409 was 1.5 mM.

The ^{1}H-NMR spectra of FK409 in 0.1 M PB containing 5% (v/v) DMSO-d_6 (at pD 8.1) with 10 mM Cys are shown in Figure 4. Although the spectra were very complicated, the signals at 6–7 ppm, after 20-min standing, indicated the presence of at least three degradation products of FK409. The degradation products included the main degradation product in PB without Cys [(a)→(a'), (b)→(b'), (c)→(c'), (d)→no signal, (e)→(e')]. The elimination of the signal at 5.45 ppm (d) with Cys was obviously faster than that without Cys. The accelerated elimination of the 5-hydrogen atom by Cys suggested the contribution of thiolate anion as well as hydroxyl ion in the degradation process.

The ^{1}H-NMR spectra showed that FK409 degradation was accompanied by degradation of Cys. The signals of breakdown product of Cys were shifted to lower chemical shifts than those of Cys: doublet at 3.04 ppm [2H] (Cys-β) → double of doublet at 3.19 ppm [1H] and 3.37 ppm [1H] from $-CH_2$-(Cys-β'); triplet at 3.40 ppm [1H] (Cys-α) → double of doublet at 4.05 ppm [1H] from −CH-(Cys-α'). The signals of the breakdown product were consistent with those of an oxidized form of Cys (cystine).

Formation of Breakdown Products of Sulfhydryl-bearing Compounds with FK409

In order to understand the breakdown process of sulfhydryl-bearing compounds with FK409, we studied the breakdown product of Cys and GSH with FK409. In PB at pH 7.4 containing both FK409 and Cys, a white insoluble precipitate was produced. The product was confirmed to be an oxidized form of Cys (cystine) due to its poor solubility in water and comparison with an authentic sample. Next we attempted to determine breakdown products of GSH in the presence of FK409 by reverse-phase HPLC. Two main degradation products were produced, as indicated by HPLC chromatograms. One was isolated by preparative HPLC and determined to be an oxidized form of GSH (GSSG). Attempts to isolate the other product (GS-1) were not successful due to its instability. However, since the LC-UV spectrum of GS-1 showed a maximum absorption at about 335 nm, GS-1 was probably S-nitrosoglutathione (11). S-Nitrosothiols such as S-nitrosoglutathione are known to be unstable in the presence of O_2 and form disulfides readily (12). We confirmed the formation of a product having a similar LC-UV spectrum to that of GS-1 in PB containing both FK409 and Cys.

Figure 5 shows the time-dependent formation of GSSG and GS-1 and time-dependent degradation of FK409. The concentrations of GSSG and GS-1 increased with progress of FK409 degradation. The peak area ratio of GS-1 to GSSG was constant at 1.6–1.7 for 60 min, and decreased slightly to 1.1 after 2 hr with a decrease of remaining FK409. Though FK409 decomposed almost completely after 2 hr, GS-1 remains in the solution.

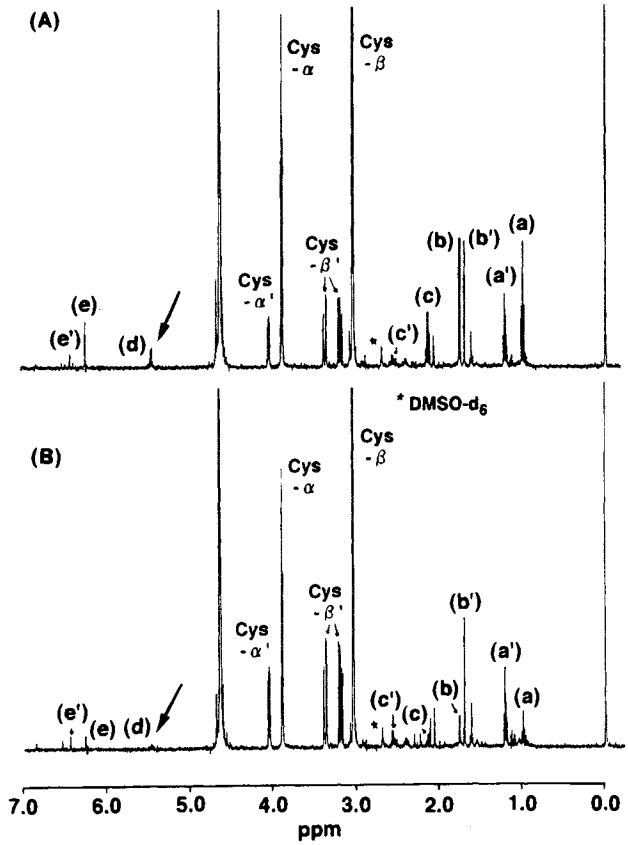


Fig. 4. Time-dependent ^{1}H-NMR spectral change of FK409 in 0.1 M PB (pD 8.1) containing 5% (v/v) DMSO-d_6 at 37°C with 10 mM Cys. (A) and (B) indicate NMR spectra after 7- and 20-min standing, respectively. The initial concentration of FK409 was 1.5 mM.

DISCUSSION

We have recently reported that both FK409 degradation and its NO release were spontaneous (4) and accelerated by thiol compounds such as Cys and GSH in a concentration-dependent manner (5). Since sulfhydryl groups exist in high concentrations *in vivo* as thiol compounds, including free Cys and GSH molecules, or Cys residues in many polypeptides, it is very important to understand the interaction profile between thiol compounds and FK409. In this paper, we studied how sulfhydryl-bearing compounds were involved in the NO-releasing pathway of FK409. The degradation in the presence of Cys showed a pH-dependency accelerating with an increase in pH above pH 5.4. Since the sulfhydryl group of Cys has an acidic

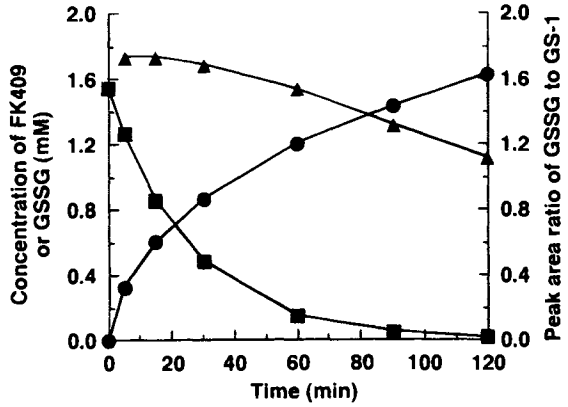
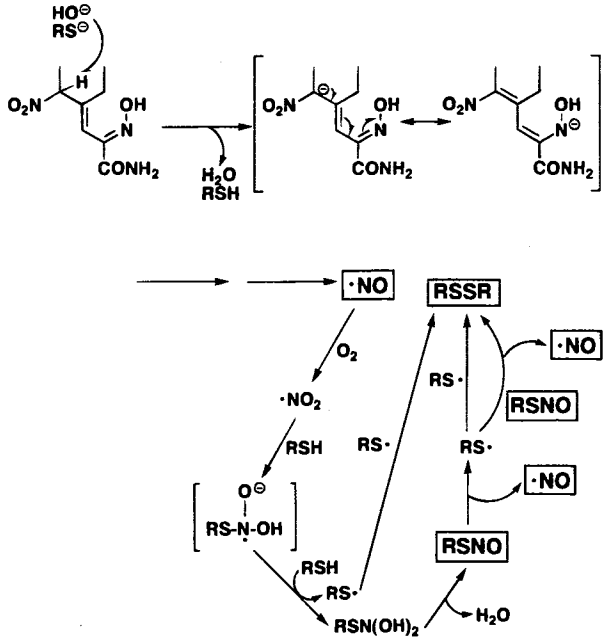


Fig. 5. Time-dependent degradation of FK409 (■), and formation of GSSG (●) and GS-1 (▲) in PB (pH 7.4) in the presence of both 1.5 mM FK409 and 10 mM GSH at 37°C. The amounts of GS-1 were represented by the peak area ratio of GS-1 to GSSG in HPLC chromatograms.

proton, the concentration of thiolate anion of Cys increases with increasing pH. These results suggested that the thiolate anion accelerated FK409 degradation by hydroxyl ion, that is, FK409 degradation in the presence of Cys involved a thiolate anion-dependent process. We proposed recently that the first step in FK409 degradation with spontaneous NO release was the deprotonation of the α-hydrogen atom of the nitro moiety (at the 5-position) by hydroxyl ion (8). Therefore, thiolate anion was also expected to act as a base, like hydroxyl ion, which subtracts the 5-hydrogen atom.

In order to clarify the prediction, we investigated the degradation of FK409 with or without Cys by means of ^{1}H-NMR spectroscopy. Figure 3 and 4 indicated that FK409 degradation was clearly accelerated in the presence of Cys. At least three main degradation products of FK409, including one major degradation product without Cys, were observed in the ^{1}H-NMR spectra. After 20-min standing with Cys, the signal of the 5-hydrogen atom of FK409 had almost disappeared. This accelerated removal of the 5-hydrogen atom by Cys suggests a thiolate anion-dependent degradation of FK409, in addition to the hydroxyl ion-dependent one. Therefore, ^{1}H-NMR experiments also suggest that the thiolate anion of Cys probably also causes degradation by acting as a base, removing the 5-hydrogen atom. The high acidity of the 5-hydrogen atom facilitates its removal in the presence of bases such as hydroxyl ion or thiolate anion. These findings support the view that proton-abstraction is the first step of FK409 degradation with NO generation in both the presence, and absence, of sulfhydryl-containing compounds. Therefore, it is suggested that NO generation from FK409 is enhanced *in vivo,* since sulfhydryl groups exist in high concentration, in comparison to the spontaneous NO release, and that this acceleration of NO release leads to the potent biological activities of FK409.

The ^{1}H-NMR spectra indicated that FK409 degradation was accompanied by degradation of thiol compounds. We observed the formation of a white insoluble precipitate, which was presumed to be cystine, in solutions containing both FK409 and Cys. We predicted the breakdown products of sulfhydryl-bearing compounds by studying the reaction of GSH with FK409 since reverse-phase HPLC techniques facilitated the determination of breakdown products of GSH. The HPLC experiments showed formation of GSSG and an other product (GS-1), which was probably S-nitrosoglutathione. Since thiol compounds react readily with NO to form S-nitrosothiols (12), the reaction of GSH with NO released from FK409 results in formation of GSSG and GS-1 (S-nitrosoglutathione). The reaction of FK409 with Cys also presumably undergoes a similar reaction due to the formation of cystine and probably S-nitrosocystein, which has a similar LC-UV spectrum to that of GS-1. From these results, the proposed NO-releasing pathway of FK409 in the presence of sulfhydryl-bearing compounds is shown in Scheme 1. The deprotonation reaction of the 5-hydrogen atom by both hydroxyl ion and thiolate anion led to NO generation from FK409. Nitrogen dioxide (NO_2), generated by oxidation of the released NO, reacts with thiols to form S-nitrosothiols, which is shown to be an intermediate, and NO and disulfide forms from breakdown of S-nitrosothiols (13). The stabilization of NO, which is very unstable in physiological conditions, owing to conversion into S-nitrosothiols, of great importance to control the pharmacological action of NO.



Scheme 1 Proposed NO-releasing pathway of FK409 in the presence of sulfhydryl-bearing compounds

We measured the time-dependent formation of GSSG and GS-1 in the presence of FK409. Their concentrations increased with FK409 degradation (Figure 5). The peak area ratio of GS-1 to GSSG after 5 min of incubation was about 1.7. The ratio decreased with a decrease of remaining FK409. After 2 hr of incubation, FK409 decomposed almost completely, however, GS-1 remained in solution. Sulfhydryl groups exist in high concentration *in vivo* as free Cys and GSH molecules, or Cys residues in many polypeptides, and react readily with NO under physiological conditions to form S-nitrosothiols (11,12). Therefore, these results suggest NO released from FK409 may readily react with sulfhydryl groups existing *in vivo* to form S-nitrosothiols. Both low molecular weight S-nitrosothiols and S-nitrosoalbumin in plasma possess endothelium-derived relaxing factor (EDRF)-like properties *in vivo* (14,15). Therefore, sulfhydryl-containing compounds in biological systems may play important roles in not only acceleration of NO release from FK409, but also control of biological functions, of FK409 involving both the duration and distribution of the drug action by trapping NO released from FK409 as S-nitrosothiols.

In conclusion, the acceleration effect of sulfhydryl-containing compounds on FK409 degradation is due to the deprotonation reaction of the hydrogen atom at the 5-position by thiolate anion as well as hydroxyl ion. The deprotonation process could well be an essential step for both FK409 degradation and NO release *in vivo* where sulfhydryl groups exist in high concentration.

ACKNOWLEDGMENTS

The authors are greatly indebted to Dr. K. Yoshida for encouragement and discussion throughout these studies. They also wish to thank Dr. D. Barrett, New Drug Research Laboratories, of their company, for reviewing the manuscript.

REFERENCES

1. M. Hino, M. Iwami, M. Okamoto, K. Yoshida, H. Haruta, M. Okuhara, J. Hosoda, M. Kohsaka, H. Aoki, and H. Imanaka. FK409, a novel vasodilator isolated from the acid-treated fermentation broth of *Streptomyces griseosporeus*. I. Taxonomy, fermentation, isolation and physico-chemical and biological characteristics. J. Antibiotics, **42**:1578–1583 (1989).
2. Y. Kita, T. Sugimoto, Y. Hirasawa, K. Yoshida and K. Maeda. Close correlation of the cardioprotective effect of FK409, a spontaneous NO releaser, with an increase in plasma cyclic GMP level. Br. J. Pharmacol., **113**:5–6 (1994).
3. K. D. Schultz, K. Schultz and G. Schultz. Sodium nitroprusside and other smooth muscle-relaxants increase cyclic GMP levels in rat ductus deferens. Nature, **265**:750–751 (1977).
4. Y. Kita, Y. Hirasawa, K. Maeda, M. Nishio and K. Yoshida. Spontaneous nitric oxide release accounts for the potent pharmacological actions of FK409. Eur. J. Pharmacol. **257**:123–130 (1994).
5. S. Fukuyama, Y. Hirasawa, D. Cox, S. Koda and Y. Kita. Acceleration of nitric oxide (NO) release from FK409, a spontaneous NO releaser, in the presence of sulfhydryl-bearing compounds. Pharm. Res. **12**:1948–1952 (1995).
6. M. Feelisch and E. Noack. Correlation between nitric oxide formation during degradation of organic nitrates and activation of guanylate cyclase. Eur. J. Pharmacol. **139**:19–30 (1987).
7. M. Feelisch. The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solution. J. Cardiovasc. Pharmacol. **17 (Suppl.3)**:S25–S33 (1991).
8. S. Fukuyama, Y. Kita, Y. Hirasawa, T. Azuma, A. Sato, Y. Morokoshi, S. Koda, T. Yasuda, S. Oka and H. Sakurai. A new nitric oxide (NO) releaser: spontaneous NO release from FK409. Free Rad. Res., **23**:443–452 (1995).
9. G. L. Ellman. A colorimetric method for determining low concentrations of mercaptans. Arch. Biochem. Biophys. **74**:443–450 (1958).
10. Y. Kita, S. Fukuyama and Y. Hirasawa. Close correlation between NO formation from and biological activities of NO releasers in rats. Jpn. J. Pharmacol. **69**:69–74 (1995).
11. J. S. Stamler, D. I. Simon, J. A. Osborne, M. E. Mullins, O. Jaraki, T. Michel, D. J. Singel and J. Loscalzo. S-nitrosylation of proteins by nitric oxide: synthesis and characterization of novel biologically active compounds. Proc. Natl. Acad. Sci. USA. **89**:444–448 (1991).
12. L. J. Ignarro, H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz and C. A. Gruetter. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J. Pharmacol. Exp. Ther. **218**:739–749 (1981).
13. W. A. Pryor, D. F. Church, C. K. Govindan and G. Crank. Oxidation of thiols by nitric oxide and nitrogen dioxide: synthetic utility and toxicological implications. J. Org. Chem., **47**:156–159 (1982).
14. J. F. Keaney, D. I. Simon, J. S. Stamler, O. Jaraki, J. Scharfstein, J. A. Vita and J. Loscalzo. NO forms an adduct with serum albumin that has endothelium-derived relaxing factor-like properties. J. Clin. Invest. **91**:1582–1589 (1993).
15. P. R. Myers, R. L. Minor Jr., R. Guerra Jr., J. N. Bates and D. G. Harrison. Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature **345**:161–163 (1990).