Close Correlation between Nitric Oxide (NO) Formation from NO Releasers and the Biological Activities of These Agents in Rats

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Received May 13, 1995 Accepted June 23, 1995

ABSTRACT—The aim of this study was to clarify the difference in the profiles of nitric oxide (NO) formation of three NO releasers and to examine the correlation between NO formation from these drugs and their biological activities in rats. (±)-(E)-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (FK409) and 3-morpholinosydnonimine (SIN-1) spontaneously generated nitrite, an oxidative product of NO, in sodium phosphate buffer (PB) solution. On the other hand, sodium nitroprusside (SNP) did not generate nitrite. The rank order of the concentrations of nitrite generated was SIN-1 > FK409 > SNP. In biological studies using rats, these drugs showed anti-platelet effects and in vitro vasorelaxant and hypotensive effects with potencies in the rank order of FK409 > SIN-1 > SNP and SNP > FK409 > SIN-1, respectively. These drugs generated nitrite with concentrations in the rank order of FK409 > SIN-1 > SNP and SNP > FK409 > SIN-1 in rat plasma and in PB solution with L-cysteine (Cys), respectively. In conclusion, three NO releasers liberate NO with NO-releasing rates of different rank orders under different incubation conditions, and the anti-platelet effects and vasorelaxant and hypotensive effects of these NO releasers closely correlate with NO formation from the compounds in the plasma and PB solution with Cys, respectively, but not with that in PB solution without Cys.

Keywords: FK409, 3-Morpholinosydnonimine (SIN-1), Sodium nitroprusside (SNP), Nitric oxide (NO)

(±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexenamide (FK409, Fig. 1) is a structurally unique vasodilator discovered from the fermentation products of Streptomyces griseosporeus (1). FK409 has produced a potent vasorelaxation mediated by the elevation of cyclic GMP in isolated dog coronary artery (2), rabbit aorta (3) and rat aorta (4). In addition, FK409 has shown a potent inhibition of platelet aggregation in human (5) and rat (6) platelet-rich plasma. Recently, we have reported that these biological actions of FK409 can be accounted for by spontaneous nitric oxide (NO) release following decomposition of the compound (5). However, the NO-releasing pathway of FK409 remains unclear.

3-Morpholinosydnonimine (SIN-1, Fig. 1) and sodium nitroprusside (SNP, Fig. 1) have been also reported as NO releasers that liberate NO spontaneously (7, 8). Three NO releasers including FK409 have different structures, so it is considered that the profiles of NO formation of the compounds are different. It has not been reported whether NO formation from different types of spontaneous NO releaser correlates with the activities of the compounds in several biological studies.

The purpose of this study was to clarify the difference in the profiles of NO formation of these NO releasers under several incubation conditions by measuring the concentration of nitrite, an oxidative product of NO, and examine the correlation between NO formation from these drugs and their biological activities. In biological studies, we evaluated in vitro anti-platelet and vasorelaxant activities and in vivo hypotensive activities.

MATERIALS AND METHODS

Materials

FK409 and SIN-1 were synthesized by Fujisawa Pharmaceutical Co. (Osaka). Trisodium citrate, adenosine 5'-
Diphosphate (ADP), tripotassium EDTA and (-)-nor-
epinephrine hydrochloride were purchased from Sigma
Chemical Co. (St. Louis, MO, USA). SNP, L-cysteine
(Cys), sulfanilic acid, N-(naphthyl)-ethylenediamine, L-
ascorbic acid and papaverine hydrochloride were pur-
chased from Nacalai Tesque Co. (Kyoto).

Nitrite analysis
For the determination of nitrite concentrations in 0.1
M sodium phosphate buffer (PB, pH 7.4) solution, each
drug was dissolved in PB solution without or with 25 mM
Cys at a concentration of 1.5 mM and immediately incu-
bated at 37°C. For the determination of nitrite concentra-
tions in the plasma, blood from male Sprague-Dawley
(SD) rats (Nihon SLC Co., Shizuoka) weighing 310-360
g, which were anesthetized with diethyl ether, was col-
lected from the abdominal aorta into plastic vessels con-
taining 4% tripotassium EDTA (1/10 volume). Plasma
was obtained from the supernatant fraction after cen-
trifugation of the blood at 1000 x g for 15 min. Each drug
was dissolved in the plasma (final drug concentration: 1.5
mM) and was incubated at 37°C immediately. Prior to in-
cubation and at various time intervals, the concentrations
of nitrite were determined by diazotization. To 0.05 ml of
PB solution or plasma containing each drug, 3.95 ml of
HCl (0.5 N for PB solution and 0.05 N for plasma), 0.5
ml of 0.2% sulfanilic acid and 0.5 ml of 0.1% N-(1-
naphthyl)ethylenediamine were added subsequently (11).
Absorbance of a purple dye at 548 nm was measured with
a spectrophotometer (UV-2200; Shimadzu Co., Kyoto).
For the standard curve, sodium nitrite was used under the
same experimental conditions. In these experiments, the
initial drug concentration (1.5 mM) was established in
order to detect nitrite generated from the drug at any
time.

In vitro platelet aggregation study
Blood from male SD rats weighing 295-360 g, which
were anesthetized with diethyl ether, was collected from
the abdominal aorta into plastic vessels containing 2.2%
trisodium citrate (1/10 volume), according to the previ-
ous report (12). Platelet-rich plasma was obtained from
the supernatant fraction after centrifugation of blood at
200 x g for 10 min. The effect of each drug on platelet ag-
gregation was determined by Born's turbidimetric
method (13) using an aggregometer (Hema Tracer 801;-
Niko Bioscience Co., Tokyo). 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 in-
duced by the addition of 5 µl of ADP (final concentra-
tion: 2.0 µM). To evaluate platelet aggregation, the maxi-
mum 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 % inhi-
bition of ADP-induced platelet aggregation compared
with vehicle treatment. The IC50 value expressed the drug
concentration required to produce 50% inhibition. The
IC50 value was logarithmically calculated for the platelet-
rich plasma from each rat.

In vitro vasorelaxant study
The thoracic aorta from male SD rats weighing
265-325 g 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 CaCl2, 1.0 mM MgCl2,
11.9 mM NaHCO3, 0.4 mM NaH2PO4 and 5.6 mM dext-
rose. Isometric tension was measured with a force-dis-
placement transducer (UL-10GR; Shinkoh, Tokyo) con-
nected to an amplifier (AP-630G; Nihon Kohden, Tokyo)
and was recorded with a polygraph (Recti-Horiz-8K;
Sanei, Tokyo). The tissue bath solution was maintained
at 37°C and bubbled with a 95% O2 and 5% CO2 gas
mixture. After the resting tension was adjusted to 0.5 g,
the strip was contracted with 0.25 ml of (-)-norepineph-
rine (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 distilled water, was added to the organ bath cumulatively. Finally, 0.25 ml of papaverine (final concentration: 100 μM) was added to the organ bath. Relaxation with papaverine was taken as 100%. The EC50 value expressed the drug concentration required to produce 50% relaxation. The EC50 value was computed by regression analysis logistically for the isolated aorta from each rat.

Hypotensive study

After male SD rats weighing 285 – 380 g were anesthetized with urethane (1.0 g/kg, s.c.) dissolved in saline, a polyethylene cannula filled with heparin solution was inserted into the left femoral artery of the rat to measure mean blood pressure. Another polyethylene cannula filled with saline was inserted into the left femoral vein of the rat to administer each drug. Mean blood pressure was measured with a pressure transducer (TR-400T, Nihon Kohden) connected to an amplifier and was recorded on a polygraph. Each drug, dissolved in saline, was administered intravenously after the stabilization of mean blood pressure. The hypotensive effects of each drug were expressed as the maximum change in mean blood pressure compared with the pre-administration value. The ED35 value expressed the drug dose required to produce a -35% change in mean blood pressure. The ED35 value was logarithmically calculated.

RESULTS

Nitrite generation in PB solution without Cys

As shown in Fig. 2, FK409 and SIN-1 spontaneously generated nitrite, an oxidative product of NO, immediately when they were dissolved in PB solution and incubated at 37°C. The first-order generation of nitrite was observed for FK409 and SIN-1, the rate constants for the ln ([nitrite]o – [nitrite]) versus time plots by 240 min being 0.0109±0.0001 1/min (r=0.972±0.010) and 0.0600±0.0010 1/min (r=0.999±0.001), respectively. Thus, the generation rate of nitrite of SIN-1 was approximately 6 times larger than that of FK409. On the other hand, SNP did not generate nitrite in PB solution during a 30-min incubation. The rank order of the concentrations of nitrite generated in PB solution during the 30-min incubation was SIN-1 > FK409 > SNP.

In vitro anti-platelet effects

Figure 3 presents the inhibitory effects of FK409 (0.32–32 μM), SIN-1 (3.2–320 μM) and SNP (10–1000 μM) on ADP-induced platelet aggregation in rat platelet-rich plasma. These drugs concentration-dependently suppressed this aggregation. The IC50 values of FK409, SIN-1 and SNP were 4.32±0.95, 24.3±1.5 and 97.8±39.2 μM, respectively.

In vitro vasorelaxant effects

In isolated rat aorta contracted with norepinephrine,
cumulative addition of FK409 (0.032–10 nM), SIN-1 (3.2–1000 nM) or SNP (0.032–10 nM) induced a concentration-dependent relaxation (Fig. 4a). The EC50 values of FK409, SIN-1 and SNP were 1.01 ± 0.26, 76.0 ± 22.2 and 0.419 ± 0.053 nM, respectively.

Hypotensive effects

Figure 4b presents the maximum changes in mean blood pressure by FK409 (0.010–1.0 mg/kg, i.v.), SIN-1 (1.0–10 mg/kg, i.v.) and SNP (0.032–1.0 mg/kg, i.v.) in anesthetized rats. Mean blood pressure decreased immediately after intravenous administration of these drugs and showed the maximum change within 5 min (data not shown). The ED35 values of FK409, SIN-1 and SNP were 0.023, 6.1 and 0.0066 mg/kg.

Fig. 5. Time-dependent generation of nitrite from FK409 (●), SIN-1 (▲) and SNP (■) in rat plasma at 37°C. The initial concentration of each drug was 1.5 mM. Each value represents the mean±S.E.M. for three experiments.

Nitrite generation in rat plasma

As shown in Fig. 5, FK409 and SIN-1 time-dependently generated nitrite in rat plasma. SNP could also generate nitrite time-dependently in rat plasma in contrast to in PB solution. However, the nitrite generation from SNP was slower than those of FK409 and SIN-1. The first-order generation of nitrite was observed for FK409 and SIN-1, the rate constants being 0.0875 ± 0.0023 1/min (r = 0.997 ± 0.001) and 0.0302 ± 0.0002 1/min (r = 0.998 ± 0.001), respectively. Thus, the generation rate of nitrite from FK409 is approximately 3 times larger than that from SIN-1. The rank order of the concentrations of nitrite generated in rat plasma during the 5-min incubation was FK409 > SIN-1 > SNP.

Nitrite generation in PB solution with Cys

As shown in Fig. 6, FK409, SIN-1 and SNP time-dependently generated nitrite in PB solution with 25 mM Cys. The first-order generation of nitrite was observed for these drugs, the rate constants being 0.0411 ± 0.0038 1/min (r = 0.981 ± 0.009), 0.0188 ± 0.0030 1/min (r = 0.965 ± 0.014) and 0.0410 ± 0.0032 1/min (r = 0.961 ± 0.011) for FK409, SIN-1 and SNP, respectively. Thus, the generation rates of nitrite from FK409 and SNP are ap-
proximately 2 times larger than that from SIN-1. The rank order of the concentrations of nitrite generated in PB solution with 25 mM Cys during the 30-min incubation was SNP > FK409 > SIN-1.

Fig. 6. Time-dependent generation of nitrite from FK409 (○), SIN-1 (▲) and SNP (●) in PB solution with 25 mM Cys at 37°C. The initial concentration of each drug was 1.5 mM. Each value represents the mean ± S.E.M. for three experiments.

Table 1. The rate constants for the first-order generation of nitrite from FK409, SIN-1 and SNP

| Drugs     | in PB (1/min) | in rat plasma (1/min) | in PB with 25 mM Cys (1/min) |
|-----------|---------------|-----------------------|------------------------------|
| FK409     | 0.0109 ± 0.0001 | 0.0875 ± 0.0023        | 0.0411 ± 0.0038              |
| SIN-1     | 0.0600 ± 0.0010 | 0.0302 ± 0.0002        | 0.0188 ± 0.0030              |
| SNP       | 0.0000 ± 0.0000 | not calculatedb        | 0.0410 ± 0.0032              |

Each value represents the mean ± S.E.M. for three experiments. aNitrite could not be measured during the experimental period. bThe [nitrite]a value could not be determined during the experimental period because nitrite generation from SNP was slow.

DISCUSSION

FK409 (5), SIN-1 and SNP (7, 8) have been reported to release NO spontaneously in solution. Actually, FK409 and SIN-1 spontaneously generated nitrite, an oxidative product of NO, with rates in the rank order of SIN-1 > FK409 in PB solution. However, SNP did not generate nitrite spontaneously in our experiments. Recently, SNP has been reported to release NO spontaneously only when a solution of the compound is irradiated with visible light (14). Our result is in agreement with the report that SNP does not release NO spontaneously without irradiation with visible light. However, SNP showed biological actions in the present studies. In particular, SNP had the most potent vasorelaxant and hypotensive activities among the three NO releasers tested. FK409 showed more potent anti-platelet, vasorelaxant and hypotensive effects than SIN-1. Taking into account these results, NO formation from these NO releasers in PB solution can not account for the biological activities of the compounds. Therefore, NO formations from these NO releasers must be evaluated under other conditions to account for their biological activities.

Nitrite generation in plasma and in PB solution with Cys was evaluated. Cys, which is a typical thiol compound containing a sulfhydryl group in its structure, was used as a reducing agent in this study, because reducing agents with sulfhydryl groups in their structures are abundantly present in rabbit blood vessels (14) and rat heart, liver and muscle (15). The concentration of Cys used was approximately equal to that (10–20 mM) of the sulfhydryl groups in human blood (16). SNP generated nitrite under these conditions in contrast to in PB without Cys. In particular, SNP generated the highest concentration of nitrite during the 30-min incubation in PB solution with Cys. The rank order of the concentrations of nitrite generated during the 30-min incubation was SNP > FK409 > SIN-1. In rat plasma, the rank order of the concentrations generated during 5 min, which is close to the drug incubation time (2 min) in the platelet aggregation study, was FK409 > SIN-1 > SNP. From these results, anti-platelet effects and vasorelaxant and hypotensive effects of these NO releasers closely correlate with NO formation from the compounds in the plasma and PB solution with Cys, respectively, but not with that in PB solution without Cys.

Considering that the rank orders of NO-releasing rates of three NO releasers are different under the three incubation conditions tested in the present studies, the profiles of NO formation of these drugs are probably different. Recently, SNP has been reported to release NO through reduction in the presence of thiol compounds such as Cys (14). On the other hand, NO formation from SIN-1 is not influenced by the presence of Cys (17); however, oxidation of the compound by oxygen in solution is required (18). Table 1 shows the rate constants for the first-order generation of nitrite, an oxidative product of NO, from the three NO releasers under three incubation conditions. Although SNP released NO in rat plasma, the compound released NO more rapidly in PB with Cys, and these results are in agreement with the report by Bates et al. (14). In contrast to the report by Bohn and Schönafinger (17), NO formation from SIN-1 was influenced by the presence of Cys. The generation rate of nitrite from SIN-1 decreased in the presence of Cys. In addition, the
decomposition rate of SIN-1 was also reduced in the presence of Cys (data not shown). The generation rates of nitrite from SIN-1 decreased in the rank order of PB > rat plasma > PB with Cys, in contrast to those from SNP. NO formation from FK409 was also influenced by the presence of Cys. The generation rate of nitrite from FK409 increased in the presence of Cys, like that of SNP. However, the generation rate of nitrite from FK409 was largest in rat plasma, unlike that from SNP. Thus, the three NO releasers have different profiles of NO formation and liberate NO with NO-releasing rates of different rank orders under different incubation conditions. Therefore, it is relevant that these NO releasers have different activities in different biological studies. From our data, anti-platelet activities and vasorelaxant and hypotensive activities of these drugs closely reflected NO formation in the plasma and in PB with Cys, respectively. Furthermore, it is reasonable to consider that thiol compounds with sulfhydryl groups are abundantly present in the aorta and resistant blood vessels. These observations agree with those by Bates et al. (14) and Sedlak and Lindsay (15).

In conclusion, three NO releasers, FK409, SIN-1 and SNP, have different profiles of NO formation and liberate NO with NO-releasing rates of different rank orders under different incubation conditions. In addition, the anti-platelet effects and vasorelaxant and hypotensive effects of these NO releasers closely correlate with NO formation from these compounds in the plasma and PB solution with Cys, respectively. Therefore, caution should be taken to consider the different profiles of formation and liberation of NO from these compounds when they are used.

Acknowledgment
We are greatly indebted to Dr. Keizo Yoshida for encouragement and discussions throughout these studies.

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