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Full Paper

Nicorandil Elevates Tissue cGMP Levels in a Nitric-Oxide-Independent Manner

Yukiko Minamiyama[1,2,*], Shigekazu Takemura[2], Seikan Hai[2], Shigefumi Suehiro[3], Shigeru Okada[1], and Yoshihiko Funae[4]

[1]*Department of Anti-Aging Food Sciences, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Shikata-cho, Okayama 700-8558, Japan*

Departments of [2]Hepato-Biliary-Pancreatic Surgery, [3]Cardiovascular Surgery, and [4]Chemical Biology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan

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Abstract. The K^+ channel opener nicorandil is a hybrid compound that contains nitrate in its structure. It has been reported that nicorandil can relax vascular tissue in vitro via a mechanism that involves activation of K_{ATP} channels and stimulation of soluble guanylyl cyclase. However, it is not known whether the increase of cGMP levels occurs through an elevation of nitric oxide (NO). The aim of the present study was to determine whether NO release was a direct effect of nicorandil. We reported here that nicorandil did not generate NO using ozone chemiluminescence detection methods in human or rat liver microsomes (P450-rich fractions) with addition of NADPH. However, nicorandil elevated cGMP levels in rat liver, aorta, and human coronary smooth muscle cells in vitro. The elevation was not inhibited by the NO trapping agent carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO). These results suggest that nicorandil elevates cGMP without NO generation.

Keywords: nicorandil, nitric oxide (NO), cGMP, P450

Introduction

Nicorandil (SG-75) is an organic nitrate vasodilator like nitroglycerin (NTG). It has been reported to be effective against stable angina (1). It has been well established that long-term administration of NTG results in tolerance (2, 3). Unlike NTG, long-term administration of nicorandil containing a nitrate structure does not result in tolerance and exerts its pharmacodynamic effect through two different modes of action: increase of cyclic GMP (cGMP) and activation of K^+ channels in smooth muscle cells. The latter mechanism could explain the lack of development of tolerance that has been demonstrated clearly in various animal experiments (4). Therefore, cross tolerance between nicorandil and NTG does not occur at dosages of 10 mg, twice a day (5). However, it is not known if the increases in cGMP is dependent on nitric oxide (•NO)-released or not. The relaxing effect of organic nitrates on vascular smooth muscle is considered to be due to a stimulation of soluble guanylyl cyclase by nitric oxide derived from organic nitrate through bioactivation catalyzed by various enzymes such as glutathione *S*-transferase, cytochrome P-450, and possibly esterases (6 – 8). Mitochondrial aldehyde dehydrogenase (ALDH-2) was recently identified to be essential for the bioactivation of glyceryl trinitrate (9). There are many reports regarding the bioconversion from organic nitrates to NO. There could be multiple effector enzymes involved in the bioconversion. We have reported that the cytochrome P450 reductase system specifically participates in NO formation from organic nitrates such as NTG and isosorbide dinitrate (ISDN) (10). The current paper investigates NO release after nicorandil administration, which is used as a nitrate compound and induced by the P450 system in various tissues.

*Corresponding author. yukiko-m@md.okayama-u.ac.jp
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Materials and Methods

Chemicals

NADPH was purchased from Oriental Yeast Co. (Tokyo). Carboxy-2-phenyl-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide (carboxy-PTIO), a specific NO scavenger, was obtained from Dojindo (Kumamoto). Isobutyl methyl xanthine (IBMX), 1-benzyl-3-(hydroxymethyl-2-furyl)indazole (YC-1), and other reagents used were of analytical grade from Wako Pure Chemicals Co. (Osaka). [1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; Sigma, St. Louis, MO, USA) was used to selectively inhibit soluble guanylyl cyclase (sGC). Nicorandil [*N*-(2-hydroxyethyl)nicotinamide nitrate ester (SG-75)] and its main denitrated metabolite SG-86 [*N*-(2-hydroxyethyl)nicotinamide] were a gift from Chugai Pharmaceutical Co., Ltd. (Tokyo). Microsomes from human lymphoblasts transfected with CYP1A2, 2A6, 2C9, 2D6, 2E1, 2J2, 3A4, or 4A11 genes were obtained from Gentest Co. (Woburn, MA, USA). Isosorbide dinitrate (ISDN) was obtained from Eisai Co. (Tokyo). NTG was obtained from Nihon Kayaku Co. (Tokyo).

Animals

Male Wistar rats (220 – 240 g; SLC, Co., Shizuoka) were anesthetized with 5 g/kg urethane and sacrificed. Blood was drawn from the abdominal aorta into a heparinized syringe, and then ice-cold saline (50 ml) was perfused through the abdominal aorta. Liver, thoracic aorta, and heart tissue were immediately isolated and frozen in liquid nitrogen.

Experiments were performed according to the Guide for the Care and Use of Laboratory Animals approved by the authorities on experimental animal research at the local committee of the University.

SG-75-induced NO formation in CYP-expressing microsomes of human lymphoblast cells

A CYP-expressing microsome preparation (100 pmol) was suspended in 0.5 ml (total volume) phosphate-buffered saline (PBS) (pH 7.4) and incubated with 3.3 mM G6P, 0.5 U/ml G6PDH, 1 mM NADPH, 3.3 mM $MgCl_2 \cdot 6H_2O$, and 1 mM SG-75, 100 μM ISDN, or 100 μM NTG for 15 min at 37°C. After incubation, samples were immediately kept on ice and centrifuged at 100,000 × *g* for 60 min at 4°C. Since NO released from drugs is immediately converted to NO_2^- under aerobic conditions, supernatants were used for the analysis of NO_2^- and NO_3^- by HPLC (10).

SG-75-induced NO formation in human liver microsomes, rat liver homogenates, aorta, heart, and plasma

A preparation (200 μg protein/ml) of liver microsomes, cytosol, total liver homogenates, 10 – 20 mg of aortic ring or a whole heart, or 100 μl of plasma was suspended in 0.5 ml (total volume) of PBS (pH 7.4) and incubated with 400 μM of NADPH and 100 μM of SG-75. To assess NO release from SG-75, samples were incubated in 12-ml amber glass vials up to 15 min at 37°C. NO released during the metabolism of SG-75 in these samples was measured using a chemiluminescence NO analyzer (MODEL-280 NOA; SIEVERS, Boulder, CO, USA). The vials were then sealed with aluminium caps with Teflon septa and purged with argon for 3 min. NO gas in the headspace was measured by flushing individual vials with N_2. N_2 was drawn via a vacuum through a needle into the reaction mixture and the purged gas was directed through a second needle directly into the NO analyzer. After NO gas production had been determined for all samples, vascular tissue was homogenized and protein levels were measured. The NO analyzer was calibrated with known concentrations of NO (Fig. 1). Standard NO gas was detected in a dose-dependent manner. After incubation, samples were immediately kept on ice and centrifuged at 12,000 × *g* for 15 min at 4°C. Supernatants of reaction mixtures after the experiments were also used for the analyses of nitrite and nitrate by HPLC (10).

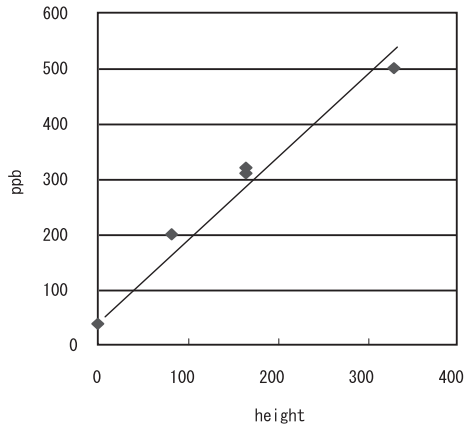

Fig. 1. Calibration for the chemiluminescence detection of •NO gas. NO gas (200, 300, 500 ppb) was injected into an O_3 chemiluminescence detector. These concentrations of NO were detectable (500 ppb: almost equal to 20 fmol/ml).

NO generation and cGMP production in rat perfused-liver

Livers from fed, male Wistar rats (230 – 250 g) were perfused in situ in the nonrecirculating mode as described by Pastor and Billiar (11). We employed single-pass perfusion with antegrade flow because this model preserves normal lobular microcirculation of the liver and physiological flow direction. The model also

avoids the problem of substrate recycling from perivenous to periportal hepatocytes. An NO electrode (NO-501; InterMedical, Nagoya) was attached to the outflow cannula placed in the inferior caval vein (IVC). Krebs-Henseleit bicarbonate buffer containing 1.2 g/l glucose, 75 mg/l sodium taurocholate, and 1 mM IBMX (a non-selective phosphodiesterase inhibitor), oxygenated with 95% oxygen and 5% carbon dioxide mixture, was used as perfusion buffer. Perfusion flow rate (3 – 3.5 ml/min per g liver), portal pressure (14 – 16 mmHg), and NO (effluent media) were monitored throughout. After 15 min of preperfusion with the perfusion buffer, 100 μl of drugs [SG-75, NTG, or *S*-nitroso-*N*-acetyl-penicillamine (SNAP)] was injected through the route of a cannula near the liver.

Drug infusions were performed with 1) SG-75 at 10, 30, or 100 μmol/min or a bolus injection of 9 or 18 μmol; 2) sodium nitroprusside (SNP) at 3 μmol/min; 3) an NTG bolus injection of 1.2 or 6 μmol; 4) SNAP at 0.03 μmol. A specific NO-sensitive electrode was placed into the outlet IVC. NTG, SNAP, and SNP were used as NO donors in the current study. To measure cGMP concentrations after SG-75 infusion, the effluent perfusate was collected in 2.0-s fractions over a period of every 120 s following injection.

Nicorandil-induced cGMP formation in human coronary arterial smooth muscle cells and the isolated rat aorta

Human coronary artery smooth muscle cells (hSMCs) in the 1st or 2nd passage were purchased from Clonetics (Walkersville, MD, USA). Cells were grown in MCDB 131 media (CellGro, Herndon, VA, USA) containing 5% fetal bovine serum (FBS), 50 μg/ml gentamycin, 50 ng/ml amphotericin B, 5 g/ml insulin, 0.5 ng/ml EGF, and 2 ng/ml bFGF. Media was changed every 2 – 3 days. Cells were grown in a humidified chamber with 95% O_2 / 5% CO_2 at 37°C to 90% – 95% confluence prior to sub-culture. Cells were used between passages 3 and 5. For the experiments, cell cultures were in HEPES-modified Tyrode solution, with various drugs added.

For cGMP determination, confluent hSMCs (10-cm dish) in HEPES-modified Tyrode buffer were pre-warmed to 37°C for 3 min. Then, 0.5 mM IBMX was added with or without 100 μM carboxy-PTIO; and samples were incubated with vehicle solution, SG-75 (1, 3 mM), SG-86 (10 mM), SNP (10 μM), or YC-1 (10 μM), which is an NO-independent soluble guanylyl cyclase activator, for 10 min at 37°C. Subsequently, proteins were precipitated using 1 ml of ice-cold 6% trichloroacetic acid (TCA) and then isolated by centrifugation at 12,000 × *g* for 10 min. The supernatant was collected for cGMP measurement. cGMP levels were normalized to the protein content of each sample as determined by the BCA assay (Pierce Chemical, Rockford, IL, USA). Proteins were measured after solubilization of TCA-precipitated proteins with 0.2 M NaOH.

Rat aortic rings (1 cm in length) were incubated in HEPES-modified Tyrode solution containing 0.5 mM IBMX for 15 min with vehicle solution, SG-75 (1, 3, 10 mM), or SNP (10 and 100 μM) with or without 100 μM carboxy-PTIO at 37°C. To examine the involvement of sGC activity, some of the experiments were performed with 1 μM of ODQ, a sGC inhibitor. After the experiments, samples were immediately frozen in liquid nitrogen and stored at −80°C until the cGMP assay was performed. Concentrations of cGMP were measured in aorta extracts using a commercial ELISA kit (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis

Unless otherwise stated, data are presented as means ± S.E.M. Results were considered significant at $P<0.05$. Statistical analysis was performed by using unpaired Student's *t*-tests or analysis of variance (ANOVA) for repeated measures followed by Fisher's test for post hoc analysis.

Results

SG-75-induced NO formation in transfected human CYP isoforms

We observed the microsomal preparations from CYP-isoform-transfected lymphoblast to catalyze NO formation from SG-75, ISDN, or NTG as a substrate (Fig. 2). Some CYP isoforms had the effective potential of NO_2^- formation from ISDN or NTG. However, any CYP isoforms had little catalytic activity to generate NO_2^- from SG-75. Microsomes- or NADPH-free system did not generate NO_2^-.

SG-75-induced NO formation in human liver microsomes, rat liver cytosol, liver homogenates, a whole heart, and aortic ring

None of the samples containing NADPH (Table 1) or those without NADPH (data not shown) showed SG-75-induced NO gas formation, although 10 μM of NTG instead of SG-75 generated 12.5 ± 0.5 pmol/mg protein (22.5 ppb) of NO gas in the aorta. Human liver microsomes did not generate NO gas from SG-75 (data not shown). The levels of NO_2^- in the reaction buffer 15 min after incubation were also not different from basal levels. The levels of total NOx ($NO_2^- + NO_3^-$) in the reaction buffer 15 min after incubation increased in the liver cytosol, aorta, plasma, and heart samples but not in

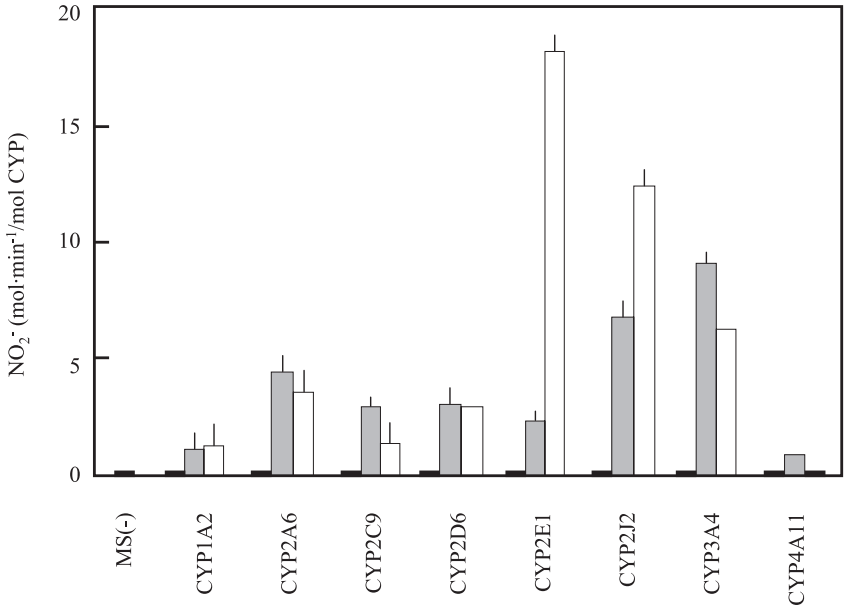

Fig. 2. SG-75-derived NO formation in human CYP isoforms-transfected microsomes as catalysts. Each CYP transfected microsome (100 pmol) was suspended in 0.5 ml of PBS (pH 7.4) and incubated as described in the Materials and Methods section. After incubation, samples were immediately centrifuged at 100,000 × *g* for 60 min at 4°C. The supernatant was used for the analysis of NO_2^- by HPLC. Closed column, SG-75; dotted column, ISDN; open column, NTG. Data represent means of triplicate determinations and S.E.M.

Table 1. NO detection after SG-75-treated samples

Substrate	Samples	NO (ppb)	$NO_2^- + NO_3^-$ (nmol/mg protein)	$NO_2^- + NO_3^-$ (μM) (in the reaction mixture)
SG-75 (100 μM)	Liver homogenate	nd	0.09 ± 0.01	1.64 ± 0.05 (1.49 ± 0.03)
	Liver cytosol	nd	0.42 ± 0.01	5.28 ± 0.12 (1.57 ± 0.02)
	Aorta	nd	9.76 ± 0.02*	4.88 ± 0.11 (1.54 ± 0.03)
	Plasma	nd	0.33 ± 0.01	22.1 ± 3.54 (13.8 ± 2.59)
	Heart	nd	0.34 ± 0.02	4.11 ± 0.04 (1.60 ± 0.05)
NTG (10 μM)	Aorta	22.5 ± 0.6	—	—

A preparation (200 μg protein/ml) of liver microsomes, cytosol, total liver homogenates, or 10 – 20 mg of aortic ring or a whole heart, or 100 μl of plasma was suspended in 0.5 ml (total volume) of phosphate buffered-saline (PBS, pH 7.4) and incubated with 400 μM of NADPH and 100 μM of SG-75. To assess gas phase for NO release and liquid phase for NOx (nitrite and nitrate) from SG-75, samples were incubated and treated as described in the Materials and Methods section. For the positive control, aorta and NTG (10 μM) were used. Each value is a means ± S.E.M. nd: not detected; parenthesis shows values before the incubation in the reaction mixture. *P<0.05, as compared with other tissues.

the liver homogenate sample. The levels per mg protein markedly increased in aorta samples as compared with the other samples.

NO generation in perfused liver

Figure 3 shows representative figures of NO generation in perfused rat liver using the NO electrode. After one injection of NTG or SNAP, NO could be detected in the outlet perfusate samples. However, no NO could be detected with SG-75 (Fig. 4, left). Infusion of SNP, an NO donor, increased NO; and when the infusion was stopped, NO decreased in the outlet perfusate (Fig. 4, right). However, no NO was detected with SG-75 infusion.

cGMP levels in the perfused liver and the aorta

SG-75 increased cGMP levels dose-dependently in both the perfused liver (Fig. 5) and isolated aorta (Fig. 6). In the aorta, SNP dose-dependently increased cGMP, but the mechanism for this was not dependent

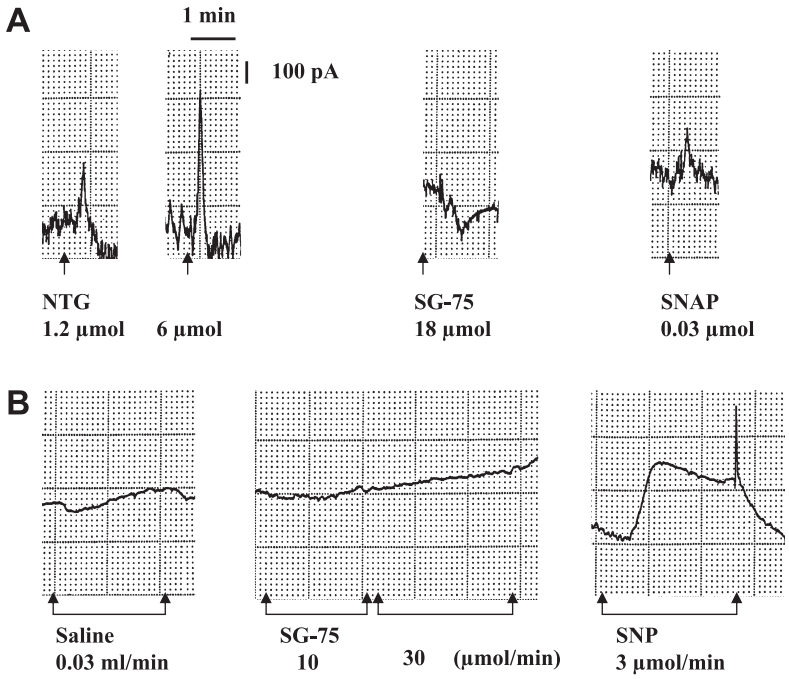

Fig. 3. Representative traces of NO electrode detection in rat perfused liver. Derived •NO was measured by an •NO electrode on the perfused liver. Traces are representative of a bolus injection of drug (A) and an infused injection (B). After 15 min of pre-perfusion with the perfusion buffer, each drug was injected through the route near the liver.

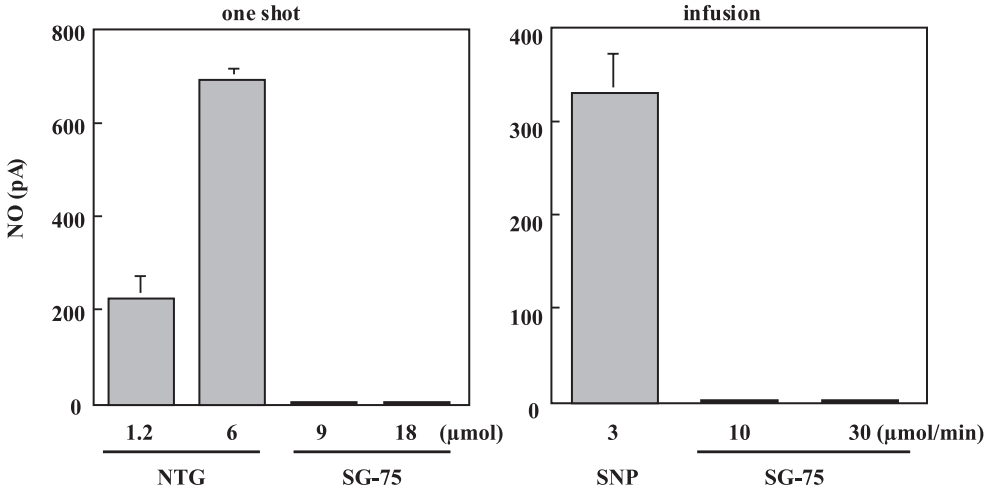

Fig. 4. NO electrode detection in rat perfused liver. Rat liver was perfused as described in the Materials and Methods section. NO detection and drug injection were performed as described in Fig. 3. Left panel shows the results of a bolus injection of drug, and the right panel shows the results of the infused drug. Each values is a mean ± S.E.M. (n = 4 – 5).

on NO. ODQ inhibited cGMP elevation by SG-75 (10 mM).

cGMP levels in human coronary arterial smooth muscle cells

SG-75 and SG-86 increased cGMP levels in coronary smooth muscle cells (Fig. 7). However, the increase of cGMP was not inhibited by carboxy-PTIO, an NO trapper. SNP increased cGMP levels, which were abolished by carboxy-PTIO.

Discussion

SG-75 did not generate NO gas in liver microsomes,

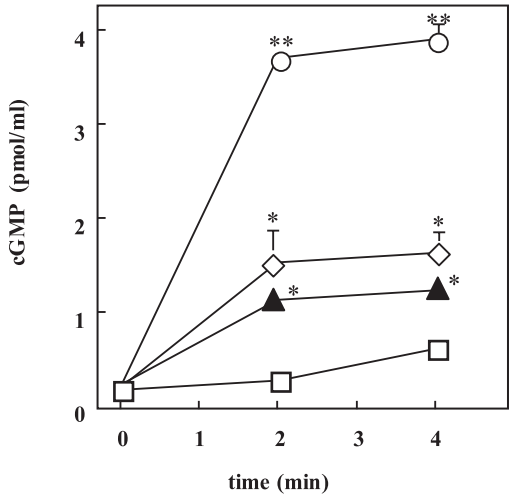

Fig. 5. cGMP levels of rat perfused liver. Rat liver was perfused as described in the Materials and Methods section. To measure cGMP concentrations after SG-75 or SNP infusion, the effluent perfusate was collected in 2.0-s fractions over a period of 120 s following every injection. Squares, SG-75 at 10 μmol/min; diamonds, SG-75 at 30 μmol/min; circles, SG-75 at 100 μmol/min; triangles, SNP at 3 μmol/min. Each value is a mean ± S.E.M. (n = 4 – 5). *P<0.05, **P<0.01, as compared with the control levels (0 min).

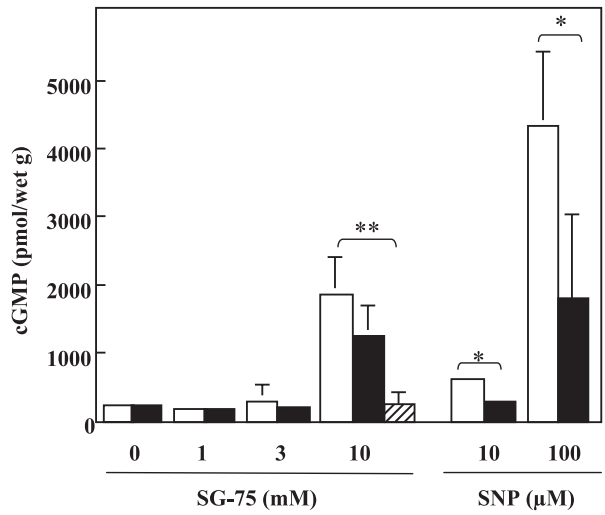

Fig. 6. Effect of SG-75 on cGMP levels of the aorta. Rat aortic rings (1 cm in length) were treated as described in the Materials and Methods section. To inhibit sGC activity, some of the experiments were performed with 1 μM of ODQ. After the experiments, samples were stored at −80°C until the cGMP assay was performed. Each value is a mean ± S.E.M. (n = 5). Open column, carboxy-PTIO-free; closed column, +carboxy-PTIO; diagonally shaded column for SG-75 at 10 mM, +ODQ. *P<0.05, **P<0.01.

enriched P450 enzyme fractions, or in homogenates and tissue containing fractions of other cellular compartments. However, cGMP levels increased NO-independently in SG-75-treated tissue. These results suggest that NO from SG-75-treated samples was not detected in any

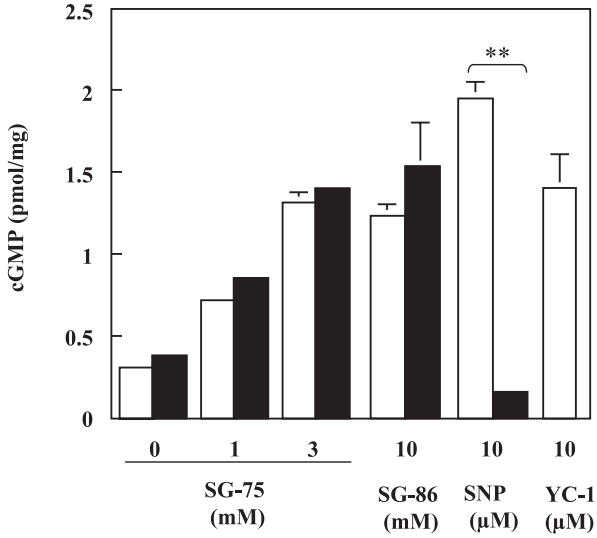

Fig. 7. cGMP levels of human coronary arterial smooth muscle cells. Confluent hSMC (10-cm dish) in HEPES-modified Tyrode buffer were treated as described in the Materials and Methods section. Each value is a mean ± S.E.M. (n = 5). Open column, carboxy-PTIO-free; closed column, +carboxy-PTIO. **P<0.01.

subcellular fractions (extra-microsomal fractions).

Sakai et al. reported that when 100 μM of SG-75 was incubated with a mixture of mitochondria and NADPH, there was a significant generation of NO (12). However, the detected products were NOx (nitrite and nitrate) and even the generated quantity was only about 1 μmol/g mitochondria protein (estimated concentration of about 100 nM) from 100 μM of SG-75. Therefore, the generated levels were only one thousandth of the SG-75 concentration and there was no direct evidence of NO generation. We examined NO generation in the mM range of SG-75 in liver homogenates and microsome, a whole heart, an aortic ring, the perfused liver, and human coronary smooth muscle cells using several techniques. However, NO was not detected from SG-75 by any methods that included the high sensitivity ozone chemilluminescence method (detection level of ppb order or fM). It is reasonable to assume that NO could not be detected by a less sensitive electrode method than the chemiluminescence. NO formation could be detected from other organic nitrates (SNP and NTG) in all detection methods. In contrast, cGMP levels increased in SG-75-treated tissue and the NO-trapper carboxy-PTIO did not inhibit the increase in cGMP. Furthermore, although SG-86, which is denitrated SG-75, was weakly reactive, SG-86 treatment elevated cGMP in some tissues in a NO-independent manner.

The levels of total NOx (more than μM order) increased in some tissues, as shown in Table 1. We cannot interpret the data at present, but this phenomenon

may be explained by the denitration from SG-75, which resulted in direct NO_3^- (or NO_2^-) generation. In addition, if SG-75 generates NO in the NADPH-P450 system, there should be nitrate tolerance as with NTG and ISDN. However, there is no report of tolerance for SG-75. Therefore, failure of NO biotransformation from SG-75 may be another supportive evidence for absence of nitrate tolerance (13).

These results suggest that SG-75 increases cGMP levels in an NO-independent manner in the tissues examined. It was reported that high concentrations of SG-75 were required to activate GC (14). Our results also indicated a high concentration of SG-75 was required for cGMP elevation as compared with the concentration of SNP (approximately 1:300) (Figs. 6 and 7). However, the results from liver perfusion showed that the potency of SG-75 to elevate cGMP was not much different from that of SNP (approximately1:3) (Fig. 5). It is considered that this experimental model simulates the in vivo situation and operating K^+_{ATP} channel and sheer stress-induced eNOS activation may contribute to the high cGMP production. The increase of cGMP may be also due to direct activation of a soluble guanylyl cyclase like YC-1 and/or due to K^+-channel opening because SG-75 does not have an inhibitory effect on phosphodiesterase (15). These findings are in agreement with a previous report demonstrating that ODQ, a soluble guanylyl cyclase inhibitor, completely blocked SG-75-induced vascular relaxation (16). However, other K^+-channel openers such as diazoxide, minoxidil, and cromakalim did not increase cGMP and cAMP (17). Pinacidil also had no effect on cGMP and cAMP (18, 19). Therefore, the effect of nicorandil on the increase of cGMP might not be due to opening of K^+-channels. In conclusion, SG-75 NO-independently increases cGMP levels via a direct activation of soluble guanylyl cyclase. It is of high interest to examine its activating mechanism and its structure-activity correlation.

Acknowledgments

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