

Possible mechanism of bronchoprotection by SIN-1 in anaesthetized guinea pigs: roles of nitric oxide and peroxynitrite

H. KANAZAWA, K. HIRATA and J. YOSHIKAWA

The First Department of Internal Medicine, Osaka City University Medical School, Osaka, Japan

Summary

Background S-morpholinosydnonimine (SIN-1) is thought to generate peroxynitrite. Recent reports suggested that peroxynitrite possessed a potent vascular relaxant activity via guanylate cyclase activation. However, no previous studies have examined the relaxant effect of peroxynitrite on airway smooth muscle.

Objective To determine the mechanism of bronchoprotection by SIN-1, considering in particular the involvement of nitric oxide (NO) and peroxynitrite.

Methods Peroxynitrite formation was assayed by monitoring the oxidizing activity of dihydrorhodamine 123, and NO was measured polarographically as a redox current *in vitro*. We examined the effect of SIN-1 delivered to the airway by ultrasonic nebulization against bronchoconstriction induced by acetylcholine in anaesthetized guinea pigs.

Results SIN-1 produced peroxynitrite in a time- and concentration-dependent manner, but did not produce NO *in vitro*. However, when mixed with glutathione (GSH) and bronchoalveolar lavage fluid (BALF), peroxynitrite formation by SIN-1 was inhibited and SIN-1 induced the release of NO. SNAP (S-nitroso-N-acetyl-penicillamine) and SIN-1 each inhibited acetylcholine-induced bronchoconstriction in a dose-dependent manner *in vivo*. Though GSH alone did not have any effect on baseline airway resistance and acetylcholine-induced bronchoconstriction, pretreatment with GSH significantly enhanced SNAP- and SIN-1-induced bronchoprotection. In addition, pretreatment with carboxy-PTIO, a NO scavenger, completely inhibited bronchoprotective effect of SNAP on acetylcholine-induced bronchoconstriction, but partially inhibited SIN-1-induced bronchoprotection.

Conclusion These findings demonstrated that SIN-1 is a potent peroxynitrite-releasing compound and caused significant bronchoprotection against acetylcholine. The mechanism of bronchoprotection by SIN-1 appears to be mediated by peroxynitrite but also at least in part through NO regeneration, which may involve GSH and airway thiols as a consequence of exposure to peroxynitrite.

Keywords: bronchoprotection, glutathione, nitric oxide, peroxynitrite, thiol

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Introduction

There is an increasing evidence that nitric oxide (NO) plays an important role in the regulation of airway smooth muscle

[1]. NO increases the level of intracellular cGMP and induces relaxation of airway smooth muscle preparations [2]. It was found that NO reversed methacholine-induced bronchoconstriction in guinea pigs [3], and that NO synthesis inhibitors induced hyperresponsiveness of guinea pig airways to histamine both *in vitro* and *in vivo* [4]. Peroxynitrite is a highly reactive species that was initially

Correspondence: H. Kanazawa, The First Department of Internal Medicine, Osaka City University Medical School, 1-4-3, Asahi-machi, Abenoku, Osaka, 545–8585, Japan.

considered to be an important mediator of the cytotoxic effects of the interaction of NO and superoxide anion [5]. However, it has recently been recognized that peroxynitrite may also have signalling mediator-like actions in vascular preparations. Recent studies have suggested that peroxynitrite stimulates guanylate cyclase and induces accumulation of cGMP in vascular endothelial and smooth muscle cells [6,7]. Thus, peroxynitrite caused relaxation of isolated canine coronary arteries and bovine pulmonary arteries through a cGMP-dependent mechanism [8,9]. These findings suggested that peroxynitrite possessed a potent vascular relaxant activity. However, no previous studies have examined the relaxant effect of peroxynitrite on airway smooth muscle. We speculated that the physiological effects of peroxynitrite in the airway should be examined using a releasing compound of peroxynitrite in an *in vivo* experimental model for two reasons. One is that peroxynitrite has a short half-life [10]. The other is that epithelial cells lining the respiratory airways can be an important source of antioxidant enzymes [11] and that the thin layer of epithelial lining fluid may contain various antioxidant substances, including vitamin E, reduced glutathione (GSH) and vitamin C [12]. Therefore, we thought that single administration of peroxynitrite into the airway was scavenged by antioxidants and might not exhibit the sufficient physiological effects. S-morpholinonydnonimine (SIN-1) is thought to generate equimolar amounts of superoxide anion and NO, which may rapidly interact to form peroxynitrite. The present study was designed to determine the mechanism of bronchoprotection by SIN-1 in anaesthetized guinea pigs, considering in particular the involvement of peroxynitrite and NO.

Methods

Measurement of pulmonary resistance

Male Hartley guinea pigs weighing 400–500 g were used. Under sodium pentobarbital anaesthesia (50 mg/kg i.p., Abbott Laboratories, South Natick, IL, USA), artificial ventilation was performed through a tracheal cannula connected to a constant volume ventilator (Model 680, Harvard Apparatus Co., North Chicago, MA, USA) at a rate of 60 breaths/min. The tidal volume was set at 6 mL/kg. Airflow was monitored continuously with a pneumotachograph (TV-241T, Nihon Koden Co., Tokyo, Japan) connected to a differential pressure transducer (TP-602T, Nihon Koden Co.). The tidal volume was calculated by electrical integration of airflow. A fluid-filled polyethylene catheter was introduced into the oesophagus to measure oesophageal pressure as an approximation of pleural pressure. Intratracheal pressure was measured using a polyethylene catheter inserted into a short tube connecting the tracheal cannula to

the pneumotachograph. Transpulmonary pressure (defined as the difference between the intratracheal and oesophageal pressures) was measured with a differential pressure transducer. Total pulmonary resistance (R_L) was calculated using methods previously described [13]. Before experiments were performed, guinea pigs were allowed 20 min to recover from the preparation procedure. To prevent alveolar atelectasis, a large inflation of three tidal volumes was performed every 5 min by occluding the expiratory valve. Drug aerosols (mass median aerodynamic diameter: 1.8 μ m; geometric SD 2 μ m; output 1.5 mL/min) were generated by an ultrasonic nebulizer (TUR-3200; Nihon Koden) placed on the inspiratory line of the ventilator.

Isolation of bronchoalveolar lavage fluid (BALF): guinea pigs were killed by exsanguination through the carotid artery. Saline (5 mL at 37°C) was then instilled into the lung and lavage was repeated three times. Cells in recovered BAL were sedimented by centrifugation at 200 g for 10 min at 4°C, and BALF was obtained as the supernatant.

Peroxyntirite and NO measurement

SIN-1 was dissolved in water and SNAP (S-nitroso-N-acetyl-penicillamine) was dissolved in ethanol just before used as 10^{-1} mol/L solutions and further dilutions were made in 0.9% saline. Peroxynitrite formation from SIN-1 was assayed by monitoring rhodamine formation at 500 nm in reaction volumes containing dihydrorhodamine 123 (50 μ mol/L), potassium phosphate (20 mmol/L, PH 7.4) and varying amounts of SIN-1 at 37°C. A standard curve of the oxidizing activity of dihydrorhodamine 123 was constructed by employing authentic peroxynitrite. Evolution of NO was measured with a commercially available NO meter (Iso-NO MARK II, World Precision Instruments, Maner, Germany) [14]. Accurate NO measurements were taken over a range of NO concentrations from 1 nmol/L to 20 μ mol/L. This probe polarographically measures the concentration of NO gas in aqueous solutions. The NO concentration is displayed as a redox current. The sensor probe housing is covered with a gas-permeable membrane. NO diffusing through the membrane is oxidized at the working platinum electrode, resulting in an electrical current. The current flow is proportional to the rate of diffusion through the membrane, which is, in turn, proportional to the NO concentration at the membrane outer surface.

Bronchoprotective effects of SIN-1 and SNAP against acetylcholine

After measuring baseline R_L (100% R_L), guinea pigs were exposed to SIN-1 (10^{-7} – 10^{-3} mol/L, 40 breaths at each concentration) or SNAP (10^{-7} – 10^{-3} mol/L, 40 breaths at each concentration). Thirty minutes after exposure to SIN-1

or 10 minutes after exposure to SNAP, guinea pigs were exposed to acetylcholine (5×10^{-3} mol/L, 40 breaths). In subsequent experiments, guinea pigs were exposed to GSH (1×10^{-3} mol/L, 60 breaths) or carboxy-PTIO (10^{-4} or 10^{-3} mol/L, 40 breaths at each concentration) 10 min prior to SIN-1 or SNAP administration.

Drugs

Acetylcholine and glutathione were purchased from Sigma Chemical Co. (St Louis, MO, USA). SIN-1, SNAP, peroxy-nitrite and carboxy-PTIO were purchased from Wako Chemical Industries Ltd (Osaka, Japan), and rhodamine 123 and dihydrorhodamine 123 from Molecular Probes (Eugene, OR, USA).

Statistical analysis

All values are expressed as the mean $\pm$ SEM. Statistical analysis was performed by analysis of variance (ANOVA) followed by Scheffe's test. *P*-values less than 0.05 were considered significant. Percentage bronchoprotection was calculated as:

$$\frac{\% \text{ increase of maximal } R_L \text{ (without agent)} - \% \text{ increase of maximal } R_L \text{ (with agent)}}{\% \text{ increase of maximal } R_L \text{ (without agent)}} \times 100$$

Results

Peroxy-nitrite and NO measurement

The formation of peroxy-nitrite by autooxidation of SIN-1 was confirmed by the oxidation of dihydrorhodamine 123

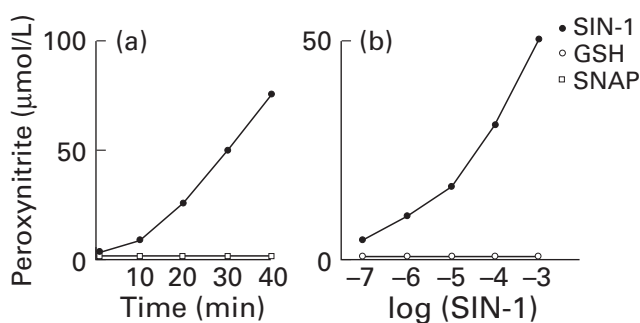


Fig. 1. (a) Time course of peroxy-nitrite formation from SIN-1 (10^{-3} mol/L) or SNAP (10^{-3} mol/L). (b) Dose-response curves of peroxy-nitrite formation from SIN-1. SIN-1 alone produced peroxy-nitrite in a dose-dependent manner after 30 minutes' incubation. Production of peroxy-nitrite from SIN-1 was abolished by addition of GSH (10^{-3} mol/L). Results are expressed as means ($n = 8$, SD was 5% in all cases)

(Fig. 1). SIN-1 oxidized dihydrorhodamine 123 to rhodamine 123 in a time- and concentration-dependent manner and this oxidation was completely inhibited by GSH (1 mmol/L). The oxidation of dihydrorhodamine 123 is mediated by peroxy-nitrite, and not by either NO or superoxide alone [15]. Actually, SNAP, a potent NO donor, did not oxidize dihydrorhodamine 123. The time courses of NO production by SNAP and SIN-1 are shown in Fig. 2. In the absence of GSH, NO generation from SNAP reached a peak value within 5 min, but SIN-1 did not generate NO. In contrast, when mixed with GSH (1 mM), SNAP generated a larger amount of NO, and addition of GSH (1 mmol/L) to SIN-1 (1 mmol/L) led to the continuous and sustained release of NO. In addition, peroxy-nitrite formation from SIN-1 was significantly inhibited by addition of BALF, but SIN-1 with BALF induced NO production (Fig. 3). In subsequent experiments, SIN-1 was used for exposure to peroxy-nitrite, and we used SNAP for exposure to NO.

Bronchoprotective effects of SNAP and SIN-1

SNAP and SIN-1 inhibit acetylcholine-induced bronchoconstriction in a dose-dependent manner (Fig. 4). Although GSH alone had no effect on baseline R_L and acetylcholine-induced bronchoconstriction, pretreatment with GSH significantly enhanced SNAP- and SIN-1-induced bronchoprotection (Fig. 5). In addition, carboxy-PTIO, a NO scavenger,

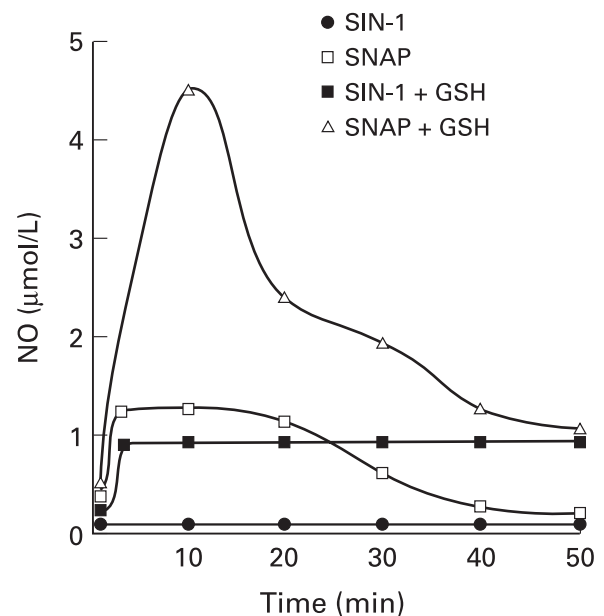


Fig. 2. Evolution of nitric oxide following addition of SIN-1 (10^{-3} mol/L), SNAP (10^{-3} mol/L), SIN-1 plus GSH (10^{-3} mol/L), and SNAP plus GSH. Results are expressed as means ($n = 8$, SD was 5% in all cases)

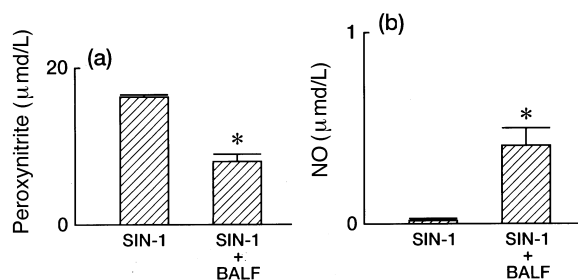


Fig. 3. (a) Peroxynitrite formation from SIN-1 with BALF. SIN-1 (10^{-5} mol/L) produced nearly $16 \mu\text{mol/L}$ peroxynitrite after 30 minutes' incubation. However, peroxynitrite formation was significantly inhibited by addition of BALF ($50 \mu\text{L}$) to the reaction medium (total $200 \mu\text{L}$) ($n = 5$). (b) Maximal NO production from SIN-1 with BALF. SIN-1 with BALF induced NO production ($n = 5$). Statistical comparison performed by ANOVA, * $P < 0.01$ vs SIN-1 alone

had no effect on baseline R_L and acetylcholine-induced bronchoconstriction, but pretreatment with carboxy-PTIO (10^{-4} mol/L) completely inhibited bronchoprotective effect of SNAP (10^{-4} mol/L) on acetylcholine-induced bronchoconstriction. Moreover, pretreatment with carboxy-PTIO (10^{-4} , 10^{-3} mol/L) partially inhibited bronchoprotective effect of SIN-1 (10^{-5} mol/L) against acetylcholine (Fig. 6).

Discussion

The present findings demonstrated that SIN-1 is a potent peroxynitrite-releasing compound which caused a significant bronchoprotection against acetylcholine. Previous studies suggested that relaxation of airway smooth muscle elicited by SIN-1 was inhibited by soluble guanylate cyclase inhibitor [16,17]. These findings provide strong evidence that peroxynitrite exerts bronchoprotective effects via guanylate cyclase-dependent pathway. We thought that NO is one of the most likely candidates for the bronchoprotection by SIN-1. However, the NO scavenger, carboxy-PTIO, completely reversed the bronchoprotection by SNAP but partially reversed the bronchoprotection by SIN-1. Therefore, peroxynitrite must be responsible for the bronchoprotection by SIN-1. Moreover, in this study, we found that exogenous GSH and carboxy-PTIO did not have any effect on baseline R_L and acetylcholine-induced bronchoconstriction. These facts indicated that endogenous NO and peroxynitrite is not involved in the maintenance of airway calibre or airway hyperresponsiveness to acetylcholine in healthy guinea pigs.

Our current understanding of the mechanisms by which peroxynitrite leads to smooth muscle relaxation stems largely from studies with vascular smooth muscle. Previous studies with vascular smooth muscle supported the hypothesis that stimulation of soluble guanylate cyclase is the

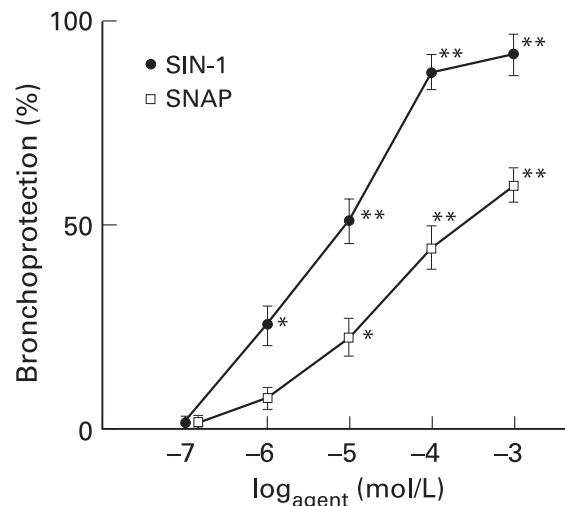


Fig. 4. Bronchoprotective effects of SIN-1 and SNAP against acetylcholine. Each point represents the mean $\pm$ SEM for six animals. The baseline R_L was $0.18 \pm 0.02 \text{ cm H}_2\text{O/mL/s}$ and administration of acetylcholine (5×10^{-3} mol/L) resulted in a significant increase in R_L ($0.50 \pm 0.05 \text{ cm H}_2\text{O/mL/s}$). Statistical comparison performed by ANOVA, * $P < 0.05$, ** $P < 0.01$ vs solvent alone

initial event in peroxynitrite-induced relaxation [18]. Moreover, peroxynitrite produces hypotensive and vasodilator responses in anaesthetized rats that are unlikely to be due to the formation of circulating S-nitrosothiols (RSNO) [19]. However, our findings in this study on how exogenous peroxynitrite elicits bronchoprotection, resulted in considering roles for peroxynitrite itself and what are thought to be some of its primary chemically reactive decomposition products.

Previous studies suggested that exposure of solutions containing GSH to peroxynitrite caused time-dependent generation of NO associated with the formation of a product of GSH resembling S-nitro-GSH (GSNO_2) [9,20], which possessed potent vascular relaxant activity. Actually, the findings of the present study demonstrated that SIN-1 plus GSH induced production of NO *in vitro*, and that pretreatment with GSH potentiated SIN-1-induced bronchoprotection. Moreover, we also found that a NO scavenger partially inhibited SIN-1-induced bronchoprotection. It is thus possible that GSNO_2 produced from the interaction of SIN-1 with GSH is an intermediate in the regeneration of NO. In this study, we found that SIN-1 induced production of NO in the presence of BALF. Given the present understanding of interactions of peroxynitrite with airway thiols (RSH), our findings suggest that nitrated thiols, particularly RSNO_2 , may be an important intermediate in thiol-dependent regeneration of NO. The mechanism of bronchoprotection by SIN-1 appears to be mediated at

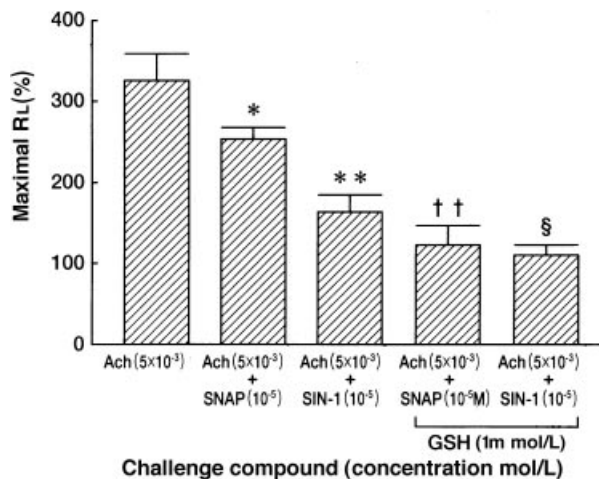


Fig. 5. Effect of GSH on SIN-1- and SNAP-induced bronchoprotection against acetylcholine. Each bar represents the mean (SEM for seven animals). Ach = acetylcholine, GSH = glutathione). Statistical comparison performed by ANOVA, * $P < 0.05$, ** $P < 0.01$ vs Ach alone, †† $P < 0.01$ vs Ach + SNAP, § $P < 0.05$ vs Ach + SIN-1

least in part through NO regeneration, which may involve airway thiols including GSH, resulting from initial exposure to peroxynitrite (Fig. 7). However, exposure to peroxynitrite derived from SIN-1 resulted in cellular injury and death of pulmonary epithelial cells [21]. Large amounts of peroxynitrite may be directly damaging to tissues and possibly result in abnormal regulation of airway function. Interestingly, the concentration of NO derivatives in sputum in patients with asthma correlates significantly with the percentages of shed epithelial cells [22], which may be evidence for the involvement of peroxynitrite.

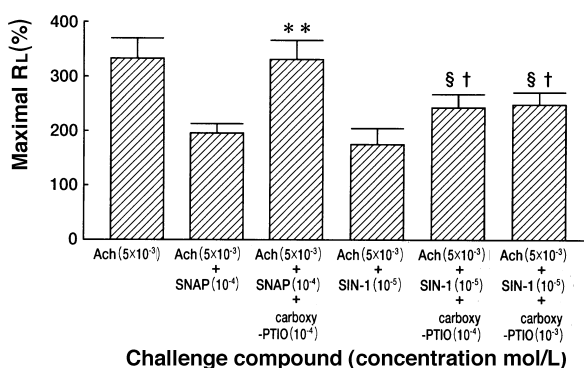


Fig. 6. Effect of carboxy-PTIO on SIN-1- and SNAP-induced bronchoprotection against acetylcholine. Each bar represents the mean (SEM for seven animals). Ach = acetylcholine. Statistical comparison performed by ANOVA, ** $P < 0.01$ vs Ach + SNAP, † $P < 0.05$ vs Ach + SIN-1, § $P < 0.05$ vs Ach alone

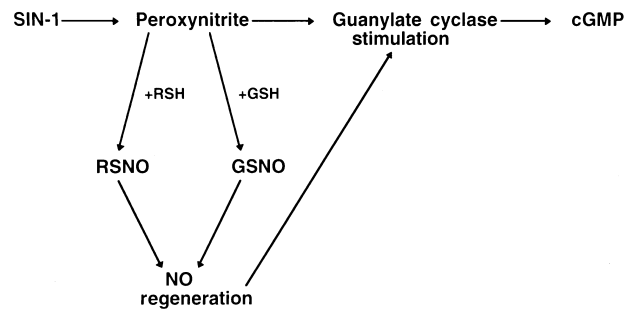


Fig. 7. Possible mechanism of SIN-1-induced guanylate cyclase stimulation

In summary, the mechanism of bronchoprotection by SIN-1 appears to be mediated by peroxynitrite, but also at least in part through NO regeneration. Further studies will be required to determine whether the levels of peroxynitrite formed in pathological states play an important role for the regulation of airway responses.

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