

Administration of SIN-1 Induces Guinea Pig Airway Hyperresponsiveness through Inactivation of Airway Neutral Endopeptidase

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Key Words

Peroxynitrite · Neutral endopeptidase · Substance P · Endothelin-1 · Airway hyperresponsiveness

Abstract

Background: Peroxynitrite plays an important role in the pathogenesis of airway inflammation. We have already found that peroxynitrite may contribute to decreased β_2 -adrenoceptor responses in airway smooth muscle. However, it is not known whether peroxynitrite can alter neutral endopeptidase (EC 3.4.24.11; NEP) activity in the airways. This study was designed to determine whether peroxynitrite induces airway hyperresponsiveness to substance P (SP) and endothelin-1 (ET-1) through the inactivation of airway NEP. **Methods:** We examined whether the administration of S-morpholinosydnonimine (SIN-1), a compound that releases peroxynitrite, increased bronchoconstrictor responses to SP and ET-1 in anesthetized guinea pigs. In addition, we assayed NEP activity in the airways of SIN-1-exposed guinea pigs. **Results:** Though SIN-1 (10^{-7} M) alone had no effect on pulmonary resistance, pretreatment with SIN-1 significantly enhanced SP- and ET-1-induced bronchoconstriction. Pretreatment with phosphoramidon, an NEP inhibitor, also enhanced SP- and ET-1-induced bronchoconstriction. However, simultaneous administration of phosphoramidon and SIN-1 had no additive effect on SP- and ET-1-induced bronchoconstriction. Peroxynitrite formation by SIN-1 was completely inhibited by N-

acetylcysteine (NAC) and glutathione (GSH) in vitro, and pretreatment with NAC and GSH significantly reversed the potentiation by SIN-1 of SP-induced bronchoconstriction. In addition, the NEP activity of the trachea after SIN-1 exposure was significantly reduced compared to the level in control guinea pigs (solvent for SIN-1: 30.0 ± 4.2 fmol·min $^{-1}$ ·mg tissue $^{-1}$; 10^{-7} M SIN-1; 15.5 ± 4.5 fmol·min $^{-1}$ ·mg tissue $^{-1}$, $p < 0.05$). **Conclusions:** These findings suggest that peroxynitrite induces airway hyperresponsiveness to SP and ET-1 through the inactivation of airway NEP, and that peroxynitrite is an important mediator of the alterations in airway functions.

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Introduction

Substance P (SP) and Endothelin-1 (ET-1) are two small bioactive peptides in the lung. These peptides are known to induce a variety of biological effects including airway smooth muscle contraction [1, 2]. Although these peptides are extremely potent biomolecules, their physiologic effects in vitro and in vivo appear to be limited by their rapid degradation. SP and ET-1 can be degraded by several peptidases, including neutral endopeptidase (EC 3.4.24.11; NEP) [3, 4]. NEP is a cell-membrane-bound peptidase that is present in the airway epithelium and smooth muscle of many species, including humans and guinea pigs [5]. Recently, several studies have demonstrated that NEP is an important modulator of SP-induced bronchoconstriction, mucus secretion

and cholinergic neurotransmission [6]. Although these peptides are subject to degradation by NEP, the relative importance of these degradative mechanisms in limiting airway effects is also influenced by the locus of NEP relative to the site of peptide administration or release. For example, when SP is administered to guinea pigs by aerosol, NEP inhibitors enhance its bronchoconstrictor activity, since NEP is predominantly an epithelial enzyme [7].

Peroxynitrite is formed by the reaction of NO with superoxide anion [8]. In many pathologic conditions, including inflammation, simultaneous cellular production of superoxide anion and NO may occur, potentially leading to the continuous formation of peroxynitrite. Peroxynitrite is an extremely potent oxidant that can cause lipid peroxidation, DNA damage and alterations of protein functions in vitro [9]. Recently, nitrogen-derived oxidants were shown to be formed in human acute lung injury, suggesting the possibility of an important role for peroxynitrite in inflammatory lung disease [10]. Most recently, we suggested that peroxynitrite derived from S-morpholinosydnonimine (SIN-1) might contribute to the decreased β_2 -adrenoceptor responses in airway smooth muscle [11]. Therefore, we speculated that peroxynitrite might also alter airway NEP activity. In this study, we examined whether the administration of SIN-1 inactivated airway NEP or increased bronchoconstrictor responses to SP and ET-1 in anesthetized guinea pigs.

Materials and Methods

Pulmonary Resistance Measurement

Hardy male guinea pigs (400–500 g) were used in this study. All guinea pigs were provided with food and water, and kept in a temperature- and humidity-controlled environment at the Institute for Laboratory Animal Research of the Osaka City University Medical School. They were anesthetized using sodium pentobarbital (50 mg/kg, intraperitoneally; Abbott Laboratories, North Chicago, Ill., USA) and then ventilated artificially with a tracheal cannula using a constant-volume ventilator (Model 680; Harvard Apparatus Co., South Natick, Mass., USA) at a frequency of 60 breaths/min. Tidal volume was set at 6 ml/kg. Airflow was monitored continuously using a pneumotachograph (TV241T; Nihon Koden Co., Tokyo, Japan) connected to a differential pressure transducer (TP-602T; Nihon Koden). The tidal volume was determined by electrical integration of the airflow. A fluid-filled polyethylene catheter was introduced into the esophagus to measure esophageal 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 esophageal pressures) was measured with a differential pressure transducer. Pulmonary resistance (R_L) was calculated as previously described [12]. Aerosols of test agents were generated with an ultrasonic nebulizer and delivered into the airways

by the ventilator. All the protocols used in this study were approved by the Osaka City University Animal Research Committee, which is responsible for ensuring the proper use of experimental animals.

Peroxynitrite Measurement in vitro

Authentic peroxynitrite readily oxidizes dihydrorhodamine 123. Peroxynitrite formation by SIN-1 and S-nitroso-N-acetyl penicillamine (SNAP) was assayed by monitoring rhodamine formation at 500 nm in reactive mixtures containing dihydrorhodamine 123 (123 (50 μ M), potassium phosphate (20 mM, pH 7.4) and various amounts of SIN-1 (10^{-7} , 10^{-5} or 10^{-3} M) at 37°C.

Effect of SIN-1 on ET-1- and SP-Induced Bronchoconstriction

After measuring baseline R_L (100% R_L), the guinea pigs were exposed to SIN-1 (10^{-7} M, 40 breaths). Thirty minutes after the exposure to SIN-1, the guinea pigs were exposed to ET-1 (10^{-10} – 10^{-8} M, 40 breaths at each concentration) and SP (10^{-5} – 10^{-3} M, 40 breaths at each concentration). In addition, the guinea pigs were exposed to phosphoramidon (1×10^{-4} M, 60 breaths) 10 min before ET-1 and SP administration. In another set of experiments, guinea pigs were exposed to N-acetylcysteine (NAC) (1 mM, 60 breaths) or glutathione (GSH) (1 mM, 60 breaths) 10 min before SIN-1 administration.

NEP Assay

NEP activity was assayed in the guinea pig tracheas 30 min after the administration of solvent or SIN-1 as previously described [13]. Briefly, each trachea was homogenized in 2 ml of buffer consisting of 50 mM tri (hydroxymethyl)-aminomethane (pH 7.4), 1 mM phenylmethylsulfonyl fluoride and 0.1% Triton X-100 in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 12,000 g . The sample supernatant from each guinea pig, 150 μ l of buffer, 20 μ l leucine aminopeptidase (0.1 U) and 10 μ l of the substrate glutaryl-(Ala)₂-Phe-*p*-nitroanilide (1 mg/ml) were used in the assay. All reagents were obtained from Sigma Chemical Co., St. Louis, Mo., USA. In the assay, the substrate was cleaved at the Ala-Phe peptide bond by NEP. The Phe-*p*-nitroanilide formed was then cleaved by leucine aminopeptidase to produce the chromophore *p*-nitroanilide. Leucine aminopeptidase will not hydrolyze the substrate without prior cleavage by NEP. The optical density resulting from the release of the chromophore *p*-nitroanilide at 0 and 15 min after the addition of substrate was 405 nm at 25°C. The difference between time points was used to calculate activity, expressed as femtomoles per minute per milligram of tissue. Each sample was assayed in triplicate.

Drugs

NAC and GSH were purchased from Sigma. SIN-1, SNAP and peroxynitrite were purchased from Wako Chemical Industries Ltd. (Osaka, Japan), and rhodamine 123 and dihydrorhodamine 123 from Molecular Probes (Eugene, Oreg., USA).

Statistical Analysis

All values are expressed as the mean $\pm$ SEM. Statistical analysis was performed by analysis of variance followed by Scheffé's test. $p < 0.05$ was considered significant.

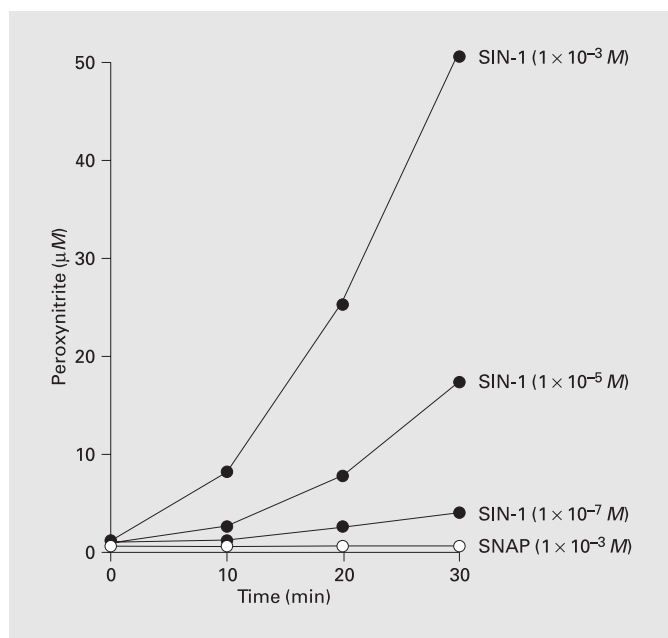


Fig. 1. Time course of peroxynitrite formation by SIN-1 and SNAP. A standard curve of the oxidizing activity of dihydrorhodamine 123 was constructed employing authentic peroxynitrite. Results are expressed as means ($n = 8$, SD was $<5\%$ in all cases).

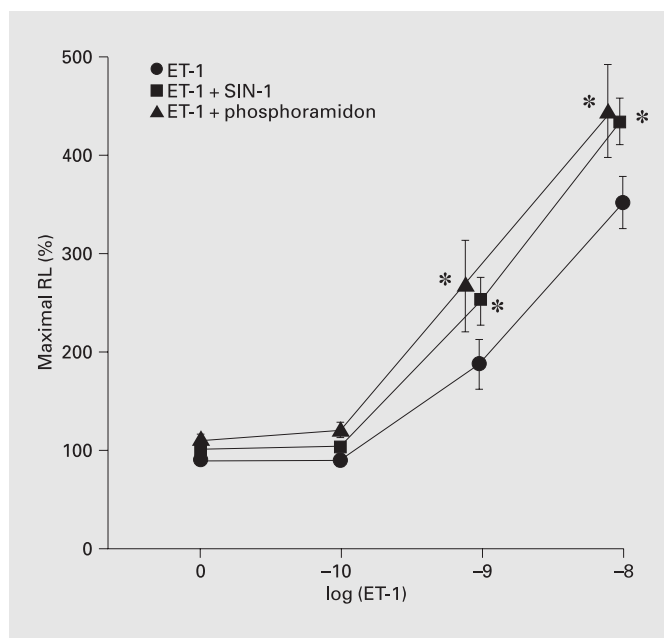


Fig. 2. Effects of SIN-1 and phosphoramidon on ET-1-induced bronchoconstriction. Each point represents the mean $\pm$ SEM for 6 animals. * $p < 0.05$ versus ET-1 alone. Percent of maximal R_L was calculated as follows: maximal R_L [with ET-1]/maximal R_L [without ET-1] $\times 100$.

Results

The formation of peroxynitrite by the auto-oxidation of SIN-1 was confirmed by the oxidation of dihydrorhodamine 123 (fig. 1). SIN-1 oxidized dihydrorhodamine 123 to rhodamine 123 in a time- and concentration-dependent manner. The oxidation of dihydrorhodamine 123 is mediated by peroxynitrite, and not by either NO or superoxide anion alone [14]. In fact, SNAP did not oxidize dihydrorhodamine 123. We utilized SIN-1 for exposure to peroxynitrite in the following experiments.

ET-1 induced bronchoconstriction in a dose-dependent manner (fig. 2). Though neither SIN-1 (10^{-7} M) nor phosphoramidon (10^{-4} M) had any effect on baseline R_L , pretreatment with either phosphoramidon or SIN-1 enhanced ET-1-induced bronchoconstriction. In addition, SP also induced bronchoconstriction in a dose-dependent manner (fig. 3), and pretreatment with either phosphoramidon or SIN-1 markedly enhanced SP-induced bronchoconstriction. However, simultaneous administration of phosphoramidon and SIN-1 had no additive effect on ET-1- and SP-induced bronchoconstriction (table 1). Peroxynitrite formation from SIN-1 (10^{-3} M) was completely inhibited by NAC (10^{-3} M) or GSH (10^{-3} M) in vitro (fig. 4a). Neither NAC nor GSH

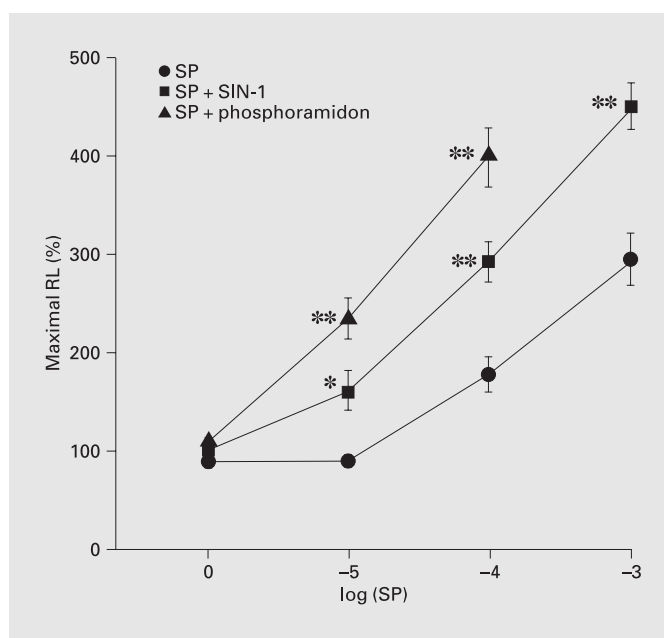


Fig. 3. Effects of SIN-1 and phosphoramidon on SP-induced bronchoconstriction. Each point represents the mean $\pm$ SEM for 6 animals. * $p < 0.05$, ** $p < 0.05$ versus SP alone.

Fig. 4. a Peroxynitrite formation by SIN-1 with NAC and GSH. Peroxynitrite formation by SIN-1 (10^{-3} M) was completely inhibited by the addition of NAC or GSH to the reaction medium. Results are expressed as means ($n = 8$, SD was $<5\%$ in all cases). **b** Effects of NAC and GSH on SP-induced bronchoconstriction in the presence of SIN-1. Each point represents the mean $\pm$ SEM for 6 animals.

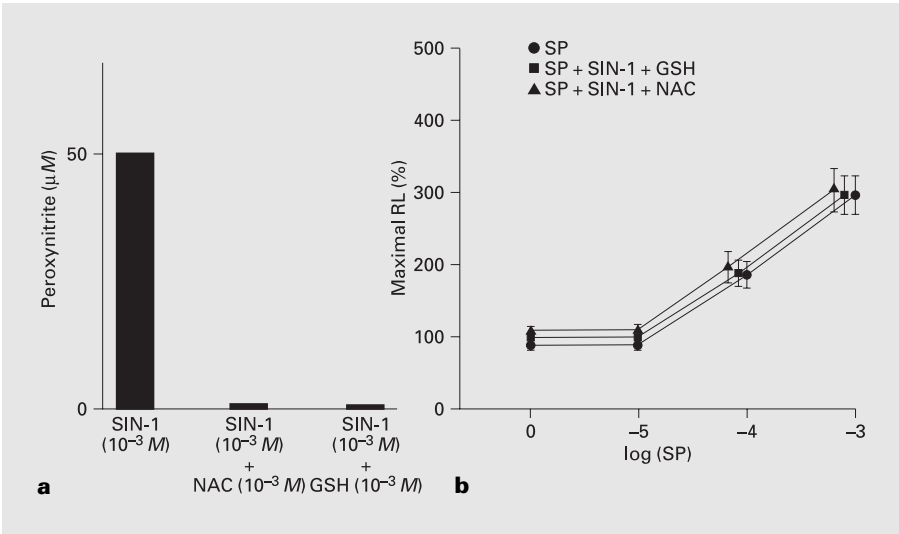


Table 1. Effect of SIN-1 on ET-1 and SP-induced bronchoconstriction

	Percent of maximal R _L , %
ET-1 (1×10^{-8} M)	350 $\pm$ 20
With phosphoramidon (1×10^{-4} M)	440 $\pm$ 50 ^a
With SIN-1 (1×10^{-7} M)	430 $\pm$ 30 ^a
With phosphoramidon (1×10^{-4} M) and SIN-1 (1×10^{-7} M)	440 $\pm$ 35 ^a
Substance P (1×10^{-4} M)	185 $\pm$ 15
With phosphoramidon (1×10^{-4} M)	400 $\pm$ 30 ^b
With SIN-1 (1×10^{-7} M)	290 $\pm$ 25 ^b
With phosphoramidon (1×10^{-4} M) and SIN-1 (1×10^{-7} M)	410 $\pm$ 35 ^b

Each value represents the mean $\pm$ SEM for 6 animals.

^a $p < 0.05$ versus ET-1 alone.

^b $p < 0.01$ versus SP alone.

had any effect on SP-induced bronchoconstriction. However, pretreatment with either NAC or GSH significantly inhibited the potentiation by SIN-1 of SP-induced bronchoconstriction (fig. 4b).

In addition, the NEP activity of the trachea after SIN-1 exposure was significantly reduced compared to the level in solvent-exposed control guinea pigs (solvent: 30.0 ± 4.2 fmol $\cdot$ min $^{-1}$ $\cdot$ mg tissue $^{-1}$; 10^{-6} M SIN-1: 13.0 ± 4.0 fmol $\cdot$ min $^{-1}$ $\cdot$ mg tissue $^{-1}$; 10^{-7} M SIN-1: 15.5 ± 4.5 fmol $\cdot$ min $^{-1}$ $\cdot$ mg tissue $^{-1}$).

Discussion

SIN-1 is thought to generate equimolar amounts of superoxide anion and NO, which may rapidly interact to form peroxynitrite. Therefore, SIN-1 has been utilized in previous studies for the exposure of various kinds of pulmonary cells to peroxynitrite [15, 16]. However, there are no data indicating that SIN-1 actually causes the release of peroxynitrite in vivo. Though nitrotyrosine staining of the airways could be used to determine this, recent work has demonstrated that nitrotyrosine formation does not necessarily implicate peroxynitrite formation [17]. In this study, we showed that SIN-1 generates peroxynitrite in vitro, and that a decrease in NEP activity after peroxynitrite adminis-

tration is the cause of the observed augmented SP- and ET-1-induced airway responses.

ET-1 is a 21-amino-acid peptide isolated from porcine cultured endothelial cells. Experimental findings have shown that ET-1 has a potent contractile effect on airway smooth muscle both in vivo and in vitro. Phosphoramidon is a potent and selective inhibitor of NEP with a nanomolar affinity constant for purified NEP as well as for NEP-like activity in guinea pig airway [18]. Previous studies have indicated that epithelium removal potentiates the response to ET-1 in guinea pig tracheas [19], and that the treatment of guinea pigs with phosphoramidon markedly potentiates the bronchoconstrictor responses evoked by aerosol administration of ET-1 in vivo [20]. In addition, phosphoramidon did not further potentiate bronchoconstrictor responses to ET-1 in an epithelium removal model of guinea pig tracheas. Moreover, other researchers have provided further data in support of a modulator role for epithelium-derived NEP on ET-1-induced contraction in guinea pig tracheas. For example, recombinant human NEP decreased ET-1-induced bronchoconstriction [21]. In this study, we found that peroxynitrite administration enhanced SP- and ET-1-induced bronchoconstriction and that in the presence of phosphoramidon, responses to SP and ET-1 in peroxynitrite-exposed and control guinea pigs were not different. We also found that pretreatment with phosphoramidon in guinea pigs exposed to peroxynitrite further potentiated the bronchoconstrictor responses to SP, but not to ET-1. It seems likely that SP is more sensitive to airway NEP than ET-1.

We recently found that peroxynitrite derived from SIN-1 significantly inhibited acetylcholine-induced bronchoconstriction in a dose-dependent manner [11]. However, 10^{-7} M SIN-1 had no effect on basal R_L or acetylcholine-induced bronchoconstriction. Therefore, this dose of SIN-1 was used in all experiments in this study. A previous study revealed that cigarette smoke enhanced airway responsiveness to SP in vivo by inactivating airway NEP via free radicals [22]. Peroxynitrite might influence NEP activity in the airways, since peroxynitrite is a stronger oxidant than either NO or superoxide anion [23]. Interestingly, the concentration of NO derivatives in the sputum of patients with asthma correlates significantly with the percentage of shed epithelial cells [24], which may be evidence for the involvement of peroxynitrite. In fact, intratracheally administered peroxynitrite induced epithelial damage and airway hyperresponsiveness in guinea pigs [25]. However, exposure to a low concentration of peroxynitrite did not induce the death of pulmonary epithelial cells in vitro [15]. The concentration of peroxynitrite obtained from SIN-1 (10^{-7} M for 30 min) used in this study was quite low, and thus probably did not induce the death of airway epithelial cells. It seems likely that peroxynitrite induced alterations in protein functions in airway epithelial cells.

In conclusion, SIN-1 is a potent peroxynitrite-releasing compound which directly inactivated NEP in guinea pig airways. Peroxynitrite may be an important regulator in the control of airway functions.

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