

Original Contribution

# Effects of inducible nitric oxide synthase inhibitors on asthma depending on administration schedule

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## Abstract

The effectiveness of two inducible nitric oxide synthase (iNOS) inhibitors on allergic airway inflammation was investigated under different administration schedules. Rats sensitized to ovalbumin (OVA) were exposed to OVA for 3 consecutive days. Both iNOS inhibitors showed markedly different effects between two pretreatment schedules: pretreatment before each of three OVA exposures (S1) and before the third exposure alone (S2). S1 pretreatment resulted in higher pulmonary resistance than triple OVA alone. This potentiation was associated with increased eosinophil infiltration and malondialdehyde levels in the lungs, which were suppressed by superoxide dismutases (SODs) but not by methylprednisolone. However, the S2 administration of both iNOS inhibitors completely suppressed the airway response. Administration by schedule S1 completely suppressed plasma nitrite and nitrate levels, but that by S2 caused only a slight suppression. The triple OVA exposures resulted in the upregulation of iNOS in alveolar macrophages and arginase activity, Mn- and Cu/Zn-SOD expression, and nitrotyrosine and lipid peroxide deposition in the airway. However, inhibitors administered by schedule S1 suppressed this upregulation, but further potentiated nitrotyrosine, which in turn was inhibited by SOD. Although iNOS inhibitors may be beneficial for asthma, repeated administration may be detrimental because of extensive reduction of NO and downregulation of SOD.

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Allergic asthma is considered a chronic and complex inflammatory disorder of the airways. The characteristic features of this disorder are allergen-induced immediate airway response (IAR) and late airway response (LAR) [1]. Based on the findings

of studies on bronchoalveolar lavage fluid (BALF) and histological analysis of lung tissue, the mechanisms for LAR are considered to be causally related to the infiltration of eosinophils and other inflammatory cells into the bronchial submucosa [2,3]. It has been suggested that various mediators, including cysteinyl leukotrienes (CysLTs) and nitric oxide (NO), play an important role in LAR [4–6]. NO has been found to play an important role as a signal molecule as well as a cytotoxic or regulatory effector molecule of the innate immune response [7]. NO is synthesized on demand for short periods after the enzymatic activation of constitutively expressed endothelial NO synthase (eNOS; type 3) or neuronal NO synthase (nNOS; type 1) [8,9]. In contrast, inducible NO synthase (iNOS; type 2) has been suggested to be responsible for the profound and long-lasting production of NO. Whereas the NO produced by

**Abbreviations:** AG, aminoguanidine; BALF, bronchoalveolar lavage fluid; Cu/Zn-SOD, copper/zinc superoxide dismutase; CysLT<sub>1</sub>R, cysteinyl leukotriene type 1 receptor; EDTA, ethylenediaminetetraacetic acid; IAR, immediate airway response; iNOS, inducible nitric oxide synthase; i.p., intraperitoneally; i.t., intratracheally; LAR, late airway response; LPS, lipopolysaccharide; MDA, malondialdehyde; Mn-SOD, manganese superoxide dismutase; MP, methylprednisolone; NO, nitric oxide; NOx, nitrite + nitrate; p.o., per os; OVA, ovalbumin; R<sub>L</sub>, pulmonary resistance; s.c., subcutaneously; SOD, lecithinized superoxide dismutase.

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stimulation with constitutive NO synthases may have antagonistic effects on bronchoconstriction induced by bradykinin and histamine [10], the huge amounts of NO generated by iNOS may be detrimental to the host [11]. However, a study by Schuling et al. has suggested that the NO produced by iNOS can have dual effects, an antagonistic action against bronchoconstriction and an enhancement of cellular infiltration into the bronchial submucosa [12]. It has been reported that NO levels are elevated in air exhaled by asthmatic patients [13,14] and that iNOS expression is upregulated in the airways of asthmatics [15,16]. In another study, the NO exhaled by asthmatics has been shown to be reduced by the oral administration of a selective iNOS inhibitor, but no significant change was seen in pulmonary function tests [17]. In consideration of these various results, intervention with a selective iNOS inhibitor for the treatment of asthmatics remains a matter of controversy [18].

It is generally thought that NOS is able to contribute to superoxide generation under certain conditions [19,20]. The formation of superoxide anion is closely controlled by antioxidant enzymes, superoxide dismutases (SODs), which are important components of the antioxidant defense system [21]. These include Cu/Zn-SOD (SOD1), Mn-SOD (SOD2), and extracellular SOD (SOD3). The inhibition of iNOS by its inhibitors may influence not only NO production but also superoxide generation, which may result in an unbalanced generation of NO and superoxide. However, there have been very few reports concerning the relationship between iNOS and SOD expression in allergic airway inflammation [22,23]. In addition, although one study has reported that allergic inflammation in the lungs of iNOS-deficient mice was inhibited [11], other studies reported that cellular infiltration was increased in experiments using these mice [24,25]. Consequently, there have been conflicting results in this mouse model [11,24,25]. To clarify the role of iNOS in allergic airway disorders, we examined iNOS and SOD expression in the lungs after there was repeated inhalation of an antigen and evaluated the effects of two selective iNOS inhibitors on these airway responses [26,27]. Using the repeated-antigen-exposure model in rats, we originally hypothesized that continuous inhibition of iNOS by repeated administration of a selective iNOS inhibitor might suppress the allergic airway inflammation more strongly than a single administration. Unexpectedly, however, such persistent inhibition of NO production resulted in more severe airway inflammation than that by the antigen alone. Consequently, we tried to examine the effects of selective iNOS inhibitors on NO production, iNOS protein expression and activity, arginase activity, SOD expression, and superoxide production by administering them according to different schedules in the repeated-antigen-exposure model.

## Materials and methods

### *Sensitization of rats*

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the School of Medicine,

Fukuoka University. Male Brown Norway rats (Seakku-Yoshitomi, Fukuoka, Japan) that were 6–8 weeks of age and weighed around 250g were used for the study. The rats were actively sensitized against ovalbumin (OVA) by subcutaneous (s.c.) injection of sterile normal saline (1 ml) containing 1 mg OVA (grade II; Sigma, St. Louis, MO, USA) and 200 mg aluminum hydroxide (Sigma). *Bordetella pertussis* vaccine containing  $6 \times 10^9$  heat-killed bacilli (Wako, Osaka, Japan) was given intraperitoneally (i.p.) as an adjuvant. Three days later, sterile saline (1 ml) containing 1 mg OVA and 200 mg aluminum hydroxide was injected (s.c.) for a booster effect. The animals selected for these studies were used 14 to 28 days after the first injection.

### *Measurement of pulmonary resistance ( $R_L$ )*

The rats were anesthetized i.p. with urethane (1 g/kg, 25% wt/vol).  $R_L$  was monitored using a PULMOS-II system (M.I.P.S., Osaka, Japan) [28]. Briefly, the tip of the tracheal tube was inserted into the trachea and the transpulmonary pressure was determined by monitoring the difference between the pressure in the external end of the tracheal cannula and that in the esophageal cannula. A Fleisch pneumotachograph and a differential transducer were used to monitor the respiratory flow rate.  $R_L$  was estimated under artificial ventilation with a Harvard Apparatus rodent respirator (Millis, MA, USA) at a respiration rate of 65/min and tidal volume of 3.5 ml.

### *Experimental design and effects of inhibitors*

Sensitized rats were repeatedly exposed to the antigen to study the effects of the number of OVA exposures, i.e., single, double, and triple, on time-course changes in  $R_L$ , according to the previous report [28]. In the triple OVA exposure experiment, the sensitized rats were challenged by inhalation of 0.25% OVA aerosol for two successive days (days 1 and 2), as shown in Fig. 1. For this purpose, the rats were placed for 20 min, on each occasion, in a 10-L Plexiglas chamber connected to a Comfort-Mini ultrasonic nebulizer (Model-10; Sin-Ei Industries, Ageo, Saitama, Japan). On day 3, a final OVA challenge was performed by intratracheal (i.t.) administration of 0.1 ml of 1.7% OVA solution to estimate the time course in  $R_L$  and histological changes. In the double OVA exposure, the rats were challenged by a single day of inhalation as described above, followed by i.t. administration. In the single OVA exposure, i.t. administration alone was done. To examine the role of iNOS in the time-course changes in  $R_L$ , one of the two selective iNOS inhibitors was administered to the rats before each challenge. To inhibit the iNOS activity for longer periods, the iNOS inhibitor was administered twice a day instead of once. In this case, the second administration was done approximately 12 h after the first, which was performed 30 min or 1 h before the OVA exposure. In contrast, either of the iNOS inhibitors was administered only before the last (third) OVA exposure. OVA grade V (Sigma)

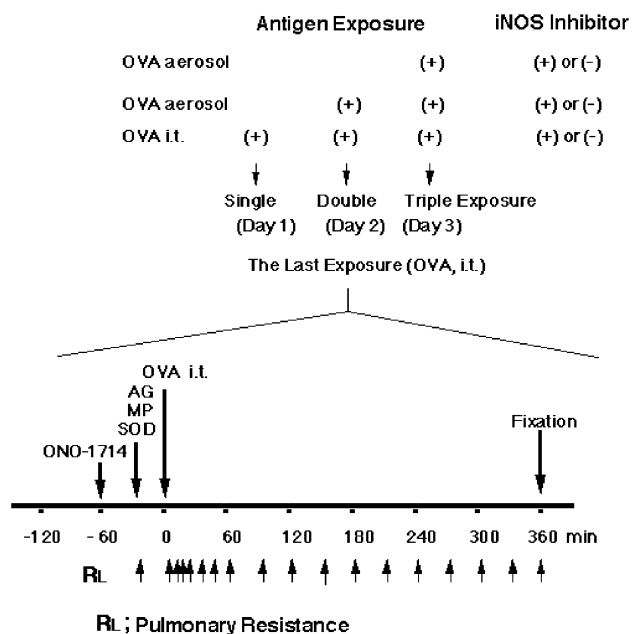


Fig. 1. Experimental schedules for the exposure of sensitized rats to OVA for up to 3 consecutive days. Certain rats were exposed to the antigen by inhalation of 0.25% OVA aerosol for 20 min. The final challenge was conducted by an instillation i.t. of 0.1 ml of 1.7% OVA solution and then  $R_L$  was measured for up to 6 h after the last exposure. ONO-1714 was administered s.c. to the rats 1 h before OVA challenge. AG, MP, and SOD were administered i.p. 30 min before the challenge.

was used for OVA exposure.  $R_L$  was measured before the challenge (baseline value); at 1, 5, 10, 15, 30, 45, and 60 min after the challenge; and then every 30 min for 6 h thereafter. As a control, the same volume of saline (0.1 ml) was administered i.t. At 6 h after the i.t. administration, the rats were exsanguinated by cutting the abdominal aorta. The lungs were either fixed in situ for histological examination or removed from the rats for biochemical study. The lungs thus removed were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use.

BAL was performed via the tracheal cannula using  $2 \times 10$  ml of saline containing 1 mM EDTA. The BALF was centrifuged at 300g for 5 min at  $4^{\circ}\text{C}$  and the cell pellet resuspended in 1.0 ml sterile saline with 0.2% rat serum. The total cell count was determined by adding an equal amount of trypan blue stain and then counting the cells under a light microscope. The differential cell count was carried out from a smear preparation stained with Diff-Quik (International Reagents Corp., Kobe, Japan); 200 cells were counted at random under  $\times 200$  magnification. The cells were identified by standard morphology.

Two selective iNOS inhibitors, ONO-1714 and aminoguanidine (AG), were used in this study [26,27]. ONO-1714 ((1*S*,5*S*,6*R*,7*R*)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0]heptane hydrochloride) (a cyclic amidine analogue) was kindly provided by ONO Pharmaceutical (Osaka, Japan) and AG was purchased from Sigma. As ONO-1714 has been reported to inhibit partially purified human iNOS, eNOS, and nNOS with  $K_i$  values of 1.88, 18.8, and 3.79 nM, respectively, it can be considered 10 times more selective for iNOS than for eNOS

and twice as selective for nNOS [26,29]. ONO-1714 has also been reported to be approximately 30–40 times more selective for human iNOS than  $N^G$ -methyl-L-arginine and twice as selective as AG. To evaluate the effects of ONO-1714 and AG on  $R_L$  and on histological features, they were dissolved in sterile saline. The former was administered s.c. 1 h before the challenge and the latter was given i.p. 30 min before the challenge. Methylprednisolone (MP) dissolved in ethanol and lecithinized SOD were i.p. administered 30 min before the challenge [30]. A CysLT<sub>1</sub> receptor antagonist (pranlukast) was suspended with 0.5% carboxymethylcellulose sodium and administered intragastrically to the rats 2 h before the challenge.

#### Measurement of NOS and arginase activity

NOS and arginase activity in the lung homogenates was measured as the transformation of [ $^{14}\text{C}$ ]arginine to [ $^{14}\text{C}$ ]citrulline and [ $^{14}\text{C}$ ]ornithine, respectively, according to a modification of the Bredt and Snyder method [31]. To estimate iNOS activity, we prepared a reaction mixture containing 0.2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]arginine with specific activity of 313 mCi/mmol (NEN Life Science Products, Boston, MA, USA), 50  $\mu\text{M}$  L-arginine, 100  $\mu\text{M}$   $\beta$ -nicotinamide adenine dinucleotide phosphate, 25  $\mu\text{M}$  tetrahydrobiopterin, 2  $\mu\text{M}$  flavin adenine dinucleotide, 2  $\mu\text{M}$  flavin mononucleotide, and 1 mM EDTA without  $\text{Ca}^{2+}$ . The control consisted of the initial reaction mixture with a Tris buffer. The lungs were thawed and then homogenized in a 25 mM Tris-HCl buffer (pH 7.4) on ice using a polytron homogenizer. The homogenates were centrifuged at  $10^4g$  for 30 min at  $4^{\circ}\text{C}$  and the supernatants were used for assay. The mixtures were incubated for 20 min at  $37^{\circ}\text{C}$ . The reaction was stopped by the addition of 0.5 M perchloric acid, and [ $^{14}\text{C}$ ]arginine, [ $^{14}\text{C}$ ]ornithine, and [ $^{14}\text{C}$ ]citrulline were separated on a Shim-Pack cationic ion-exchange column (Shimadzu Model ISC-05/S0504; Kyoto, Japan) in an HPLC system (Waters Assoc., Milford, MA, USA). We used solvent A (0.2 M sodium citrate buffer containing 7% ethanol, pH 3.2) and solvent B (0.6 M sodium citrate/0.2 M borate buffer, pH 10.0). The mobile phase began with solvent A at a flow rate of 0.6 ml/min at  $50^{\circ}\text{C}$ . This was followed by a linear increase over 10 min to 100% solvent B and 5 min to 100% solvent B and then a return to solvent A on a linear gradient over 10 min. The citrulline and ornithine fractions were collected with a fraction collector (Model 201; Gilson, Villiers-Le-Bel, France). After a scintillation cocktail was added, the isotope was measured for 3 min using a scintillation counter (TRI-CARB Model 2500 TR; Packard, Meriden, CT, USA). The specific transformations to [ $^{14}\text{C}$ ]citrulline or [ $^{14}\text{C}$ ]ornithine were calculated as follows: the isotope count in the citrulline or ornithine fraction eluted from the chromatogram of the incubated mixtures containing the isoforms was subtracted from the isotope count of the fraction eluted from the control. The resulting figure was divided by the specific activity of [ $^{14}\text{C}$ ]arginine (313 mCi/mmol). The specific activities of NO synthase and arginase activity were expressed as picomoles of citrulline or ornithine formation per milligram of protein per minute, respectively.

#### Western blotting for expression of iNOS and SODs in lungs

The lungs were suspended in 5 volumes of a lysis buffer (10 mM Tris–HCl, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 1 µg/ml chymostatin, 1 mM phenylmethanesulfonyl fluoride, 5 µg/ml trypsin inhibitor, 1% SDS, at pH 7.4) and homogenized on ice using a polytron homogenizer (Kinematica, Steinhofhalde, Switzerland). The homogenates were centrifuged at  $10^4g$  for 30 min at 4°C. Protein concentrations of the supernatants were measured by the Bradford method (Pierce, Rockford, IL, USA) and then equal protein amounts from the supernatants were applied to 7.5% SDS–PAGE for iNOS and to 15% SDS–PAGE for SODs. Ten nanograms of authentic mouse macrophage iNOS (Cayman Chemicals, Ann Arbor, MI, USA) was applied to one lane for the analysis of iNOS expression. The proteins were transferred to a nitrocellulose membrane (Immobilon, Millipore Corp., Bedford, MA, USA). The membrane was incubated with the rabbit IgG antibody against iNOS (Upstate Biotechnology, Lake Placid, NY, USA) or against Cu/Zn-SOD (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1000 dilution and against Mn-SOD (StressGen Biotechnology, Victoria, BC, Canada) at a 1:3000 dilution in Tris-buffered saline with 0.1% Tween 20. The membrane was washed and incubated with donkey anti-rabbit IgG antibody coupled with horseradish peroxidase (Amersham, Buckinghamshire, UK) in Tris-buffered saline with 0.1% Tween 20 at a 1:1000 dilution. The blots were developed with the Amersham ECL system. The bands were scanned with a laser densitometer (Personal Densitometer SI; Molecular Dynamics, Sunnyvale, CA, USA).

#### Northern blotting for Cu/Zn-SOD and Mn-SOD in lungs

Total RNA was extracted from the lung homogenates using TRIzol (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's instructions. The RNA (30 µg per lane) was size fractionated by electrophoresis on an agarose/formaldehyde gel, transferred to a nylon membrane (Hybond-N; Amersham), and hybridized at 42°C for 16–18 h with a  $^{32}P$ -labeled Cu/Zn-SOD or Mn-SOD probe or a  $^{32}P$ -labeled GAPDH probe (Clontech, Palo Alto, CA, USA). To obtain both SOD probes, total RNA was isolated from rat placentas and RT-PCR was performed as previously reported with primers for Cu/Zn-SOD (5'-TTTCGAGCAGAAGGCAAGC GGTGAA-3' and 5'-AATCCCAATCACACCACAAGCCAA-3') and for Mn-SOD (5'-ATTAACGCGCAGATCATGCAG-3' and 5'-TTTCAGATAGTCAGGTCTGACGTT-3') [32]. The amplification conditions consisted of 20 cycles of 1 min at 95°C for denaturing, 1 min at 65°C for annealing, and 1 min at 72°C for extension. The sizes of the PCR products obtained were 396 bp for Cu/Zn-SOD and 483 bp for Mn-SOD. After hybridization, the blots were washed thrice with  $0.1 \times$  SCC–0.1% SDS for 15 min at 65°C. The blots were then exposed to X-ray film with an intensifying screen at –80°C and then scanned with the laser densitometer.

#### Assay for lipid peroxides in lungs

Lipid peroxides were estimated according to a previously reported method [33]. The reaction mixture containing lung homogenates, SDS, acetate buffer, and 2-thiobarbituric acid (Sigma) at pH 3.5 was heated at 95°C for 60 min. After cooling, the red pigment produced was extracted with an *n*-butanol–pyridine mixture and estimated by the absorbance at 532 nm. The amount of malondialdehyde (MDA) was calculated by the molar absorption coefficient as  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ , and the level of lipid peroxides was expressed as nmol MDA/g wet wt.

#### Histological examination

The lungs were fixed in situ by the intratracheal administration of 10% formaldehyde solution given at a pressure of 15 cm H<sub>2</sub>O. The excised lungs were stained by hematoxylin–eosin. For the immunohistochemical analysis, the sections were subjected to the labeled streptavidin–biotin technique that involved overnight incubation at 4°C with anti-nitrotyrosine monoclonal antibody (Cayman Chemicals; 1:50 dilution), monoclonal antibody against oxidized low-density lipoprotein (DLH3; 1:100 dilution), anti-iNOS antibody (1:100 dilution), or antibody against Mn-SOD (1:200 dilution) [34]. After a wash with PBS, the Envision system was applied to the sections using the protocol provided by the manufacturer (Dako, Glostrup, Denmark). Visualization was performed by reaction with a solution of new fuchsin and naphthol AS-BI phosphate or 3,3'-diaminobenzidine solution for the studies with anti-nitrotyrosine and anti-DLH3 antibody. The sections were treated with 3,3'-diaminobenzidine tetrachloride–H<sub>2</sub>O<sub>2</sub> for the studies with anti-iNOS and anti-Mn-SOD antibody. Counterstaining was performed using Mayer's hematoxylin.

#### Statistical analysis

Data are reported as the means  $\pm$  SEM. The statistical analysis was performed using the general linear models procedure in SAS (Statistical Analysis System; SAS, Cary, NC, USA). Multiple-stage analyses were done using the Tukey–Kramer test. A *p* value of less than 0.05 was considered significant.

## Results

#### Effects of selective iNOS inhibitors on $R_L$ after OVA exposure

In the single OVA exposure, the rats were challenged by the i.t. administration of 1.7% OVA solution on day 1 without any previous inhalation of OVA aerosol. As shown in Fig. 2A, when rats were given 0.1 ml saline i.t., no significant change in  $R_L$  was observed (control). On the other hand, i.t. instillation of 0.1 ml 1.7% OVA into the sensitized rats resulted in a rapid increase in  $R_L$ , a form of IAR, within 10 min of challenge, and uncertain LAR. Pretreatment with ONO-1714 (0.03 mg/kg, s.c.) 1 h before challenge resulted in a tendency for decrease in

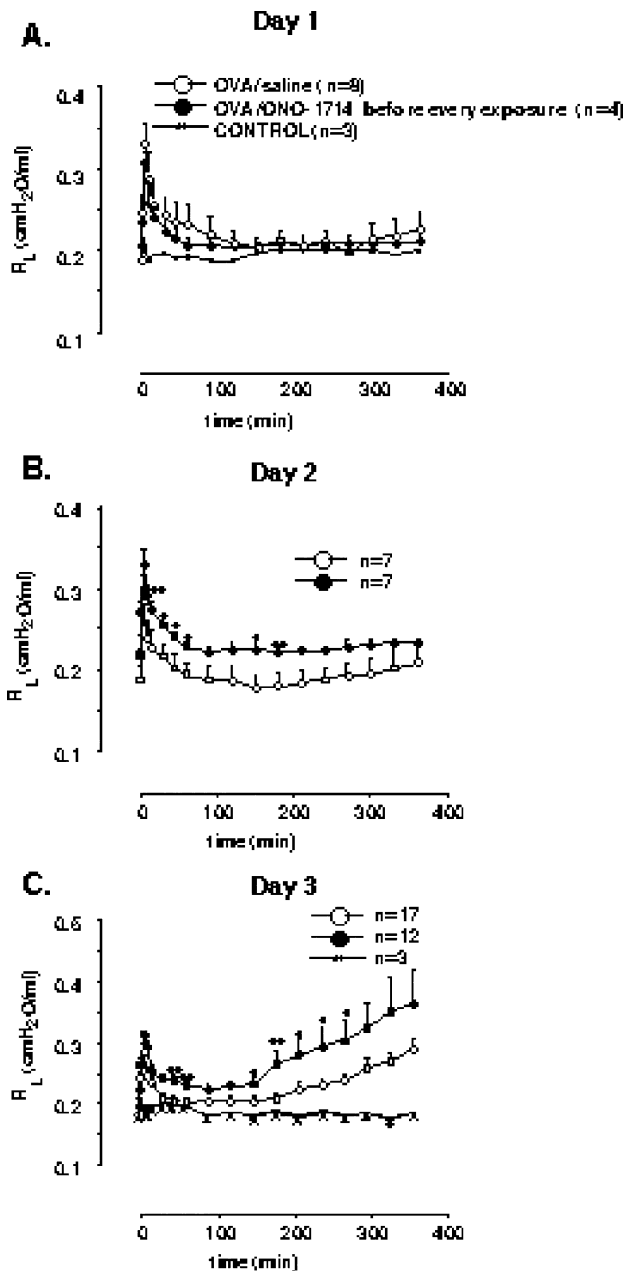


Fig. 2. Time course of  $R_L$  changes after OVA exposure and the effects of ONO-1714. (A) The time course of  $R_L$  after the first OVA exposure after pretreatment with ONO-1714 (0.03 mg/kg, s.c.) or without pretreatment (saline 1 ml/kg) 1 h before the challenge (day 1). As a control, the same volume of saline (0.1 ml) was administered i.t. (B) The time course of  $R_L$  changes after the second OVA exposure with or without ONO-1714 1 h before every OVA exposure (day 2). The rats had also inhaled OVA aerosol the previous day. (C) The time course of  $R_L$  changes after the third OVA exposure with or without ONO-1714 before every exposure (day 3). The rats had inhaled OVA aerosol on each of the 2 previous days. Data are expressed as the  $R_L$  values (cm H<sub>2</sub>O/ml/s) and presented as the means  $\pm$  SEM. \* $p$  < 0.05 and \*\* $p$  < 0.01 between OVA/saline and OVA/ONO-1714.

$R_L$  on day 1. This dose was selected based on the results of our previous studies [35,36]. In contrast, the same pretreatment potentiated IAR in the rats that received the double OVA exposure (Fig. 2B). As shown in Fig. 2C, although the rats that received a triple challenge with saline (control) did not show any significant change in  $R_L$ , the rats that received triple

exposure to OVA showed a prominent LAR after the third OVA challenge. In the rats pretreated with ONO-1714 before every challenge, the LAR was further potentiated after the third challenge (day 3). The baseline  $R_L$  level in the group pretreated with the iNOS inhibitor was of a higher value than the  $R_L$  level by the triple OVA challenge alone. Another selective iNOS inhibitor (AG; 15 mg/kg, i.p.) induced similar potentiation of LAR on day 3 (Table 1). The dose of AG was selected based on the results of our previous study [36]. As the half-lives of ONO-1714 and AG in blood have been reported to be under several hours [26,27], we next examined the effects of a more persistent inhibition of iNOS by administering either iNOS inhibitor (ONO-1714 or AG) twice a day. As shown in Fig. 3A, the persistent inhibition of iNOS by two injections a day resulted in a greater LAR after the third OVA exposure than did the use of one injection a day. In contrast, a single administration of the iNOS inhibitor only before the third OVA completely suppressed the appearance of LAR, as shown in Fig. 3B. A single administration of AG (15 mg/kg, i.p.) before the third challenge also resulted in suppression of the LAR on day 3 (Table 1). In this schedule, however, a similar administration of SOD (10 mg/kg, i.p.) did not suppress the appearance of LAR induced by the third OVA exposure (Fig. 3B). Table 1 summarizes these data. The maximum levels of IAR and LAR were not significantly influenced by the pretreatment with ONO-1714 after the first OVA challenge.

Table 1

Effects of selective iNOS inhibitors on levels of baseline  $R_L$ , IAR, and LAR

|                            | Baseline $R_L$  | IAR          | LAR           |
|----------------------------|-----------------|--------------|---------------|
| Day 1                      |                 |              |               |
| OVA/saline (7)             | 0.19 $\pm$ 0.02 | 183 $\pm$ 10 | 128 $\pm$ 8   |
| OVA/ONO-1714 (4)           | 0.20 $\pm$ 0.01 | 151 $\pm$ 4  | 104 $\pm$ 2   |
| Day 3                      |                 |              |               |
| OVA/saline (17)            | 0.19 $\pm$ 0.01 | 145 $\pm$ 5  | 157 $\pm$ 14  |
| OVA/ONO-1714 (triple) (12) | 0.22 $\pm$ 0.01 | 147 $\pm$ 6  | 171 $\pm$ 22* |
| OVA/ONO-1714 (single) (13) | 0.21 $\pm$ 0.01 | 151 $\pm$ 6  | 103 $\pm$ 1** |
| OVA/AG (triple) (5)        | 0.20 $\pm$ 0.01 | 175 $\pm$ 5  | 187 $\pm$ 17* |
| OVA/AG (single) (5)        | 0.19 $\pm$ 0.01 | 147 $\pm$ 4  | 102 $\pm$ 2** |

The baseline  $R_L$  value and the maximum levels of IAR and LAR after OVA exposure are summarized. The effects of selective iNOS inhibitors (ONO-1714 or AG) on the baseline  $R_L$  value and the maximum levels of IAR and LAR were compared with the effects of saline pretreatment only (see Fig. 2). The rats were pretreated with saline (1 ml/kg, i.p.) or ONO-1714 (0.03 mg/kg, s.c.) 1 h or AG (15 mg/kg, i.p.) 30 min before every OVA exposure. Days 1 and 3 indicate single and triple OVA exposures, respectively. After the sensitized rats were given 0.1 ml 1.7% OVA solution i.t. at the last OVA exposure,  $R_L$  was monitored for up to 6 h. Each baseline value estimated just before every OVA challenge was expressed as cm H<sub>2</sub>O/ml/s. Each value concerning IAR and LAR was divided by the value obtained before the challenge (baseline value 100%) and was expressed as a percentage of the baseline level. On day 3, the iNOS inhibitor was administered to the rats according to two administration schedules, OVA/iNOS inhibitor (triple) and OVA/iNOS inhibitor (single). The former indicates that the rats were pretreated with ONO-1714 or AG before every challenge and the latter that the rats were pretreated with ONO-1714 or AG only before the third challenge after the inhalation of OVA aerosol for 2 consecutive days. The numbers in parentheses indicate the number of animals used.

\*  $p$  < 0.05 between triple and single administration of either iNOS inhibitor.

\*\*  $p$  < 0.05 compared between OVA/saline and OVA/ONO-1714 (single) or OVA/AG (single).

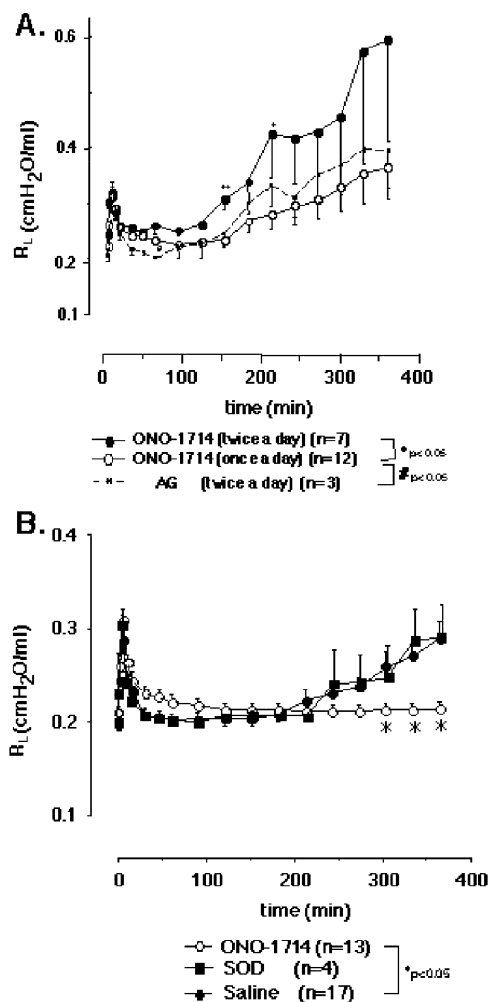


Fig. 3. Comparison among the time courses of the  $R_L$  changes on two different administration schedules: the repeated or the single administration of the iNOS inhibitors. (A) The time course of  $R_L$  after the third OVA exposure in the rats pretreated with iNOS inhibitors (ONO-1714 or AG) administered either once or twice a day before every OVA challenge. (B) Effects of single pretreatment with ONO-1714 or SOD on the time course of  $R_L$  changes after the third OVA challenge. The rats inhaled OVA aerosol for the first 2 consecutive days without pretreatment with iNOS inhibitors. SOD did not affect the time course of  $R_L$  changes after the third OVA challenge, but ONO-1714 completely inhibited the LAR. Data are expressed as the  $R_L$  values (cm H<sub>2</sub>O/ml/s) and presented as the means  $\pm$  SEM.

On day 3, the rats that had been pretreated with ONO-1714 before every challenge (OVA/ONO-1714 (triple)) showed a higher baseline  $R_L$  value than the rats that had received the OVA challenges alone (OVA/saline). Whereas pretreatment with ONO-1714 did not significantly influence the maximum level of IAR, in rats that received triple pretreatment and challenge, the maximum LAR level increased. The rats that received two aerosol challenges followed by a single pretreatment with ONO-1714 only before the third OVA challenge (OVA/ONO-1714 (single)) showed a significantly lower maximum LAR than the rats given triple OVA exposures alone (OVA/saline). The use of AG instead of ONO-1714 showed similar results.

To explain a possible mechanism for the potentiation of LAR under iNOS inhibition, we studied the effects of a

CysLT<sub>1</sub>R antagonist (pranlukast), MP, and SOD on the LAR after the third OVA exposure. The CysLT<sub>1</sub> receptor antagonist was examined as it has been reported that CysLTs play an important role in the LAR [4]. The administration of pranlukast (10 mg/kg, p.o.) with ONO-1714 before every OVA exposure did not inhibit the LAR (data not shown). The administration of MP (10 mg/kg, i.p.) with ONO-1714 before each of three OVA exposures did not significantly suppress the LAR after the third challenge, as shown in Fig. 4A. A single administration of MP only before the third challenge did not affect the LAR. However, the coadministration of SOD (10 mg/kg, i.p.) significantly suppressed the LAR by either triple or single pretreatment only before the third OVA exposure (Fig. 4B). The i.p. administration of SOD at 1 or 3 mg/kg did not suppress these LARs. Moreover, most of the rats that received i.v. administration of SOD at 10 mg/kg died by unknown etiology. Consequently, SOD was administered i.p. at 10 mg/kg in the following experiments.

#### Lipid peroxides in the lungs

As the LAR after the triple OVA exposures with the triple pretreatment with the iNOS inhibitor was markedly suppressed

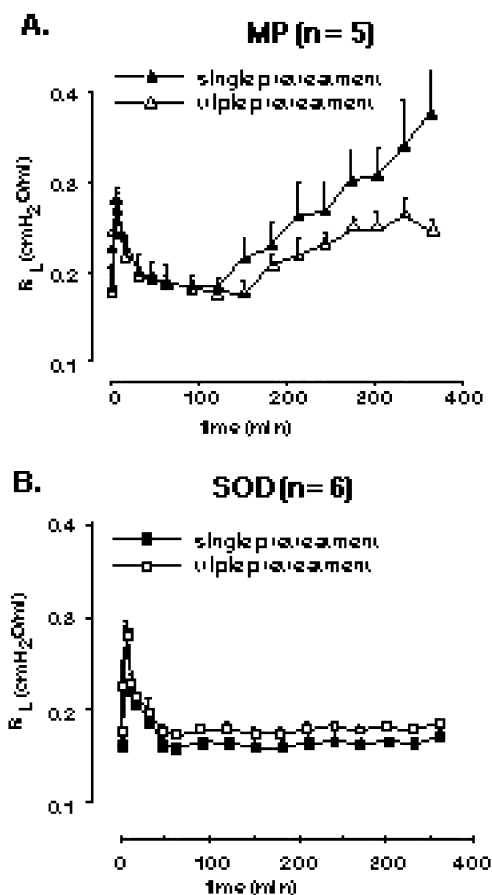


Fig. 4. The effects of MP or SOD on the time course of  $R_L$  changes after the third OVA challenge in rats given triple pretreatment with the iNOS inhibitors. (A) MP was administered 30 min before every challenge or only before the last (third) challenge. (B) SOD was similarly administered 30 min before every challenge or only before the last (third) challenge.

by SOD, it was suggested that overproduction of superoxide anion played an important role in this response. To investigate this issue, we evaluated the level of lipid peroxides as the amount of MDA in the lungs and then compared the effects of SOD with those of MP on the MDA levels. Although the MDA levels increased significantly in the lungs after the third OVA challenge in comparison with the control (saline i.t.), the addition of the iNOS inhibitor before every challenge increased the MDA level, as shown in Fig. 5. The administration of SOD before each of the three challenges significantly reduced the formation of MDA after the third OVA challenge and the third pretreatment with the iNOS inhibitor, but the administration of MP before each of the three challenges did not decrease the MDA level.

#### Cytological study of the BALF

Whereas the BALF from control rats predominantly contained macrophages (>90%), that from rats at 6 h after the third OVA challenge showed a marked increase in total cells, with a predominance of eosinophils and neutrophils, as shown in Figs. 6A and 6B. After the rats were made to inhale OVA aerosol for the first 2 days without pretreatment with an iNOS inhibitor, a single pretreatment with ONO-1714 only before the third OVA challenge significantly reduced the total number of cells and the infiltration of eosinophils in the BALF. However, under a similar condition, a single administration of SOD only before the third OVA challenge did not inhibit the number of cells in the BALF (data not shown). In contrast, the pretreatment with ONO-1714 before each of three OVA challenges increased the number of cells, especially eosinophils, in the BALF after the third challenge. Under this condition, pretreatment with either MP or SOD before every

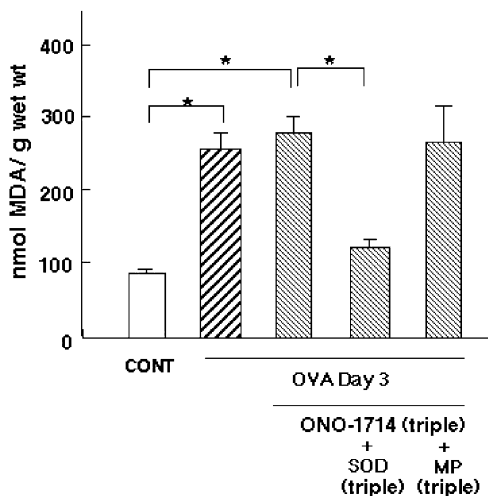


Fig. 5. Comparison of the lipid peroxides (MDA) in lungs and effects of SOD or MP on the amount of MDA. Lipid peroxides in the lungs were estimated in the control rats (CONT) and in rats after the third OVA challenge with (OVA day 3/ONO-1714 (triple)) or without (OVA day 3 alone) pretreatment with ONO-1714 before every challenge. The effects of triple pretreatment with SOD or MP on airway inflammation were compared with the levels of lipid peroxides in the lungs from the rats after the third OVA challenge with triple pretreatment of ONO-1714 before every challenge. \* $p < 0.05$ .

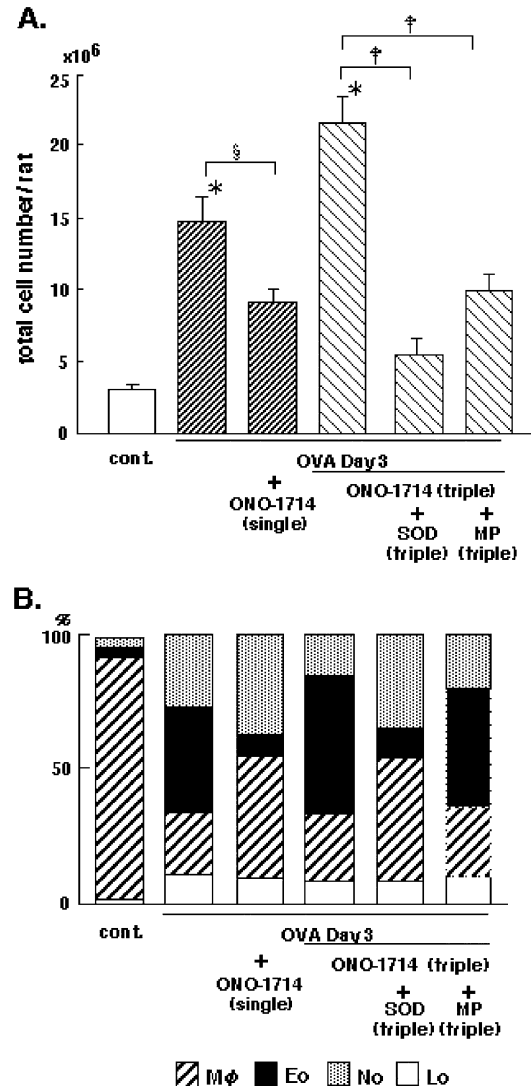


Fig. 6. A cytological analysis of inflammatory cells in BALF. (A) The total cell number in the BALF recovered from control (cont), after triple OVA challenges without (OVA day 3) or with ONO-1714 by a single administration before the third OVA, or after triple pretreatment (ONO-1714 (triple)) before each of three OVA challenges. In rats given triple pretreatment with ONO-1714, the effects of triple pretreatment with SOD or MP before each of three OVA challenges were also examined. \* $p < 0.05$  compared with the control. §, † $p < 0.05$  between the respective columns. (B) Percentages of cellular differentiation in BALF.

OVA challenge resulted in a significant reduction in the total cell number in the BALF, and pretreatment with SOD resulted in a marked reduction in eosinophils, but that with MP did not (Fig. 6B).

#### Histological studies

Fig. 7A shows representative histological findings in lungs obtained from control rats (saline i.t.) and rats 6 h after the third OVA challenge in low- and high-power fields (Figs. 7A, a and b). The infiltration of numerous inflammatory cells (mainly eosinophils and neutrophils) was seen in the bronchial submucosa in the lungs after the third challenge in contrast to the results in the controls. The pretreatment with the iNOS

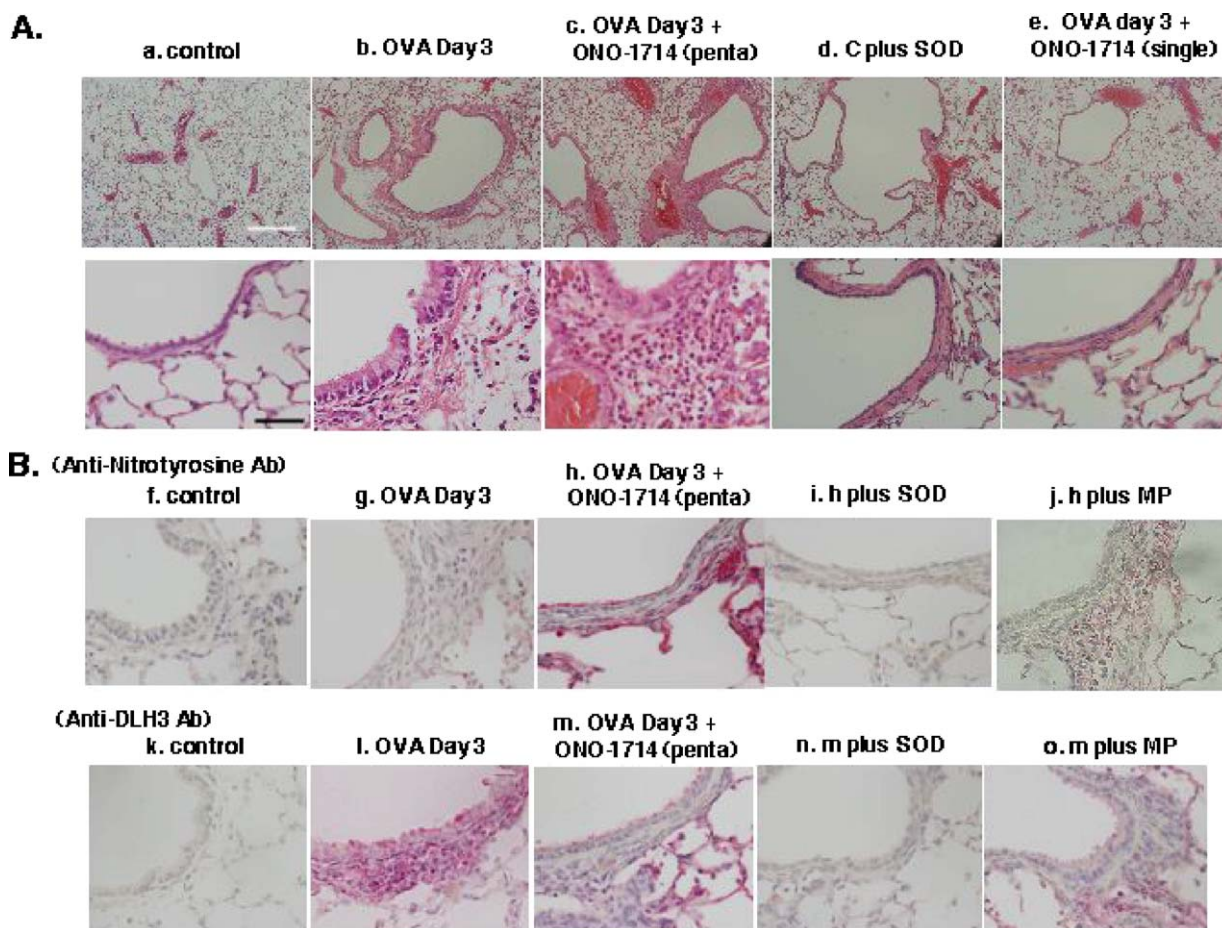


Fig. 7. Histological and immunohistochemical studies in lungs. (A) Histological study of lung specimens by hematoxylin–eosin staining. Two magnifications (low- and high-power fields) are shown. (a) Control lungs; (b) lungs after triple OVA challenges; (c) lungs after triple OVA challenges with pretreatment with ONO-1714 (0.03 mg/kg, s.c.), made twice daily (total: five times) before every challenge; (d) lungs prepared as in (c) but with SOD (10 mg/kg, i.p.) before every challenge; (e) lungs prepared as in (b) but with pretreatment by a single administration of ONO-1714 (single) only before the third OVA challenge, whereas the rats received the inhalation of OVA aerosol for the first 2 consecutive days without pretreatment. Black bar indicates 50  $\mu$ m and white bar 500  $\mu$ m. (B) Immunohistochemical study using anti-nitrotyrosine or anti-lipid peroxide antibody (DLH3). (f and k) Control lungs; (g and l) lungs after triple OVA challenges; (h and m) triple OVA challenges pretreated with ONO-1714, twice a day (total: five times), before every challenge; (i and n) plus SOD or (j and o) MP before every challenge.

inhibitor (ONO-1714) twice a day, before every challenge and 12 h later, resulted in exaggerated infiltration of inflammatory cells into the bronchial submucosa after the third OVA exposure (7A, c). Whereas the coadministration of SOD with ONO-1714 before every OVA challenge significantly suppressed the infiltration of inflammatory cells into the bronchial submucosa (7A, d), that with MP suppressed the cellular infiltration only moderately. In contrast, after the rats inhaled OVA aerosol for the first 2 days without pretreatment with an iNOS inhibitor, the single administration of ONO-1714 only before the third OVA challenge remarkably improved the histological findings (7A, e). However, a similar administration of SOD did not affect the histological findings. An immunohistochemical study showed moderate expression of nitrotyrosine and lipid peroxides in the inflammatory cells infiltrating the bronchial submucosa and luminal surface of the epithelium after the triple OVA exposures, as shown in Fig. 7B (g and l). In contrast, the administration of the iNOS inhibitor twice a day markedly potentiated the expression of nitrotyrosine after the third OVA, but did not significantly change the expression of lipid peroxides in the cells infiltrating the bronchial

submucosa and luminal surface of the epithelium (7B, h and m). The administration of SOD completely inhibited the expression of both (7B, i and n), but the administration of MP caused only moderate inhibition (7B, j and o).

#### *iNOS protein expression, plasma NOx levels, and iNOS and arginase activity in the lungs*

Fig. 8 shows a representative immunoblot for iNOS expression in the lungs subjected to saline challenge (control), triple OVA exposures, and triple OVA exposures with ONO-1714 pretreatment before each exposure. The data are summarized in the graphs ( $n = 8$ ). The repeated exposure to the antigen resulted in the elevated expression of iNOS in the lungs (Fig. 8A). Pretreatment with ONO-1714 before every challenge further increased iNOS expression in the lungs. Although calcium-independent NOS activity was significantly upregulated in the lung homogenates obtained from triple OVA exposures, the triple pretreatment with ONO-1714 inhibited the upregulation of iNOS activity (Fig. 8B). Plasma NOx (nitrite and nitrate) levels were estimated according to the HPLC–

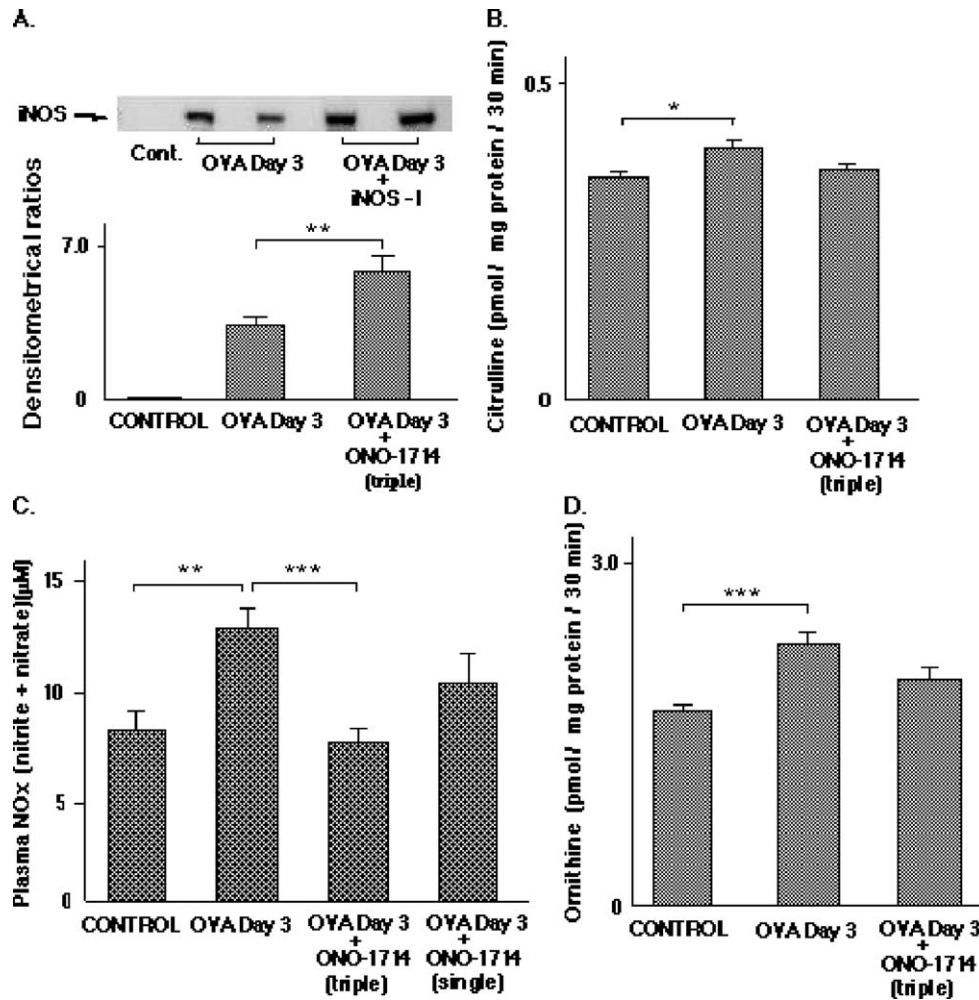


Fig. 8. Effects of triple OVA challenge on iNOS expression, iNOS activity, plasma NOx, and arginase activity in the lungs with or without triple pretreatment of ONO-1714. (A) Western blotting for iNOS in lungs. The top indicates a representative immunoblot for iNOS expression in the lungs obtained from control rats and rats at 6 h after the third OVA exposure, with or without ONO-1714 before each exposure. The bottom summarizes the relative densitometrical intensity against the band of 10 ng authentic iNOS standard ( $n = 8$ ). (B) Calcium-independent NO synthase (iNOS) activity, presented as citulline pmol/mg protein/30 min, in lung homogenates from control rats and rats at 6 h after the third OVA exposure, with or without ONO-1714 before each exposure. (C) Plasma NOx (nitrite plus nitrate) levels from control rats and the rats at 6 h after the third OVA exposure with or without ONO-1714 before every exposure and with a single administration of ONO-1714 only before the third exposure. (D) Arginase activity, presented as ornithine pmol/mg protein/30 min, in lung homogenates from control rats and the rats at 6 h after the third OVA exposure with or without ONO-1714 before every exposure. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Griess method [35]. Whereas NOx levels in the plasma from the control rats were  $8.1 \pm 0.8 \mu\text{M}$ , NOx levels in the rats after triple OVA exposures significantly increased to  $12.9 \pm 0.8 \mu\text{M}$  (Fig. 8C). Although the triple pretreatment with ONO-1714 significantly suppressed the plasma NOx levels, pretreatment with this drug only before the third challenge did not significantly inhibit the NOx. As shown in Fig. 8D, the triple OVA challenge significantly increased arginase activity in the lung homogenates and the triple pretreatment with the iNOS inhibitor decreased this activity.

#### Cu/Zn-SOD and Mn-SOD protein and mRNA expression in the lungs

As shown in Fig. 9A, the lungs of rats that received triple OVA exposures showed a significantly higher expression of Cu/Zn-SOD protein than those from the controls. Pretreatment with ONO-1714 before each of three challenges significantly

inhibited the elevation of Cu/Zn-SOD levels after the third OVA challenge (OVA day 3 + ONO-1714). The Cu/Zn-SOD mRNA level seemed to decrease in the lungs after triple OVA exposures with triple pretreatment of ONO-1714 (Fig. 9B). Whereas Mn-SOD protein and mRNA expression was significantly upregulated in the lungs after triple OVA exposures, the triple pretreatment with ONO-1714 significantly suppressed this upregulation (Figs. 9C and 9D).

#### Immunohistochemical study using anti-iNOS and anti-Mn-SOD antibody

Fig. 10A shows moderate expression of iNOS protein in histiocytes–macrophages in alveolar spaces after triple OVA exposures (10A, b), but the administration of the iNOS inhibitor potentiated the expression of iNOS in these cells (10A, c). Bronchial epithelium of control rats showed moderate expression of Mn-SOD (arrows, a in Fig. 10B).

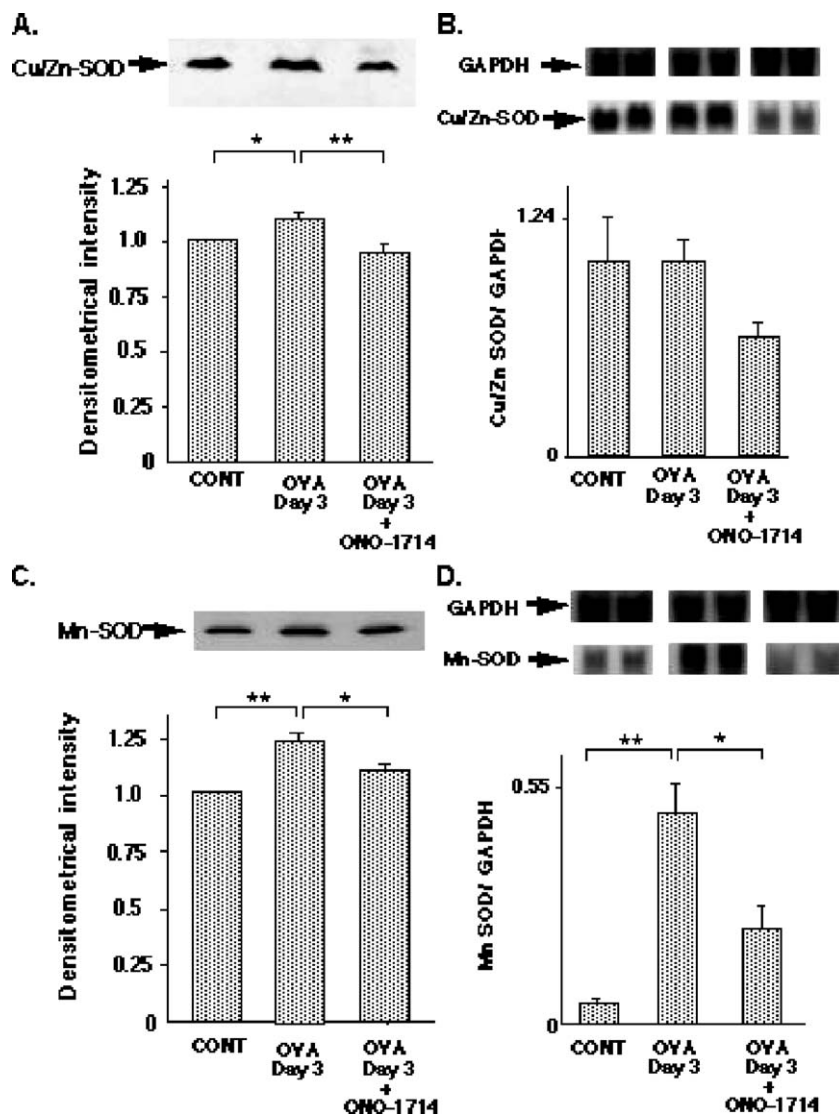


Fig. 9. Northern hybridization and Western blotting for Cu/Zn-SOD mRNA and protein and Mn-SOD mRNA and protein expression in lungs. (A) Immunoblot for Cu/Zn-SOD expression in the control lungs and in the lungs after triple OVA exposure with or without ONO-1714 pretreatment before each of three exposures. (B) Northern hybridization for Cu/Zn-SOD mRNA and the effects of ONO-1714. (C) Immunoblot for Mn-SOD expression in the control lungs and in the lungs after triple OVA exposure with or without ONO-1714 pretreatment before each of three exposures. (D) Northern hybridization for Mn-SOD mRNA expression and the effects of ONO-1714. The GAPDH mRNA expression is also shown ( $n = 5$ ). Each blot is representative of five independent experiments.

The triple OVA exposure increased Mn-SOD expression in the epithelium (arrows, 10B, b), but the administration of the iNOS inhibitor before every OVA exposure suppressed the upregulation (10B, c).

## Discussion

Although there have been numerous studies concerning the participation of NO in various inflammatory reactions, the precise role of NO in allergic asthma remains to be explained [18]. The expression of iNOS in the airway epithelium has been reported to be abundant in asthmatic patients in contrast to control subjects [16]. In another study, bronchial specimens obtained from asthmatic patients showed strong immunoreactivity for nitrotyrosine, but those from the control subjects did not [37]. In addition, treatment with inhaled steroids reduced

the expression of peroxynitrite in asthmatics. Our study also showed that iNOS expression was induced in the lungs, especially in histiocytes–macrophages, after repeated antigen exposures. However, the effects of the iNOS inhibitors on inflammation of the airway changed markedly depending on the administration schedule. In the case of triple antigen exposures, the single administration of a selective iNOS inhibitor only before the last challenge suppressed the LAR and airway inflammation. However, repeated administration of an iNOS inhibitor before every antigen exposure potentiated both reactions after the third challenge. The more persistent inhibition of iNOS accomplished by administering the inhibitor twice a day instead of once a day before every antigen challenge resulted in further potentiation of the LAR. Consequently, the repeated inhibition of iNOS before every antigen challenge had a detrimental effect in this asthma model.

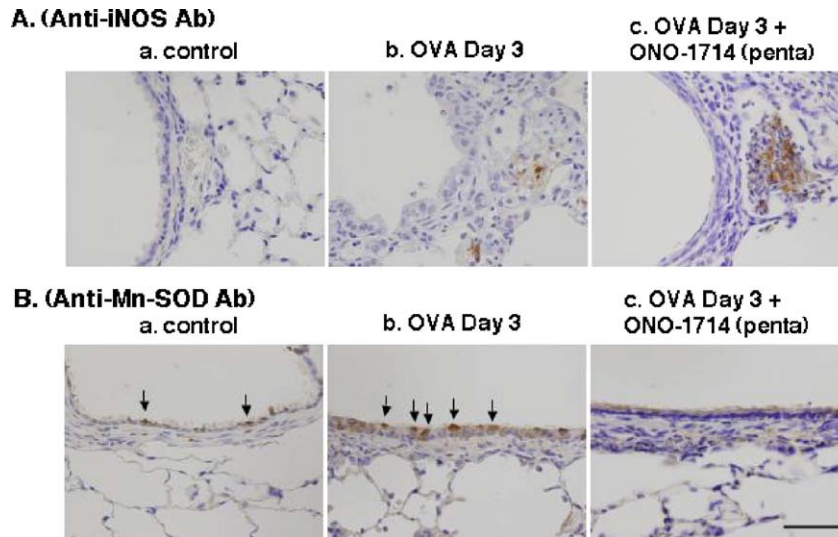


Fig. 10. Immunohistochemical study of localization of iNOS and Mn-SOD in the lungs. Immunohistochemistry using (A) anti-iNOS antibody and (B) anti-Mn-SOD antibody was performed. (a) Control lungs; (b) lungs after triple OVA challenges; (c) lungs after triple OVA challenges with pretreatment with ONO-1714 (0.03 mg/kg, s.c.) twice a day (total: five times), before every challenge. Black bar indicates 50  $\mu$ m.

The potentiation of LAR under iNOS inhibition was potently inhibited by the i.p. administration of SOD, but not by MP, which suggests that superoxide anion plays an important role in this response. A histochemical study showed that the potentiation of LAR was associated with the infiltration of numerous eosinophils and marked expression of nitrotyrosine in the airway tissues. The levels of lipid peroxides in the lung homogenates, as estimated by the amount of MDA, albeit being not so specific for lipid peroxides, were significantly elevated after triple OVA exposures, with or without the pretreatment with the iNOS inhibitor. The elevated level of

MDA was also reduced by SOD, but not by MP. The potentiation of allergic inflammation by the repeated administration of iNOS inhibitor may be consistent with the previous reports that iNOS-deficient mice showed a potentiation of lung inflammation in a hyperoxic environment after LPS injection [24,25]. Therefore, NO generated by iNOS had different effects, ranging from beneficial to detrimental, depending on the experimental conditions.

As SODs are one of the most important defense systems against oxygen stress, we examined alterations in the two types of SOD expression in the lungs after the antigen exposure.

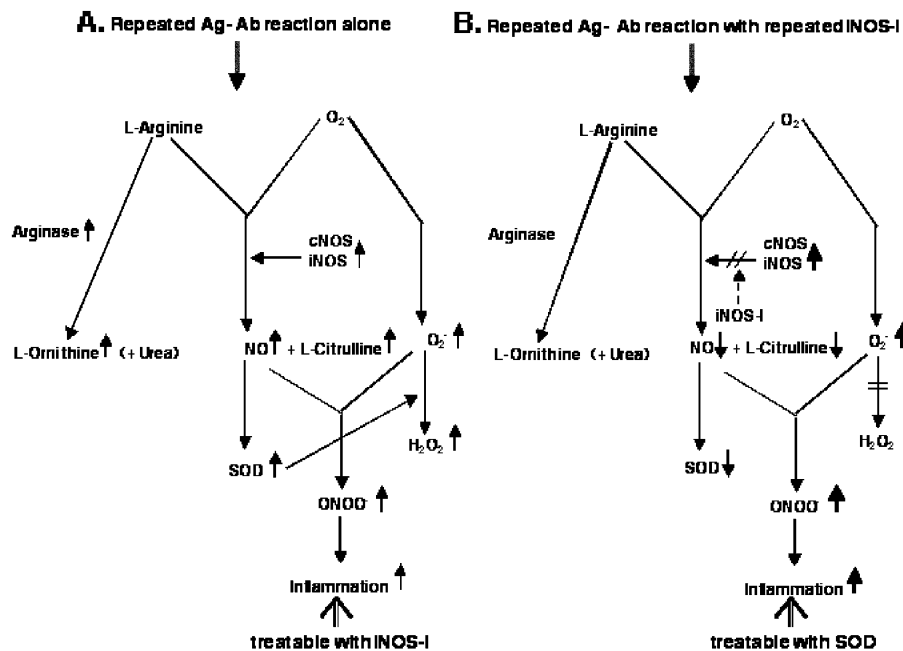


Fig. 11. Proposed pathways for allergic airway inflammation involving NO production and degradation, arginase activity, SOD expression, and superoxide anion production. (A) The repeated Ag–Ab reaction with single administration of an iNOS inhibitor (iNOS-I). An iNOS inhibitor may lead to an appropriate reduction in NO production and may be effective against the inflammation. (B) The repeated Ag–Ab reaction with repeated administration of an iNOS inhibitor. Administration of SOD may be effective against the inflammation in this condition.

Whereas Cu/Zn-SOD and Mn-SOD were upregulated in the lungs after triple antigen exposures, the upregulation of SODs was suppressed by the repeated administration of the iNOS inhibitors. The histochemical study indicated that Mn-SOD expression was upregulated in the bronchial epithelium by the antigen challenges, but the pretreatment with the iNOS inhibitor suppressed the expression. Taken together with the fact that the administration of SOD strongly suppressed airway inflammation after repeated antigen challenge during iNOS inhibition, the results suggest that iNOS-derived NO plays an important role in the regulation of endogenous SOD expression in the bronchial epithelium [21,38,39]. Frank et al. reported that mRNA and protein levels of Cu/Zn-SOD were mediated by a signal through endogenously produced NO [40]. In addition, after LPS stimulation, the inhibition of NO production suppressed the late recovery of downregulation of Cu/Zn-SOD. Therefore, a functional link between iNOS-derived NO and SOD expression is one of the mechanisms involved in preventing overproduction of peroxynitrite.

The results that SOD fully and MP partially inhibited the late elevation of pulmonary resistance, cellular infiltration of eosinophils, and deposition of nitrotyrosine and lipid peroxides in the lungs suggest that superoxide anion plays a significant role in the exacerbation of allergic airway inflammation after antigen challenge during the inhibition of iNOS. However, a single administration of an iNOS inhibitor completely inhibited the LAR after the last (third) antigen challenge, but a similar administration of SOD under the same circumstances did not. To explain this difference in effects between the two administration schedules of the iNOS inhibitors (i.e., the triple and single administration schedules), we hypothesize two types of allergic airway inflammation involving NO production and degradation, arginase activity, SOD expression, and superoxide anion production. As shown in Fig. 11A, triple OVA exposures result in induction of iNOS and arginase through repeated antigen–antibody (Ag–Ab) reaction. NO production probably through iNOS induces SOD expression, which scavenges superoxide anion. As a result, the production of peroxynitrite is restricted to a mild-to-moderate level, resulting in mild-to-moderate inflammation. A regimen of iNOS inhibitor administration such as that of a single administration only before the last challenge may lead to an appropriate reduction in NO production and may be effective against this type of inflammation. Another type of inflammation (Fig. 11B), the repeated Ag–Ab reaction with the repeated administration of an iNOS inhibitor, results in the extensive reduction of NO production and does not significantly induce arginase activity after the third OVA exposure. However, the reduced NO production further induces iNOS protein expression in alveolar macrophages, possibly as a compensatory mechanism due to functional NO deficiency [41]. As SOD expression is not upregulated because of the lack of signaling through NO, the degradation of superoxide anion is delayed. As a result, massive amounts of peroxynitrite are produced and inflammation is exaggerated. The systemic administration of SOD may be effective against this type of inflammation. In fact, in the present study, iNOS activity in the lung homogenates and plasma NOx levels were completely suppressed by the repeated

administration of the iNOS inhibitor, but the single administration before the last antigen challenge only slightly reduced the NOx levels. These results may support the hypothesis of Meurs et al. that the low level of NO induced by upregulated arginase and/or downregulation of NOS activity by a NOS inhibitor may result in the increased synthesis of the highly reactive and detrimental oxidant peroxynitrite [5]. On the basis of the combined results, superoxide anion and NO may play divergent and complex roles in the pathophysiology of asthma [42].

In conclusion, whereas a potent and selective iNOS inhibitor may prove to be a novel and beneficial treatment for allergic asthma if used appropriately, the repeated administration of this drug before repeated stimulation may show detrimental effects due to the extensive reduction of NO production and downregulation of SOD expression.

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