The effect of ascorbic acid on β-adrenergic-mediated bronchodilator responses in the presence of endogenous nitric oxide

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ABSTRACT

The role played by the interaction of nitric oxide (NO) and endothelins (ET) in the modulation of airway function, and the modulation of β-adrenergic-mediated bronchodilator responses by ascorbic acid in the presence or absence of endogenous NO induced by ET in anesthetized guinea-pigs was examined. Endothelins induced a concentration-dependent increase in respiratory resistance, and pretreatment with the NO synthase inhibitor, Nω-nitro-L-arginine methylster, (L-NAME), significantly increased ET-mediated bronchoconstriction. In addition, ET-3-mediated bronchoconstriction following pretreatment with L-NAME was reversed by L-arginine. These findings suggest that the response to the administration of ET-3 can be attributed to the release of NO from the guinea-pig airway. Neither inhaled isoproterenol nor salbutamol significantly affected ET-3-mediated bronchoconstriction, but pretreatment with L-NAME markedly enhanced isoproterenol-mediated bronchodilatation. These findings suggest that endogenous NO induced by ET-3 may attenuate isoproterenol-mediated bronchodilator responses. Ascorbic acid, an antioxidant, enhanced isoproterenol-mediated bronchodilator responses following precontraction with ET-3, but pretreatment with L-NAME reduced this effect of ascorbic acid. The findings suggest that ascorbic acid attenuates the effect of endogenous NO on β-adrenergic-mediated bronchodilator response via its antioxidant activity. Since 10^{-7}mol/L ET-1 does not induce the release of NO from the guinea-pig airway, ascorbic acid cannot enhance isoproterenol-mediated bronchodilator responses. It seems likely that endogenous NO plays an important regulatory role in modulating β-adrenergic-mediated bronchodilator responses in the airway.

Key words: antioxidant, β-adrenergic function, endothelin, guinea-pig airway, nitric oxide.

INTRODUCTION

The endothelins (ET) are a group of physiologically active peptides. Recent studies have demonstrated the existence of three separate ET genes in the human genome encoding for three specific peptides, namely ET-1, ET-2 and ET-3. Recent observations suggest that these peptides are released from human airway epithelial cell cultures in response to endogenous and exogenous signals. In addition, endothelin receptors are present on the airway smooth muscle of animals and humans, and their stimulation by endothelins may result in bronchoconstriction. Nitric oxide (NO) is formed from the semi-essential amino acid L-arginine by the action of the enzyme NO synthase. NO has been shown to elicit a number of biological responses in the lung including arterial vasodilatation and bronchodilatation. NO and related compounds increase the level of intracellular cyclic guanosine monophosphate (cGMP) and induce relaxation of airway smooth muscle in both humans and guinea-pigs. In blood vessels, ET induces strong and long-lasting constriction in vivo and in vitro. It has been shown that the three forms of ET activate ETB receptors at similar concentrations, whereas ET-1 and ET-2 activate ETA receptors at concentrations lower than does ET-3. ET relaxes vascular smooth muscle, which induces the release of NO by activating ETB receptors. These findings suggest that NO may act as a physiological antagonist of ET in blood vessel walls. We have attempted to determine the role played by the interaction of ET and NO in modulation of airway function, and whether ascorbic acid modulates β-adrenergic-mediated bronchodilator responses in the presence or absence of endogenous NO induced by ET in anesthetized guinea-pigs.
METHODS

Measurement of pulmonary resistance
Male Hartley guinea-pigs weighing 400–500 g were used. Under sodium pentobarbital anesthesia (50 mg/kg, i.p.; Abbott Laboratories, IL, USA), artificial ventilation was performed through a tracheal cannula connected to a constant-volume ventilator (Model 680, Harvard Apparatus Co., 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 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 the esophageal pressure) was measured with a differential pressure transducer. Total pulmonary resistance ($R_L$) was calculated using methods previously described.10 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.

Bronchial provocation
Airway resistance was determined by exposing guinea-pigs to increasing concentrations of bronchoconstricting agents administered directly into the airway via the endotracheal tube. Drug aerosols (mass median aerodynamic diameter, 1.8 μm; geometric standard deviation, 2 μm; output, 1.5 mL/min) were generated by an ultrasonic nebulizer (TUR-3200, Nihon Koden, Japan) placed on the inspiratory line of the ventilator. Concentration-response curves were obtained using the following method: after a control challenge with the solvent used to prepare the bronchoconstrictor agents, an aerosol of this agent was administered throughout several series of 30 breaths, each series was separated by 5 min intervals. Respiratory resistance was measured 30 s after the end of each nebulization, when the bronchoconstrictor response reached its maximum.

The effect of L-NAME on ET-1-, ET-2- or ET-3-induced bronchoconstriction
In one series of experiments, concentration curves for the response to endothelins between $10^{-10}$ and $10^{-6}$ mol/L (30 breaths at each concentration) were recorded for two groups of six guinea-pigs each. One group was administered aerosolized $N_\text{O}$-nitro-L-arginine methyl ester (L-NAME) and the other aerosolized saline. In another series of experiments, pulmonary resistance in response to $10^{-7}$ mol/L ET-3 was recorded. Groups of six guinea-pigs each were given either ET-3 and L-NAME ($10^{-4}$ mol/L, 30 breaths), or ET-3, L-NAME and L-Arginine ($10^{-3}$ mol/L, 30 breaths).

Effect of L-NAME and ascorbic acid on isoproterenol- or salbutamol-induced bronchodilation
In each group, guinea-pigs were randomly allocated to pretreatment with either aerosolized L-NAME (administered 20 min before ET-inhalation) or saline administered under the same conditions. We recorded the pulmonary resistance in response to isoproterenol ($10^{-4}$ mol/L, 30 breaths) 20 min after ET-3-administration. In additional experiments we also recorded the pulmonary resistance in response to the simultaneous aerosolized administration of $10^{-4}$ mol/L isoproterenol or salbutamol and $10^{-3}$ mol/L ascorbic acid.

Drugs
L-Arginine was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). L-NAME, salbutamol and isoproterenol were obtained from Sigma Chemical Company (St Louis, MO, USA). ET-1, ET-2 and ET-3 were purchased from the Peptide Institution, Inc. (Osaka, Japan).

Statistical analysis
All values are expressed as mean ± s.e.m. Statistical analysis was performed by one-way analysis of variance followed by Fisher’s exact test. Statistical significance was indicated by $P<0.05$.

RESULTS
Effect of L-NAME on ET-1-, ET-2- or ET-3-induced bronchoconstriction
Baseline respiratory resistance did not differ significantly in guinea-pigs administered L-NAME, ascorbic acid, or saline. In the absence of pretreatment with L-NAME, aerosolized ET induced concentration-dependent bronchoconstriction in guinea-pigs in vivo, and the order of contractile ability was ET-1>ET-2>ET-3 (Fig. 1). After pretreatment with L-NAME ($10^{-4}$ mol/L), aerosolized ET significantly increased bronchocontractile responses compared with those in guinea-pigs administered saline. However, high doses of ET-1 and ET-2 ($10^{-7}$ mol/L and $10^{-4}$ mol/L, respectively) did not have this effect. ET-3 ($10^{-7}$ mol/L)-induced bronchoconstriction was enhanced by pretreatment with L-NAME and was reversed by L-arginine ($10^{-3}$ mol/L; Fig. 2).
Effect of L-NAME and ascorbic acid on isoproterenol- or salbutamol-induced bronchodilator response

Aerosolized ET-1 and ET-3 each rapidly induced maximal constriction and subsequently maintained constant airway tone. Neither administration of aerosolized ascorbic acid nor isoproterenol significantly inhibited ET-3-induced bronchoconstriction, but in the presence of ascorbic acid, isoproterenol markedly inhibited ET-3-induced bronchoconstriction (Fig. 3). However, after treatment with L-NAME, aerosolized isoproterenol significantly inhibited ET-3-induced bronchoconstriction, and no additive bronchodilatative effect was obtained by simultaneous aerosolized administration of ascorbic acid (Fig. 4). Salbutamol, another β-adrenergic agent, also did not significantly inhibit ET-3-induced bronchoconstriction, and the simultaneous aerosolized administration of salbutamol and ascorbic acid markedly inhibited ET-3-induced bronchoconstriction (Fig. 5). Aerosolized ET-1 (10^7 mol/L) induced bronchoconstriction to the same extent as did ET-3 (10^7 mol/L). However, isoproterenol did not significantly inhibit ET-1-induced bronchoconstriction, and the bronchodilatative effect of salbutamol was not affected by ascorbic acid (Fig. 6).

**Fig. 1** Dose-response curves of bronchoconstriction induced with (a) endothelin (ET-1), (b) ET-2 and (c) ET-3 with (n) or without (d) the nitric oxide synthase inhibitor (L-NAME; 10^-4 mol/L). Values for guinea-pigs administered endothelin only were significantly different (P<0.05, **P<0.01).

**Fig. 2** The effects of L-arginine and nitric oxide synthase inhibitor (L-NAME) on endothelin 3 (ET-3)-induced bronchoconstriction. Each group represents the mean ± s.e.m. for six animals.

**Fig. 3** The bronchodilatative effect of isoproterenol on endothelin-3 (ET-3)-induced bronchoconstriction with (n) or without (d) ascorbic acid (10^-3 mol/L). Neither isoproterenol nor ascorbic acid significantly affected pulmonary resistance (R_l) caused by precontraction with ET-3. Simultaneous administration of aerosols of isoproterenol and ascorbic acid (o) potentiated the decrease in R_l (P<0.05, **P<0.01).
DISCUSSION

Inhaled ET-1 and ET-3 each caused concentration-dependent increases in $R_L$. In addition, pretreatment with the NO synthase inhibitor, L-NAME, significantly increased ET-mediated bronchoconstriction. These findings suggest that ET-1 and ET-3 induce the release of NO in the guinea-pig airway. However, high-dose ET-1 did not induce the release of NO. This agrees with and enhances findings from experiments using isolated guinea-pig airways. Battistini et al. reported that at concentrations up to $10^{8}$ mol/L, ET-1 induced transient relaxations prior to the contractile response. Administration of the NO precursor, L-arginine, given after the administration of L-NAME, reversed the increased $R_L$ in response to ET-3. These findings suggest that ET-3 administration induces the release of NO or a related molecule which counteracts the bronchocontractile response to ET-3. However, whether ET-3 can directly stimulate NO release from guinea-pig airway remains to be determined.

Neither inhaled isoproterenol nor salbutamol significantly affected ET-3-induced bronchoconstriction, but pretreatment with L-NAME markedly enhanced isoproterenol-mediated relaxation. We therefore suggest that endogenous NO induced by ET-3 may attenuate isoproterenol-mediated relaxation responses. Ascorbic acid, an antioxidant, enhanced isoproterenol-mediated bronchodilator response following pretreatment with ET-3, but pretreatment with L-NAME reduced the effect of ascorbic acid. These findings suggest that ascorbic acid attenuates the effect of endogenous NO on $\beta$-adrenergic-mediated bronchodilator responses via its antioxidant activity. Since $10^{-7}$ mol/L ET-1 does not induce the release of NO from guinea-pig airway, ascorbic acid cannot possibly enhance isoproterenol-mediated bronchodilator responses. Endogenous NO may have beneficial effects by relaxing airway smooth muscle, but may also have deleterious effects when produced in high concentrations. It is a potent vasodilator and might contribute to hyperemia in asthma patients. NO may also increase the exudation of plasma from leaky postcapillary venules, which could lead to increased mucus production and airway obstruction.

**Fig. 4** The bronchodilative effect of isoproterenol on endothelin-3 (ET-3)-induced bronchoconstriction with (s) or without (d) ascorbic acid ($10^{-3}$ mol/L) following pretreatment with nitric oxide synthase inhibitor (L-NAME). Following pretreatment with L-NAME, isoproterenol significantly decreased pulmonary resistance ($R_L$) caused by precontraction with ET-3. Additionally, simultaneous administration of aerosols of isoproterenol and ascorbic acid did not significantly potentiate the decrease in $R_L$.

**Fig. 5** The bronchodilative effect of salbutamol on endothelin-3 (ET-3)-induced bronchoconstriction with (s) or without (d) ascorbic acid ($10^{-3}$ mol/L). Salbutamol did not effect pulmonary resistance ($R_L$) following pretreatment with ET-3. Simultaneous administration of aerosols of salbutamol and ascorbic acid potentiated the decrease in $R_L$ (*$P<0.05$, **$P<0.01$).

**Fig. 6** The bronchodilative effect of isoproterenol on endothelin-1 (ET-1)-induced bronchoconstriction with (s) or without (d) ascorbic acid ($10^{-3}$ mol/L). Isoproterenol did not significantly potentiate the decrease in pulmonary resistance ($R_L$).
capillary venules in the airway. Indeed, inhibition of endogenous NO production significantly reduces plasma exudation and inflammation in the airway. Under normal conditions, NO can interact with the superoxide anion to protect lung cells. However, NO and the superoxide anion react rapidly to form peroxynitrite, a strong oxidant that may contribute to lung injury, when cytokines have increased production of both NO and the superoxide anion. The finding that nitrotyrosine, which is formed by the reaction of peroxynitrite with aromatic amino acids, is present in lungs of patients with adult respiratory distress syndrome, and attenuation of oxidative stress suggest a role for NO, via peroxynitrite production, in mediation of lung damage. Peroxynitrite can directly interfere with the protein components of membrane receptor systems and alter membrane properties, via lipid peroxidation, leading to indirect alterations of receptor structure or diminished coupling of receptors to effector proteins. Direct oxidative modification of receptor systems may include redox modification of critical thiol/disulphide groups, as has been shown for β-adrenergic receptors. Adrenergic receptors in airway smooth muscle are more sensitive to oxidative damage than muscarinic receptors. This difference has been proposed to result in oxidative stress-induced autonomic imbalance, possibly contributing to airway hyper-reactivity, as in patients with asthma. Ascorbic acid affects airway hyper-reactivity. Increases in ascorbic acid concentrations have been associated with increased pulmonary functions, suggesting that ascorbic acid may attenuate deterioration of lung function related to oxidative stress. Insufficient dietary micronutrient antioxidants may influence the clinical course of asthma: supplement anti-oxidants, especially ascorbic acid, may be beneficial for some forms of airway hyper-reactivity. Recent studies suggest that the NO in exhaled air is formed preferentially in the airway, and that exhaled NO concentration is higher in asthmatic patients than in normal subjects. Endogenous NO may play a very important regulatory role in airway function, and may be involved in the pathophysiology of airway disease. While inhibition of endogenous NO using arginine analogs and NO synthase inhibitor is likely to lead to some clinical problems, such as hypertension, it seems possible that certain airway diseases might be ameliorated by antioxidants, including those administered as aerosols. Optimal functioning of a number of plasma membrane proteins in the respiratory tract, including receptors (proteins involved in signal transduction) and ion channels, may require the presence of strong antioxidants. Antioxidant therapy thus appears to be an exciting new treatment for airway inflammatory disease.

REFERENCES