Effects of Carbocysteine on Antigen-Induced Increases in Cough Sensitivity and Bronchial Responsiveness in Guinea Pigs

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ABSTRACT

Carbocysteine is a mucoactive drug and is being used for both acute and chronic infectious airway diseases. Although carbocysteine can repair the damage of epithelial cells caused by exposure to various agents, the effects of this agent on allergic airway diseases such as asthma and eosinophilic bronchitis has been widely accepted that damage of the airway epithelium has an important role in the development and maintenance of bronchial hyperresponsiveness in asthma (Djukanovic et al., 1990). Evidence has been shown in many reports on human and animal models in which bronchial hyperresponsiveness develops as a result of exposure to various bronchoconstrictor stimuli (Tsukagoshi et al., 1985; Huang et al., 1989) and other agents known to damage the airway mucosa. For example, upper respiratory tract viral infections that cause inflammation and desquamation of the airway epithelium (Jacoby et al., 1988; Dusser et al., 1989; Miura et al., 1989) and mechanically remove the epithelial layer (Djokic et al., 1989; Fine et al., 1989), induce bronchial hyperresponsiveness. In these studies, it has been demonstrated that bronchial hyperresponsiveness caused by epithelial damage is associated with a decrease in neutral endopeptidase (NEP) activity in the tracheal tissue, but it did not influence the bronchial hyperresponsiveness or bronchoalveolar lavage cell component. These findings suggest that carbocysteine promotes the repair of damaged epithelium by allergic reaction and may be useful in allergic airway diseases accompanied by isolated chronic coughing, especially eosinophilic bronchitis without asthma and tracheobronchitis with cough hypersensitivity.

Bronchial asthma is a chronic allergic disease of the airways characterized by bronchial hyperresponsiveness (Sterk and Bel, 1989). Even though the precise mechanisms underlying bronchial hyperresponsiveness are still poorly understood, it has been widely accepted that damage of the airway epithelium has an important role in the development and maintenance of bronchial hyperresponsiveness in asthma (Djukanovic et al., 1990). Evidence has been shown in many reports on human and animal models in which bronchial hyperresponsiveness develops as a result of exposure to various bronchoconstrictor stimuli (Tsukagoshi et al., 1985; Huang et al., 1989) and other agents known to damage the airway mucosa. For example, upper respiratory tract viral infections that cause inflammation and desquamation of the airway epithelium (Jacoby et al., 1988; Dusser et al., 1989; Miura et al., 1989) and mechanically remove the epithelial layer (Djokic et al., 1989; Fine et al., 1989), induce bronchial hyperresponsiveness. In these studies, it has been demonstrated that bronchial hyperresponsiveness caused by epithelial damage is associated with a decrease in neutral endopeptidase (NEP) activity in the tracheal tissue, but it did not influence the bronchial hyperresponsiveness or bronchoalveolar lavage cell component. These findings suggest that carbocysteine promotes the repair of damaged epithelium by allergic reaction and may be useful in allergic airway diseases accompanied by isolated chronic coughing, especially eosinophilic bronchitis without asthma and tracheobronchitis with cough hypersensitivity.

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ABBREVIATIONS: NEP, neutral endopeptidase; NC, negative control; PC, positive control; N, control; N100, carbocysteine; BAL, bronchoalveolar lavage; OA, ovalbumin; Al(OH)3, aluminum hydroxide; Suc-Ala-Ala-Phe-p NA, succinyl-alanyl-alanyl-phenylalanyl-para-nitroanilide; C10, C30, and C100, animals given 1.0 ml/kg of carbocysteine solution at concentrations of 10, 30, and 100 mg/ml, respectively.
epithelium, an effect that may contribute to its mucoregulatory action (Colombo et al., 1994). More recently, it has been shown that carbocysteine can ameliorate the damage of epithelial cells and mucociliary clearance caused by exposure to various agents known to damage the airway mucosa in animals (Okamura et al., 1987; Katoh and Soejima, 1992), and improve impaired mucociliary clearance in patients with chronic bronchitis (Oghara et al., 1982). However, the efficacy of carbocysteine in allergic airway diseases (such as asthma and eosinophilic airway disorders accompanied by chronic coughing like eosinophilic bronchitis without asthma (Fujimura et al., 2000) and eosinophilic tracheobronchitis with cough hypersensitivity (Fujimura et al., 2000)) is not clear. In this study, we investigated the effects of carbocysteine on antigen-induced cough hypersensitivity, bronchial hyperresponsiveness, and decreases in airway NEP activity in guinea pigs.

Materials and Methods

Animals. Male, albino, Hartley-strain guinea pigs weighing 200 to 220 g each were obtained from Sankyou Laboratory Service (Toyama, Japan). They were quarantined in the Animal Research Center of Kanazawa University. All the animal procedures in this study complied with the standards set out in the Guideline for the Care and Use of Laboratory Animals at the Takara-machi Campus of Kanazawa University.

Experimental Protocol 1. Actively sensitized guinea pigs were assigned into five groups: negative control (NC), positive control (PC), and carbocysteine (C10, C30, and C100) groups (n = 8 for each group). Animals in the NC group were challenged with aerosolized saline, and the other groups with aerosolized antigen. Every 12 h after the challenge, guinea pigs in groups NC and PC were given 1.0 ml/kg of vehicle, whereas those in groups C10, C30, and C100 were given 1.0 ml/kg of carbocysteine solution at concentrations of 10, 30, and 100 mg/ml, respectively, via intraperitoneal injection. Cough sensitivity to inhaled capsaicin and bronchial responsiveness to inhaled methacholine were measured at 48 and 72 h after challenge with either the antigen or the saline, respectively. After the measurement of bronchial responsiveness, bronchoalveolar lavage (BAL) was carried out, and then tracheal samples were taken to measure the NEP activity.

Experimental Protocol 2. Naive guinea pigs were kept in the same surroundings as the active sensitization groups and separated into control (N) and carbocysteine (N100) groups. Animals were challenged with aerosolized physiological saline. Guinea pigs in group N were given 1.0 ml/kg of vehicle and those in group N100 were given 1.0 ml/kg of 100 mg/ml carbocysteine solution every 12 h after the saline inhalation via intraperitoneal injection. Cough sensitivity to capsaicin and bronchial responsiveness to methacholine were measured at 48 and 72 h after challenge. BAL was performed after measuring bronchial responsiveness.

Active Sensitization and Antigen Challenge. Guinea pigs were actively sensitized by the method reported by Muraki et al. (1994). Each animal was given an intraperitoneal administration of 2 mg of ovalbumin (OA) and 100 mg of aluminum hydroxide [Al(OH)3] 2 days after intraperitoneal administration of 30 mg/kg cyclophosphamide. Three weeks later, boosting was carried out by intraperitoneal administration of 10 µg of OA and 100 mg of Al(OH)3. Three weeks after the boosting, actively sensitized guinea pigs were challenged with an aerosolized OA solution under spontaneous breathing at 20 min after an intraperitoneal administration of diphenhydramine (20 mg/kg). Conscious guinea pigs were placed in a dual chamber plethysmograph (head chamber volume, 1520 ml) (model PMUA-SAR, Buxco Electronics, Sharon, CT). Animals were challenged with 10 mg/ml OA aerosol for 90 s (head chamber only, 0.08 ml/min output). The aerosol was generated by a Devilbus 646 nebulizer (Devbiass Co., Somerset, PA) operated by compressed air at 7.57 l/min (Minipon 54B-588, Origin Medical Industry Co., Ltd., Tokyo, Japan).

Cough Sensitivity. Each conscious guinea pig was placed in an airtight custom-built transparent plastic box consisting of a head chamber (1600 ml volume) isolated from a body chamber, and pressure in the body chamber was recorded. Coughs were detected as a transient change in the pressure (a rapid inspiration followed by rapid expiration). To disregard motion- and sneezing-related changes in the pressure, movements of the guinea pigs were visually monitored. Coughs were counted by a trained observer and recognized by the characteristic animal posture and the pressure transducer recordings. Increasing concentrations of capsaicin solution (10−8, 10−6, 10−4 M) were inhaled for 2 min from a Devilbus 646 nebulizer (Devbiass Co., Somerset, PA) operated by compressed air at 1.6 l/min (Iwaki Air Pump AP-115AN, Iwaki Co., Ltd., Tokyo, Japan). The nebulizer output was 0.037 ml/min. The number of coughs was counted during a 2 min inhalation of each capsaicin solution and for additional 1 min. The total number of coughs during the 3-min period was recorded on the inhalation of each concentration of capsaicin.

Bronchial Responsiveness. Guinea pigs were anesthetized by an intraperitoneal injection of 75 mg/kg of sodium pentobarbital and placed in a supine position. After the trachea was cannulated with a polyethylene tube (outside diameter, 2.5 mm; inside diameter, 2.1 mm), the animals were artificially ventilated using a small animal respirator (model 1680, Harvard Apparatus Co., Inc., South Natick, MA) adjusted to a tidal volume of 10 ml/kg at a rate of 60 strokes/min. Ascending doses of methacholine solution (50, 100, 200, and 400 µg/ml) were delivered for 20 s by an ultrasonic nebulizer (NE-U06, Omron, Kyoto, Japan) at 5-min intervals. The nebulizer generated the aerosol at a rate of 15.2 µl/min. The changes in lung resistance to insufflation, the lateral pressure of the tracheal tube (pressure at the airway opening: cm H2O), were measured using a differential pressure transducer (model TP-603T, Nikon Koden Kogyo Co., Ltd., Tokyo, Japan).

BAL. BAL was performed immediately after completion of the measurement of bronchial responsiveness to methacholine. Through the tracheal cannula the lungs were lavaged with 10 ml of saline 2 times (total: 20 ml). The cells in BAL fluid were stained with Turk solution and counted in duplicate in a hemocytometer (in a Burker chamber). Differential cell counts were made on a smear prepared by cytocentrifuge and stained with Wright-Giemsa.

Tracheal Samples. Tracheal segments, weighing 100 to 200 mg each, of actively sensitized guinea pig were resected and isolated after performance of BAL. The removed trachea was soaked in saline and homogenized by an ultrasonic homogenizer (SONIFIER 250, Branson Ultrasonics, Danbury, CT). The sample was filtrated through gauze and centrifuged at 5000 rpm for 2 min. The supernatant was diluted up to volume with saline depending on tracheal tissue weight (final volume: 0.04 ml/mg) and used as a tracheal sample.

NEP Activity. NEP activity was determined by a two-step reaction method using the substrate succinyl-alanyl-alanyl-phenylalanyl-para-nitroanilide (Suc-Ala-Ala-Phe-p NA) (Van der Velden et al., 1994). One hundred microliters of tracheal samples were incubated with Suc-Ala-Ala-Phe-p NA (final concentration: 4 mmol/l in Tris-HCl, pH 7.4) in the presence or absence of phosphoramidon (final concentration: 2 µmol/l). The reaction (total volume: 250 µl) was measured in duplicate in a 96-well microtiter plate. The increase in specific absorbance at 405 nm (as a result of the accumulation of free p-nitroaniline) was determined at several time points (0–48 h) using a plate reader (EAR 340AT, SLT-Labinstruments, Göddiq, Austria). Several concentrations of aminopeptidase solution were used to construct the standard curve. NEP activity was determined as the activity that could be inhibited by phosphoramidon and was expressed as units per milligram in reference to the standard curve.
One unit represents the enzyme activity that is able to separate 1 μmol/min p NA from Suc-Ala-Ala-Phe-p NA at pH 7.4 at 25°C.

**Preparation of Drugs.** The following chemicals were used: sodium pentobarbital (Abbott Laboratories, North Chicago, IL), methacholine (Wako Pure Chemical Ind., Osaka, Japan), diphenhydramine (Wako Pure Chemical Ind.), ovalbumin (Sigma, St. Louis, MO), Al(OH)$_3$ (Wako Pure Chemical Ind.), dimethyl sulfoxide (Wako Pure Chemical Ind.), physiological saline (Otsuka Pharmaceutical Co., Ltd., Osaka, Japan), capsaicin (Sigma), phosphoramidon (Sigma), cyclophosphamide (Shionogi Co., Ltd., Osaka, Japan), aminopeptidase (Sigma), Tris hydrochloride (Sigma), Suc-Ala-Ala-Phe-p NA (Sigma), and carbocysteine (Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan).

**Statistical Analysis.** All data are shown as mean ± S.E.M. Differences between any pair of groups were analyzed using the Mann-Whitney U test. A p value less than 0.05 was considered significant.

### Results

**Cough Sensitivity.** Figure 1A shows the effect of carbocysteine on the coughs elicited by aerosolized capsaicin in actively sensitized guinea pigs. The number of coughs elicited by an aerosol of capsaicin (10$^{-6}$ and 10$^{-4}$ M) was significantly increased in the PC group compared with the NC group, showing antigen-induced cough hypersensitivity. Carbocysteine inhibited the antigen-induced increase in the number of coughs dose dependently. Figure 1B shows the effect of carbocysteine on the number of capsaicin-induced coughs in naive guinea pigs. Carbocysteine did not alter the number of coughs at any concentration of capsaicin aerosol.

**Bronchial Responsiveness.** Bronchial responsiveness to inhaled methacholine and the effect of carbocysteine in actively sensitized guinea pigs are shown in Fig. 2A. It was not detected in the alteration of hyperresponsiveness to methacholine between any pair of the PC group and a carbocysteine group, whereas the bronchial responsiveness of the PC group was significantly heightened when compared with that of the NC group. Likewise, there was no significant difference in bronchial responsiveness to methacholine between the N and N100 groups in naive guinea pigs (Fig. 2B).

**BAL Cells.** The effect of carbocysteine on BAL cell count in actively sensitized guinea pigs is shown in Fig. 3A. The number of BAL eosinophils was significantly increased in the PC group compared with the NC group. There were no significant differences in the number of macrophages, lymphocytes, or neutrophils or the total cell count between the PC and NC groups. No dose of carbocysteine significantly changed the cell counts when compared with the PC group. Likewise, there were no significant differences in the cell counts between the N and N100 groups in naive guinea pigs (Fig. 3B).

**NEP Activity.** Figure 4 shows the NEP activity of the tracheal tissue from actively sensitized guinea pigs. NEP activity was significantly decreased in the PC group compared with the NC group. Carbocysteine dose dependently prevented a decrease in NEP activity of tracheal tissue following antigen challenge.

### Discussion

Carbocysteine is a mucoregulatory drug characterized by a spectrum of activities other than a direct effect on mucus secretion (Yasuoka et al., 1986; Barga et al., 1990; Colombo et al., 1994). Normalization of mucociliary clearance by carbocysteine probably results from its ability to repair the damaged airway epithelium (Ogihara et al., 1982; Okamura et al., 1987; Katoh and Soejima, 1992). On the other hand, mucus hypersecretion and injury to airway epithelium are common findings of asthma. However, reports concerning the effects of carbocysteine on human asthma or animal models of allergic airway diseases have been very rare (Asti et al., 1987; Katoh and Soejima, 1992). On the basis of that experimental evidence, we hypothesized that carbocysteine would be effective against asthma and eosinophilic airway diseases accompanied by isolated coughs such as eosinophilic bronchitis without asthma (Gibson et al., 1989) and eosinophilic tracheobronchitis with cough hypersensitivity (Fujimura et al., 2000), by encouragement of restoring damaged epithelium. Therefore, to verify this hypothesis, we tested carbocysteine in a commonly used experimental model of airway allergic inflammation, which is associated with both nonspecific bronchial hyperresponsiveness (Muraki et al., 1994) and cough receptor hypersensitivity (Liu et al., 2001).

The present study clearly showed that carbocysteine reduced the increase of cough sensitivity in inhaled capsaicin and restored the depressed NEP activity of the tracheal tissue following antigen challenge. Carbocysteine did not influ-
ence the capsaicin cough sensitivity or bronchial responsiveness to methacholine in naive guinea pigs, or the antigen-induced bronchial hyperresponsiveness to methacholine in sensitized animals. These findings indicate that antigen-induced inactivation of NEP is an underlying mechanism of antigen-induced cough hypersensitivity, whereas it is not a contributor to antigen-induced bronchial hyperresponsiveness, at least in our study design. Carbocysteine may inhibit antigen-induced cough hypersensitivity by promoting restoration from the antigen-induced NEP inactivation.

Several findings suggest that coughing induced by capsaicin is mediated by selective excitation of nonmyelinated C-fibers and by the subsequent release of sensory neuropeptides such as tachykinins (substance P, neurokinin A, and neurokinin B). Tachykinins cause many airway responses, including smooth muscle contraction (Sekikawa et al., 1987b), gland secretion (Wagner et al., 1999), and increased vascular permeability (Dusser et al., 1989), and they potentiate cholinergic neurotransmission (Sekikawa et al., 1987b; Belvisi et al., 1994; Hey et al., 1996). On the other hand, Kohrogi et al. (1988) reported that NEP inhibitors potentiated the cough response to inhaled capsaicin in naive guinea pigs. NEP, a membrane-bound enzyme, is located on the surfaces of multiple cells, including nerves, smooth muscle, epithelium, and glands in airways, thus providing multiple potential sites for degrading neuropeptides when they are released from nerves (Sekikawa et al., 1987a).

As shown in the present study, the cough response to inhaled capsaicin is increased after challenge with an antigen in actively sensitized guinea pigs. Liu et al. (2001) have reported that bronchodilators, the β2-adrenoceptor agonist procaterol and the anticholinergic agent atropine have no effect on the antigen-induced increased cough response in sensitized guinea pigs. Moreover, in this study, we have shown that NEP activity of the tracheal tissue is decreased after challenge with an antigen in actively sensitized guinea pigs. We believe that the antigen-induced increase in the cough response is due to increased cough receptor sensitivity associated with results from decreased NEP activity, and it is independent of the bronchoconstrictor response or nonspecific bronchial responsiveness at least to methacholine. Furthermore, the results that carbocysteine restored depressed NEP activity of the tracheal tissue and inhibited the increase of cough sensitivity following antigen challenge support our standpoints.

In many previous studies, it has been shown that airway epithelial damage enhances bronchoconstriction due to inhaled tachykinins by decreasing NEP activity (Jacoby et al., 1988; Dusser et al., 1989; Cheung et al., 1992, 1993). However, the results regarding the influence of epithelial injury...
One unit represents the enzyme activity that is able to separate 1 μmol/min p-nitroanilide from Suc-Ala-Ala-Phe- p NA at pH 7.4 at 25°C. Each column represents mean ± S.E.M. *p < 0.01.

or NEP activity on nonspecific bronchial responsiveness are controversial. Miura et al. (1989) showed that inoculation of the type C influenza virus caused airway epithelial damage and increased bronchial responsiveness to acetylcholine in dogs. Chiba and Misawa (1995) reported that intravenous treatment with a NEP inhibitor, phosphoramidon, induced bronchial hyperresponsiveness to inhaled acetylcholine in nonsensitized rats. In addition, the authors (Chiba and Misawa, 1994) reported that bronchial responsiveness to inhaled acetylcholine was significantly increased by pretreatment with the NEP inhibitor phosphoramidon, but the NEP inhibitor-induced effect was no longer observed after antigen challenge in sensitized rats. Chiba and Misawa (1994) also demonstrated that airway NEP activity was significantly decreased in the antigen-challenged rats. Conversely, Cheung et al. (1992, 1993) showed that there was no significant difference in bronchial hyperresponsiveness to methacholine after pretreatment with a NEP inhibitor, thiorphan, compared with a placebo in nonasthmatic (Cheung et al., 1992) and asthmatic subjects (Cheung et al., 1993). In nonsensitized guinea pigs, Fine et al. (1989) reported that the contractile response to acetylcholine was increased in tracheal tissue denuded of epithelium, whereas Dusser et al. (1989) showed that viral respiratory infection by the Sendai virus did not affect the response to acetylcholine despite decreasing the NEP activity in the airway epithelium. Likewise, carbocysteine did not modulate the increase of bronchial responsiveness to methacholine induced by antigen challenge in actively sensitized guinea pigs regardless of restoring the NEP activity of tracheal tissue in this study. In contrast, Asti et al. (1995) showed that pretreatment of carbocysteine, given as an aerosol, was able to reduce the increase in bronchial responsiveness to acetylcholine induced by cigarette smoke in nonsensitized guinea pigs, but the mechanism of this phenomenon is not clear. In short, these findings suggest that the role of NEP in nonspecific bronchial hyperresponsiveness may be different between species and/or employed experimental systems. There is considered to be little relationship between airway NEP activity and nonspecific bronchial responsiveness, at least in humans and actively sensitized guinea pigs.

Airway eosinophilia does not always induce bronchial hyper-responsiveness. Gibson et al. (1989) have shown that bronchial responsiveness is not heightened in patients with isolated chronic coughs and sputum eosinophilia. This airway disorder has been called eosinophilic bronchitis without asthma, in which BAL eosinophilia and eosinophil infiltration into bronchial mucosa are the same as bronchial asthma (Gibson et al., 1998). Recently, it has been shown that cough sensitivity is increased (Brightling et al., 2000), and only corticosteroids are effective for cough (Gibson et al., 1989, 1998) in that disorder. On the other hand, we have independently proposed eosinophilic tracheobronchitis with cough hypersensitivity associated with global atopic tendency, abbreviated as atopic cough (Fujimura et al., 2000), as a cause of isolated chronic nonproductive cough. The presence of eosinophil infiltration in the submucosa of trachea and bronchi and the absence of BAL eosinophilia are characteristic in atopic cough (Fujimura et al., 2000). In this disorder, bronchial responsiveness is within normal limits (Fujimura et al., 1994, 1997, 2000), airway cough sensitivity is heightened (Fujimura et al., 1994, 1997, 2000), bronchodilators are ineffective (Fujimura et al., 1994, 1997, 2000), and histamine H1-antagonists and corticosteroids are effective (Fujimura et al., 1994, 1997, 2000). We have reported some cases of atopic cough in which a single inhalation challenge with environmental fungus antigens caused both coughing and increased cough sensitivity to capsaicin (Ogawa et al., 1998, 1999, 2000). Thus, we hypothesized that a single antigen inhalation can cause airway cough hypersensitivity, and Liu et al. (2001) have proven this hypothesis using the same guinea pig model as this study. Accordingly, the present results suggest that carbocysteine may be useful for the treatment of chronic coughing based on airway cough hypersensitivity associated with airway allergies and decreased NEP activity. Further studies are needed to elucidate the efficacy of carbocysteine in the treatment of isolated coughs of various causes, such as postnasal drip-induced coughs and gastroesophageal reflux-associated coughs, in addition to eosinophilic bronchitis without asthma and atopic cough.

In conclusion, the present study clearly demonstrated that carbocysteine prevents the airway cough hypersensitivity, but not nonspecific bronchial hyperresponsiveness following antigen challenge by means of promoting restoration from the antigen-induced NEP inactivation in sensitized guinea pigs. These findings suggest that carbocysteine may be useful in allergic airway diseases with cough hypersensitivity, such as eosinophilic bronchitis without asthma (Gibson et al., 1989) and atopic cough (Fujimura et al., 2000), rather than bronchial asthma.

References


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