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Intratracheal dosing with disodium cromoglycate inhibits late asthmatic response by attenuating eicosanoid production in guinea pigs

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Abstract

Disodium cromoglycate is an anti-asthmatic drug that has mast cell-stabilizing effects and other anti-inflammatory effects. However, the mechanisms of its anti-inflammatory effects are unclear. In this study, we evaluated effects of disodium cromoglycate on eosinophilia, early and late asthmatic responses, and production of arachidonic acid metabolites in guinea pig lungs. Guinea pigs were alternately sensitized and challenged by exposure to mists of ovalbumin+$Al(OH)_3$ and ovalbumin, respectively. Disodium cromoglycate (0.5–2 mg/0.1 ml/animal) administered intratracheally before the fifth challenge dose-dependently inhibited asthmatic response, but early asthmatic response was not affected. Disodium cromoglycate at 2 mg/animal potently suppressed increases in cysteinyl leukotrienes (CysLTs) and thromboxane A_2 in the lung during late asthmatic response. Eosinophilia was slightly reduced by disodium cromoglycate. The inhibitory effect of disodium cromoglycate on late asthmatic response is apparently due to inhibition of the release of arachidonic acid metabolites, some of which may be derived from eosinophils that infiltrate the lung.

Keywords: Disodium cromoglycate; Late asthmatic response; Cysteinyl leukotriene; Thromboxane A_2; Eosinophil; (Guinea pig)

1. Introduction

When patients with allergic asthma are given an inhalation challenge of aerosolised allergen, they develop airway obstruction consisting of early and late asthmatic responses (Booij-Noord et al., 1972). In contrast to induction mechanisms of early asthmatic response, which are based on constriction of the airway smooth muscle, it has been suggested that those of late asthmatic response are closely related to airway inflammation resulting from recruitment and activation of inflammatory cells in the lung (Varner and Lemanske, 1995). Several drugs have been found to suppress the induction of late asthmatic response. Corticosteroids strongly abolish late asthmatic response via a wide range of anti-inflammatory actions (Pepys et al., 1974; Cockcroft and Murdock, 1987), although they have also been found to induce a variety of side effects (Pedersen, 1995). In addition, antagonists of cysteinyl leukotriene 1 ($CysLT_1$) receptors and thromboxane A_2 TP receptors have been shown to effectively suppress late asthmatic response (Taylor et al., 1991, Rasmussen et al., 1992; Hamilton et al., 1998; Obase et al., 1998; Dogne et al., 2002). However, these antagonists do not inhibit late asthmatic response as strongly as corticosteroids.

Disodium cromoglycate is a drug with a wide margin of safety and which is effective therapy for asthma in children and adults (Morrison-Smith and Pizarro, 1972). One of the mechanisms by which disodium cromoglycate suppresses asthma is the prevention of anaphylactic release of chemical mediators from mast cells. Indeed, release of histamine, prostaglandin D_2 and/or cysteinyl leukotrienes from mast cells harvested from rat peritoneum (Kusner et al., 1973), human lung (Leung et al., 1988) and monkey lung (Wells et al., 1986) is inhibited by treating these cells with disodium cromoglycate. However, it has also been reported that disodium cromoglycate attenuates late asthmatic response

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and airway hyperresponsiveness (Cockcroft and Murdock, 1987; Hoag and McFadden, 1991), the induction mechanisms of which cannot be explained simply by the release of chemical mediators from mast cells induced by antigen–antibody reaction on the cell. Mechanisms of anti-inflammatory effects of disodium cromoglycate other than its mast cell-stabilizing effect have been examined. In an in vivo study, disodium cromoglycate suppressed cellular inflammation in the lung tissue of asthmatic patients (Diaz et al., 1984), probably via inhibition of the expression of adhesion molecules, as suggested by results of another study (Hoshino and Nakamura, 1997). However, the details of anti-inflammatory mechanisms of disodium cromoglycate leading to inhibition of late asthmatic response and airway hyperresponsiveness are unclear.

Because disodium cromoglycate is only slightly absorbed in the gastrointestinal tract, for treatment of bronchial asthma, the powdered drug is administered into the mouth using a special inhaler. In studies using guinea pigs (Hutson et al., 1988; Sugasawa et al., 1991), mice (Cieslewicz et al., 1999) and rats (Yamawaki et al., 1997), aerosolised mists have been administered into the nostrils because these animals breathe through the nose rather than through the oral cavity. However, we have found that only approximately 15% of mist generated by an ultrasonic nebulizer and administered into the nostrils arrives at the lung (Nabe et al., 1997a), indicating that direct intratracheal administration is a more efficient delivery method for the evaluation of anti-asthmatic drugs.

In the present study, we evaluated whether disodium cromoglycate suppresses the induction of early and late asthmatic responses in a guinea pig model of allergic asthma that we previously established (Nabe et al., 1997b, 1998). Treatment with disodium cromoglycate was performed using a newly developed intratracheal administration method. The mechanisms of the inhibitory effects of disodium cromoglycate on late asthmatic response were analyzed by examining its effects on eosinophilia and production of arachidonic acid metabolites, cysteinyl leukotrienes and thromboxane A_2 in the lung during the late phase response.

2. Materials and methods

2.1. Animals

Male 4-week-old Hartley guinea pigs (Japan SLC, Hamamatsu, Japan) were used. The animals were housed in an air-conditioned room at 23±1 °C and 60±10% humidity, with lights on from 8:00 a.m. to 8:00 p.m., for at least 1 week after purchase. They were fed a standard laboratory diet and given water ad libitum.

This animal study was approved by the Experimental Animal Research Committee of the Kyoto Pharmaceutical University.

2.2. Reagents

Reagents and their sources were as follows: disodium cromoglycate and lidocaine hydrochloride (Xylocaine®, Fujisawa Pharm., Osaka, Japan); mepyramine maleate (Sigma, St. Louis, MO, USA); ovalbumin, Triton X-100® and H_2O_2 (Wako, Osaka, Japan); 3,3′,5,5′-tetramethylbenzidine (Dohjindo Lab., Kumamoto, Japan); KBr (Merck, Darmstadt, Germany); and sucrose (Nacalai Tesque, Kyoto, Japan).

$Al(OH)_3$ gel was prepared with 0.25 N NaOH and 0.25 N $Al_2(SO_4)_3$ as described elsewhere (Nabe et al., 1997a). The average of diameter of the gel particles was 0.43 μm when measured by a laser diffraction particle size analyzer (SALD-2000A, Shimadzu, Kyoto, Japan).

Ovalbumin+$Al(OH)_3$, which was used for sensitization, was prepared as previously reported (Nabe et al., 1997a,b). Ovalbumin solution, which was used for challenge, was prepared at a concentration of 16 mg/ml.

2.3. Sensitization and challenge with antigen

Sensitization and challenge by inhalation of the mists of ovalbumin+$Al(OH)_3$ and ovalbumin, respectively, were performed using a previously described method (Nabe et al., 1997b, 1998, 2002; Yamashita et al., 1999). Briefly, conscious guinea pigs were sensitized by inhalation of the ovalbumin+$Al(OH)_3$ mist at a dose of 15 μg ovalbumin/750 μg $Al(OH)_3$/animal/time once every 2 weeks for the first two times. Then, the animal was subjected to inhale the ovalbumin mist (10 μg/animal/time) and the ovalbumin+$Al(OH)_3$ mist alternately for challenge and sensitization, respectively, once every 2 weeks until the fourth or fifth challenge (sensitized challenged group). For a negative control group, guinea pigs that had been sensitized and challenged as described above were subjected to inhale physiological saline mist instead of ovalbumin mist at the time corresponding to the fifth challenge (sensitized non-challenged group).

In preliminary experiments, more than 60% of the sensitized guinea pigs died immediately (within 20 min) following the first challenge, and the second challenge induced severe anaphylactic symptoms including laboured breathing and cyanosis in the majority of the animals. Therefore, in the present experiments, all animals were administered with mepyramine prior to the respective first to third challenges. Because very few animals exhibited these severe symptoms at the fourth or fifth challenge, no mepyramine treatment was performed at these provocations.

2.4. Intratracheal administration of disodium cromoglycate

Intratracheal administration was performed using a conscious guinea pig without surgery (Fig. 1). After

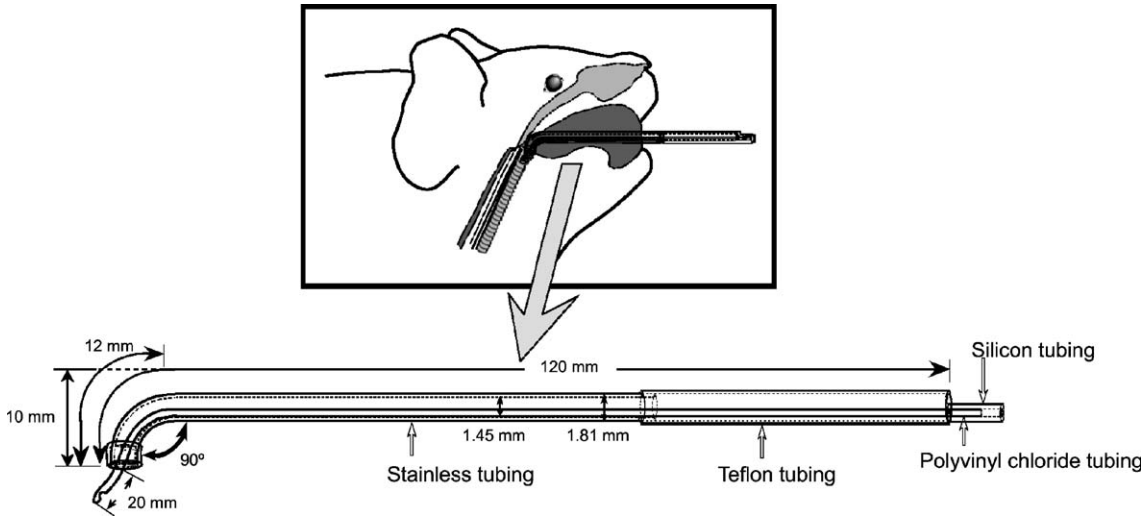

Fig. 1. Schematic illustration of the present method of intratracheal administration of disodium cromoglycate solution through a catheter inserted into an oral cannula. The tip of the oral cannula was positioned at the top of the trachea and then the tracheal catheter was inserted into the oral cannula, followed by insertion approximately 2 cm into the trachea. Disodium cromoglycate or the vehicle solution was dripped from the tip of the catheter into the trachea at a dose of 100 μl/animal.

removing food sticking to the inner surface of the oral cavity with cotton wool, the mucosal surface of the oral cavity and larynx was topically anaesthetized by painting of 1% lidocaine solution absorbed into cotton wool. Then, stainless steel tubing (external diameter, 1.81 mm; internal diameter, 1.45 mm; length, 90 mm), the tip of which was bent at an angle of 90°, was inserted into the oral cavity. The tip of the oral cannula was properly attached to the opening of the trachea at the larynx. Teflon tubing (length, 30 mm) was attached to the other end of the oral cannula, and the inner surface of the Teflon tubing was monitored for moisture indicating expiration by the guinea pig through the oral cannula. Polyvinyl chloride tubing (external diameter, 1.0 mm; Atom indwelling feeding tube for infant 3Fr, NS-510-03, Atom Medical, Tokyo, Japan) was inserted into the oral cannula and then approximately 20 mm into the trachea. After confirming that the guinea pig was breathing through the polyvinyl chloride tubing, disodium cromoglycate (0.5, 1 and 2 mg/animal) or vehicle (physiological saline) was instilled into the tube with a pipet at a dose of 100 μl/animal.

A solution of Evans blue adsorbed on bovine serum albumin was intratracheally administered at a dose of 1 mg Evans blue/5 mg bovine serum albumin/0.1 ml/animal; 5.2±0.6% of the dye was deposited at the trachea and main bronchi, and 58.9±6.1% was deposited at the lung (n=5). The rest of the Evans blue was detected at the oral cavity, larynx and esophagus: 2.6±0.3%, 12.8±2.6% and 0.8±0.3% (n=5), respectively. Approximately 20% of the instilled dye reached the stomach or could not be collected.

Intratracheal administration of disodium cromoglycate was performed either 10 min before or 160 min after the antigen challenge.

2.5. Measurement of the pulmonary function

Specific airway resistance (sRaw) was measured using a two-chambered, double-flow plethysmograph system (Pulmos-I·II·III, M.I.P.S., Osaka, Japan) according to the method of Pennock et al. (1979) before and after the fifth antigen challenge under nonanaesthesia.

2.6. Bronchoalveolar lavage and measurement of cysteinyl leukotrienes and thromboxane A_2 in bronchoalveolar lavage fluid

We evaluated whether the inhibitory effect of disodium cromoglycate on LAR is due to the suppression of production of CysLTs and thromboxane A_2, which contribute to the induction of late asthmatic response in this model (Yamashita et al., 1999). Before and 5 h after the fifth ovalbumin challenge, sensitized guinea pigs were sacrificed by drawing blood from the abdominal aorta under pentobarbital (40 mg/kg, i.p.) anaesthesia. After perfusion of the lung with phosphate buffered saline (PBS) via the pulmonary artery and isolation of the lung with the trachea, BAL was performed with PBS (10 ml×2). The supernatant recovered after centrifugation was used to measure cysteinyl leukotrienes and thromboxane B_2 by enzyme immunoassay (EIA), after removal of protein with 80% ethanol as described by Yamasaki et al. (2001). The leukotriene $C_4/D_4/E_4$ EIA system was purchased from Amersham (Buckinghamshire, UK). The cross-reactivity of the rat anti-leukotriene $C_4/D_4/E_4$ antibody in the system for leukotriene C_4, leukotriene D_4 and leukotriene E_4 was 100%, 100% and 70%, respectively. The standard curve was prepared using leukotriene C_4. An EIA kit for measurement of thromboxane B_2 was purchased from Cayman Chem. (Ann Arbor, MI, USA).

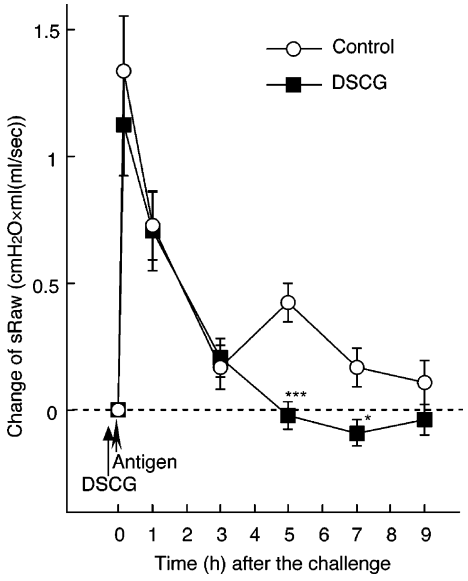

Fig. 2. Effect of pretreatment with disodium cromoglycate on antigen-induced early and late increases of specific airway resistance (sRaw) in sensitized guinea pigs. Disodium cromoglycate (2 mg/animal) was intratracheally administered 10 min before the antigen challenge. Each point represents the mean±S.E.M. of 11 to 13 animals. (*) and (***): significantly different from the control; $P<0.05$ and $P<0.001$, respectively.

2.7. Isolation of the lung and measurement of eosinophil peroxidase (EPO) activity in the lung homogenate

We have reported that although the number of eosinophils does not increase in bronchoalveolar lavage 5 h after ovalbumin challenge, at the peak of late asthmatic response, EPO activity considerably increases at that time point (Nabe et al., 1998). Thus, in order to evaluate effects of disodium cromoglycate on eosinophilia during late asthmatic response, we used increased EPO activity in the lung homogenate to indicate lung eosinophilia.

Following bronchoalveolar lavage, all lung lobes and cells recovered by bronchoalveolar lavage were combined and homogenized (4 °C, 12 000 rpm, 20 s×2) in 12 ml of an assay buffer consisting of 0.3 M sucrose in 50 mM acetate buffer (pH 5.4) and were then homogenized again (4 °C, 8000 rpm, 20 s×2) in the presence of 0.2% Triton X-100®. EPO activity in the homogenate was measured as previously described (Nabe et al., 1998, 2002; Yamashita et al., 1999). A 50-μl aliquot of the 100-fold diluted homogenate was added to 200 μl of the assay buffer with or without 5.3 mM KBr in a polypropylene tube. After adding 100 μl of 3.9 mM 3,3′,5,5′-tetramethylbenzidine containing 2.8 mM H_2O_2 and mixing well, the mixture was incubated for 5 min at 21 °C. The peroxidase reaction was terminated by adding 200 μl of 1 N H_2SO_4. After centrifugation, 200 μl of the supernatant was transferred onto a microtiter plate, and the optical density at 450 nm was measured. EPO activity was expressed as ΔOD±KBr/lung. This EPO assay was originally reported by Bozeman et al. (1990) and modified by Tagari et al. (1993). Bozeman et al. (1990) reported that this method using bromide ion is specific for EPO.

2.8. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA). If significance was detected, individual group differences were evaluated by Bonferroni's multiple test. A probability value (P) of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Effects on early and late asthmatic responses

Consistent with our previous reports (Nabe et al., 1997b, 1998, 2002; Yamashita et al., 1999), the fifth antigen challenge induced time course changes in sRaw with clearly separate early and late asthmatic responses: peak sRaws occurred at 10 min in early asthmatic response and at 5 or 7 h in late asthmatic response after the challenge (Fig. 2). When disodium cromoglycate was intratracheally administered 10 min before the challenge at a dose of 2 mg/animal, late asthmatic response was completely inhibited although early asthmatic response was not affected (Fig. 2). As shown in area under response curves for sRaw, the inhibitory effect of disodium cromoglycate on late asthmatic response was dose-dependent at 0.5 to 2 mg/animal (Fig. 3).

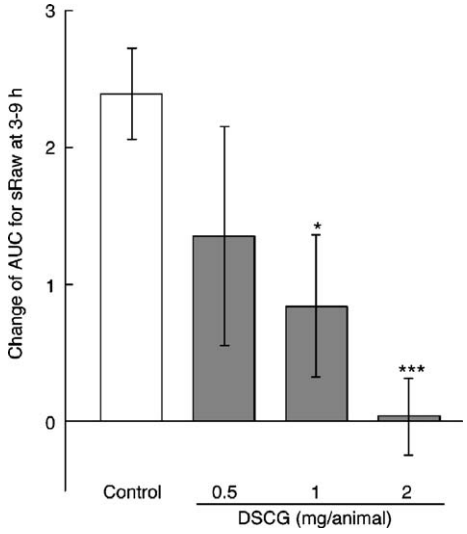

Fig. 3. Dose–response effect of pretreatment with disodium cromoglycate on antigen-induced late increase of specific airway resistance (sRaw) in sensitized guinea pigs. Data are represented as area under the response curve (AUC) for sRaw at 3 to 9 h after the challenge. Disodium cromoglycate (0.5–2 mg/animal) was intratracheally administered 10 min before the antigen challenge. Each column represents the mean±S.E.M. of 9 to 16 animals. (*) and (***): significantly different from the control; $P<0.05$ and $P<0.001$, respectively.

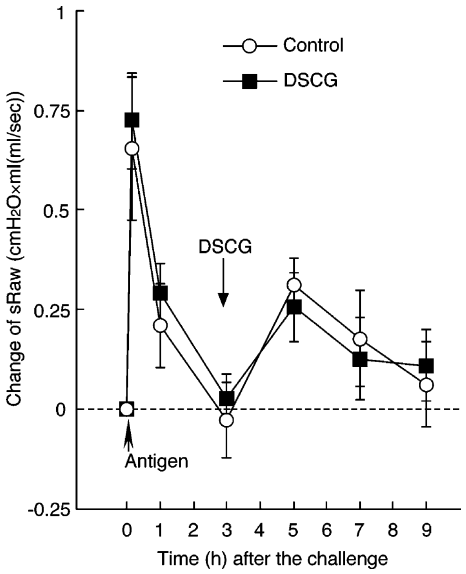

Fig. 4. Effect of posttreatment with disodium cromoglycate on antigen-induced late increase of specific airway resistance (sRaw) in sensitized guinea pigs. Disodium cromoglycate (2 mg/animal) was intratracheally administered 160 min after the antigen challenge. Each point represents the mean±S.E.M. of 11 to 13 animals.

When disodium cromoglycate was administered 160 min after the challenge, late asthmatic response was not affected (Fig. 4).

3.2. Effect on the production of cysteinyl leukotrienes and thromboxane A_2

Levels of cysteinyl leukotrienes and thromboxane B_2 in bronchoalveolar lavage tended to increase at 5 h after the challenge, at the peak of late asthmatic response. These increases in arachidonic acid metabolites were completely suppressed by pretreatment with disodium cromoglycate at 2 mg/animal (Fig. 5).

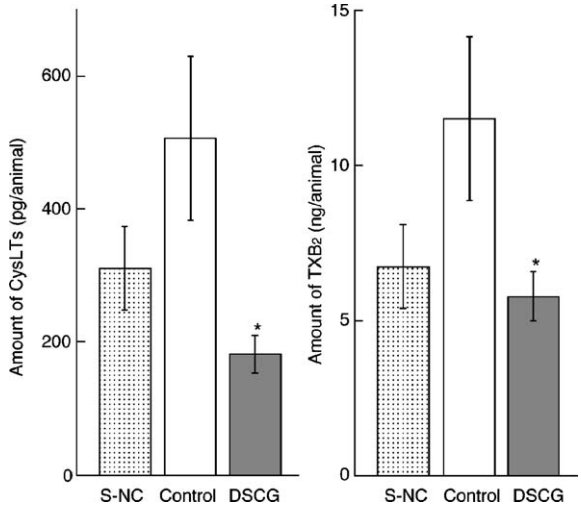

Fig. 5. Effect of disodium cromoglycate on antigen-induced increases of levels of cysteinyl leukotrienes (CysLTs) and thromboxane B_2 (TxB_2) in bronchoalveolar lavage fluid (BALF) during late asthmatic response in sensitized guinea pigs. Disodium cromoglycate (2 mg/animal) was intratracheally administered 10 min before the antigen challenge. BAL was performed 5 h after the challenge. Each column represents the mean±S.E.M. of 12 to 14 animals. (*): significantly different from the control; $P<0.05$. S-NC: sensitized nonchallenged animals.

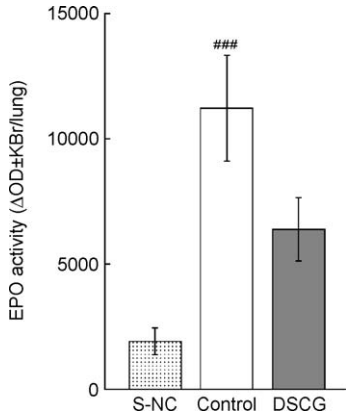

Fig. 6. Effect of disodium cromoglycate on antigen-induced increase of eosinophil peroxidase (EPO) activity in lung homogenate during late asthmatic response in sensitized guinea pigs. Disodium cromoglycate (2 mg/animal) was intratracheally administered 10 min before the antigen challenge. The lung was isolated 5 h after the challenge. Each column represents the mean±S.E.M. of 14 animals. S-NC: sensitized nonchallenged animals. (###): significantly different from S-NC; $P<0.001$.

3.3. Effect on pulmonary eosinophilia

Consistent with our previous reports (Nabe et al., 1998, 2002; Yamashita et al., 1999), pulmonary eosinophilia (detected by increased EPO activity in the lung homogenate) was clearly observed 5 h after the challenge. Pretreatment with disodium cromoglycate at 2 mg/animal tended to suppress the eosinophilia by approximately 40% (Fig. 6).

4. Discussion

For asthma therapy, anti-asthmatic drugs are generally inhaled from the mouth but not from the nostrils. Similar to the clinical setting, the present novel method of intratracheal administration allowed us to efficiently deliver disodium cromoglycate to the lung using a small amount of the drug. In addition, this method requires neither surgery nor general anaesthesia. Thus, the intratracheal administration appears more effective than inhalation of disodium cromoglycate mist through the nasal cavity for evaluation of effects on lung function in experimental asthma. When disodium cromoglycate was intratracheally administered 10 min before the antigen inhalation challenge, the induction of late asthmatic response was dose-dependently suppressed, and the inhibitory rate at 2 mg/animal was nearly 100%. However, early asthmatic response was not affected by the pretreatment. Intratracheal administration of disodium cromoglycate at 160 min (after completion of early asthmatic response) showed no effect on late asthmatic response.

Although disodium cromoglycate has been shown to suppress release of anaphylactic chemical mediators from mast cells harvested from human (Leung et al., 1988) and monkey lungs (Wells et al., 1986) and rat peritoneum (Kusner et al., 1973), disodium cromoglycate has not been found to affect release of these mediators from guinea pig lung fragments or mast cells (Hashimoto et al., 1987; Nabe et al., 1992; Lau et al., 1994). Early asthmatic response is apparently caused mainly by airway smooth muscle constriction induced by mast cell-derived chemical mediators such as histamine, cysteinyl leukotrienes and prostaglandin D_2 (Varner and Lemanske, 1995). Thus, there is no contradiction between the lack of effect of disodium cromoglycate on early asthmatic response and its lack of effect on the release of anaphylactic chemical mediators from mast cells in guinea pigs. On the other hand, posttreatment with disodium cromoglycate did not have a suppressive effect on late asthmatic response. The contrast between this result and the effect of pretreatment suggests that disodium cromoglycate inhibits pro-inflammatory event(s) that occur until 160 min after the challenge that induces late asthmatic response. We speculate that induction of late asthmatic response is not initiated after completion of early asthmatic response but is dependent on sequential events that occur during early asthmatic response.

We previously found that frequency of induction of late asthmatic response is reduced by pretreatment with pranlukast (a $CysLT_1$ receptor antagonist) and seratrodast (a TP receptor antagonist) by approximately 45% and 40%, respectively (Yamashita et al., 1999), suggesting that both cysteinyl leukotrienes and thromboxane A_2 participate in the pathogenesis of late asthmaitc response. Consequently, in the present study, we examined effects of disodium cromoglycate on the production of these arachidonic acid metabolites in the lung during late asthmatic response. Disodium cromoglycate completely inhibited increases in these metabolites in bronchoalveolar lavage fluid after challenge, suggesting that inhibition of late asthmatic response by disodium cromoglycate is closely associated with suppression of production of cysteinyl leukotrienes and thromboxane A_2. The previous finding that early asthmatic response in this model was also partly inhibited by pranlukast and seratorodast (Yamashita et al., 1999) suggests that cysteinyl leukotrienes and thromboxane A_2 are produced immediately after antigen challenge, leading to the induction of early asthmatic response, which peaks at 10 min. The lack of effect of disodium cromoglycate pretreatment on early asthmatic response suggests that disodium cromoglycate does not affect the immediate production of cysteinyl leukotrienes and thromboxane A_2 and that the mechanisms which produce these metabolites during early and late asthmatic responses are induced independently of each other. Thus, we speculate that disodium cromoglycate inhibits infiltration (into the pulmonary tissue) or activation of inflammatory cells that produce cysteinyl leukotrienes and thromboxane A_2.

The following previous findings suggest that eosinophils infiltrated into the lung and produce cysteinyl leukotrienes and thromboxane A_2 in this model: (1) numbers of eosinophils markedly increase in the lung during late asthmatic response (Nabe et al., 1998, 2002; Yamashita et al., 1999); (2) depletion of eosinophils by an anti-interleukin-5 antibody suppresses late asthmatic response by approximately 50% (Nabe et al., 2002); (3) guinea pig eosinophils can produce a large amount of thromboxane A_2 and a small but significant amount of cysteinyl leukotrienes (Giembycz et al., 1990; Nabe et al., 2000). In addition to these findings, in a bronchial biopsy study of asthmatic patients, treatment with disodium cromoglycate reduced expression of adhesion molecules such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (Hoshino and Nakamura, 1997), which are thought to be closely involved in the recruitment of eosinophils into the lung. Therefore, in the present study, we examined whether pretreatment with disodium cromoglycate inhibits antigen-induced pulmonary eosinophilia occurring at 5 h, when late asthmatic response peaks. Disodium cromoglycate tended to suppress eosinophilia by approximately 40%, implying that the inhibition of late asthmatic response by disodium cromoglycate involves reduction of eosinophilia and that infiltrated eosinophils are a source of cysteinyl leukotrienes and thromboxane A_2, leading to the induction of late asthmatic response. However, whereas disodium cromoglycate completely inhibited late asthmatic response, disodium cromoglycate only moderately suppressed eosinophilia, suggesting that cells other than eosinophils are sources of arachidonic acid metabolites. In humans, basophils secrete cysteinyl leukotrienes at levels equivalent to mast cells; levels of both basophils and mast cells are 10 to 100 times greater than those of eosinophils (MacGlashan et al., 2002). Thus, basophils and/or mast cells that migrate into the lung may be sources of arachidonic acid metabolites leading to the induction of late asthmatic response.

There have been conflicting results regarding the effectiveness of disodium cromoglycate on asthmatic responses in ovalbumin-induced asthmatic models of guinea pigs. Consistent with the present finding, Sugasawa et al. (1991) reported that inhalation of 1% disodium cromoglycate for 2 min on two occasions did not affect early asthmatic response but significantly inhibited late asthmatic response. However, in another study, inhalation of an aerosolised solution of 1% disodium cromoglycate before challenge inhibited both early and late asthmatic responses and inhalation of 1% disodium cromoglycate after completion of early asthmatic response inhibited late asthmatic response (Hutson et al., 1988). The reasons for these differences in the effectiveness of disodium cromoglycate on early and late asthmatic responses are unclear. We speculate that they are due to differences in sensitization/challenge protocol and in the method of administering disodium cromoglycate.

In conclusion, intratracheal administration of disodium cromoglycate potently suppressed induction of late asthmatic response, a characteristic feature of severe asthma. This inhibition appears to be based on anti-inflammatory actions, including reduced generation of cysteinyl leukotrienes and thromboxane A_2 in pulmonary tissue. The present findings further indicate that disodium cromoglycate can provide clinical benefits in therapy for severe asthma.

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