

Effect of cromolyn on adenosine-induced airway microvascular leakage in sensitized rats

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ABSTRACT: Inhalation of adenosine causes bronchoconstriction in asthmatic subjects, but the effect of this purine nucleotide on airway vascular permeability is unknown.

In order to determine whether adenosine produces airway microvascular leakage and, if so, to examine the effect of cromolyn (sodium cromoglycate (SCG)) on this extravasation of Evans blue was measured in the airways of ovalbumin-sensitized Brown Norway rats.

Inhaled adenosine caused microvascular leakage in sensitized but not in non-sensitized rats, and the response was abolished by capsaicin pretreatment or the tachykinin neurokinin-1 receptor antagonist FK888. Adenosine-induced vascular leakage became apparent in nonsensitized rats when treated with phosphoramidon, and airway neutral endopeptidase activity was lower in sensitized than in non-sensitized animals. The extravasation induced by adenosine in sensitized rats was dose dependently inhibited by SCG aerosols. SCG likewise inhibited microvascular responses to substance P, but had no effect on those to platelet-activating factor.

These results suggest that: 1) adenosine induces airway microvascular leakage in sensitized rats through stimulation of neurokinin-1 receptors; 2) this effect is associated with a sensitization-induced decrease in neutral endopeptidase activity; and 3) sodium cromoglycate inhibits adenosine-induced extravasation, presumably *via* functional antagonism of tachykinins.

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Adenosine is a naturally occurring purine nucleotide formed by the cleavage of adenosine 5'-monophosphate by 5'-nucleotidase or by the catabolism of *S*-adenosylhomocysteine. Based on the findings that adenosine can be released from mast cells in both early- and late-phase asthmatic responses *via* antigen/immunoglobulin E (IgE) interaction after allergen bronchoprovocation [1] and that inhaled adenosine causes bronchoconstriction in asthmatics but not in normal subjects [2], this purine nucleotide is thought to play a role in the pathophysiology of asthma. Increased microvascular permeability and plasma protein extravasation in the airways might contribute to mucosal oedema, inflammatory cell infiltration and epithelial cell desquamation, which are characteristic features of asthma [3]. Previous studies have shown that adenosine induces plasma extravasation in rat skin [4] and hamster cheek pouch [5], but its effect on airway microvascular permeability is unknown.

Cromolyn (sodium cromoglycate (SCG)) is an antiallergic drug and has been widely used in the treatment of asthma [6, 7]. Although SCG has long been recognized as a mast cell-stabilizing agent [8], its mechanism of action in asthma is still unclear. CROSSMAN *et al.* [9] showed that oedema formation in the human skin produced by substance P was reduced by SCG, suggesting that SCG may modify tachykinin actions. Therefore, the purposes of the

present study were: 1) to determine whether adenosine produces airway vascular leakage; 2) to elucidate the possible involvement of tachykinins in the effect of adenosine; and 3) to determine whether SCG inhibits the effect of adenosine and, if so, what its mechanism of action is.

Neurogenic inflammation in the airway is modulated by the membrane-bound enzyme neutral endopeptidase (NEP, EC 3.4.24.11) [10]. Respiratory viral infections [11] and inhalation of occupational irritants [12] and cigarette smoke [13] exaggerate neurogenic inflammation by inhibiting endogenous NEP activity. Thus, the role of NEP in the vascular action of adenosine was also examined using phosphoramidon, a specific inhibitor of NEP [14], and by measuring the activity of NEP.

Materials and methods

Animals

Pathogen-free male Brown Norway rats (SLC Japan Co., Hamamatsu, Japan), weighing 250–300 g were used. The rats were kept in a temperature-controlled environment with standard laboratory food and water freely available for 3 days prior to the experiment. All experimental procedures were approved by the Committee on Animal Research of Tokyo Women's Medical University.

Study design

The rats were sensitized by intraperitoneal injection of a 1 mL suspension of 1 mg ovalbumin (Sigma Chemical Co., St Louis, MO, USA) and 100 mg aluminium hydroxide (Wako Chemical Co., Osaka, Japan) in saline on three consecutive days. For nonsensitized controls, the rats were given 1 mL saline alone in a similar manner. Twenty-one days after the initial injection, the animals were anaesthetized with intraperitoneal sodium pentobarbital (40 mg·kg⁻¹), and the larynx and upper trachea exposed. The trachea was incised below the larynx, and a cannula inserted 4 mm into the trachea. The rats were then artificially ventilated at a frequency of 70 breaths·min⁻¹ and at a tidal volume of 10 mL·kg⁻¹. A polyethylene catheter was inserted into the left carotid artery to monitor blood pressure using a pressure transducer (model TP-300T; Nihon Kohden, Tokyo, Japan).

In order to measure vascular extravasation, Evans blue (3% solution in 0.9% NaCl, Sigma) was used. Immediately after injection of the dye into the femoral vein (30 mg·kg⁻¹), aerosols of adenosine (Sigma) dissolved in saline were given for 2 min *via* the tracheal cannula using an ultrasonic nebulizer (aerosol delivery rate 0.2 mL·min⁻¹, Pulmo-Sonic, model-25; De Vilbiss Co., Somerset, PA, USA). The chest was opened 5 min after the completion of adenosine inhalation, a cannula inserted into the ascending aorta through the left ventricle and the systemic circulation perfused with phosphate-buffered saline at a pressure of 13.3 kPa (100 mmHg). After 2 min of perfusion, the right ventricle was opened and the pulmonary circulation perfused with additional phosphate-buffered saline for 1 min. The trachea and lungs were dissected out *en bloc*, the parenchyma gently scraped off and extraneous tissue removed. Then, the trachea, main bronchi and intrapulmonary airways were separated from each other. After blotting on absorbent paper, tissues were weighed and incubated in 3 mL of formamide (Kanto Chemical Co., Tokyo, Japan) for 18 h at 50°C to extract the extravasated Evans blue.

Firstly, regional differences in airway microvascular response to adenosine (3,000 µg·kg⁻¹) was studied in sensitized and nonsensitized rats. In evaluating the dose-response relationship, the effects of increasing doses of adenosine (100–3,000 µg·kg⁻¹) on tracheal vascular permeability were examined in sensitized rats. In the control experiment, the rats received saline alone. Secondly, in order to detect the possible involvement of tachykinins in the adenosine action, the rats were pretreated subcutaneously with 25, 50 and 100 mg·kg⁻¹ capsaicin (Sigma) dissolved in 10% Tween 80 and 10% ethanol in saline on the three consecutive days before sensitization [15]. A separate experiment showed that this procedure completely blocked vascular leakage induced by inhaled capsaicin (10⁻⁵ and 10⁻⁴ M), thus confirming capsaicin desensitization. Using these rats, the microvascular response to inhaled adenosine (3,000 µg·kg⁻¹) was studied. The effect of FK888, a selective neurokinin-1 (NK₁) receptor antagonist [16], on the adenosine action was also examined. In order to do this, FK888 (10 mg·kg⁻¹) was injected intravenously, and 15 min later aerosols of adenosine (3,000 µg·kg⁻¹) were given.

It is possible that airway vascular leakage induced by adenosine in sensitized rats could be related to alterations in endogenous NEP activity. To test this hypothesis, the

microvascular responses of sensitized and nonsensitized rats pretreated with phosphoramidon were studied. The rats were given intravenous phosphoramidon (2.5 mg·kg⁻¹, Sigma), and 5 min later the effect of adenosine (3,000 µg·kg⁻¹) on tracheal vascular leakage was determined. The dose of phosphoramidon was chosen based on a previous study [17], and preliminary experiments showed that phosphoramidon alone had no effect on microvascular permeability.

The effect of SCG (Fujisawa Pharmaceutical Co., Osaka, Japan) on the increase in vascular permeability caused by adenosine was then studied. Various doses of SCG (1–100 mg·kg⁻¹) were given by inhalation, and 5 min later intravenous Evans blue and inhaled adenosine (3,000 µg·kg⁻¹) were given consecutively. Since the inhibitory effect of SCG on the adenosine-induced vascular leakage reached a plateau at a dose of 30 mg·kg⁻¹, it was determined whether this dose of SCG likewise inhibits the responses to other stimuli. Five min after SCG (30 mg·kg⁻¹) administration, Evans blue was injected and then various doses of substance P (3–300 µg·kg⁻¹; Peninsula Laboratories, Inc., Belmont, CA, USA) or platelet-activating factor (PAF) L- α -phosphatidylcholine- β -acetyl- γ -O-hexadecyl, 10–1,000 µg·kg⁻¹; Sigma) were given by inhalation. In preliminary experiments, 100 µg·kg⁻¹ substance P, 300 µg·kg⁻¹ PAF and 1,000 µg·kg⁻¹ adenosine were equipotent in increasing vascular permeability in the rat trachea. Substance P was dissolved in 0.9% NaCl, and PAF was prepared as a stock solution (1 mg·mL⁻¹) in 95% ethanol, stored at -80°C and then diluted to the final concentration in 0.25% bovine serum albumin in 0.9% NaCl. Separate studies showed that SCG at doses of up to 100 mg·kg⁻¹ *per se* did not change the baseline microvascular permeability of the rat trachea.

Measurement of vascular permeability

Extravasation of Evans blue-labelled macromolecules from the microcirculation in the airways was quantified by measuring the optical density of the formamide extracts at a wavelength of 620 nm using a spectrophotometer (Ultrospec Plus; Pharmacia LKB Biochrom, London, UK). The amount of Evans blue extravasated into the tissues, expressed in ng·mg wet weight⁻¹, was interpolated from a standard curve of Evans blue concentrations (0.1–5 µg·mL⁻¹). A previous report demonstrated a close correlation between exudation of Evans blue, which binds to plasma albumin, and the leakage of radioiodinated serum albumin [18].

Measurement of neutral endopeptidase activity

The NEP activity was measured using a modification of the procedure of LLORENS *et al.* [19]. Briefly, the tracheae from sensitized and nonsensitized rats were minced and homogenized. The homogenates were incubated with a radiolabelled enkephalin analogue (³H-Tyr-d-Ala²-leucine enkephalin, 20 nM, Amersham, Tokyo, Japan) in 125 mM NaCl containing 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer (pH 7.4) for 40 min at 37°C. Degraded ³H-Tyr-d-Ala²-leucine enkephalin was chromatographically separated from intact peptide and the radioactivity in each fraction was determined in a scintillation counter. The NEP activity

was then determined by calculating the ratio of cleaved enkephalin to total $^3\text{H-Tyr-d-Ala}^2$ -leucine enkephalin, adjusted to the total protein content of the tissue, and enkephalin degradation expressed as $\text{fmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$.

Statistical analysis

All data were expressed as $\text{mean}\pm\text{SEM}$. The mean values of the spectrophotometric measurements of Evans blue extravasation were analysed by means of two-way analysis of variance. Comparisons between means in each condition were performed by means of Scheffe's F-test, n referring to the number of rats from which tracheae were taken and a p -value <0.05 was considered statistically significant.

Results

There was no significant change in mean blood pressure after inhalation of adenosine or SCG aerosols compared with saline in sensitized or nonsensitized Brown Norway rats. As demonstrated in figure 1, inhalation of adenosine ($3,000\ \mu\text{g}\cdot\text{kg}^{-1}$) caused microvascular leakage of Evans blue in the trachea and main bronchi but not the intrapulmonary airways in rats sensitized with ovalbumin, whereas it was without effect in nonsensitized animals. Therefore, only trachea were studied in the subsequent experiments. The adenosine-induced increase in tracheal microvascular permeability was dose-dependent (fig. 2), with significant vascular leakage observed at adenosine doses of $\geq 300\ \mu\text{g}\cdot\text{kg}^{-1}$, and at $3,000\ \mu\text{g}\cdot\text{kg}^{-1}$ adenosine the increase over control was $736\pm 49\%$ ($p<0.01$, $n=9$). Pretreatment of animals with capsaicin or FK888 abolished the vascular response to $3,000\ \mu\text{g}\cdot\text{kg}^{-1}$ adenosine ($p<0.01$, $n=9$ for both).

In nonsensitized rats, pretreatment with phosphoramidon potentiated the adenosine ($3,000\ \mu\text{g}\cdot\text{kg}^{-1}$)-induced microvascular permeability by $626\pm 53\%$ ($p<0.01$, $n=10$). In contrast, phosphoramidon did not further increase the vascular response to adenosine in sensitized animals (fig. 3). The activity of NEP in tracheal tissues was $327\pm 80\ \text{fmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ in sensitized rats ($n=9$) and $981\pm 92\ \text{fmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ in nonsensitized animals ($n=10$). There was a significant difference between these values ($p<0.01$).

As shown in figure 4, prior inhalation of SCG aerosols reduced the adenosine ($3,000\ \mu\text{g}\cdot\text{kg}^{-1}$)-induced microvascular leakage in a dose-dependent manner. The maximal inhibition was $73\pm 5\%$ ($p<0.01$, $n=10$), and the dose of SCG required to produce a half-maximal effect was $15\pm 3\ \text{mg}\cdot\text{kg}^{-1}$ ($n=10$). Inhalation of substance P or PAF produced a dose-dependent increase in microvascular leakage. Pretreatment with SCG aerosols ($30\ \text{mg}\cdot\text{kg}^{-1}$) inhibited the response of microvascular permeability to substance P at $100\ \mu\text{g}\cdot\text{kg}^{-1}$ and $300\ \mu\text{g}\cdot\text{kg}^{-1}$ by $69\pm 6\%$ ($p<0.01$, $n=8$) and $77\pm 8\%$ ($p<0.01$, $n=8$), respectively, but failed to affect PAF-induced vascular extravasation (fig. 5).

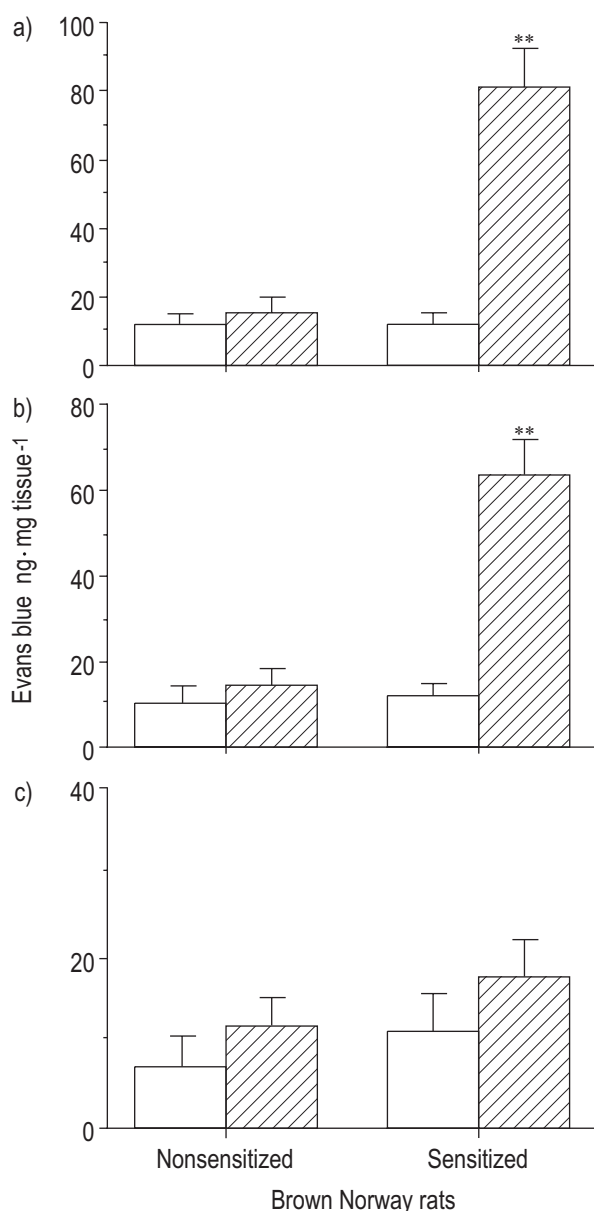


Fig. 1. – Effect of inhaled adenosine aerosols (▨; $3,000\ \mu\text{g}\cdot\text{kg}^{-1}$) on airway microvascular permeability in nonsensitized and ovalbumin-sensitized Brown Norway rats. □ : saline alone. Vascular extravasation was evaluated by measuring the amount of Evans blue extravasated into: a) the trachea; b) the main bronchi; and c) intrapulmonary airways. Data are presented as $\text{mean}\pm\text{SEM}$ ($n=9$). **: $p<0.01$ versus corresponding control (saline).

Discussion

In the present study, plasma protein extravasation was assessed by measuring the tissue accumulation of Evans blue, which binds to plasma albumin. It is known that there is a close correlation between the exudation of Evans blue and that of radioiodinated serum albumin [18]. Based on ultrastructural, pathological and pharmacological studies, the site of airway microvascular leakage appears to be the postcapillary venules, whose endothelial cells contract in response to spasmogens, resulting in the formation of intercellular gaps [20]. Under this experimental condition, there were four major findings: firstly, inhalation of adenosine increases airway plasma protein extravasation

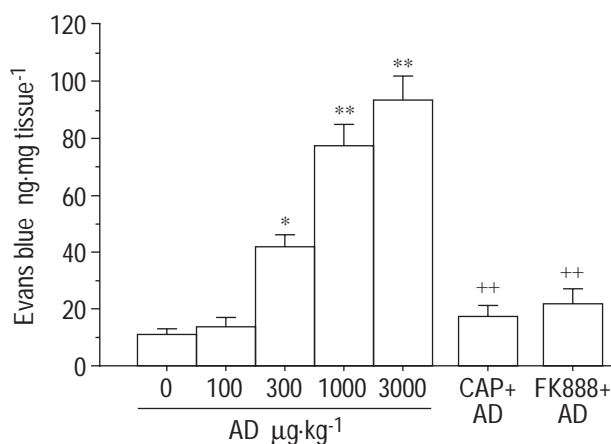


Fig. 2. – Effects of various doses of inhaled adenosine (AD) aerosols on tracheal microvascular permeability in sensitized rats. Some animals were pretreated with capsaicin (CAP) injection or FK888, and then given inhaled AD at 3,000 $\mu\text{g}\cdot\text{kg}^{-1}$. Data are presented as mean \pm SEM (n=9). *: p<0.01, **: p<0.001 versus control (saline); ++: p<0.01 versus 3,000 $\mu\text{g}\cdot\text{kg}^{-1}$ AD alone.

in sensitized Brown Norway rats; secondly, the response to adenosine is mediated by tachykinin stimulation of NK₁ receptors; thirdly, sensitization decreases endogenous NEP activity, thereby potentiating the effect of adenosine; and fourthly, prior inhalation of SCG inhibits adenosine-induced vascular leakage, presumably through a functional antagonism of tachykinins.

Adenosine is a purine nucleotide released from mast cells through an IgE-dependent mechanism, and may play a role in the pathophysiology of allergic asthma. It has been shown that inhaled adenosine causes bronchoconstriction in patients with asthma but not in normal subjects [2], but the reason for this discrepancy remains unknown. It was found that inhalation of adenosine aerosols dose dependently produced tracheobronchial extravasation of Evans blue only in ovalbumin-sensitized rats. This is the first demonstration that adenosine is capable of increasing airway microvascular permeability. Subsequently, in order to elucidate the mechanism of adenosine action, the possible involvement of airway neurogenic inflammation was studied.

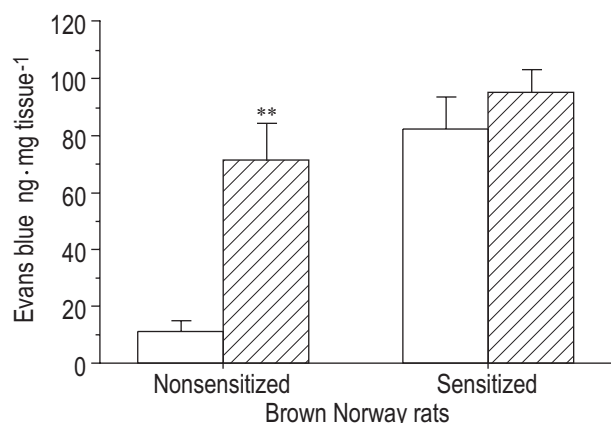


Fig. 3. – Effect of phosphoramidate (▨) on tracheal vascular leakage induced by adenosine (□) aerosols in nonsensitized and sensitized rats. After intravenous injection of Phosphoramidon (2.5 $\text{mg}\cdot\text{kg}^{-1}$), Evans blue and adenosine (3,000 $\mu\text{g}\cdot\text{kg}^{-1}$) were consecutively given. Data are presented as mean \pm SEM (n=10). **: p<0.01 versus AD alone.

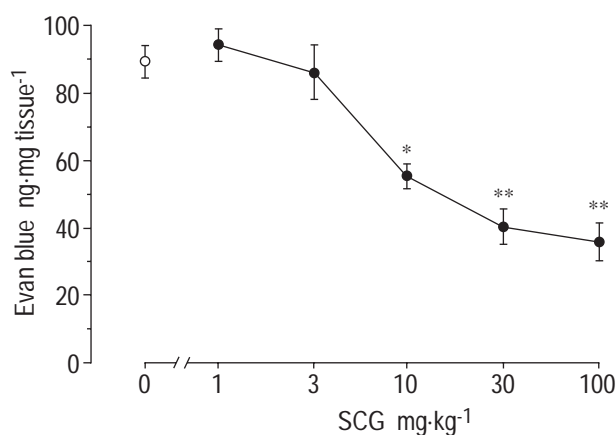


Fig. 4. – Dose-dependent effects of sodium cromoglycate (SCG) on tracheal vascular leakage induced by adenosine aerosols in sensitized rats. The rats received various doses of SCG aerosol by inhalation, and 5 min later Evans blue and adenosine (3,000 $\mu\text{g}\cdot\text{kg}^{-1}$) were consecutively given. Data are presented as mean \pm SEM (n=10). *: p<0.05; **: p<0.01 versus adenosine alone.

Stimulation of unmyelinated sensory nerves in the airway mucosa causes the release of tachykinins [21], which, in turn, produce plasma protein extravasation in rat airways [22]. To date, at least three subtypes of tachykinin receptor have been identified, denoted NK₁, NK₂ and NK₃ [23], and plasma extravasation caused by sensory nerve stimulation in the rat trachea is mediated by NK₁ receptor activation. PIEDIMONTE *et al.* [24] showed that CP-99,994, an antagonist of NK₁ receptors, inhibited plasma extravasation in the rat trachea produced by substance P and by stimulation of sensory nerves with capsaicin. In the present study, pretreatment of rats with capsaicin injection to deplete endogenous tachykinins or intravenous administration of the selective NK₁ receptor antagonist FK888 [16] abolished adenosine-induced plasma extravasation. These findings suggest that adenosine may stimulate the release of tachykinins from sensory nerves, which consequently activate NK₁ receptors on the endothelial cells of the postcapillary venules. In agreement with this notion, MEADE *et al.* [25] have recently shown that sensory nerve stimulation is involved in adenosine A₃ receptor-mediated bronchoconstriction in rats, but the involvement of this receptor subtype was not tested in the present experimental system.

In nonsensitized rats, adenosine produced vascular leakage in the trachea and main bronchi but had little effect in intrapulmonary airways. Similarly to this finding, previous studies have shown that plasma exudation induced by inflammatory mediators and antigen challenge is observed mainly in the large airways [26, 27]. This regional difference could be due to the difference in distribution of adenosine receptors and/or tachykinin-containing nerves between the central and peripheral airways. It is known that the membrane-bound NEPs are present in airway tissues [28], where the enzyme cleaves and inactivates tachykinins including substance P [29] and thus limits neurogenic inflammation [10]. Conversely, the decrease in endogenous NEP activity may result in the potentiation of neurogenic inflammation. In the present study, inhibition of NEP activity with phosphoramidon markedly potentiated adenosine-induced microvascular leakage in

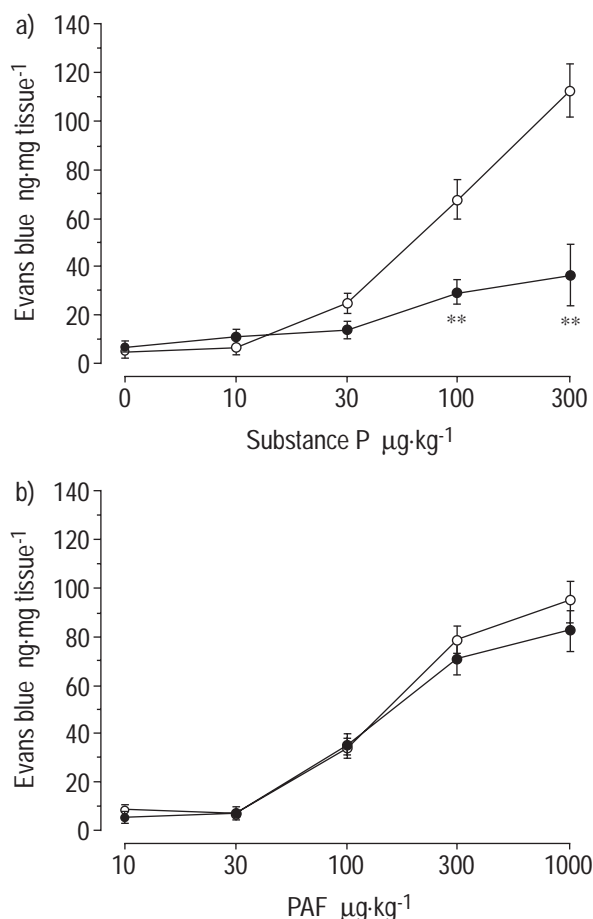


Fig. 5. – Effect of sodium cromoglycate (SCG) on tracheal vascular leakage induced by inhaled: a) substance P; and b) platelet-activating factor (PAF) in sensitized rats. The rats received SCG aerosols (30 mg·kg⁻¹) by inhalation, and 5 min later Evans blue and substance P (3–300 µg·kg⁻¹) or PAF 10–1,000 µg·kg⁻¹) were consecutively given (●). In the control experiments, the rats received substance P or PAF alone (○). Data are presented as mean±SEM (n=8). **: p<0.01 versus control.

nonsensitized rats, but was without effect in sensitized rats. Furthermore, it was found that NEP activity in tracheal tissues in sensitized animals was significantly less than that in nonsensitized ones. Therefore, the difference in microvascular responses to adenosine between sensitized and nonsensitized rats may be explained by the difference in endogenous NEP activity.

SCG was originally introduced as a mast cell-stabilizing drug and the first of a novel class of therapeutic agents for the treatment of allergic diseases [8]. However, its mast cell-stabilizing property is unlikely to explain the therapeutic efficacy of SCG in the treatment of asthma, because many compounds that are far more potent in stabilizing mast cells have failed to show therapeutic usefulness [30]. More recently, SCG has been shown to inhibit bronchoconstrictor responses evoked by irritants including bradykinin [31] and sulphur dioxide [32]. Because bradykinin and sulphur dioxide have no direct action on airway smooth muscle cells but stimulate autonomic nerve fibres, SCG may have the capacity to inhibit neurally-mediated airway responses. In addition, SCG inhibits substance P-induced oedema formation in the human skin [9] and bronchospasm in man [33]. These ob-

servations raised the possibility that SCG might reduce the neurogenic inflammation mediated by capsaicin-sensitive sensory nerves in the airways; this effect is probably associated with the inhibition of tachykinin release and/or postjunctional action of SCG in antagonizing tachykinin receptors. In the present study, inhalation of substance P aerosols caused an increase in microvascular permeability, an effect that was inhibited by SCG to the same extent as observed in the experiment with adenosine aerosols. Thus, the site of SCG action may be postjunctional, and the authors speculate that this drug might antagonize tachykinin receptors. This hypothesis is supported by a previous autoradiographic study demonstrating that SCG inhibits the binding of substance P to several tissues *in vitro* [9].

It is possible that SCG could have exerted the effects demonstrated in a manner independent of the stimulus, *i.e.* by acting directly on vascular endothelial cells and protecting against agonist-induced opening of intercellular gap junctions. However, this possibility seems unlikely, because, in contrast to its inhibitory effects on adenosine- and substance P-induced extravasation, SCG had no effect on the response to PAF, which is believed to directly open the gap junctions of endothelial cells and cause extravasation of macromolecules [34].

Airway inflammation, characterized by vascular leakage and infiltration of inflammatory cells, may be an important factor in the pathogenesis of asthma. Vascular leakage facilitates airway oedema, which may consequently produce epithelial cell damage, bronchospasm and airway obstruction [3]. The present results suggest that, when endogenous neutral endopeptidase activity is decreased after antigen sensitization, adenosine causes airway microvascular leakage through a tachykinin-dependent mechanism and that sodium cromoglycate inhibits this effect, presumably by acting at a postjunctional level to selectively inhibit tachykinin action on vascular endothelial cells. This novel anti-inflammatory effect could play a part in the therapeutic efficacy of sodium cromoglycate in inflammatory airway diseases.

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