

## Capsaicin Sensitive Afferents Contribute to Acute Airway Edema following Tracheal Instillation of Hydrochloric Acid or Gastric Juice in the Rat

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The formation of acute edema in the tracheobronchial mucosa following local instillation of hydrochloric acid or gastric juice was studied in rats. Protein extravasation using the Evans blue technique was measured and used to indicate edema formation. A pH dependent Evans blue extravasation was observed whereby pH 2 produced a small, pH 1.5 an intermediate, and pH 1 a pronounced effect. Also, gastric juice (pH 1.3 ± 0.1) induced a marked Evans blue extravasation in the tracheobronchial mucosa. Rats which had been pretreated with capsaicin (100 mg/kg s.c.) had a much lower Evans blue extravasation following local instillation of both hydrochloric acid and gastric juice. Thus, the increase in Evans blue content induced by acid at pH 1.5 was abolished while about 50% of the response remained at pH 1. The protein extravasation caused by gastric juice was reduced by about 70% after capsaicin treatment. Terbutaline (73 nmol/kg, iv) or enprophylline (25 μmol/kg, iv) injected intravenously 10 min before, as well as hydrocortisone injected immediately after the intratracheal instillation of gastric juice, did not influence the magnitude of edema formation. Beta-methasone (0.25 mg/kg ip) pretreatment 24 h prior to the gastric juice exposure enhanced tracheal extravasation. In conclusion, acute protein extravasation in the lower airway mucosa induced by hydrochloric acid or gastric juice is mainly dependent on capsaicin sensitive sensory nerves, suggesting that local release of mediators, such as tachykinins, play a key role in this reaction. (Key words: Complications: aspiration. Lung: airway edema; aspiration; capsaicin. Mediators: tachykinins.)

REGURGITATION AND SUBSEQUENT aspiration of gastric juice into the lower airways is a feared complication in anesthesia. Mendelson, in 1946,<sup>1</sup> described two different entities in the aspiration syndrome based both on clinical observations and experimental work on rabbits. The first type was due to aspiration of liquid material, which produced an asthma-like condition with distinct clinical, roentgenologic, and pathologic features. The second type was caused by aspiration of solid material

producing laryngeal and bronchial obstruction with massive atelectasis and suffocation. Mendelson pointed out the importance of acid in the induction of the aspiration syndrome. Subsequently, Teabeault<sup>2</sup> found that the aspirated material must have a pH of <2.4 to cause aspiration pneumonitis. In other studies, the critical pH has been found to be in a similar range in both experimental animals<sup>3,4</sup> and humans.<sup>5</sup> Aspiration of either hydrochloric acid or gastric juice is associated with both acute symptoms<sup>6-8</sup> and late pathological changes.<sup>1,4,9-13</sup> The histopathologic changes, which include edema, exudate, aggregation of a large number of neutrophils and hemolyzed red blood cells, and necrotizing and hemorrhagic lesions, seem to be maximal after 48 h.<sup>4,9</sup> However, in only 3 min after aspiration, there are extensive areas of pulmonary atelectasis, capillary congestion in the alveolar walls,<sup>11</sup> mild interstitial edema, and various degrees of intraalveolar hemorrhage.<sup>14</sup> There are also acute symptoms, such as bronchospasm,<sup>7,8</sup> after aspiration of any material, liquid or solid. A large dose of atropine and cervical vagotomy can, however, diminish the bronchoconstrictor response to aspiration,<sup>7,8</sup> indicating involvement of a vagally mediated parasympathetic reflex mechanism elicited by airway irritation.

Increasing evidence suggests that multiple neuropeptides released from peripheral branches of sensory nerves, located under and within the lining epithelium, around blood vessels, and within the tracheobronchial smooth muscle layer, are involved in the mediation of several local protective responses in the airways.<sup>15</sup> The tachykinin family of neuropeptides (Substance P [SP], neurokinin A [NKA] and neuropeptide K [NPK]), as well as calcitonin gene-related peptide (CGRP), are present in a subpopulation of afferent neurons in the airways of several species, including rats<sup>16,17</sup> and humans.<sup>15,16,18</sup> Tachykinins and CGRP are released from sensory nerves in the guinea pig lung *in vitro* after irritation with capsaicin, the pungent agent in hot peppers, as well as after histamine and bradykinin.<sup>19-21</sup> Pretreatment with a high dose of capsaicin is associated with an almost total and long-lasting depletion of tachykinins and CGRP in the airways.<sup>15</sup> Following systemic or local administration, tachykinins are active in induc-

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ing mucosal edema in many tissues, such as the airway mucosa.<sup>22</sup> Thus, tachykinins released from peripheral branches of sensory nerves in the lower airways may be involved in responses such as mucosal edema formation due to plasma extravasation,<sup>22,23</sup> as well as bronchoconstriction,<sup>24,25</sup> while both tachykinins and CGRP may contribute to local vasodilation.<sup>25,26</sup>

The present study was performed to investigate whether capsaicin sensitive afferent nerves are involved in the formation of acute mucosal edema in the lower airways following local instillation of hydrochloric acid or gastric juice.

### Materials and Methods

Ninety-seven male rats (250–350 g) were used for the tracheal instillation experiments. Three other rats were used for collection of gastric juice.

The study was approved by the Ethics Committee at the Karolinska Institute.

### CAPSAICIN PRETREATMENT

Thirty-two rats were pretreated with capsaicin dissolved in 10% ethanol and 10% Tween 80. A total dose of 100 mg/kg capsaicin from a stock solution of 10 mg/ml was injected subcutaneously. The total dose was divided into three portions (20 mg/kg + 30 mg/kg + 50 mg/kg) injected on three consecutive days. On all three occasions, the rats were anesthetized in a glass box with ether before capsaicin administration. To counteract respiratory impairment associated with capsaicin administration, the rats received pretreatment with terbutaline (0.1 mg/kg) and theophylline (10 mg/kg) 5 min prior to the capsaicin injections.<sup>27</sup> The rats were used in the tracheal edema experiments 1 week after completion of the capsaicin pretreatment, since, at that time, the content of tachykinins and CGRP in sensory nerves is markedly reduced.<sup>15,19</sup> The effectiveness of capsaicin pretreatment was controlled by installing one drop of a 0.1 mg/ml capsaicin solution into one eye. Absence of wiping with the forepaws and blepharospasm was taken as an indication of successful capsaicin desensitization. In addition, a small piece of the trachea from each animal was subjected to analysis of the presence of SP-immunoreactive nerve fibers using immunohistochemistry.<sup>19</sup> In all capsaicin treated animals, an almost total loss of SP-immunoreactive nerves was observed.

### COLLECTION OF GASTRIC JUICE

Three rats were anesthetized with pentobarbital (50 mg/kg ip). The abdominal wall was opened and the stomach exposed. After closure of the pylorus with a ligature, the ventricular fundus was opened and the in-

terior of the stomach carefully rinsed with saline. A polyethylene tube (PE 150) was introduced into the ventricular lumen. The fundus and the abdominal wall were then closed with sutures. Stimulation of gastric juice secretion was performed with iv injection of pentagastrin (50 µg/kg). The secreted gastric juice was collected by means of aspiration with a 2-ml syringe. The aspirate was centrifuged at 3000 rpm for 10 min in order to separate the gastric juice from larger particulate matter. The gastric juice was then removed with a pipette and the pH was measured using a pH meter (Radiometer, Copenhagen). The pH of the gastric juice was  $1.3 \pm 0.1$ .

### TRACHEAL INSTILLATION EXPERIMENTS

The rats were anesthetized with pentobarbital (40 mg/kg ip). To monitor the cardiovascular status of the animals before intratracheal injections, blood pressure and heart rate were recorded using a catheter in the left carotid artery and a Statham pressure transducer connected to a tachygraph unit and a Grass Polygraph 7E (Grass Instruments Co., MA). After careful surgical exposure of the trachea Evans blue (20 mg/kg, 10 mg/ml dissolved in saline) was injected into a femoral vein. The rats were subsequently raised to a 60° head-up angle, and the cricothyroid membrane was penetrated with a thin needle attached to a Hamilton microsyringe. Twenty-five microliters of saline, hydrochloric acid at different pH, or gastric juice was then slowly injected during 5 s into the tracheal lumen in a caudal direction within 2 min after the iv administration of Evans blue. Five minutes after the intratracheal injection, the thorax was opened and the animals were manually perfused with 60 ml of saline *via* the aorta for 30 s. A 5-mm piece of the trachea just above the carina and a piece of a stem bronchus were dissected out. The pieces were pressed between filter papers to remove excess fluid, weighed, placed in Ellerman tubes, and frozen at -20° C before analysis of the Evans blue content.

Six control rats were analyzed after tracheal instillation of 25 µl saline (table 1). In one part of the study, hydrochloric acid of different pH (pH 1, 1.5, and 2) was injected into the trachea of normal (n = 23) and capsaicin pretreated (n = 23) rats (table 1). In other series of experiments, gastric juice was injected into the trachea of nine normal and nine capsaicin pretreated animals, as well as into normal rats that had been pretreated with other drugs (table 1). Thus, five rats were pretreated with terbutaline (Bricanyl®, Draco, Sweden) (73 nmol/kg iv), and seven rats received enprophylline (Draco, Sweden) (25 µmol/kg, iv) 10 min before the intratracheal injection. Five rats were pretreated with betamethasone (Betapred®, Glaxo, England) (250 µg/kg ip)

TABLE I. Pretreatment

	Normal	Capsaicin	Terbutaline	Enpropyphylline	Hydrocortisone	Beta-methasone	24 h
Saline	6						
Hydrochloric acid							
pH 1	7	7					
pH 1.5	9	10					
pH 2	7	6					
Gastric juice	9	9	5	7	5	5	5

Experimental protocol indicating the number of animals used in the different groups. Saline was injected into the tracheas of six normal rats. Hydrochloric acid of pH 1, 1.5, and 2 was injected into the tracheas of normal rats and rats pretreated with capsaicin (total dose 100 mg/kg, s.c.) 1 week prior to the experiment. Gastric juice was injected into the tracheas of normal rats and rats which had received various types of pretreatment: capsaicin (see above), terbutaline (73

nmol/kg, iv), and enpropyphylline (25 µg/kg, iv) were both administered 10 min prior to the intratracheal injections, hydrocortisone (5 mg/kg, iv) was administered immediately after the intratracheal injections while betamethasone (0.25 mg/kg, ip) was given 24 h before the intratracheal injections. 24 h indicates five rats whose extravasation was studied with Evans blue 24 h after the intratracheal injection of gastric juice.

24 h before, while, in five other rats, hydrocortisone (Solu-Cortef®, Upjohn, MI) (5 mg/kg iv) was administered immediately after the intratracheal injection of

gastric juice. Finally, five rats were given Evans blue 24 h after the gastric juice instillation into the trachea, and perfused with saline 5 min later.

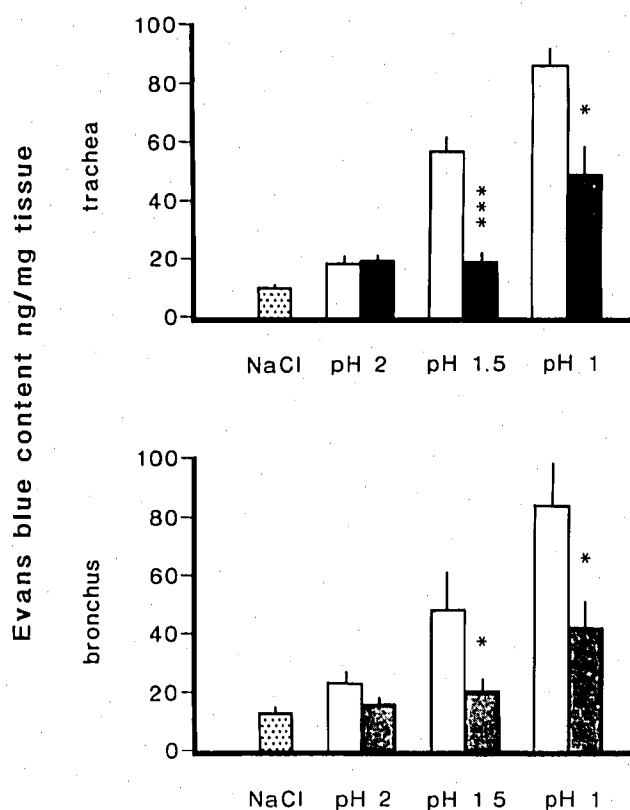


FIG. 1. Evans blue content (ng/mg tissue) in the trachea (upper panel) and stem bronchi (lower panel) from rats 5 min after local tracheal injection of 25 µl saline or hydrochloric acid at different pH. Open bars represent untreated animals and filled bars responses in rats which had been pretreated with capsaicin (100 mg/kg s.c. 1 week prior to the experiments). Data are given as means ± SEM (n = 6-10). \*P < 0.05, \*\*\*P < 0.001 using Mann-Whitney's U-test comparing untreated and capsaicin-treated animals.

#### EXTRACTION AND QUANTIFICATION OF EVANS BLUE

The tracheal and bronchial pieces were placed in 2.0 ml formamide and incubated for 24 h at 50° C in a water bath.<sup>28</sup> A Shimadzu RF 540 spectrofluorometer was used for measurements of the red fluorescence of Evans blue.<sup>29</sup> The excitation wavelength was 620 nm (band width 10 nm) and the emission wavelength 687 nm (band width 10 nm), which was found to be the wavelength exhibiting the maximal emission when tested before analysis. Calculations were based on standards of Evans blue in formamide. There is a linear relationship between fluorescence and Evans blue concentration up to a concentration of at least 200 ng/ml.<sup>29</sup>

#### STATISTICAL METHODS

The mean and standard error of the mean (SEM) were used throughout the study. Mann-Whitney's U-test and the Kruskal-Wallis analysis of variance with multiple comparisons were used to test statistical significance.

#### Results

Five minutes after intratracheal instillation of hydrochloric acid or gastric juice, a marked increase in tracheobronchial Evans blue content was observed compared to saline injections (figs. 1, 2). Twenty-four hours after intratracheal application of gastric juice, no increased Evans blue extravasation was present compared to control rats into which saline was injected (fig. 2).

A relationship existed between pH of the hydrochloric acid and the magnitude of Evans blue extravasation.

sation in trachea and bronchi. Thus, a marked extravasation was observed at pH 1 and 1.5, but not at pH 2.0, compared to saline injections (fig. 1). After capsaicin pretreatment, the Evans blue extravasation in both trachea and bronchi was significantly smaller when acid at pH 1 ( $P < 0.05$ ) and 1.5 ( $P < 0.001$  and  $P < 0.05$ , respectively) was injected. Thus, whereas the increase in Evans blue content upon injection with acid at pH 1.5 was virtually abolished after capsaicin pretreatment, a considerable response still remained in trachea (50%) and bronchi (42%) at pH 1 (fig. 1).

After intratracheal injection of gastric juice (pH 1.3), capsaicin pretreatment markedly ( $P < 0.001$ ) decreased the Evans blue extravasation response by 62% in the trachea and 70% in the bronchi (fig. 2). Intravenous terbutaline pretreatment caused a slight, but not significant, inhibition of the gastric juice induced extravasation, while enprophylline and hydrocortisone did not effect this response (fig. 2). Betamethasone given 24 h prior to the intratracheal injection of gastric juice significantly ( $P < 0.05$ ) enhanced (by 51%) the Evans blue extravasation in the trachea, but not in bronchi (fig. 2).

Terbutaline and enprophylline treatment was associated with a rapid decrease in the arterial blood pressure and an increase in heart rate. Thus, the mean arterial blood pressure had decreased by  $18 \pm 13$  mmHg ( $n = 5$ ) and  $16 \pm 10$  mmHg ( $n = 7$ ), respectively, while the heart rate had increased by  $42 \pm 8$  ( $n = 5$ ) and  $66 \pm 10$  ( $n = 7$ ) beats/min, respectively, as measured 10 min after the intravenous injection of the two drugs just prior to the tracheal instillations. The maximal fall in blood pressure induced by terbutaline and enprophylline during this 10-min period was  $32 \pm 6$  mmHg ( $n = 5$ ) and  $45 \pm 6$  mmHg ( $n = 7$ ), respectively. Pretreatment with hydrocortisone and betamethasone did not influence blood pressure or heart rate compared to untreated animals (data not shown).

### Discussion

In the present study, we have demonstrated and characterized an acute, pH-dependent protein extravasation in the lower airways upon local instillation of acidic solutions. Evans blue, when injected into the systemic circulation, rapidly binds to macromolecules, *i.e.*, serum albumin.<sup>29</sup> An increased tissue content of the Evans blue dye is, therefore, considered to reflect increased extravasation of plasma proteins through postcapillary venules, subsequently leading to edema formation.<sup>29</sup> The tracheo-bronchial Evans blue extravasation produced by hydrochloric acid and gastric juice was an acute response, since no increased extravasation was present 24 h after the intratracheal injections. The magnitude of

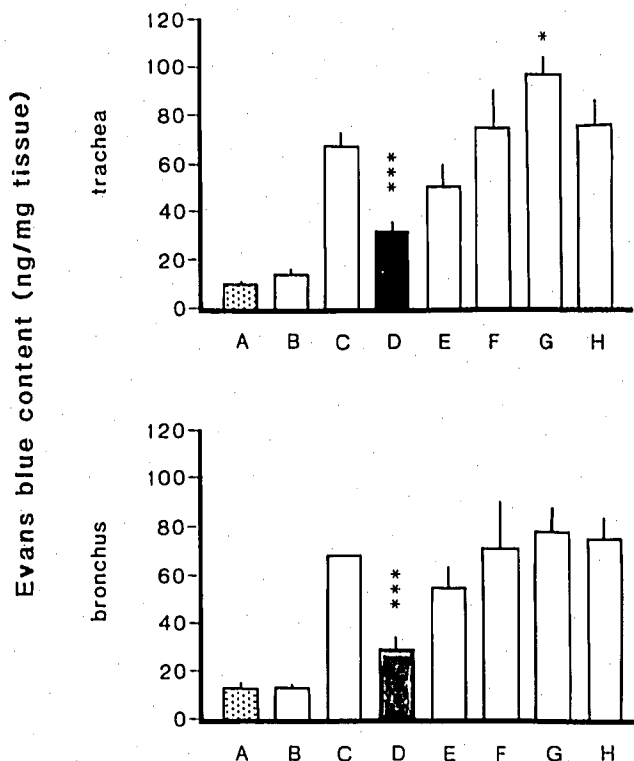


FIG. 2. Evans blue content (ng/mg tissue) in the rat trachea (upper panel) and stem bronchi (lower panel) after local tracheal instillation of (A) 25  $\mu$ l saline or (B-H) 25  $\mu$ l gastric juice. A, and C-H represent observations 5 min, and B, 24 h after tracheal injections. A-C represent control animals, D were pretreated with capsaicin (100 mg/kg s.c. 1 week prior to the experiments), E were pretreated with terbutaline (73 nmol/kg iv) and F with enprophylline 25  $\mu$ mol/kg iv) 10 min prior to experiments.<sup>31</sup> G were pretreated with betamethasone (250  $\mu$ g/kg ip) 24 h before the experiments and H with hydrocortisone (5 mg/kg iv) immediately after the intratracheal injection of gastric juice. Data are presented as means  $\pm$  SEM ( $n = 5-9$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$  using Kruskal-Wallis analysis of variance comparing group C (normal animals injected with gastric juice) with group D-H.

the acute airway edema, represented by Evans blue extravasation, had a similar pH-dependence, as has been established also for histopathological changes and mortality.<sup>3-6</sup>

The tracheo-bronchial edema formation upon instillation of acid in our study was, to a large extent, dependent on the functional integrity of a special population of sensory nerves, since, in the capsaicin pretreated animals, the Evans blue extravasation was absent at pH 1.5 and considerably reduced at pH 1. Protein extravasation in the rat airways induced by antidromic vagal nerve stimulation or, for instance, inhalation of cigarette smoke or ether vapor, as well as the flare reaction induced by histamine or allergen challenge in the human skin, can also be abolished by capsaicin pretreatment, suggesting that these sensory nerves are activated

by a variety of chemical stimuli.<sup>15</sup> Furthermore, electrophysiological recordings from single vagal C-fiber afferents have indicated an increase in electrical activity upon inhalation of sulfur dioxide,<sup>30</sup> as well as together with intravascular administration of capsaicin.<sup>31</sup> The capsaicin pretreatment seems to specifically affect peptides in a population of sensory nerves of the lung. Thus, tachykinin- and CGRP-like immunoreactivities are depleted, while both biochemical and histochemical evidence suggest that the cholinergic, sympathetic, and vasoactive intestinal polypeptide (VIP)-containing systems are intact after capsaicin pretreatment.<sup>15,19</sup>

Activation of capsaicin sensitive afferents and local release of neuropeptides<sup>19-21,25</sup> seem to induce protein extravasation,<sup>22,23,25</sup> increased blood flow,<sup>25,26</sup> and bronchoconstriction.<sup>24,25</sup> Protein extravasation is readily induced by tachykinins, while CGRP induces no<sup>32</sup> or minor<sup>33</sup> Evans blue extravasation *per se*. CGRP, however, potentiates the edema induced by tachykinins, probably due to its potent vasodilatory property.<sup>34</sup> A vasodilator response in the trachea upon vagal nerve stimulation and capsaicin aerosol still remains after pretreatment with a ganglionic blocking agent, to prevent parasympathetic vasodilator mechanisms, suggesting an involvement of local release of sensory neuropeptides with potent vasodilator properties, such as tachykinins and CGRP.<sup>26</sup>

Tachykinins, especially NKA, have been shown to cause powerful contraction of airway smooth muscle, both *in vitro* and *in vivo*, while CGRP seemingly lacks bronchoconstrictor effects.<sup>15,19,25</sup> The non-cholinergic bronchoconstriction in the guinea pig induced by vagal nerve stimulation, iv administered capsaicin, or inhalation of ether is abolished after capsaicin pretreatment, indicating that changes in bronchial smooth muscle tone are also dependent on the existence of an intact innervation with peptide containing C-fiber afferents.<sup>15,24</sup> SP analogues with tachykinin antagonistic properties have been shown to diminish both the protein extravasation and bronchoconstriction elicited by activation of capsaicin sensitive afferents, which further supports an involvement of tachykinins in these responses.<sup>15</sup> It is, therefore, likely that irritant agents are capable of initiating central reflexes, including cough and secretion,<sup>31</sup> but also local non-cholinergic respys, such as protein extravasation, which are likely to be at least partially mediated by release of tachykinins and/or CGRP from peripheral branches of sensory nerves.<sup>15</sup> The relative importance of different mediators for the protein extravasation response remains to be established using specific antagonists. Interestingly, gastroesophageal reflux of gastric juice has been proposed as a factor capable of initiating cough, hoarseness, and

asthma, especially nocturnal asthma and wheezing.<sup>35</sup> A reflex induced bronchoconstriction may be elicited either *via* a central vagal reflex from afferents in the esophagus,<sup>36,37</sup> or by subsequent aspiration of small amounts of gastric juice into the airways,<sup>1,8,38</sup> causing local and/or central reflexes. In addition, airway edema formation could contribute to reflex induced symptoms. About 50% of the Evans blue extravasation in the trachea and bronchi induced by hydrochloric acid at pH 1, as well as 30-40% of the response to gastric juice, remained in the capsaicin treated animals. It is, therefore, likely that an acid solution at pH 1, in addition to activating capsaicin-sensitive sensory nerves, also produces protein extravasation *via* other mechanisms, such as, presumably, by a direct burning effect. It seems clear from earlier studies on vascular permeability in rat trachea that intubation evokes massive protein extravasation *via* mechanisms which are resistant to capsaicin treatment.<sup>23</sup>

Terbutaline, which is a selective  $\beta_2$ -adrenoceptor agonist known to inhibit bradykinin induced increase in vascular permeability to macromolecules,<sup>39</sup> and enprophylline, which reduces leakage elicited by capsaicin and leukotrienes,<sup>40</sup> did not significantly change the Evans blue extravasation in the rat trachea induced by instillation of gastric juice. It has previously been shown that local treatment with terbutaline did not affect the tracheal protein extravasation elicited by exposure to cigarette smoke, while the massive Evans blue leakage induced by mechanical stimulation of the tracheal mucosa (intubation was reduced).<sup>23</sup> The reported effectiveness of both terbutaline and enprophylline in the present doses as inhibitors of protein leakage in guinea pig trachea<sup>40</sup> may either indicate important differences between species and/or mechanisms of edema formation. In this context, it is of interest that the presently used doses of terbutaline and enprophylline caused marked initial fall in blood pressure and subsequent tachycardia, which also could have influenced the extravasation reaction due to changes in local perfusion pressure.

The beneficial effects of treatment with steroids after aspiration of acidic material has been a controversial issue.<sup>4,10,12,13</sup> In the present study, an immediate intravenous injection of hydrocortisone did not affect the acute protein extravasation induced by gastric juice. In fact, an increased tracheal Evans blue extravasation response was present in the rats which were treated with betamethasone 24 h prior to gastric juice injection. In another study, acute edema formation in the trachea upon vagal nerve stimulation in rats was also enhanced by glucocorticoid pretreatment.<sup>23</sup> Thus, it seems possible that, under certain circumstances, steroids might

enhance protein extravasation. Furthermore, corticosteroid therapy has been reported to promote the development of diffuse interstitial lung fibrosis induced by the chemical irritant butylated hydroxytoluene.<sup>41</sup>

In conclusion, the present data indicate that activation of peptide containing capsaicin sensitive afferents represents a key step in the mediation of the acute protein extravasation in the tracheobronchial wall upon local instillation of hydrochloric acid and gastric juice in the rat. This reaction does not seem to be reduced by pretreatment with terbutaline, enprophylline, or steroids. Whether a similar reaction occurs upon aspiration of gastric material in humans remains to be established, although it seems clear that tachykinin and CGRP containing sensory nerves are also present in human lower airways.<sup>15</sup>

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