Zoological Studies **41**(1): 13-22 (2002)

Zoological Studies

Morphological Alterations in the Trachea of Capsaicin-pretreated Rat during Postnatal Development

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(Accepted September 2, 2001)

Shang-Ming Yu and Kwan-Hwa Lin (2002) Morphological alterations in the trachea of capsaicin-pretreated rat during postnatal development. *Zoological Studies* **41**(1): 13-22. The purpose of this study was to investigate morphological changes of the mucosa layer of the trachea in rats pretreated with capsaicin neonatally. Paraformaldehyde-lysine-periodate- and osmium-fixed plastic sections were prepared from 17 capsaicin pretreated rats and 15 controls and studied at the light microscopic level. The results indicate that no significant differences occurred in the mean cross-sectional epithelial thickness of the tracheal mucosa between 1 wk after neonatal capsaicin treatment (20.40 ± 0.48 μm) and sham treatment (21.80 ± 0.80 μm). Cross-sectional epithelial thickness (18.81 ± 0.39 μm) continued to show no significant difference through 2 wk after neonatal capsaicin treatment, but the epithelial thickness significantly ($p < 0.05$) increased at 2 wk after sham treatment (40.05 ± 1.80 μm). Furthermore, cross-sectional epithelial thickness also significantly ($p < 0.05$) increased at 1 mo after sham treatment (41.24 ± 1.20 μm) compared to that with neonatal capsaicin treatment (24.79 ± 0.54 μm). At the light microscopic level, large vacuoles were strikingly prominent in the tracheal epithelium at 1 wk after neonatal capsaicin treatment, as were many small vacuoles at 2 wk. Another striking change was that apical cytoplasmic blebbing of goblet cells had increased moderately at 2 wk after neonatal capsaicin treatment and had increased markedly at 1 mo. Substance P-like immunoreactivity (IR) was not discernible at 1 wk after sham or capsaicin treatment. Substance P-like IR was slightly immunolabeled at 2 wk and increased markedly at 1 mo after sham treatment. In contrast, substance P-like IR was not immunolabeled at either 2 wk or 1 mo after neonatal capsaicin treatment. The present study suggests that neonatal capsaicin treatment results in epidermal thinning of the trachea during postnatal development. The blockade of the elastic fibers containing substance P-like IR implies involvement of the elastic fibers in the histologic response to capsaicin's effect on the depletion of substance P. Moreover, formation of apical cytoplasmic blebbing of goblet cells indicates hindrance by capsaicin of mucus secretion and clearance. http://www.sinica.edu.tw/zool/zoolstud/41.1/13.pdf

Key words: Capsaicin, Immunocytochemistry, Postnatal development, Substance P, Trachea.

In mammalian anatomical airways, immunocytochemical labeling has localized many bioactive peptides to cells and/or sensory nerve endings. These peptides include substance P (Wharton et al. 1979, Springall et al. 1990, Bauman et al. 1999), neuropeptide tyrosine (NPY) (Sheppard et al. 1984a), bombesin (Wharton et al. 1978), cholecystokinin (Ghatei et al. 1982), leuenkephalin (Cutz et al. 1981), calcitonin (Becker et al. 1980), calcitonin-gene related peptide (Cadieux et al. 1986, Holzer 1988, Luts et al. 1990), dopamine-ß-hydroxylase (Sheppard et al. 1983), S-100 (Sheppard et al. 1983), protein gene product 9.5 (Baluk et al. 1992), and neuron-specific enolase (NSE) (Sheppard et al. 1984a, b). These neuropeptides may act as neurotransmitters or modulators in the central and peripheral nervous systems. Sensory fibers containing these neuropeptides, especially substance P and related tachykinins, have been found to have strong actions on vascular permeability and airway smooth muscle contraction (Nilsson et al. 1977),

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glandular secretions (Lundberg et al. 1988, Kuo et al. 1990), and possibly ciliary motion (Uddman and Sundler 1986). It is also known that such effects are reduced after administration of substance P antagonists (Lundberg et al. 1983a), or tachykinin antagonists (Morimoto et al. 1992, Murai et al. 1992), or pretreatment with capsaicin, an extract of the pungent red pepper (Lundberg and Saria 1983). Previous investigations have demonstrated that substance P-like immunolabeling is localized in the epithelium and bronchial smooth muscle of the lower respiratory tract (Lundberg et al. 1984). Capsaicin pretreatment reduces the number of substance P-immunoreactive axons in the trachea. Numerous studies have also demonstrated that neonatal capsaicin treatment causes degeneration of the sensory neurons of the vagus nerve (Jancsó et al. 1977, Gamse et al. 1980) and depletion of substance P in the vagus nerve (Jessell et al. 1978, Gamse et al. 1981a). In addition, neonatal capsaicin treatment desensitizes pain receptors of the sensory nerve to chemical stimuli (Jancsó 1955) and prevents neurogenic inflammatory responses (Jancsó et al. 1967). It has further been shown that, in the rat, the epithelial thickness of the skin is reduced after denervation (Chiang et al. 1998, Hsieh and Lin 1999). No evidence has been reported of changes of the pseudostratified epithelial thickness of tracheal mucosa after sensory denervation. It is of interest to reexamine by light microscopy morphological alterations and to correlate these changes in epithelial thickness of the tracheal mucosa by morphometry with responses to neonatal capsaicin pretreatment during postnatal development.

MATERIALS AND METHODS

Light microscopy

Sprague-Dawley rats were administered a single subcutaneous injection of capsaicin (50 mg/kg body weight, prepared in a vehicle of 10% ethanol, 10% Tween 80, and 80% isotonic saline) on the 2nd day of life. Pups from other litter mates injected with the same volume of vehicle or those untreated served as controls (n = 15). At 1 wk (n = 6), 2 wk (n = 6), and 1 mo (n = 5) after capsaicin treatment, animals were killed by decapitation. The number within brackets indicates the number of animals in that age group used for this study. The middle portion of each trachea was removed after decapitation and fixed in McLean and Nakane fixative (1974) followed by osmium fixation (Yu 1993a, b) in 0.1 M phosphate buffer overnight at 4 ˚C. Superficial mucus was removed by gently rinsing and shaking with 0.1 M phosphate buffer prior to fixation. After fixation, tissues were washed in 0.1 M phosphate buffer, immersed in 1% osmium tetroxide, dehydrated in a graded ethanol series to absolute ethanol, immersed in propylene oxide, infiltrated with Epon-Araldite/propylene oxide (1:1), and cured in freshly prepared Epon-Araldite resin. After polymerization, the plastic tissues were sectioned at 1-2 µm with glass knives on an ultramicrotome for light microscopy. Sections were mounted on slides within a circle marked with a diamond pen. Sections were then stained with toluidine blue.

Immunocytochemistry

The plastic-embedded tissues were sectioned at 1-2 µm with glass knives on an ultramicrotome for immmunocytochemical staining. Sections were deplasticized in saturated sodium hydroxide in absolute alcohol (1:3) for 7 min, immersed in 3% hydrogen peroxide, and incubated in 10% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 10 min. Immunocytochemical staining was performed as follows: (1) incubation in rabbit anti-substance P (A/SP, diluted 1:50-1:100, BioGenex Laboratories, San Ramon, CA, USA) for 14-16 h, in 0.01 M PBS; (2) incubation in biotinylated goat anti-rabbit IgG (Vectastain kit, Vector Labs, Burlingame, CA, USA) for 2 h; (3) incubation in ABC reagent (avidin-biotin-peroxidase complex, Vectastain kit, Vector Labs) for 2 h; (4) treatment with 3, 3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA, 0.3 mg/ml) in 0.05 M Tris buffer containing 0.002% hydrogen peroxide, pH 7.6 until immunoreactive sites were visible. All incubations were carried out at room temperature. Finally, sections were dehydrated in ethanol, cleared in xylene, and covered with a coverslip.

In control samples, the 1st antibody, A/SP, was replaced by (1) normal rabbit serum or (2) PBS.

Morphometry

Quantitative measurements of the plastic sections (thickness 1-2 µm) were made using the Image-Pro Plus Data Analysis Program (Media Cybernetics, Silver Spring, MD, USA). No correction was made for shrinkage between fresh tissue and structures measured through the WinFast

T230 TV video system in the plastic sections. All data were stored in the computer and analyzed by SPSS for Windows. Statistical comparisons of cross-sectional area in multiple groups were made using one-way analysis of variance (one-way ANOVA). Post hoc analysis for significance between any 2 groups used the Student-Newman-Kuels test.

RESULTS

Morphometry: quantitative analysis

As shown in figure 1, there were no significant differences in the mean cross-sectional epithelial thickness of the tracheal mucosa between 1 wk after neonatal capsaicin treatment (20.40 ± 0.48 μm) and sham treatment (21.80 ± 0.80 μm). Cross-sectional epithelial thickness (18.81 ± 0.39 μm) continued to show no significant difference through 2 wk after neonatal capsaicin treatment, but epithelial thickness had significantly ($p < 0.05$) increased at 2 wk after sham treatment (40.05 ± 1.80 μm). Furthermore, cross-sectional epithelial thickness had also significantly ($p < 0.05$) increased at 1 mo after sham treatment (41.24 ± 1.20 μm) compared to that after neonatal capsaicin treatment (24.79 ± 0.54 μm).

Light microscopy

At 1 wk after sham treatment, light microscopic observations revealed that the pseudostratified mucosal epithelium of the trachea consisted of 4 cell types: basal cells, intermediate cells, goblet cells, and ciliated cells. Ciliated cells contained many cilia and a slightly pale cytoplasm. The goblet cells contained slightly intensely stained cytoplasm and lacked cilia. Basal cells rest on the basement membrane. At the light microscopic level, the basement membrane was located at the junction between the respiratory pseudostratified columnar ciliated epithelium and the lamina propria. Intermediate cells were located slightly above the basal cell layer. At 1 wk after sham

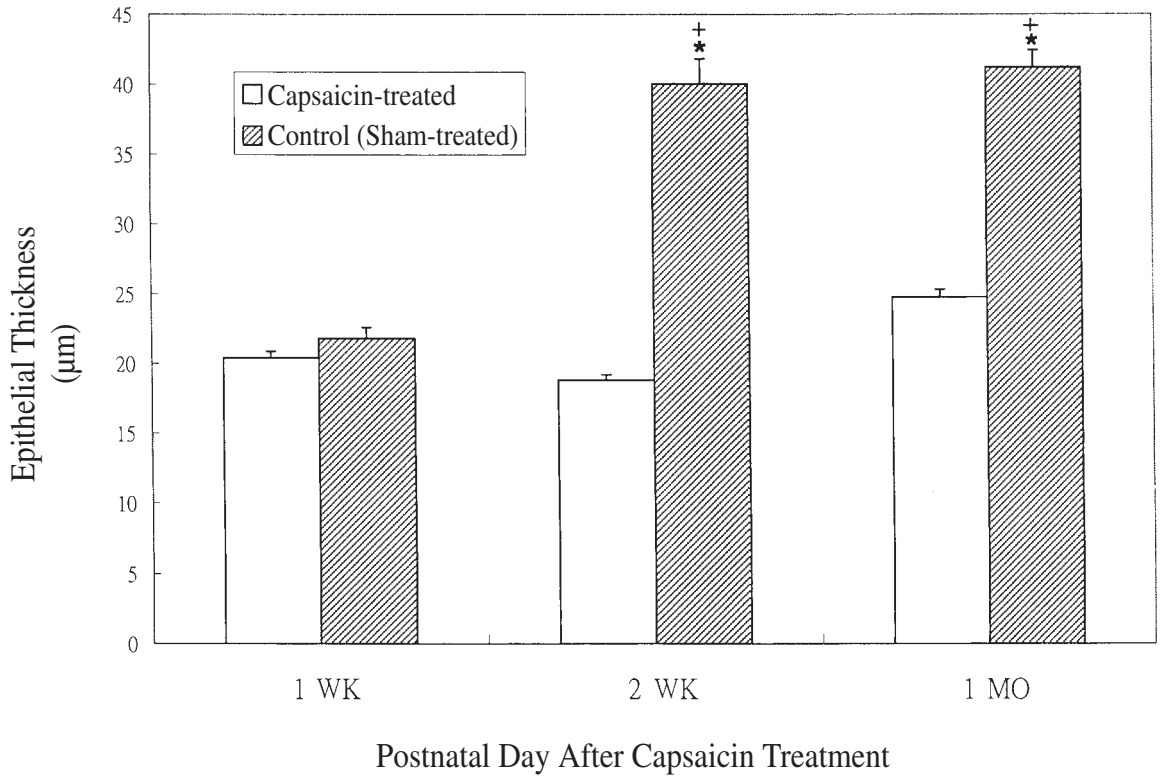

Fig. 1. Mean cross-sectional epithelial thickness of the trachea with standard error of mean. * denotes $p < 0.05$ for data compared with the control (sham-treated) group, + denotes $p < 0.05$ for data compared with the capsaicin-treated group.

treatment, ciliated cells were randomly distributed between goblet cells (Fig. 2a). At 2 wk after sham treatment, the mucosa of the trachea had formed dome-shaped protuberances among which were intermingled ciliated and goblet cells. The mucosal epithelium of the trachea was thicker at 2 wk than that at 1 wk. Ciliated cells possessed prominent cilia, and their cytoplasm was faintly stained. The ciliated cells extended from the basement membrane to the luminal surface of the epithelium. Goblet cells contained intensely basophilic cytoplasm. Goblet cells tapered off near the apical surface or at the base. Most goblet cells showed a slight dome at the upper surface and extended from the apical epithelial surface to the basal cell layer. Basal cells and intermediate cells remained in their positions (Fig. 2b). At 1 mo after sham treatment, the mucosal surface of the trachea was mainly covered by the prominent cilia of ciliated cells. Goblet cells were deeply interposed between the ciliated cells in the mucosal epithelium and occasionally contained blebbings of cytoplasm on the apical surface. The cytoplasm continued to be faintly stained in ciliated cells and intensely stained in goblet cells (Fig. 2c). At 1 wk after neonatal capsaicin treatment, the mucosal surface was covered by ciliated and goblet cells. Many large vacuoles were found in the spaces between the cells (Fig. 2d). At 2 wk after neonatal capsaicin treatment, apical blebbing of the cytoplasm of goblet cells had increased moderately. In addition to the large vacuoles, many small vacuoles were also observed among ciliated cells and goblet cells. These small vacuoles were located beneath the apical surface. All epithelial cells revealed an intensely basophilic cytoplasm. The epithelial thickness of the trachea was not well developed in comparison with that of the sham-treated ones (Fig. 2e). At 1 mo after neonatal capsaicin treatment, apical blebbing of the cytoplasm of goblet cells had increased markedly. The epithelial thickness of the trachea was moderately developed. This epithelial thickness was measured from the bottom of the apical blebbing of goblet cells to the basement membrane. The large and small vacuoles were slightly reduced in both size and number at this stage. The cytoplasm became faintly stained in ciliated cells and intensely stained in goblet cells (Fig. 2f).

Substance P-like immunoreactivity

Substance P-like immunopositive dot-like structures appeared and were localized in the lamina propria of the tracheal mucosa. Under electron microscopic investigation, these densely dotted structures were revealed to be elastic fibers (Yu, unpubl. data).

No discernible substance P-like immunoreactivity (IR) was immunolabeled in the trachea at 1 wk after sham treatment (Fig. 3a). At 2 wk after sham treatment, substance P-like IR was slightly immunolabeled in the trachea (Fig. 3b). Furthermore, at 1 mo after sham treatment, substance-P-like IR had markedly increased in the trachea (Fig. 3c). However, no discernible substance P-like IR was immunolabeled in the trachea at 1 wk (Fig. 3d), 2 wk (Fig. 3e), or 1 mo (Fig. 3f) after neonatal capsaicin treatment.

DISCUSSION

Substance P (SP), a tachykinin, putatively functions as a sensory neurotransmitter and may play a role in mediating neurogenic inflammation in the airways (Baluk et al. 1992, Maggi et al. 1993, Lundberg 1996). Substance P in the rat originates in the nodose and jugular ganglia (Uddman and Sundler 1986). SP-immunoreactive (IR) nerve fibers are located at the base of the epithelium of the tracheal mucosa (McDonald et al. 1988). The vagal nerves contain many SP-IR nerve fibers (Lundberg et al. 1978 1980). Electrical stimulation of the vagus nerves increases vascular permeability in rat trachea presumably by releasing substance P or other tackykinins from sensory nerve endings (Lundberg et al. 1983). The vagal nerves are depleted of their SP contents by capsaicin pretreatment (Gamse et al. 1981b), which appears to result in selective degeneration of chemosensitive C-fiber afferents (Jancsó et al. 1977). Surgically evoked degeneration of a single vagal trunk causes a decrease in neurogenic inflammation produced by the irritant, capsaicin, in the bronchi of the rat (Huang et al. 1995). Previous investigations have demonstrated that SP-IR axons are very slender and long and are arranged in a network located close to the bases of epithelial cells or between epithelial cells through thick sections. Our study employed paraformaldehyde-lysine-periodate- and osmium-fixed plastic semi-thin sections (thickness 1-2 μm) for substance P-like immunocytochemical staining. Such a semithin sectioned tissue preparation entirely differs from paraffin sections or whole mounts used previously. Although the survival and positive immunoreactive labeling of hormone

Fig. 2. Light micrographs of tracheal epithelium. (a) At 1 wk after sham treatment, the epithelial layer of the mucosa of the trachea is composed of 4 cell types: basal cells (double arrows), intermediate cells (double arrowheads), goblet cells (arrowhead), and ciliated cells (arrow). The intercellular space was not discernible. (b) At 2 wk after sham treatment, the mucosa of the trachea had formed dome-shaped protuberances among which were intermingled ciliated cells (arrow) and the goblet cells (arrowhead). (c) At 1 mo after sham treatment, the mucosal surface of the trachea was mainly covered by the cilia of ciliated cells (arrow), and goblet cells (arrowhead) were interposed among the ciliated cells. Note the apical blebbing of the cytoplasm of the goblet cell (double arrows). (d) At 1 wk after neonatal capsaicin treatment, the mucosa was covered by ciliated cells (arrow) and goblet cells (arrowhead). Note the large vacuoles in the intercellular space (asterisk). (e) At 2 wk after neonatal capsaicin treatment, the apical blebbing (arrowhead) had noticeably increased. Note the large vacuoles (asterisk) and small vacuoles (double arrowheads) between ciliated cells (arrow) and goblet cells. (f) At 1 mo after neonatal capsaicin treatment, blebbing of the cytoplasm of goblet cells (arrowhead) had markedly increased. Note that the large vacuoles (asterisk) and the small vacuoles are reduced in size. EP: epithelium; LP: lamina propria. Bar = 10 μm.

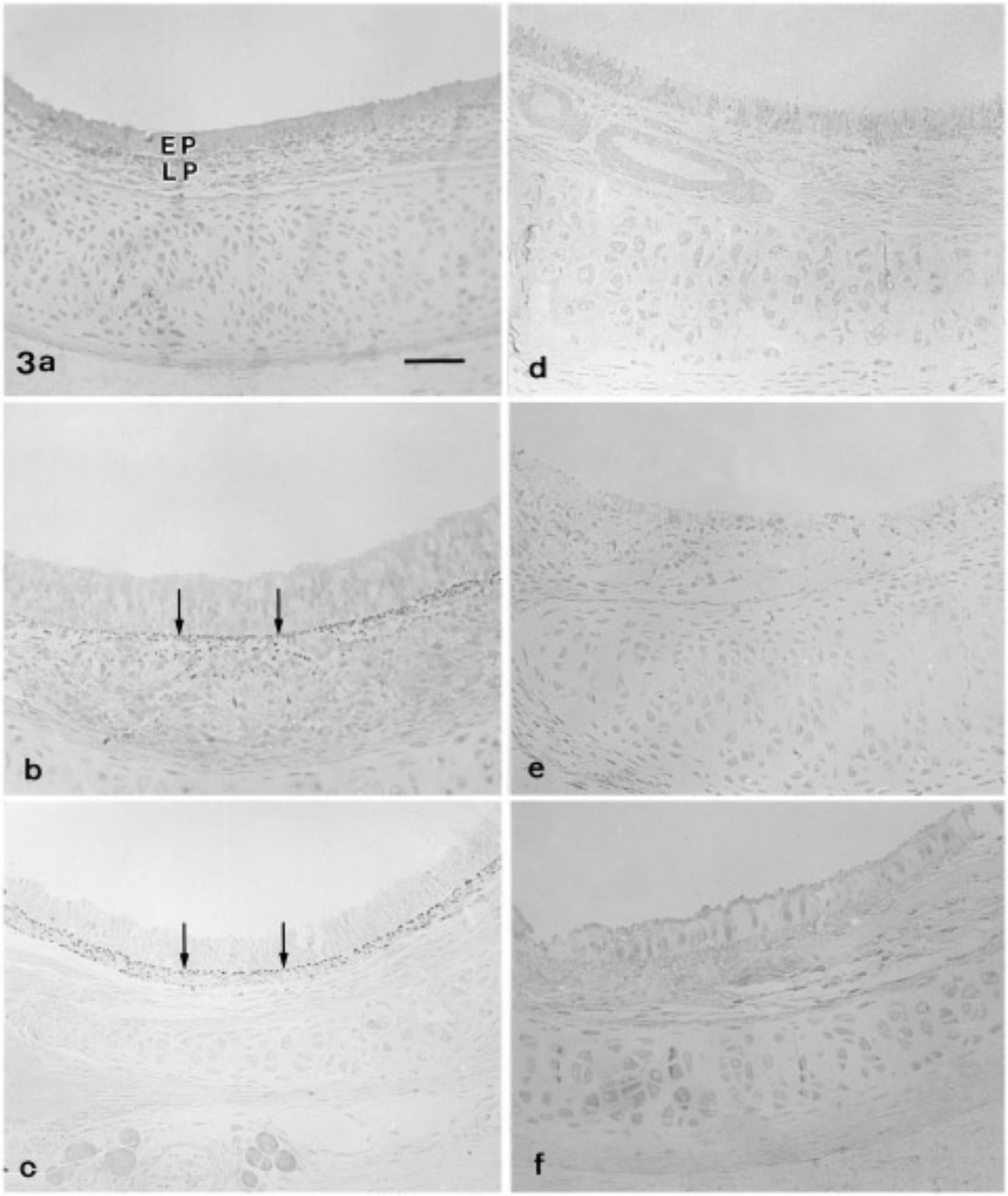

Fig. 3. Substance P-like immunoreactivity (IR) in the trachea. (a) No discernible substance P-like IR was immunolabeled in the trachea at 1 wk after sham treatment. (b) Substance P-like IR (arrows) was slightly immunolabeled in the trachea at 2 wk after sham treatment. (c) Substance-P-like IR (arrows) had markedly increased in the trachea at 1 mo after sham treatment. However, no discernible substance P-like IR was immunolabeled in the trachea at 1 wk (d), 2 wk (e), or 1 mo (f) after neonatal capsaicin treatment. EP: epithelium; LP: lamina propria. Bar = 25 μm.

antigens and protein markers has been demonstrated in these osmium-fixed, plastic-embedded tissues (Yu 1993a, b), owing to the limited content of substance P, immunolabeled nerve endings were barely detectable in these semithin sections. In this study, substance P-like immunoreactivity definitely survived the harsh treatment and heat involved in osmium tetroxide fixation and epoxy embedding. Immunopositive dots appeared and were localized in the lamina propria. Under electron microscopic investigations, these dense dotted structures were identified as elastic fibers (Yu, unpubl. data). The functional significance of elastic fibers containing substance P-like immunoreactivity in capsaicin-pretreated rat trachea during postnatal development needs to be elucidated in the future.

Neonatal capsaicin treatment reduces the bronchoconstrictor response elicited in the adult guinea pig by electrical field stimulation of the vagus nerve (Martling et al. 1984). A distinct population of primary sensory neurons involved in mediation of chemogenic pain in the capsaicin-pretreated neonate rat has also been shown to selectively degenerate (Jancsó et al. 1977). It is possible that capsaicin interferes with the synthesis and release of neurohumor in pain-sensitive nerve terminals or neurons. At the electron microscopic level, the fine structure of primary sensory neurons is severely impaired, including swollen mitochondria, disorganization of the cisternae, and dilation of the perinuclear cisternae as well as that of cisternae of the rough endoplasmic reticulum. Axonal degeneration is accompanied by the appearance of many glial cells. However, capsaicin treatment does not induce degeneration of primary sensory neurons in the adult rat. These selective degenerations in capsaicin-pretreated rat are initiated by the release of substance P from peripheral branches of sensory nerve fibers (Lembeck and Holzer 1979, Rosell et al. 1981). Substance P stimulates pulmonary airway mucus secretion (Gonzales and Ballard 1989) and is also the mediator of the axon reflex in cutaneous tissue (Gamse et al. 1980). A previous ultrastructural study (Rhodin 1966) shows that lateral surfaces of basal cells send fine cytoplasmic processes into the narrow intercellular space in which free sensory nerve endings also terminate. The results of our study indicate that large and small vacuoles seem to be located in the intercellular space. These vacuoles were markedly increased at 1 and 2 wk after neonatal capsaicin treatment. Vacuolization has been shown in the nerve tissue and vacuoles corresponding to swollen vacuolated presynaptic terminals after ischemic injury (Garcia et al. 1993). A likely explanation for this alteration is that capsaicin causes depletion of substance P from elastic fibers and pheripheral branches of sensory nerve fibers. Therefore, formation of vacuoles indicates the site of degenerated nerve terminals. Previous studies have established that denervation causes a significant thinning of the denervated epidermis of the skin (Hsieh and Lin 1999). This epidermal thinning was reversed by epidermal reinnervation 3 mo after denervation. They suggested that skin innervation exerts an influence on the proliferation of keratinocytes and the thickness of the epidermis. A similar mechanism may have influenced thinning of the mucosal epithelium after neonatal capsain treatment seen in our study. Capsaicin denervation may hinder the proliferation and differentiation of mucosal epithelial cells (including basal cells, intermediate cells, ciliated cells, and goblet cells) during postnatal development.

The results of our study demonstrate that cytoplasmic blebbing on the apical surface of goblet cells is formed and maintained after neonatal capsaicin treatment. Previous scanning electron microscopy findings indicated that globular mucin-containing secretory products are trapped within the cilia of the ciliated cells after neonatal capsaicin treatment (Yu and Lin 1995). It has been postulated that capsaicin presumably hinders mucus secretion and interferes with the removal of inhaled particles by the mucociliary clearance mechanism. The impairment and blockade of mucus secretion closely parallels the disappearance of substance P-like immunoreactivity in neonatally capsaicin-pretreated trachea. Alteration of the apical cytoplasmic blebbing provides evidence to support capsaicin impairment of the delicate balance of mucus secretion and clearance in tracheal mucosa.

In conclusion, the present study suggests that neonatal capsaicin treatment results in thinning of the tracheal epithelium during postnatal development. In addition, vacuolization may be involved in the degeneration of sensory nerve endings and in the impairment of proliferation and differentiation in epithelial cells of the tracheal mucosa. Furthermore, formation of apical cytoplasmic blebbing of goblet cells is probably due to hindrance of mucus secretion and clearance by capsaicin.

Acknowledgments: This work was supported by the National Science Council (grants nos.

NSC89-2314-B-010-031, NSC89-2320-B-010-156), Taipei, Taiwan, R.O.C., and the corresponding author was presented an award by the Medical Research and Advancement Foundation in memory of Dr. Chi-Shuen Tsou.

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Zoological Studies **41**(1): 13-22 (2002)

經辣椒素處理大白鼠氣管出生後發育的形態學變化

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本研究的目的在探討初生大白鼠經辣椒素處理後的氣管內形態學和黏膜厚度的變化。十七隻經辣椒素處理和十五隻對照組的老鼠，採用三聚甲醛－離氨酸－過碘酸鹽和鐵酸固定的樹酯切片製作，並用光學顯微鏡來觀察。結果顯示，大白鼠氣管上皮的平均橫斷面厚度，在經辣椒素處理後的第一周為 20.40 ± 0.48μm 與同期對照組的 21.80 ± 0.80μm 並無顯著性的差異。氣管上皮的平均橫斷面厚度經辣椒素處理後的第二周實驗組為 18.81 ± 0.39μm 與第一周實驗組仍無顯著性的差異，但與第二周對照組的 40.05 ± 1.80μm，卻有顯著性的差異 ($p < 0.05$)。再者，氣管上皮的平均橫斷面厚度在經辣椒素處理後的第一個月為 41.24 ± 1.20μm，與同期對照組 24.79 ± 0.54μm 相較也具有顯著性的差異 ($p < 0.05$)。在光學顯微鏡下，經辣椒素處理後在第一周時主要特徵為顯著大空泡的形成，到第二周時有許多小空泡存在。另一顯著特徵為第二周時杯狀細胞的頂部具有圓形外突的細胞質略為增加，到第二周時，卻更顯著地增加。在對照組，氣管上皮的 P 物質免疫細胞化學活性，在第一周時幾乎無法辨識，在第二周時免疫細胞化學活性略為增加，而到第一個月時 P 物質免疫細胞化學活性卻顯著地增加。然而，在實驗組氣管上皮的 P 物質免疫細胞化學活性，在第一周、第二周和第一個月時幾乎無法辨識其存在。目前的實驗結果指出，初生時辣椒素處理可造成發育中氣管上皮厚度的減小。經辣椒素處理後，含有 P 物質免疫細胞化學活性的彈性纖維被抑制，可能與辣椒素排空 P 物質的效應有關。再者，杯狀細胞的頂部細胞質圓形外突的形成，可能係為黏液分泌與清除的機制受到辣椒素抑制的緣故。

關鍵詞：辣椒素，免疫細胞化學，出生後發育，P物質，氣管。

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