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Immunological localization of neuropeptide-degrading enzymes in the nasal mucosa*†

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SUMMARY

Neutral endopeptidase (NEP, EC 3.4.24.11), angiotensin-converting enzyme (ACE, EC 3.4.15.1) and carboxypeptidase N (CPN, EC 3.4.17.3) are potentially important enzymes which regulate the degradation of neuropeptides, such as bradykinin (BK) and substance P (SP), in the respiratory mucosa. Some neuropeptides are also degraded by these enzymes in vitro and in vivo. We investigated the localization of these enzymes in the human nasal mucosa by an indirect immunohistochemical technique (immunogold silver staining). NEP-immunoreactive areas were present in the epithelium, the serous cells of the submucosal glands, and the endothelial cells of small vessels. The epithelium and the serous cells were the predominant areas of NEP immunoreactivity in the nasal mucosa. ACE-immunoreactive areas were seen in the outer layer of the epithelium, the endothelial cells of vessels, and widely distributed in the superficial lamina propria. The endothelial cells of the vessels showed maximum positive intensity to ACE. CPN-immunoreactive areas were observed in the epithelium, the endothelium of vessels and the superficial lamina propria, except for the gland cells. The superficial lamina propria exhibited maximum immunoreactivity for CPN. We observed that the enzymes were widely distributed in the nasal mucosa. The epithelium, including the epithelial cells and glycocalyx, contains all three enzymes. These enzymes play an important role in the mucosal immunity of the respiratory mucosa by degrading active neuropeptides. These results show that NEP secretion is regulated by a glandular, cholinergic control. On the other hand, ACE and CPN secretion are regulated by vascular permeability.

Key words: nasal mucosa, neuropeptides, neuropeptide-degrading enzymes

INTRODUCTION

Neutral endopeptidase (NEP; enkephalinase, EC 3.4.24.11), angiotensin-converting enzyme (ACE; EC 3.4.15.1) and carboxypeptidase N (CPN; EC 3.4.17.3) have been demonstated in a number of tissues and body fluids (Kerr and Kenny, 1974; Oshima et al., 1975; Danielsen et al., 1980; Hara et al., 1982; Llorens et al., 1982; Erdos et al., 1985; Taira et al., 1985; Spillartini et al., 1989; Ito et al., 1989; Hamai et al., 1990). These enzymes are potentially important enzymes in the control of neuropeptide activity in tissues and body fluids. NEP is known to be capable of metabolizing substance P (SP; Skidgel et al., 1984), neurokinin A and B (NKA, NKB; Shore and Drazen, 1982), vasoactive intestinal peptide (VIP; Goetzl et al., 1989), gastrin-releasing peptide (GRP; Erdos and Skidgel, 1989), bradykinin (BK; Gafford et al., 1983), endothelin (ET; Vijayaraghavan et al., 1990), and other peptides. ACE is also known to metabolize BK (Erdos et al., 1985), SP (Skidgel et al., 1984), NKA (Skidgel et al., 1984), and other peptides. CPN is also known as kininase I and metabolizes BK (Erdos and Solane, 1962). Recently, these neuropeptides have been considered to be important factors in the manifestation of airway hyperreactivity and neurogenic inflammation (Piper and Vane, 1978; Steranka et al., 1988; Drazen et al., 1989). NEP inhalation, for instance, is known to prevent SP-induced cough *in vivo* in guinea pigs (Kohrogi et al., 1989). Therefore, it has been suggested that NEP, ACE and CPN can control these airway reactions. The existence of these enzymes in the upper airway mucosa has already been confirmed (Fitz et al., 1972; Johnson et al., 1985; Catanzaro et al., 1991). The purpose of this study was to determine the existence as well as the exact location of these enzymes.

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MATERIAL AND METHODS

Human nasal mucosa was obtained from patients undergoing surgery for nasal obstruction. We did not know the patients' disease, infection, allergy, or hypertrophy. Nine specimen were obtained and divided into three groups each for immunohistochemical detection of the three enzymes.

Tissues were freshly fixed in 4% formaldehyde at 4°C for 4 h. After fixation, the samples were stored in PBS at 4°C before paraffin embedding. The immunogold silver-enhancing technique was used. Six-micron sections were cleared in xylene and a graded series of ethanol, washed in water, PBS, and then pretreated with 5% non-immune goat serum and 0.5% bovine serum albumin in PBS.

Sections were incubated in the first antibody at 4°C for 20 h in a humid chamber. The first antibody for NEP was MEK-5, which was produced in rats, and obtained from Genentech (California, USA). The monoclonal antibody for ACE was also produced in rats and was kindly provided by Dr. Erdos (College of Medicine, University of Illinois, USA). A polyclonal antibody for CPN was obtained from goats and was kindly provided by Dr. Okada (Nagoya City University, Japan). Following incubation with the first antibody, the slides were washed in PBS, and then incubated in colloidal gold-labeled goat-anti-rabbit IgG or anti-goat IgG at room temperature for 60 min. After washing with water and PBS, silver-enhancing solution was added. The development was monitored by light microscopy. The slides were washed in water and counter-stained with nuclear fast red.

RESULTS

NEP immunohistochemistry

The mucosa of the human nasal turbinate is lined by a pseudostratified columnar epithelium with an underlying superficial and deep lamina propria. In the superficial lamina propria, beneath the basement membrane, are fenestrated capillaries and tubulo-acinar, seromucous submucosal glands. The large venous sinusoids are located deeper in the mucosa. The epithelium, the serous cells of the submucosal glands, the endothelium of small veins, and myo-epithelial layer of arterioles were stained positively with MEK-5 (Figures 1A, C). Addition of recombinant NEP completely inhibited staining of these structures. No positively stained goblet cells were noted. In all glands, the serous cells were stained positive (Figures 1A, C). In contrast, the mucous cells were negative (Figures 1B, D).

ACE immunohistochemistry

ACE-positive staining was detected between the epithelial cells and in the glycocalyx, but not in the epithelial cells, and a very intense staining in the interstitium just beneath the basement membrane as well as in the endothelium of veins and arteries was noted (Figures 2A, C). The endothelial cells of the vessels showed maximum positive staining for ACE.

Addition of human serum ACE completely prevented staining of all structures (Figures 2B, D). In the glands, the inner lumen of ducts was occasionally positive; ACE-positive gland cells were not noted.

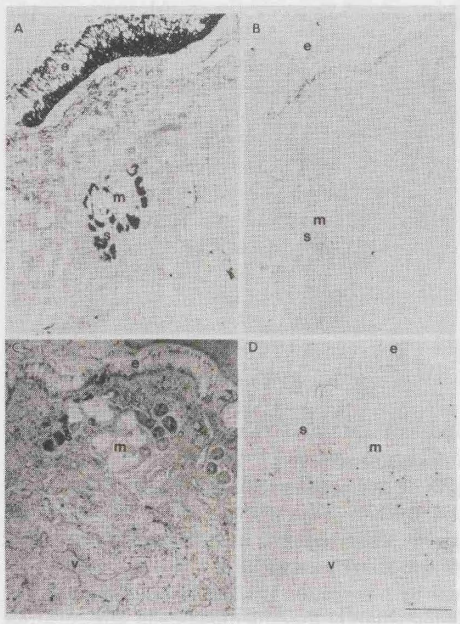

Figure 1. NEP immunohistochemistry.
(A) NEP immunoreactivity was seen in the epithelium (e) and serous cells (s) of the gland. Mucous cells (m) were not stained positive.
(B) NEP immunoreactivity was absent after pre-incubation with first antibody and recombinant NEP.
(C) Positive areas were also seen in the epithelium (e) and the serous cells (s) of the glands.
(D) NEP immunoreactivity was abolished after pre-incubation with first antibody and recombinant NEP.

Figure 2. ACE immunohistochemistry.
(A) ACE immunoreactivity was seen in the glycocalyx, just beneath the basement membrane (b), occasional gland (g) duct, and endothelium of veins (v).
(B) ACE immunoreactivity was completely lost after pre-incubation with first antibody and human serum ACE.
(C) ACE immunoreactivity was seen in the glycocalyx, just beneath the basement membrane (b), interstitium, and endothelium of veins (v; arrow heads).
(D) ACE immunoreactivity was completely absent after pre-incubation with first antibody and human serum ACE.

CPN immunohistochemistry

The same distribution of CPN was seen in each tissue. Epithelial cells, superficial lamina propria interstitium, the endothelium of veins and arteries, and the gland ducts just beneath the epithelium stained positively with the CPN antibody (Figures 3A, C). The superficial lamina propria exhibited

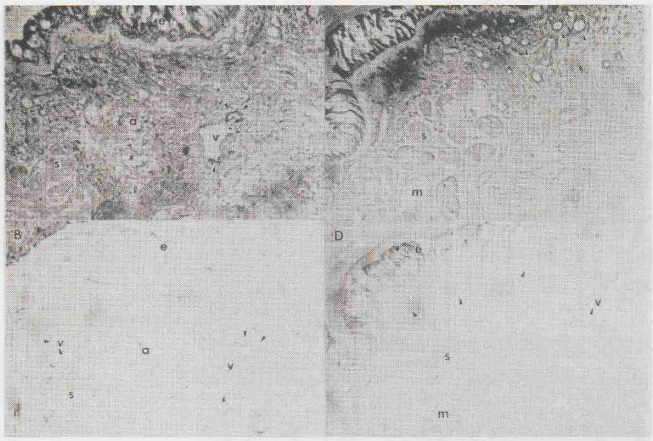

Figure 3. CPN immunohistochemistry.
(A) CPN immunoreactivity was seen in the epithelium (e), superficial lamina propria, and endothelium of the vein (v).
(B) CPN immunoreactivity was completely abolished after pre-incubation with first antibody and human serum CPN.
(C) CPN immunoreactivity was seen in the epithelium (e), superficial lamina propria and endothelium of the superficial small vein (v; arrow heads).
(D) CPN immunoreactivity was completely lost after pre-incubation with first antibody and human serum CPN.

maximum immunoreactivity for CPN. Addition of human serum CPN completely inhibited staining of all these structures (Figures 3B, D). In the epithelium, basal cells stained relatively weak. Gland cells were not stained at all.

DISCUSSION

SP induces cough in guinea pigs *in vivo* (Kohrogi et al., 1989), and endogenous tachykinins, including SP, are released from C-fibers in the epithelium, whereas BK is present in the glycocalyx as circulating peptide. Three enzymes, NEP, ACE and CPN, degrade active peptides such as SP and BK, which cause hyperreactivity and neurogenic inflammation in the airways (Piper and Vane, 1978; Steranka et al., 1988; Drazen et al., 1989). Surprisingly, all these enzymes are located in in the glycocalyx, the epithelial cells, and the basal cells. In the epithelium, NEP is located in the epithelial cells and the basal cells, ACE is present only in the glycocalyx as circulating enzyme, and CPN is located in the epithelial cells. These findings indicate that the epithelium is an important site in the manifestation of airway hyperreactivity and airway neurogenic inflammation, which is caused by some active peptides.

In the respiratory tract, the secretagogue control is divided into two regulatory fashions, glandular and vascular control. In the lamina propria, only NEP is present in the glands, whereas ACE and CPN are located mainly in the vessels (Table 1). This indicates that NEP is synthesized in the nasal mucosa itself and exists in the glands, whereas ACE and CPN are circulating enzymes and are transported to the nasal mucosa by blood vessels. Actually, NEP-mRNA has been identified in the nasal mucosa, especially in the epithelium and the serous cells of the submucosal glands, by Baraniuk et al. (1991a). Therefore, we think that NEP has an important role in the control of glandular secretion rather than vascular permeability. On the other hand, ACE and CPN have been called kininases because metabolism of BK is controlled by ACE and CPN. BK is an important inflammatory factor in inducing vascular permeability in the airways (Baraniuk et al., 1991b) and other tissues (Regoli and Barabe, 1980). Hence, it is suggested that ACE and CPN have important roles in the control of vascular permeability, whereas NEP plays an important role in the control of glandular secretion.

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Table 1. Distribution of neuropeptide-degrading enzymes in the nasal mucosa.

	epithelium				lamina propria								
					gland			vessel				interstitum	
	glyco-calyx	collum-nar cell	basal cell	goblet cell	serous	mucous	duct	arteriole	middle arteriole	middle venule	sinusoid	upper	lower
neutral endopeptidase (NEP)	-	+	+++	-	++	-	-	+	-	+	-	-	-
angiotensin-converting enzyme (ACE)	++	-	-	-	-	-	+	++	++	++	++	+++	-
carboxy-peptidase N (CPN)	++	++	++	-	-	-	-	++	-	++	-	++	-

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