

Human nasal mucosal carboxypeptidase: Activity, location, and release

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Background: Carboxypeptidases (CPs), such as carboxypeptidase N (CPN) (kininase I, E.C.3.4.17.3), may regulate peptide-mediated vasodilation and vascular permeability in respiratory mucosa by degrading proinflammatory peptides such as bradykinin, anaphylatoxins, and neuropeptides during allergic and nonallergic inflammation. The sources of CP activity in human nasal secretions were investigated.

Methods: Well-characterized human nasal provocation and secretion analysis methods were used. Potential sources of CPN in human nasal mucosa were identified by immunohistochemistry. CP activity was defined as DL-2-mercaptopmethyl-3-guanidinoethylthiopropionic acid inhibitable Bz-Gly-Lys degradation. CP activity was measured in nasal mucosal homogenates and nasal lavage fluids induced by methacholine, histamine, and allergen nasal provocation.

Results: CPN-immunoreactive material was localized to the glycocalyx of the epithelium, some vessels, and gland ducts near the epithelial basement membrane but not to submucosal gland cells. CP activity in human nasal lavage fluid after saline nasal provocation was 0.10 ± 0.04 U/L. Histamine provoked secretion of significantly more CP activity (3.84 ± 0.99 U/L; $p < 0.01$ vs saline). Methacholine did not significantly increase secretion (0.54 ± 0.22 U/L). After nasal allergen challenge, CP activity was at a maximum between 11 and 20 minutes, and CP activity correlated with IgG concentration ($r = 0.91$, $p < 0.01$), a marker for proteins of plasma origin, suggesting that CP activity originated in plasma.

Conclusions: These data suggest that plasma is the predominant source of CP activity secreted from human nasal mucosa and that plasma extravasation and interstitial fluid exudation across the epithelium are the primary processes regulating its appearance in nasal secretions. (*J ALLERGY CLIN IMMUNOL* 1995;96:924-31.)

Key words: Carboxypeptidase, immunohistochemistry, nasal mucosa, nasal secretion, vascular permeability

Basic carboxypeptidases (CPs), such as carboxypeptidase N (CPN), carboxypeptidase B (CPB), carboxypeptidase H (CPH), and carboxypeptidase M

(CPM), cleave the C-terminal arginine or lysine from substrates. CPN (kininase I, peptidyl-L-arginine hydrolase, EC 3.4.17.3.) cleaves bradykinin at the C-terminal arginine in plasma² and is also active on anaphylatoxins³ and fibrinopeptides.⁴ CPN is a soluble metalloenzyme in plasma and other biologic fluids.^{2, 5-7} CPB is a soluble pancreatic carboxypeptidase.⁸ CPH is located in secretory granules in the brain and the pituitary and adrenal glands.⁹ CPM is a membrane-bound CP that may be present in the placenta and lungs.^{10, 11}

CP enzymes may act in concert with angiotensin converting enzyme (ACE)¹² and neutral endopeptidase (NEP)¹³ to regulate the activities of bradykinin and other peptides in the respiratory tract.¹⁴ Bradykinin is generated in allergic and viral respiratory inflammation¹⁴⁻¹⁷ and stimulates vascular permeability.^{14-16, 18} To determine the potential role(s) of CP activity as an inflammation-modulating activity, the distribution, activity, and sources

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Abbreviations used

ACE:	Angiotensin converting enzyme
BSA:	Bovine serum albumin
CPB:	Carboxypeptidase B
CPH:	Carboxypeptidase H
CPM:	Carboxypeptidase M
CPN:	Carboxypeptidase N
MC:	Methacholine
MERGETPA:	DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid
NEP:	Neutral endopeptidase
NLF:	Nasal lavage fluid
SP:	Substance P
TP:	Total protein

of CP activity were determined in human nasal mucosa.

METHODS

Tissue handling

Human nasal mucosa samples were obtained at the time of surgery from patients with nasal obstruction.¹⁶ No patient had experienced a recent respiratory tract infection. At the time of surgery, 2% tetracaine HCl and 0.25% phenylephrine HCl were applied topically to the nasal surface. The turbinates were injected with 2 to 4 ml of 1% lidocaine with 1:100,000 epinephrine. The nasal mucosa was dissected from the conchal bone.

Human CPN standard

Plasma CPN was purified from citrated human plasma, as previously described.¹⁹

Anti-human CPN antibody

Polyclonal antibodies to purified human plasma CPN were raised in rabbits and were partially purified by ammonium sulfate precipitation at 45% saturation, as described.²⁰

CPN immunohistochemistry^{12, 13}

Fresh tissues ($n = 3$) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) at 4° C for 4 hours. After fixation, samples were stored in PBS at 4° C before being embedded in paraffin. Six-micron-thick paraffin sections were cleared in xylene and graded alcohols and then washed in water and PBS, followed by 5% nonimmune goat serum and 0.5% bovine serum albumin (BSA) in PBS. Sections were incubated with anti-human CPN antiserum (1:500 diluted by 1% BSA in PBS, Calbiochem, La Jolla, Calif.). Incubations were performed at 4° C for 20 hours in a humidified chamber. The slides were washed in PBS and then incubated with colloidal gold-labeled goat anti-rabbit IgG (Amersham, Arlington Hts, Ill.; 1:40 dilution

in 1% BSA in PBS) at room temperature for 60 minutes. After washing three times in PBS for 5 minutes each and three times in distilled water for 3 minutes each at room temperature, silver enhancing solution (Biocell; Ted Pella, Redding, Calif.) was added. The development of the stained slides was monitored by light microscopy. The slides were washed in distilled water and were counterstained with nuclear fast red.

Nasal mucosal extract preparation

Fresh nasal mucosa samples for CPN enzymatic assays²¹ were frozen in 2-methyl-butane on dry ice for 20 seconds and then stored at -70° C. Frozen turbinate tissue from single individuals was weighed (average wet weight of 478 ± 39 mg with a range of 168 to 848 mg, $n = 21$). The tissue was finely minced with scissors and suspended in 10 volumes (wet tissue wt/vol) of 0.32 mol/L sucrose in pH 7.4, 50 mmol/L Tris HCl, and homogenized with a polytron homogenizer (STD Tissue-izer; Tekmar, Cincinnati, Ohio) at 4° C at a setting of 6 for 30 seconds.^{12, 13} The homogenates were centrifuged at 1000 g for 10 minutes at 4° C, and the supernatants were collected. The pellets were suspended in the same buffer and recentrifuged at 1000 g for 10 minutes. The pellets, which contained connective tissue fragments, were discarded. Both supernatants were combined and designated as "S1." Fifteen S1 supernatants were centrifuged at 25,000 g for 60 minutes at 4° C to yield the "S2" supernatant and "P2" pellet.

CP activity

CP activity was defined as DL-2-mercaptomethyl-3-guanidinoethylthiopropionic acid (MERGETPA, Calbiochem) inhibitable Bz-Gly-Lys degrading activity.²¹ Samples (50 μ l) were preincubated for 2 hours at 4° C in the presence of CoCl₂ (1 mmol/L) with 50 μ l of pH 7.5, 0.25 mol Tris HCl and 80 μ l of water in glass tubes. CoCl₂ was added to maximize CP activity. After preincubation, 50 μ l of 5 mmol/L Bz-Gly-Lys (Peninsula Laboratory, Belmont, Calif.) enzyme substrate was added and incubated for 2 hours at 37° C. Reactions were terminated by adding 250 μ l of HCl and reducing the temperature to 4° C. Ethyl acetate (1500 μ l) was added to each tube, vortexed for 15 seconds, and then centrifuged at 500 g for 5 minutes. From the top organic layer, 1 ml of solution was transferred to another glass tube and the solution evaporated. Water was added to each tube and mixed well, and the absorbance at 228 nm was measured against a water reference tube. Sample blanks were prepared by adding 100 nmol/L (final concentration) MERGETPA to the sample and buffer solutions before the substrate. The CPN catalytic activities (units per liter) of individual samples (b) were calculated as follows:

$$b = (A_{228}/t \times 1000)/(9.8 \times 0.91 \times 0.67 \times V) = 3360$$

$$\times A_{228}/t \text{ (U/L)}$$

where the absorption coefficient of Bz-Gly was $\epsilon_{228} = 0.98 \text{ L} \times \text{mmol}^{-1} \times \text{mm}^{-1}$; the fraction of Bz-Gly extracted in ethyl acetate was 0.91; the fraction of ethyl acetate transferred was 0.67; V was the sample volume (0.05 ml); A_{228} was the changes in absorbance at 228 nm; and t was the duration of the incubation with substrate (120 minutes). Total sample activity minus sample activity with MERGETPA present equalled the MERGETPA-inhibitable, or CP, activity.

MERGETPA inhibition of Bz-Gly-Lys substrate degradation defined CP activity in plasma. The degradation was linearly related to time and was dose-dependent between 10 nmol/L and 10 mmol/L. The inhibitory concentration of 50% for MERGETPA was 100 nmol/L.

Nasal challenges in vivo

A total of 18 adult subjects (nine men, nine women) between the ages of 24 and 53 years were studied. No subject was examined within 3 weeks after recovering from an upper respiratory tract infection, and no subject was experiencing nasal symptoms at the time of study. No medications were taken within 48 hours of the nasal challenge. Atopic subjects ($n = 14$) were defined as having perennial ($n = 3$) or seasonal ($n = 11$) symptoms and had positive responses to relevant allergens at the time of nasal challenge, as determined by skin prick tests with a battery of aeroallergens. A positive epicutaneous skin test response was defined as a wheal 4 mm greater than controls. Seasonal atopic subjects were studied out of their allergy season. Nonatopic subjects ($n = 4$) had no allergic symptoms and had skin test negative responses.

Nasal challenges were performed as previously reported.²²⁻²⁶ Nasal lavage fluid (NLF) was collected through 8F rubber catheters. Subjects' nasal cavities were washed with four 4 ml saline prewashes at 10-minute intervals. The nasal challenge solutions were then applied to one or both sides of the nasal cavity from a nebulizer bottle. Each 10-minute challenge period was followed by a 4 ml saline lavage. In each study, baseline secretions were collected 10 minutes after saline provocation.

In the first protocol, 10 subjects were challenged sequentially with saline and then 25 mg of methacholine.²² In the second protocol, saline challenge was followed by a unilateral challenge with 1 mg of histamine.²³ In the third protocol, eight atopic subjects received an allergen challenge as described.^{24, 25} After relevant allergen (75 protein nitrogen units) was instilled, NLF was collected every 10 minutes for 120 minutes.

NLF CP activity assay

Measurement of CP activity (above) was adapted to the dilute NLF samples. Sample and buffer volumes were adjusted to 100 μl of sample, 50 μl of Tris buffer, 20 μl of CoCl_2 , and 30 μl of water. Samples were incubated for 3 hours at 37°C.²¹

Total protein (TP) assay

Protein in nasal extract samples and NLF samples was measured by the Lowry method^{23, 27} with BSA as the standard.

Lactoferrin assay

Lactoferrin, a measure of submucosal gland serous cell exocytosis, was measured by a modified noncompetitive ELISA, as previously reported.²⁶ The assay range was between 1 and 200 ng/ml.

Albumin assay

Albumin, a measure of vascular permeability of plasma proteins and subsequent exudation across the epithelium, was measured by a direct, competitive ELISA, as previously reported.²³ The assay range was between 1 and 100 $\mu\text{g}/\text{ml}$.

IgG assay

IgG, a measure of vascular permeability of plasma proteins and subsequent exudation across the epithelium, was measured by a noncompetitive ELISA, as previously reported.²⁵ The assay range was between 1 and 100 ng/ml. IgA and albumin are highly correlated markers of plasma exudation.

Statistics

Data were expressed as the mean $\pm$ SEM. The paired Student's t test was used for statistical comparisons.

RESULTS

CPN immunohistochemistry

Specific CPN-immunoreactive materials were detected in the glycocalyx, apical aspects of epithelial cells, and, to lesser extents, in the interstitial spaces of the superficial lamina propria, proximal gland ducts just beneath the epithelium, and endothelial cells of veins and arteries (Fig. 1). Neither goblet cell vacuoles nor submucosal gland serous and mucous cells contained CPN-immunoreactive material. In all experiments, addition of human serum CPN (final concentration of 0.3 U/L) reduced specific staining, indicating the specificity of the CPN antiserum.

CP activity in nasal mucosal fractions

CP activity was detected in S2 (3.96 ± 0.19 U/gm protein) and P2 (2.61 ± 0.21 U/gm protein) fractions.

CP activity in histamine-induced NLF

Baseline CP activity after saline challenge was 0.10 ± 0.04 U/L. After histamine challenge, CP activity in NLF from the challenged, ipsilateral side was significantly increased to 3.84 ± 0.99 U/L.

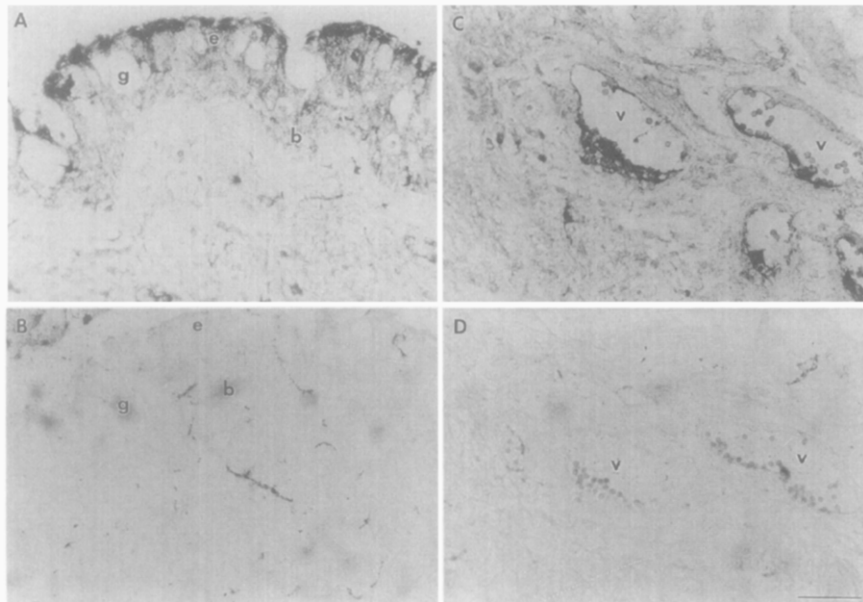


FIG. 1. CPN-immunoreactive material in the human nasal mucosa. **A**, CPN-immunoreactive material (intensely stained black material) is present in the glycocalyx and apical aspects of epithelial cells (*e*). Goblet cell vacuoles (*g*) and basal cell cytoplasm (*b*) did not stain for CPN. **B**, Adsorption control. CPN antiserum adsorbed with excess purified CPN did not identify immunoreactive structures in adjacent tissue sections. **C**, CPN immunoreactivity is noted in the endothelium and inner lumen of the veins (*v*). **D**, Adsorption control. Nuclear fast red counterstain. Bar represents 50 μ m.

($p < 0.01$). CP activity was not stimulated on the contralateral side (0.03 ± 0.02 U/L).

To determine the primary source of CP activity in NLF, TP, lactoferrin, and albumin were also measured (Fig. 2). TP is a measure of total protein released from both glandular and vascular sources. NLF CP activity was significantly correlated with TP ($r = 0.78$, $p < 0.01$) and albumin, a measure of vascular permeability²³ ($r = 0.64$, $p < 0.01$). Secretion of lactoferrin, a measure of serous cell exocytosis, glandular secretion, and cholinergic glandular function,^{22,26} was not correlated with CP activity after histamine challenge. Histamine-induced plasma extravasation^{23,26} appeared to be the predominant source of CP activity.

Methacholine-induced CP activity in NLF

Methacholine challenge modestly increased CP activity in NLF (0.54 ± 0.22 U/L), but the concentration was not significantly different from that in saline-induced secretions (Table I).

Allergen challenge-induced CP activity in NLF

Allergen provocation induced nasal mucosal CP activity release (Fig. 3). CP activity reached maximal levels in fractions collected between 11 and 20 minutes after provocation (6.95 ± 2.11 U/L). Ac-

tivity returned to the baseline rate about 2 hours after provocation.

The concentrations of TP, lactoferrin, IgG, and CP activity were measured in each allergen-induced fraction. All three markers were significantly correlated with CP activity, but IgG was most highly correlated ($r = 0.91$, $p < 0.01$) (Fig. 4), suggesting a common plasma origin for lavage fluid IgG and CP activity.

DISCUSSION

The basic CPs, CPN, CPM, CPH, and CPB, each have different enzymatic and physiologic properties¹ but are sensitive to similar activity inhibitors, including chelators,⁶ Cd^{2+} ,⁵ MERGETPA,²² and guanidinoethylmercaptosuccinic acid.²⁸

CPN (EC 3.4.17.3.; kinase I) was identified by Erdös and Solane² as a CPB-like enzyme in human plasma that inactivated bradykinin. Later it was shown to hydrolyze the COOH-terminal basic amino acid from the complement anaphylatoxins, other peptides, and proteins and earned the titles "anaphylatoxin inactivator,"^{3,4} "protaminase,"²⁹ "creatinine kinase conversion factor,"^{30,31} and "carboxypeptidase K."³¹ CPB is present only in the digestive system,⁹ whereas CPH is only in secretory granules of neurohormonal glands.^{1,9} CPM is a

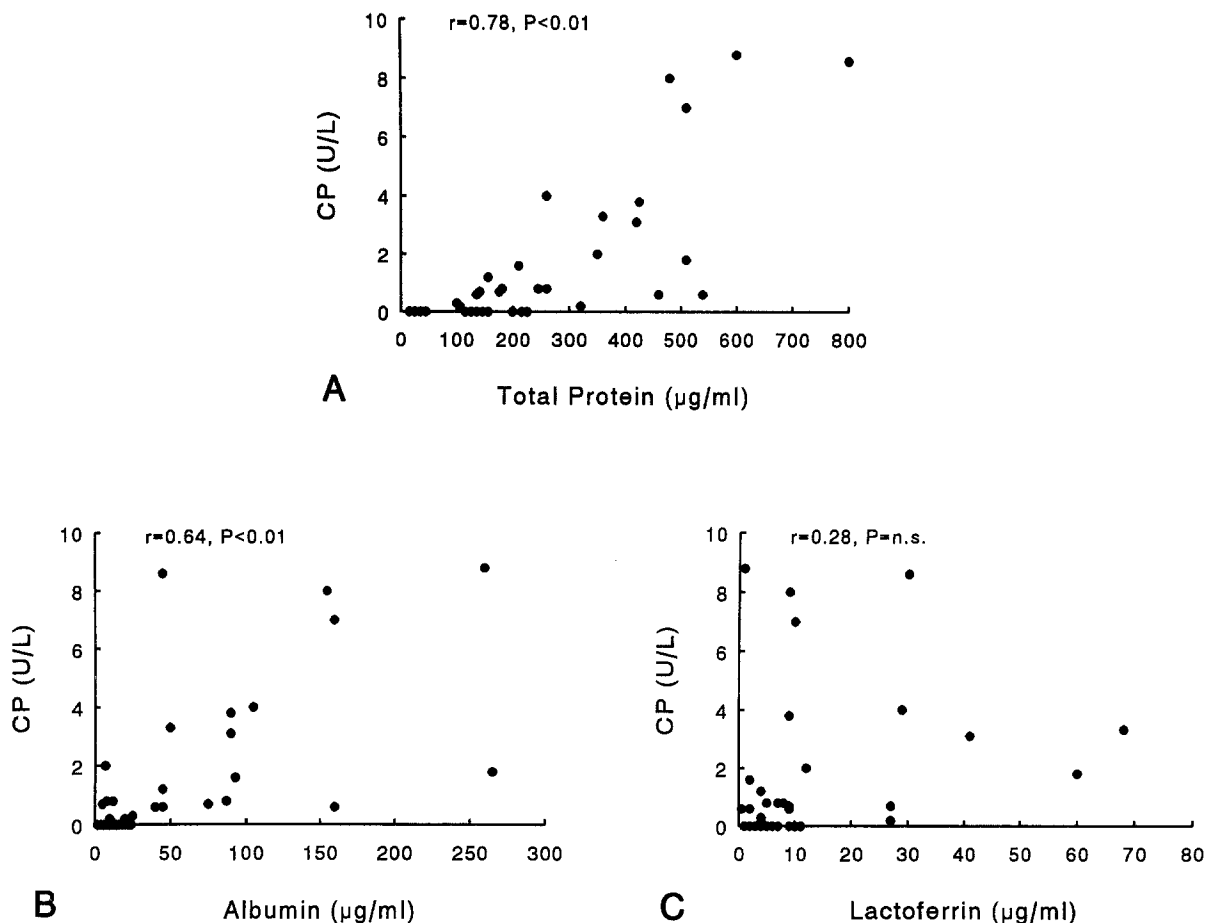


FIG. 2. Histamine-induced nasal secretions. Data and correlations are shown for secretion of CP activity and total protein (A), albumin (B), and lactoferrin (C) concentrations. Data were compared by linear regression. The r values represent the coefficients of variation.

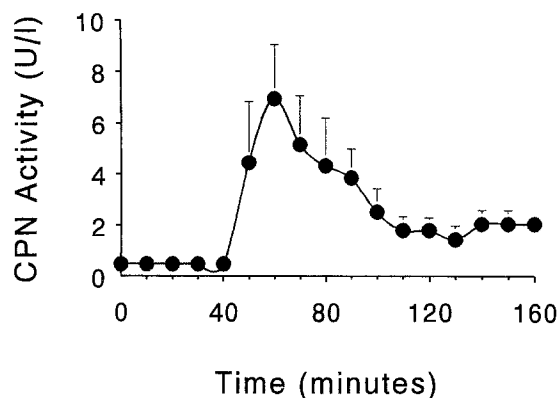


FIG. 3. CP activity in NLF after antigen challenge ($n = 8$). MERGETPA-inhibitable CPN activity is expressed as units per liter. Atopic subjects received serial saline lavages, then at 40 minutes were challenged topically with allergen. Lavages were collected at 10-minute intervals. Bars represent the standard error of the mean.

membrane-bound enzyme that is present in alveolar cells and placental microvilli.^{10, 11, 32} CPN may be the major contributor to MERGETPA-inhibitable CP activity in soluble nasal secretions, whereas CPM may contribute to membrane-bound activity.

CPN was specifically localized by immunohistochemistry to the glycocalyx and apical aspects of epithelial cells, the superficial lamina propria, gland ducts just beneath epithelium, and endothelium of vessels. The glycocalyx staining is particularly similar to that of albumin²³ and IgG.²⁵ Endothelial CPN immunoreactivity might be due to adsorbed plasma CPN. CPN in extracellular sites is probably the result of transudation through superficial, permeable vessels into the interstitium. The absence of epithelial cell cytoplasmic staining is consistent with the report by Proud et al.,³³ who

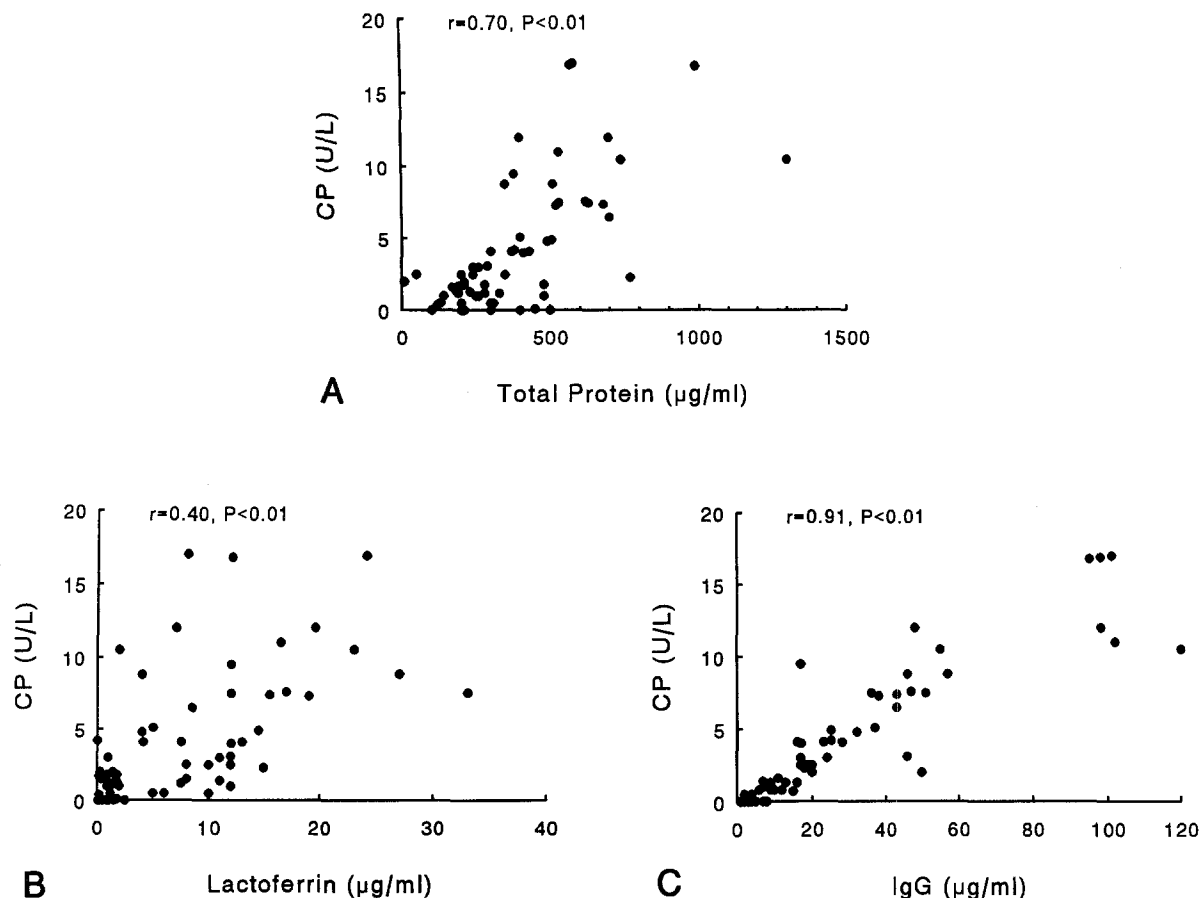


FIG. 4. Allergen-induced nasal secretions. Data and correlations are shown for secretion of CP activity and total protein (A), lactoferrin (B), and IgG (C) concentrations. Data were compared by linear regression. The r values represent the coefficients of variation.

found no CPN activity in cultured respiratory epithelial cells.

Nasal provocations with methacholine,²² histamine,²³ and allergen²⁴ were used to determine the functional routes of CP activity secretion. Previous studies have indicated that methacholine induces glandular secretion,²² histamine induces vascular permeability and recruits parasympathetic glandular secretion,²³ and allergen induces both ipsilateral vascular and glandular secretion and contralateral parasympathetic reflex-mediated glandular secretion.²⁴ Specific markers identify the sources of secreted proteins. Lactoferrin and lysozyme are exocytosed from serous cells of submucosal glands and are markers of glandular secretion.²⁶ Albumin²³ and IgG²⁶ are released by plasma extravasation from vessels into the interstitial spaces, followed by exudation across the epithelium.

Histamine stimulated significant CP activity release on the ipsilateral side but none on the

contralateral side (reflex-mediated). This is consistent with histamine-induced albumin and IgG release and suggests that plasma extravasation is the source of CPN in vivo.²³ CP activity release was well correlated with albumin but not lactoferrin concentrations in histamine-induced lavage fluids, which also supports this contention. The absence of contralateral CP secretion suggests the absence of a glandular origin.^{22, 23}

Methacholine²² did not induce significant secretion of CP activity. This is consistent with the immunohistochemical absence of a glandular source of CP and the pattern of histamine-induced secretions.

Allergen challenge also supported a plasma origin because CP activity was highly correlated with IgG secretion.^{24, 25} IgG and albumin secretion are highly correlated markers of plasma extravasation.^{22, 23} Proud et al.^{15, 17} have previously demonstrated CPN exudation after allergen challenge in the human nose with much the same profile of

release. Differences in CP activities are due to differences in substrate (Bz-Gly-Lys vs bradykinin), assay sensitivity, methods of lavage collection, collection times, and the inclusion of the time factor in the definition of CP units. CP activity paralleled that of the vascular proteins albumin and IgG²⁴ and was released relatively earlier than the glandular products lactoferrin, lysozyme,²⁴ and NEP.¹³ The prolonged duration of CP exudation demonstrates the relatively long duration of the release of this component of the "immediate" phase that follows mast cell degranulation.

These data suggest that CP activity has a plasma origin³⁴ and enters the superficial lamina propria by vascular permeability from superficial permeable vessels. Like albumin and IgG, CPN-immunoreactive material may cross the epithelium as a component of the extracellular fluid to enter the nasal epithelial lining fluid. This process was promoted by histamine. Glandular secretion does not appear to be a significant route for CP activity secretion. CPN may degrade bradykinin, anaphylatoxins, or other vasoactive peptides to reduce their effects, and so limit the duration of peptide-induced vascular permeability events. This permeability-induced flux of plasma CPN may be an important mechanism in regulation of peptide actions in vivo.

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