Neutral endopeptidase 24.11 in human neutrophils: Cleavage of chemotactic peptide

(enkephalinase/fMet-Leu-Phe/bradykinin/immunocytochemistry/leukocyte)

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ABSTRACT Membrane metallo-endopeptidase (NEP; neutral endopeptidase, kidney-brush-border neutral proteinase, enkephalinase, EC 3.4.24.11) cleaves peptides at the amino side of hydrophobic amino acids. While the enzyme is known to be in organs such as kidney and brain, we found it in human neutrophils. These cells cleaved the NEP substrate glutaryl (Glut)-Ala-Ala-Phe-(4-methoxynaphthylamine) (Glut-Ala-Ala-Phe-MNA) at a rate of 9.5 nmol·hr^{-1} per 10^6 cells, and phosphoramidon (1 μM) inhibited the hydrolysis by 90%. Intact neutrophils from donors who smoked had NEP activities about twice that of nonsmokers. Subcellular fractionation and sucrose density gradient centrifugation of lysed neutrophils showed that most of the NEP activity was membrane bound. A washed membrane fraction from human neutrophils rapidly cleaved 0.5 mM Glut-Ala-Ala-Phe-MNA (96 nmol·min^{-1}·mg^{-1}) and the hydrolysis was inhibited by phosphoramidon and by specific antiserum to human renal NEP. The washed membrane fraction also rapidly cleaved 0.1 mM bradykinin (34 nmol·min^{-1}mg^{-1}) and 0.1 mM fMet-Leu-Phe (49 nmol·min^{-1}·mg^{-1}). The membrane-bound enzyme cleaved the peptide substrates at the same site as the homogeneous human renal NEP, and phosphoramidon and thiorphan inhibited the hydrolysis. Kinetic studies with pure human renal NEP showed that the chemotactic peptide fMet-Leu-Phe was one of the best biologically active substrates (K_m, 59 × 10^{-6} M; k_{cat}, 3654 min^{-1}). Immunocytochemistry at the light microscopic level revealed a high concentration of NEP on the cell membrane of neutrophils. This was confirmed with electron microscopy using the immunogold technique on ultrathin cryosections. These studies indicate that NEP in neutrophils may have important functions in inflammation and chemotaxis.

The membrane metallo-endopeptidase (NEP; neutral endopeptidase, kidney-brush-border neutral proteinase, enkephalinase, EC 3.4.24.11), was first found in the brush border of rabbit kidney (1) and later in many other tissues (2–7). This enzyme is identical with the enkephalinase A of brain (2, 3). The richest source of NEP appears to be the kidney, and it has been purified to homogeneity from both human (4) and animal (1) kidneys. NEP cleaves peptides at the amino side of hydrophobic amino acids and thereby inactivates a variety of peptide hormones, including enkephalins (2, 3, 5), substance P, neurotensin (8), oxytocin (7), bradykinin, and angiotensins I and II (4).

The activity of NEP in blood is normally very low (9). We found, however, that serum from patients with adult respiratory distress syndrome (ARDS) contained high levels of the enzyme. The increase in serum NEP activity was particularly marked in patients with septic pneumonia (9). Because sequestration of polymorphonuclear leukocytes within the lungs is a common finding in the early stages of ARDS (10), we measured the activity of NEP in isolated human neutrophils. We found that these cells contained NEP, and the highest activity was associated with a membrane fraction from disrupted neutrophils. NEP was identified and localized on the cell membrane by immunohistochemistry. Both the purified human renal enzyme and that in neutrophil membranes cleaved the chemotactic tripeptide fMet-Leu-Phe. These studies with human leukocytes complement our earlier observation that NEP may be a marker for lung damage in ARDS and suggest a potentially important function for the enzyme in inflammation.

MATERIALS AND METHODS

The substrates glutarylalanylalanylphenylalanyl (4-methoxynaphthylamine) (Glut-Ala-Ala-Phe-MNA), and methoxysuccinylalanylalanylprolylvalyl-(7-amino 4-methyl coumarin) (MeO-Suc-Ala-Ala-Pro-Val-AMC), and the elastase inhibitor, methoxysuccinylalanylalanylprolylvalyl-chloromethyl ketone (CK-20), were purchased from Enzyme Systems Products (Livermore, CA). N-formylmethionylleucylphenylalanine (fMet-Leu-Phe) was obtained from Sigma and Peptides International (Louisville, KY). N-Benzyloxycarbonyl-L-prolyl-L-[³H]alanine (N-Cbz-Pro-[³H]Ala) was prepared as described (11). Bestatin and phosphoramidon were obtained from Peninsula Laboratories (Belmont, CA). Thiorphan was kindly provided by J. Berger (Schering Corp., Bloomfield, NJ), and captopril was donated by Z. Horovitz (Squibb, Princeton, NJ). Human NEP was purified to homogeneity from kidney as described (4).

Collection of Human Neutrophils. Neutrophils from heparinized donor blood were isolated by centrifugation over histopaque cushions (12). Donors were segregated into two groups: those who abstained from smoking and those who smoked more than one pack of cigarettes a day. The cell pellet contained 98% neutrophils with >98% viability. The cells were washed once in phosphate-buffered saline (PBS; 0.01 M sodium phosphate, pH 7.2/0.15 M NaCl) and were recentrifuged at 200 × g. This procedure yielded ≈2 × 10^7 cells from 15 ml of whole blood. For measurement of NEP activity in intact neutrophils, aliquots of the resuspended cells were diluted 1:10 with PBS, counted in a hemacytometer, and assayed immediately.

Abbreviations: NEP, neutral metallo-endopeptidase; CK-20, methoxy-succinyl-Ala-Ala-Pro-Val-chloromethyl ketone; MNA, 4-methoxynaphthylamine; Mes, 2-(N-morpholino)ethanesulfonic acid; ARDS, adult respiratory distress syndrome; MeO-Suc-Ala-Ala-Pro-Val-AMC, methoxysuccinylalanylalanylprolylvalyl-(7-amino 4-methyl coumarin); Glut, glutaryl.
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Fractionation of Neutrophils. After hypotonic lysis of erythrocytes, isolated neutrophils ($\approx 10^8$) were lysed in 5 ml of 0.34 M sucrose in 0.1 M 2-(N-morpholino)ethanesulfonic acid (Mes) buffer (pH 6.5) containing heparin (336 units/ml). The crude lysate was centrifuged at 800 × g for 15 min to remove unbroken cells and nuclear material (P_1). The supernatant (S_1) was centrifuged at 25,000 × g for 20 min to obtain a crude granular pellet (P_2) and a supernatant (S_2) fraction. The S_2 fraction was then centrifuged at 100,000 × g for 1 hr in a Beckman model L5-65 ultracentrifuge. The resulting membrane pellet (P_3) was resuspended in the sucrose/Mes buffer and centrifuged at 25,000 × g (20 min) to sediment contaminating granular material with minimal loss of membrane fragments. The supernatant was then recentrifuged at 100,000 × g for 60 min. The washed membrane pellet (P_3 washed) was resuspended in 2 ml of sucrose/Mes, aliquoted in 200-μl fractions, and frozen at −70°C until assayed.

Separation of Granules on a Sucrose Gradient. Lysed neutrophils were also fractionated by centrifugation in a continuous sucrose gradient (13) ranging from 55% sucrose at the bottom to 30% at the top in 0.1 M Mes (pH 6.5) containing heparin (5 units/ml) to inhibit aggregation of the granules. The S_1 fraction obtained from lysed neutrophils was applied to the top of the gradient and the tubes were centrifuged at 95,000 × g for 3 hr at 4°C in a swinging bucket (SW-28) rotor. After centrifugation, there were four major bands in the tube. From bottom to top, these bands consisted of the primary-fast (I), primary-slow (II), and specific (III) granular fractions (14). A fourth band (IV), on the top in the initial 3–4 mm of the gradient, contained membrane particles or the microsomal fraction, and it was collected with the top layer containing soluble material. To determine whether the enzyme activity in band IV was due to membrane-bound or soluble material, it was diluted with 0.1 M Mes (pH 6.5) and centrifuged at 100,000 × g for 45 min. Some aggregated material was usually found below band I and was not characterized further.

Inhibition by Antisera. Antisera to purified NEP (4) were raised in New Zealand White female rabbits (6). Antisera and preimmunized (control) sera were heated for 30 min at 56°C to destroy endogenous NEP activity. The sera were incubated with a washed membrane fraction (P_3) of neutrophils at 4°C for 24 hr, and the residual NEP activity was measured. The specificity of the antisera was established previously (6).

Enzyme Assays. NEP activity was measured by a two-step spectrofluorometric assay using Glut-Ala-Ala-Phe-MNA as substrate in 0.1 M Mes buffer at pH 6.5 (7). Aliquots from each sample were also incubated in the presence of the NEP inhibitor phosphoramidon (1 μM), and the difference in activity between the inhibited and noninhibited samples was taken as NEP activity. The fluorescent product was measured in a spectrophotofluorometer with excitation at 340 nm and emission at 425 nm. With intact cells, the buffer was PBS and 25,000–50,000 cells were used per assay.

The elastase activity of fractionated cells was measured spectrofluorometrically by using the substrate, MeO-Suc-(Ala)$_2$-Pro-Val-AMC (15) in 0.1 M Mes, pH 7.4/0.5 M NaCl/0.1% Triton X-100. In control experiments, the specific elastase inhibitor CK-20 (5 μM) was used. The fluorescence was measured at 380 nm excitation and 460 nm emission.

Marker Enzymes. Acid and alkaline phosphatase activities were measured spectrophotometrically with p-nitrophenol phosphate substrate (16). Prolylcarboxypeptidase activity was assayed to distinguish the lysosomal fraction from the membrane-enriched fraction. Cell fractions were incubated with N-Cbz-Pro-[³H]Ala at 37°C for 6 hr, and the [³H]alanine released was extracted and counted in a scintillation counter (17). Elastase, used as a marker for azurophil granules (18, 19), was assayed as described above.

HPLC Assay of Bradykinin and fMet-Leu-Phe Hydrolysis. An aliquot (0.4–1.7 μg of protein) of a washed membrane fraction from neutrophils (P_3) was equilibrated for 15 min on ice with bestatin and CK-20 (10 μM each) in 0.03 M Mes, pH 6.5/0.1% Triton X-100. Additional samples were preincubated with other enzyme inhibitors to assess the specificity of the assay. Bradykinin or fMet-Leu-Phe (0.1 mM) was added to initiate the reaction, and the mixture (0.1 ml final vol) was incubated at 37°C for 20–60 min. Reactions were terminated with 0.1 ml of either 100% ethanol or 20% trifluoroacetic acid, and the mixture was filtered through 0.2-μm nitrocellulose filters.

HPLC analyses of the reaction products were carried out in a Waters automated gradient system with a μBondapak C$_{18}$ column (20). Peptide products were separated with a linear gradient of increasing concentrations of CH$_3$CN/0.05% trifluoroacetic acid (solvent B) in H$_2$O/0.05% trifluoroacetic acid (solvent A) and were detected by absorbance at 214 nm. Bradykinin products were separated with a gradient of 5–49% solvent B in 20 min and products of fMet-Leu-Phe hydrolysis were separated with a gradient of 20–60% solvent B in 15 min.

Kinetic studies of the hydrolysis of fMet-Leu-Phe by purified human kidney NEP were carried out by measuring reaction rates at 10 substrate concentrations ranging from 50 to 200 μM. For determination of kinetic constants, data were plotted according to Hanes (see ref. 21) and fitted to the best straight line by linear regression.

Immunocytochemistry. Neutrophils were isolated as described above, and the pellet of intact cells was resuspended in 0.5 ml of PBS. The cells were diluted with 1 ml of 3% formaldehyde in PBS and fixed overnight. The peroxidase-antiperoxidase method (22) was used to localize NEP at the light microscopic level following the procedures outlined in a kit from DAKO (Santa Barbara, CA). A green interference filter (570-nm transmission) was used in photomicrography to enhance the contrast.

Identification and localization of NEP at the electron microscopic level was achieved by an indirect immunogold technique. Neutrophils were embedded in gelatin and 0.5-mm slices were clamp frozen in liquid nitrogen (23). Ultrathin frozen sections were cut on a Sorvall MT5000 ultramicrotome with FS1000 cryokit attachment (24). Frozen sections were transferred to 300-mesh parlodion-coated nickel grids with a drop of 2.3 M sucrose (25). NEP was localized immunochemically within the cell slices as described (26–28). Briefly, the sections were incubated for 10 min in 5% bovine serum albumin to block nonspecific binding, and then the primary antibody (1:500) was applied, and the specimens were incubated at 4°C overnight. The second antibody was affinity-purified goat anti-rabbit IgG (Boehringer-Mannheim) conjugated to 20-nm colloidal gold particles and was prepared as described (29, 30). Incubation with the second antibody anti-rabbit IgG was done for 90 min at room temperature. The sections were then washed, stained with uranyl acetate, and embedded in methyl cellulose (25, 27).

RESULTS

NEP Activity of Intact Cells. There was significant NEP activity (9.5 ± 0.9 nmol·hr^{-1} per 10^6 cells ± SEM) in intact neutrophils of healthy normal volunteers of both sexes (n = 6). In a preliminary set of experiments, neutrophils were also separated from blood collected from persons who were cigarette smokers. NEP activity in neutrophils in these samples (n = 7) was almost double that of the nonsmokers (17.3 ± 0.9). Phosphoramidon (1 μM) inhibited NEP in the intact cells of both groups an average of 90%, while the specific elastase inhibitor CK-20 (3 μM) inhibited the cleavage of the NEP substrate only by 11%.

Subcellular Distribution of NEP and Marker Enzymes. To compare the subcellular distribution of NEP to that of marker enzymes, neutrophils were lysed with heparin and fractions were isolated by differential centrifugation. The fractional

distributions of total activity for NEP and marker enzymes are shown in Fig. 1. Most of the NEP activity sedimented with the 100,000 × g pellet (P$_3$), and there was none present in the high speed supernatant (S$_3$). While 39% of the NEP activity sedimented in the P$_2$ fraction, this was probably due to sedimentation of membrane particles at the g force used (25,000 × g for 20 min). When the fractionation was repeated using relative g force of 15,000 × g for 20 min, only 10% of the activity was in the pellet. In contrast, most of the elastase activity was recovered in the 25,000 × g pellet (P$_2$), which represents the lysosomal granular fraction. Eighty-one percent of the acid phosphatase was recovered in the P$_2$ fraction, and only 7% was in the P$_3$ fraction. While much of the prolylcarboxypeptidase activity was solubilized from the lysosomes during the lysing of the cells, 38% was recovered in the P$_2$ fraction, but <10% was recovered in the P$_3$ fraction (Fig. 1).

Density Gradient Centrifugation. When neutrophil lysates were separated in a sucrose gradient, four major protein bands were obtained after centrifugation consisting of (from bottom to top) primary-fast granules (I), primary-slow granules (II), specific granules (III), and a top band (IV) containing membrane fragments and soluble proteins (Fig. 2). The majority of the NEP activity (83%) was in the top layer (IV); the remainder was distributed between the two primary granular bands (I and II) and the fraction containing the specific granules (III). Upon recentrifugation of the top layer (IV), 94% of the NEP activity resedimented at 100,000 × g with the membrane particles. The majority of the alkaline phosphatase activity (a membrane marker enzyme) was recovered in the top layer (IV) and, when recentrifuged at 100,000 × g, 74% of the activity resedimented. Elastase activity (56%) was concentrated in the primary-slow granular band (II). This fraction corresponds to band II described by Gallin (31). Of the elastase activity still present in fraction IV (12% of the total), 74% resedimented at 100,000 × g.

NEP Activity in Washed P$_3$. Neutrophil membrane fraction (P$_3$) cleaved the NEP substrate, Glut-Ala-Ala-Phe-MNA, with a high specific activity (96 nmol·min^{-1}·mg^{-1}; Table 1). The activity was almost completely inhibited by 1 μM phosphoramidon, while 5 μM CK-20, the elastase inhibitor, had no effect (Table 1). The IC$_{50}$ of phosphoramidon for neutrophil NEP was 3.3 nM, similar to the IC$_{50}$ obtained with the purified enzyme (32). Antisera to human renal NEP at a dilution of 1:200 (vol/vol) inhibited half of the neutrophil NEP activity in the P$_3$ fraction while preimmunized sera had no effect (Table 1).
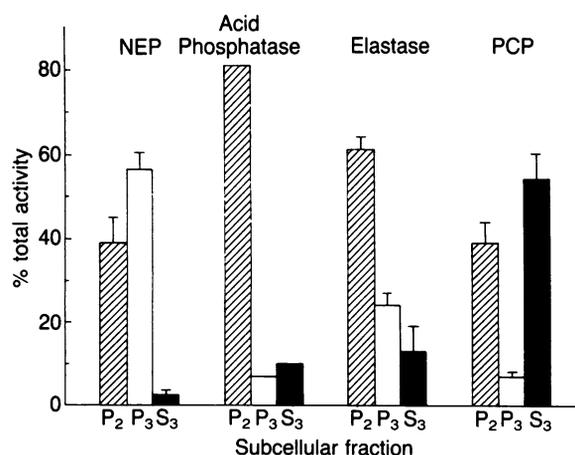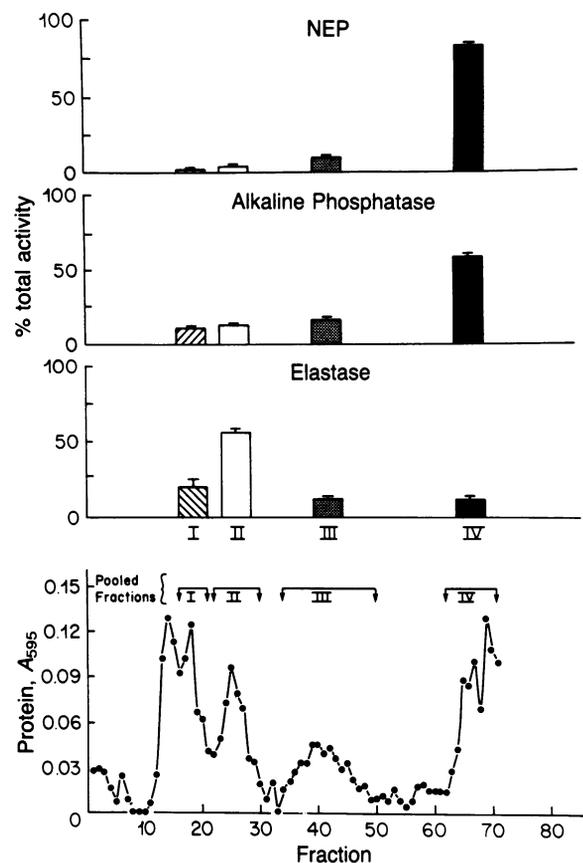

FIG. 2. Distribution of enzymes and protein content in fractions obtained after density gradient centrifugation of lysed human neutrophils. Results are the mean ± SEM for three to four different neutrophil preparations.

Hydrolysis of Bradykinin and fMet-Leu-Phe. The hydrolysis of bradykinin and fMet-Leu-Phe by the P$_3$ fraction of neutrophils was investigated because these two peptides are thought to be involved in inflammation and chemotaxis. The P$_3$ fraction was incubated with either peptide (0.1 mM) in the presence of 10 μM CK-20 and bestatin to inhibit any elastase or aminopeptidase activity. When analyzed by HPLC, the major products of bradykinin hydrolysis were the COOH-terminal dipeptide Phe^8-Arg^9 and the heptapeptide des-(Phe^8-Arg^9)-bradykinin, the same products also released by homogeneous human NEP (4). Phosphoramidon or thiorphan at a concentration of 0.1 μM inhibited the cleavage by 95% and 88%, respectively, indicating that the hydrolysis of bradykinin was due to NEP in the polymorphonuclear leukocyte membranes. Captopril (1 μM), the specific inhibitor of kininase II or angiotensin I converting enzyme, did not inhibit the hydrolysis (Table 1). Thus, while kininase II releases the COOH-terminal dipeptide from bradykinin (33), it does not participate in the hydrolysis of bradykinin by the P$_3$ fraction. The rate of bradykinin hydrolysis was 34 nmol·min^{-1}·mg^{-1} protein.

The P$_3$ fraction also readily cleaved the chemotactic peptide fMet-Leu-Phe by releasing Leu-Phe. At 0.1 mM concentration, fMet-Leu-Phe was hydrolyzed even faster than bradykinin, at a rate of 49 nmol·min^{-1}·mg^{-1}. Again, 0.1 μM phosphoramidon inhibited 91% of the activity, indicating NEP was the enzyme responsible for this cleavage.

As NEP has not been shown before to cleave fMet-Leu-Phe, we incubated purified, homogeneous human NEP with fMet-Leu-Phe and analyzed the products by HPLC. Purified NEP also released the COOH-terminal dipeptide from fMet-Leu-Phe. Kinetic studies showed that fMet-Leu-Phe is one of the best biologically active substrates with a K$_m$ of 59 × 10^{-6} M

FIG. 1. Distribution of enzymes in subcellular fractions of lysed human neutrophils. PCP, prolylcarboxypeptidase. Results are the mean ± SEM for three to seven different neutrophil preparations (except alkaline phosphatase, which is the mean of two preparations).

Table 1. NEP activity in a washed membrane fraction (P_3) of human neutrophils

Substrate	Inhibitor	% inhibition	Uninhibited activity,* nmol·min^{-1}·mg^{-1}
Glut-Ala-Ala-Phe-MNA	None	0	96 ± 23
	Phosphoramidon (1.0 μM)	95	
	CK-20 (5.0 μM)	0	
	Antiserum to NEP[†]	49	
	Preimmune serum[†]	0	
Bradykinin	None		34 ± 2
	Phosphoramidon (0.1 μM)	95	
	Thiorphan (0.1 μM)	88	
	Captopril (1.0 μM)	0	
fMet-Leu-Phe	None	0	49 ± 19
	Phosphoramidon (0.1 μM)	91	

Washed membranes (P_3) from human neutrophils were incubated with 0.5 mM Glut-Ala-Ala-Phe-MNA, 0.1 mM bradykinin, or 0.1 mM fMet-Leu-Phe.
*Results are mean values (± SEM) from three to four different neutrophil preparations.
[†]NEP antiserum or preimmune serum was incubated with the membrane fraction at a final dilution of 1:200 (vol/vol).

and a V_{max} of 40.6 μmol·min^{-1}·mg^{-1} (equivalent to a k_{cat} of 3654 min^{-1}). The specificity constant (k_{cat}/K_m) was 62 μM^{-1}·min^{-1}.

Immunocytochemical Localization. At the light microscopic level, the peroxidase-antiperoxidase technique revealed the heaviest concentration of NEP along the cell membrane of the neutrophils (Fig. 3B). Controls incubated with normal serum (Fig. 3A) or antiserum preadsorbed with NEP (Fig. 3C) lacked peroxidase substrate precipitate along the membranes of these cells. In control reactions, a few large leukocytes stained because of endogenous peroxidase activity. In some neutrophils, dark NEP-positive granules were recognized within the cytoplasm (Fig. 3B, arrowheads).

The localization of NEP along the cell membrane was confirmed at the ultrastructural level using the immunogold method on ultrathin cryosections (Fig. 4). The distribution of nonspecifically bound gold label over the nucleus and cytoplasm was approximately equal in NEP-labeled sections and in normal serum controls. No cytoplasmic organelles stained specifically for NEP. The intracellular location of NEP seen in some cells under the light microscope by the peroxidase-antiperoxidase method may indicate internalization of cell membranes. Fig. 4C is an example of NEP detected at the ultrastructural level, which suggests such internalization.

DISCUSSION

The participation of neutrophil proteases in the inflammatory process (31, 34) has been widely studied. Granular enzymes, such as elastase, collagenase, and cathepsin G, which are released upon neutrophil activation, break down bacterial cells and destroy host tissues (35), but they may have other physiological functions as well. For instance, elastase can modulate platelet function (36) and, while the physiological importance is less clear, cathepsin G can liberate the active peptide angiotensin II from angiotensin I (37).

Our study focuses on another enzyme in neutrophils, NEP, which may have an important role in the inflammatory process. NEP appears to be concentrated on the cell membrane of neutrophils as indicated by both immunocytochemistry and separation of subcellular particles. Assuming that the kinetic properties of neutrophil NEP are the same as those of the human kidney enzyme, the concentration of NEP on the membrane can be calculated from the rate of hydrolysis of bradykinin or fMet-Leu-Phe. Based on these calculations, the concentration of NEP is estimated to be ≈2 μg per mg of membrane protein of the washed P_3 fraction.

Membrane-bound NEP also occurs at several other sites in the body (6, 7, 38, 39). Although the specificity of NEP was defined with purified renal enzyme using the B chain of insulin as substrate (1), added importance of the peptidase was realized when it was localized in the brain and found to inactivate enkephalins (3). NEP was then named enkephalinase and a specific inhibitor, thiorphan, was synthesized (3). However, because this enzyme cleaves many other active peptides (2–8, 38, 39) it cannot be named after a single substrate.

This report underscores the broad specificity of NEP and describes its action on one more biologically active peptide, fMet-Leu-Phe, which is the major chemotactic peptide produced by *Escherichia coli* (40). This peptide appears to be one of the best of the biologically active substrates, even though it is among the shortest, as a protected tripeptide. The K_m
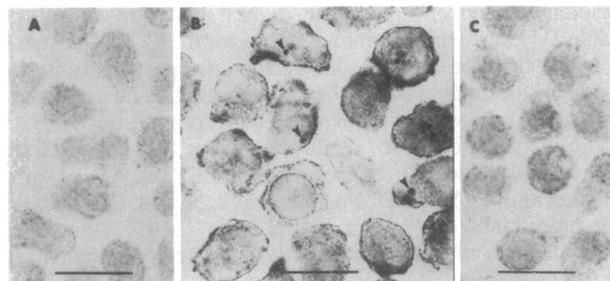

FIG. 3. Immunocytochemical localization of NEP in human neutrophils by the peroxidase-antiperoxidase technique including counterstaining with Mayer's hematoxylin. (A) Normal serum control. (B) Antiserum to NEP. (C) Antiserum preadsorbed with NEP. Arrowheads identify some intracellular loci of NEP antigen. (Bar = 10 μm.)
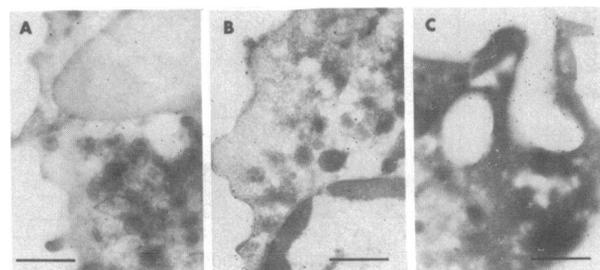

FIG. 4. Localization of NEP in neutrophils by the immunogold method applied to ultrathin cryosections. (A) Normal serum control. (B and C) Incubation with antiserum to NEP. (C) Tangential section of an invagination in the cell suggests internalization of plasmalemmal NEP. (Bar = 1 μm.)

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with purified human renal NEP was 59×10^{-6} M, lower than the K_m of [Leu]enkephalin (4, 8). It also had a relatively high specificity constant (k_{cat}/K_m) of 62 $\mu M^{-1} \cdot min^{-1}$, similar to the value of 63 $\mu M^{-1} \cdot min^{-1}$ obtained for [Leu]enkephalin (4).

NEP bound to the cell membrane in human neutrophils is very similar to, or identical with, the purified human renal enzyme as judged by several criteria. First, the NEP inhibitors phosphoramidon and thiorphan inhibited the enzyme from either source. Second, antisera to human renal NEP inhibited hydrolysis of the NEP substrate by the P_3 fraction, while preimmunized rabbit serum was inactive. Finally, immunocytochemistry using the same antisera in light and electron microscopy localized NEP primarily on the plasma membrane of neutrophils. These observations concur with the cell fractionation studies, which showed that most of the enzymatic activity was recovered in a plasma membrane-enriched fraction.

The fact that membrane-bound NEP readily cleaves the chemotactic peptide fMet-Leu-Phe suggests several functions. It has been indicated that neutrophil degranulation and chemotaxis require cleavage of chemotactic peptides (41, 42). If NEP on the neutrophil membrane is associated with the chemotactic peptide receptor, it could mediate the chemotactic signal by cleaving fMet-Leu-Phe. Alternatively, NEP may cleave the chemotactic peptide on its approach to the receptor to control the local concentration of the peptide. Neutrophil NEP might also play a direct role in exocytosis of the neutrophil granules. A recent study showed a metalloendoprotease to be involved in receptor-mediated exocytosis in mast cells and adrenal chromaffin cells (43).

Our studies were originally stimulated by the observation that in patients with ARDS, especially those complicated with septic pneumonia, the level of NEP in blood was severalfold higher than normal (9). Lung fibroblasts (44) and bronchial epithelial cells (45) contain the enzyme and could thus be the source of NEP released from damaged lungs. However, the relatively high concentration in neutrophils suggests that these cells are also a possible source for blood-borne NEP, although it is not yet known how NEP can be released from the neutrophil membrane *in vivo*, for example, by proteolytic enzymes such as elastase.

Significant NEP activity was detected in intact neutrophils when incubated with Glut-Ala-Ala-Phe-MNA, indicating that the membrane-bound NEP has access to peptide substrates in the surrounding medium. In addition, preliminary experiments showed that intact neutrophils from donors who smoked had higher NEP activity than normal. To decide whether this is a direct effect of smoking or is due to other factors would require further investigations in a much larger number of samples.

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