Identification and Characterization of Neutral Endopeptidase in Endothelial Cells from Venous or Arterial Origins*

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Neutral endopeptidase (NEP; enkephalinase, EC 3.4.24.11) is a cell membrane-associated zinc metalloprotease, which cleaves peptides like atrial natriuretic peptide (ANP) on the amino side of hydrophobic amino acids. Although NEP is mainly located in reabsorptive epithelia (kidney proximal tubule), it is also present in non-epithelial cells such as neuronal cells. As the renal NEP cannot account for the entire ANP metabolism, other locations were postulated. The present experiments show its expression in endothelial cells (EC) from arterial (bovine pulmonary, porcine, and human aorta) and venous (human umbilical, rabbit ear marginal) origins.

Three different methods were used to demonstrate the presence of the protein and its mRNA. 1) NEP enzymatic activity was estimated using both a synthetic ([D-Ala²,Leu⁶]enkephalin) and a natural substrate (bradykinin). Using the synthetic substrate, the enzymatic activity in EC was completely blocked by thiorphan, a specific NEP inhibitor with an IC₅₀ value in the nanomolar range. In contrast, captopril, bestatin, [2-guanidinoethylmercapto]succinic acid, inhibitors of angiotensin-converting enzyme, aminopeptidases, and carboxypeptidases, respectively, were 10,000 times less active, revealing an inhibition profile similar to that of the purified enzyme. Bradykinin, a natural substrate of NEP, was in part metabolized by NEP, in the presence of captopril, since 50% of the formation of the major metabolite bradykinin 1–7 was inhibited by thiorphan. 2) Immunoreactive NEP was detected on the plasma membrane of rabbit EC using a monoclonal antibody directed against the homologous renal enzyme. 3) NEP mRNA was detected by Northern blot analysis of rabbit EC as a major transcript of 3.9 kilobases. Reverse transcriptase polymerase chain reaction amplifications showed the presence of a specific transcript in all EC tested. Therefore, endothelial NEP may play an important role in the inactivation of ANP, bradykinin, and endothelins by its localization facing the circulating vasoactive peptides.

Neutral endopeptidase (NEP), also called enkephalinase (EC 3.4.24.11), is a 90-kDa membrane-bound zinc enzyme which acts on numerous substrates. Malfroy et al. showed in 1978 that it inactivated endogenous enkephalins in the central nervous system by hydrolyzing the Gly²-Phe⁴ amide bond (Malfroy et al., 1978). The enzymatic characteristics of the purified enzyme (Almenoff et al., 1981; Malfroy and Schwartz, 1982a; Fulcher et al., 1982) were subsequently found to be identical to those of a pig kidney enzyme identified several years previously by Kerr and Kenny (1974) using the B chain of insulin as substrate. They used the term "neutral endopeptidase" (NEP) for this enzyme. The gene encoding for NEP has been cloned in rat (Malfroy et al., 1987), rabbit (Devault et al., 1987), and man (Malfroy et al., 1988). As deduced from the cDNA sequences of the rat and rabbit enzymes, NEP is indeed a highly conserved protein with a great degree of homology (93%) between rat and rabbit enzymes. The human (Malfroy et al., 1988) and rat sequences are even more similar, there being only six nonconservative changes in the 742 amino acids of human and rat NEPs. Subsequently, the gene for a pre-B lymphocyte surface antigen (CD10), the common acute lymphoblastic leukemia antigen (CALLA), was cloned, and its sequence was shown to be identical to that of NEP (Letarte et al., 1988; Shipp et al., 1989).

NEP is found in the epithelial cells of various organs (Llorens and Schwartz, 1981; Gee et al., 1985; Ronco et al., 1988) and in vitro it can hydrolyze a large number of peptides. In vivo, however, its involvement in the metabolism of peptides has been established for a smaller number of them including enkephalins, substance P, bradykinin, atrial natriuretic peptide (ANP), gastrin, neurotensin, and the chemotactic peptide (for reviews see Schwartz et al., 1989; Erdös and Skidgel, 1989). ANP is a vasodilator and natriuretic factor which plays a role in the physiological regulation of blood pressure and fluid and electrolyte homeostasis (Cantin and Genest, 1985; Needleman et al., 1985). Cleavage by NEP results in its inactivation (for reviews see Schwartz et al., 1990; Gerbes and Volmar, 1990). Administration of NEP inhibitors to rats increases plasma ANP levels and the urinary excretion of cGMP. This results in sodium diuresis and a reduction in blood pressure (Bralet et al., 1990; Lafferty et al., 1989, Gros et al., 1989; Sybertz et al., 1989; Seymour et al., 1990; Lecomte et al., 1990). NEP inhibitors are also able to improve cardiac function in cases of cardiac insufficiency.

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1 The abbreviations used are: NEP, neutral endopeptidase; ANP, atrial natriuretic peptide; ACE, angiotensin I-converting enzyme; HPLC, high performance liquid chromatography; HEPRS, N-2-hydroxethylpropionitrile-N’-thioketonic acid; MEM, minimal essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase polymerase chain reaction; GEMSA, (2-guanidinoethylmercapto)succinic acid; kb, kilobase(s).
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(Northridge et al., 1989). Consequently, there has been considerable interest in identifying inhibitors of NEP activity. Several studies suggest that ANP may be metabolized within kidney, lung, liver, or blood vessels (Tang et al., 1984; Luft et al., 1986). Kidney contains the highest NEP activity and seems to be a major site of ANP metabolism. However, the precise site and the importance of kidney in ANP degradation is not clear, since ANP acts on glomerular receptors, whereas NEP is located on proximal tubular cells and since Katsube et al. (1986) and Luft et al. (1986) demonstrated that nepro-
tomoy enhanced the plasma half-life of ANF only by 2-fold. In addition, Sybertz et al. (1989) and Chiu et al. (1991) reported that the disappearance of infused ANP from the circulation of bilaterally nephrectomized rats was delayed in the presence of the NEP inhibitor SCH 39370. These observations strongly suggest that there are extrarenal sites of action for NEP inhibitors.

The endothelium is an important site for the metabolism of circulating vasoactive peptides. For example, angiotensin I is mainly metabolized in endothelial cells by angiotensin I-converting enzyme (ACE), which, like NEP, is a membrane-bound zinc enzyme (Soffer, 1976). Although NEP is believed to be mainly located in epithelial cells, Johnson et al. (1985) report the presence of a low concentration of NEP in endothelial cells. Therefore, we looked for this protein using enzyme- and immunohistochemical techniques and determined if its mRNA is expressed in cultured venous and arterial endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Enzymatic Assays—**Orthophosphoric acid and acetone (HPLC grade) were obtained from Merck (Darmstadt, Germany); bradykinin and bradykinin fragments 1-5 and 1-7 were obtained from Bachem (Budendorf, Switzerland); triethylamine was obtained from Prolabo (Paris, France). Porapak Q was obtained from Waters Associates (Milford, MA). Bestatin was a generous gift from Roger Bellon Laboratories (Neuilly-sur-Seine, France). Captopril (SQ 14225; d-3-mercapto-2-methylpropanoyl-l-proline) was from the Bristol-Myers Squibb Institute for Medical Research (Princeton, NJ). Thiorphan (20 nM) was purchased from Sigma. 

**GEMSMA** (3,000 G/M) and counted by liquid scintillation spectrometry (Aquasol-2, Du Pont-New England Nuclear).

**Cell Culture—**Gelatin and sodium bicarbonate were purchased from Bottcher (Budendorf, Switzerland) and counted by liquid scintillation spectrometry (Aquasol-2, Du Pont-New England Nuclear). Addition of 10-8 M thiorphan diminished by 99% the hydrolysis of the substrate such that it was equal to the reaction blank obtained by adding HCl before incubation.

**Bradykinin Metabolism—**Bradykinin hydrolysis by rabbit ear marginal vein endothelial cell membranes was measured by incubating 20 nM [d-Ala6,Leu7] [125I]Enkephalin (50 Ci/mm, or [125I]Bradykinin (2,3-~rolyl-3,4-~H) (110 Ci/mmol) in the presence of 10-8 M thiorphan. Puromycin (10-7 M) was added in order to prevent aminopeptidase activity degrading the substrate. Incubations were performed at 37 °C under conditions of initial velocity measurement and were stopped (after 15 or 30 min) by the addition of 25 nM of 0.3 % HCl. 

**Membrane Preparation from Tissues and Endothelial Cells—**As controls, NEP activity was measured in a particulate fraction from mouse or rat tissues and endothelial cells as follows: striata or kidneys from Swiss mice and New Zealand rabbits were dissected and homogenized in 10 volumes of cold 10 mM HCl buffer (pH 7.4). The homogenate was centrifuged (100,000 g x 90 min) and the supernatant fluid was discarded, and the pellet was superficially washed three times with 5 ml of cold buffer. It was then resuspended in the same buffer to give a protein concentration of 1 mg/ml.

After discarding the cell medium, endothelial cells were scraped in 3 ml of 1 X PBS and centrifuged (200 g x 10 min). The supernatant was discarded, and the pellet containing intact cells was sonicated in 6 ml of Tris-HCl buffer (0.05 M, pH 7.4) and then treated as described above.

**NEP Enzymatic Activity—**NEP activity was estimated according to the method of Llorens et al. (1982) by measuring the hydrolysis of 20 nM [d-Ala6,Leu7] [125I]Enkephalin in the presence or absence of 10-8 M thiorphan. Puromycin (10-7 M) was added in order to prevent aminopeptidase activity degrading the substrate. Incubations were performed at 37 °C under conditions of initial velocity measurement and were stopped (after 15 or 30 min) by the addition of 25 nM of 0.3 % HCl. 

**Immunofluorescence—**Rabbit endothelial cells were seeded in chamber slides. After 4-5 days of culture, when cells were at near confluency, the culture medium was replaced by PBS, followed by a solution of 4% paraformaldehyde in PBS. After 5-20 min of fixation, the cells still adhering to the slide were washed with PBS and subjected to routine immunofluorescence as follows: cells were incubated successively in 3% normal goat serum, the monoclonal antibody (1:100 dilution) for rabbit NEP (Fong et al., 1986) at 20 μg/ml, and finally in fluoresceinated labeled sheep anti-rabbit immunoglobulin solution (5-10 μg/ml). After each step, the cells were washed in PBS. Finally the cells were mounted in Mowiol.

2. C. Llorens-Cortes, H. Huang, P. Vicart, J.-M. Gasc, D. Paulin, and P. Corvol, unpublished results.

3. Vicart et al., submitted for publication.
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NEP mRNA Characterization by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Amplification—Kidneys and lungs from male Wistar rats (180–200 g) were dissected out and total RNA was prepared with the guanidinium CsCl method (Chirgwin et al., 1979). The method of Chomczynski and Sacchi (1987) was used for endothelial cell RNA preparation. Endothelial cell medium containing adherent cells was scraped in 10 volumes of a denaturing solution, containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, homogenized using a Potter homogenizer, and total RNA prepared as already described (Chomczynski and Sacchi, 1987). In all experiments, the absence of degradation of isolated mRNA was verified by gel electrophoresis.

For RT-PCR amplification, oligonucleotide primers derived from the human NEP cDNA sequence (reverse, exon 24: 5'-TGA AGA TCA CCA AAC CCG GCA CTT-3', position 2256-2235 base pairs and sense exon 18: 5'-GGT CAT AGG AGA CGA GAA CAC-3', position 1759-1759 base pairs, according to D'Adamo et al., 1989) were synthesized using a PTC-Mate 391 instrument (Applied Biosystems). Taq polymerase (Cetus) and 80 nM primers in 0.1 ml of 10 mM Tris-HCl buffer (pH 7.5), 50 mM KC1, 2.5 mM MgCl2, 0.5 mM dNTP, 20 pg/ml yeast tRNA, and the 32P-labeled cDNA fragment of the rat NEP described above at 5 X 106 dpm/ml. Filters were washed twice for 15 min at 42 °C in 2 X SSC, 0.1% SDS, twice for 15 min at 42 °C in 2 X SSC, 0.1% SDS and twice for 20 min in 0.2 X SSC, 0.1% SDS at 42 and 55 °C and finally autoradiographed at room temperature using Kodak X-Omat film.

**Northern blot Analysis**—Total RNA was fractionated by gel electrophoresis in 1% agarose gels containing 10% formaldehyde, stained with ethidium bromide, photographed under UV light, and transferred to Hybond N membrane (Amersham). The blot was prehybridized for 16 h in 42 °C in 2 X SSC, 0.1% SDS, twice for 15 min at 42 °C in 2 X SSC, 0.1% SDS, twice for 15 min at 42 °C in 0.2 X SSC, 0.1% SDS, and then exposed to an X-Omat film at –80 °C in the presence of intensifying screens. An image analysis system coupled to a video camera (Biocom RAG 200, Les Ulis, France) was used for densitometric analysis of the autoradiograms.

**Protein Determination**—Protein content was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

**Analysis of Data and Statistical Treatment**—For determination of IC50 values, the total curves were analyzed with an iterative computer least squares method derived from that of Parker and Wood (1971). Statistical analysis was performed using the Student's t test for paired data.

**RESULTS**

**Characterization of NEP Activity in Endothelial Cells**—NEP activity was assessed by the degradation of [d-Ala3,Leu5] [-H]enkephalin in conditions of initial velocity. The specificity of this assay was confirmed by its total inhibition in the presence of 10–7 M thiorphan, an inhibitor of NEP (Roques et al., 1980). The presence of d-alanine and the addition of 10–4 M puromycin, an aminopeptidase inhibitor, (Barclay and Phillips, 1978) prevented degradation of the substrate by aminopeptidases or dipeptidyl aminopeptidases. As there was no degradation of the substrate in the presence of thiorphan, the assay system contained no other active peptidase.

NEP activity was found in endothelial cells of venous and arterial origins (Fig. 1). NEP activity levels expressed per mg of membrane protein in endothelial cells from bovine pulmonary artery, human aorta, or human umbilical vein were within the same order of magnitude as those found in mouse striatum, the brain region richest in NEP. Endothelial NEP levels, however, were 25 and 70 times lower than those in mouse or rat kidney, respectively. Rabbit-transformed endothelial cells which originated from the ear marginal vein had the highest NEP activity of all endothelial cells tested (Fig. 1). In contrast, no significant NEP activity was detected in pig aorta EC. The very weak [d-Ala3,Leu5] [-H]enkephalin-degrading activity observed was not inhibited by 10–7 M thiorphan and was therefore likely to be due to NEP. These cells were not studied further.

The peptide-hydrolitic activity observed in endothelial cells was shown to be due to NEP by testing the inhibitory potency of different peptidase inhibitors on the hydrolysis of [d-Ala3,Leu5] [-H]enkephalin (Fig. 2). The IC50 for thiorphan was in the nanomolar range for all endothelial cells tested (2.3 ± 0.3 nM in rabbit EC, 2.4 ± 0.4 nM in human umbilical vein EC, and 1.6 ± 0.2 nM in human aorta EC), whereas GEMSA, a carboxypeptidase inhibitor (Fricker et al., 1983), bestatin, an aminopeptidase inhibitor (Umegawa et al., 1976), and captopril, an ACE inhibitor (Cushman et al., 1977), each had IC50 > 10–3 M (Fig. 2, A–C). In the same experiments, similar IC50 values for these different peptidase inhibitors (thiorphan: IC50 = 1.2 nM; GEMSA, captopril, and bestatin, IC50 > 10–4 M) were found in mouse striatum in agreement with previous data (Llorens et al., 1982; De la Baume and Schwarz, 1988).

[1H]Bradykinin Metabolism by Endothelial NEP—The degradation of [1H]bradykinin by rabbit ear marginal vein EC membranes was studied in the presence of 10–7 M captopril to inhibit ACE activity. This resulted in the production of three different metabolites which were separated by reverse phase HPLC, as shown in Fig. 3. In addition to bradykinin 1–7 and bradykinin 1–5, an unidentified degradation product migrating faster than bradykinin 1–5 was seen. 10–7 M thiorphan

**Fig. 1.** NEP activity in venous or arterial endothelial cells as compared with other tissues. NEP activity was determined with 20 nM [d-Ala3,Leu5] [-H]enkephalin, in the presence or absence of 10–7 M thiorphan in particular fractions from rat kidney and striatum, mouse striatum, rabbit marginal vein EC, human umbilical vein EC, bovine pulmonary artery EC, human and porcine aorta EC. Values (means ± S.E.) were obtained from two or three different cultures (triplicate determinations).

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Cell membranes

Rabbit ear marginal vein EC

Percent of inhibition of ([D-Ala²-Leu⁷]-enkephalin) hydrolysis

log [inhibitor] (M)

FIG. 2. Effect of different peptidase inhibitors on NEP activity in venous or arterial endothelial cells. Particulate fractions from human aorta EC (A), human umbilical vein EC (B), and rabbit ear marginal vein EC (C) were incubated with 20 nM [D-Ala²,Leu⁷]-[³H]enkephalin in the presence or absence of different concentrations of the peptidase inhibitors. Each point represents the mean of data from two to four experiments with duplicate determinations, with less than 10% of variation from one experiment to the other.

decreased bradykinin degradation by 53% (787 ± 88 versus 367 ± 85 fmol/min/mg of protein). This was due to a 50% reduction in the formation of bradykinin 1–7 (643 ± 88 versus 323 ± 75 fmol/min/mg of protein) and to a 69% reduction in the formation of the unidentified metabolite (144 ± 20 versus 44 ± 11 fmol/min/mg of protein). Bradykinin 1–5 formation was not due to NEP activity and was unaffected by the addition of thiorphan.

Immunochemical Localization of NEP in Rabbit EC—Rabbit endothelial cells cultured on histological slides displayed a faint immunofluorescence when tested with the 85A2 antibody (Fig. 4A). The staining appeared to be located at the plasma membrane. In addition, a bright dotted or stippled pattern of distribution was superimposed over the more homogeneous immunofluorescence. Some cells were not stained, probably because of a very low level of NEP expression. Two preparations of mouse immunoglobulins not related to NEP, even when used at higher concentration than 85A2, did not reveal any fluorescent signal at the plasma membrane, but only a diffuse fluorescence of the cell nuclei (Fig. 4B).

As a positive control, cryosections of rabbit kidney were used in which an intense fluorescence was observed at the brush border of the proximal tubular cells, even with a higher dilution of the anti-NEP antibody (3 µg/ml) than that used for the cultured cells (not shown).

Identification of NEP mRNA in Endothelial Cells—Endothelial cells were tested for the presence of specific NEP transcripts by two approaches, RT-PCR amplification of mRNAs from endothelial cells and Northern blot analysis.

RT-PCR Amplification—Total RNA from human and rat kidneys, rat lung, bovine pulmonary artery EC, human umbilical vein EC, and rabbit ear marginal vein EC was prepared, and the quality of the RNA was tested by fractionating it on denaturing agarose gel. At this step in each sample, the total RNA content was estimated from the amount of the 18 S ribosomal band, as assessed by densitometry (not shown).
Subsequently, each sample was subjected to RT-PCR amplification for 16, 22, or 30 cycles. The transcripts were visualized by ethidium bromide under UV light and after hybridization with a 32P-labeled rat NEP cDNA fragment, a specific transcript corresponding to the expected size (518 base pairs) for the transcript of the NEP cDNA was detected in all endothelial cells and tissues tested. Saturation of the signal was observed at 30 cycles (Fig. 5), whereas at 16 cycles (not shown), the NEP transcript could not be detected in human umbilical vein and bovine pulmonary artery endothelial cells. For these reasons, the best estimation of the signal was obtained at 22 cycles (Fig. 5). Amounts of mRNA were calculated in arbitrary units defined by the area under the peak of the NEP-specific band divided by the area under the peak of total RNA applied to the gel and used for the PCR experiment. All measurements were performed within the range in which a linear relationship exists between the autoradiogram signal and the area scanned, as well as between the amount of ethidium bromide-stained RNA and the area scanned. For comparative purposes, the amount of kidney NEP mRNA was arbitrarily assigned a value of 100, and all other values were calculated relative to it. The order of amplitude of the signal was: rat kidney (100%) > rabbit endothelial cells (75%) > rat lung (55%) > human umbilical vein endothelial cells (25%) > bovine pulmonary artery endothelial cells (13%).

*Northern Blot Analysis*—Rabbit ear marginal vein endothelial cells express an NEP mRNA of 3.9 kb, whereas human umbilical vein endothelial cells and bovine pulmonary artery endothelial cells express NEP mRNA of 5.1 and 4.8 kb, respectively (Fig. 6). In comparison, Fig. 6 shows the expected NEP mRNAs expressed in rat kidney and lung (3.8, 4.6, and 6.0 kb), the major transcript being 3.8 kb.

By scanning the band corresponding to the major transcript in each tissue and by relating it to the amount of total RNA as estimated by scanning the 18 S ribosomal band, NEP mRNA levels were quantified and expressed as described above. NEP mRNA levels estimated by RT-PCR amplification and by Northern blot analysis correlated well (r = 0.995, p < 0.01) (not shown).

**FIG. 6.** Expression of NEP mRNAs in venous or arterial endothelial cells as compared with that in rat lung and kidney. Total RNA (15 µg/lane), prepared from rat kidney (lane 1), rat lung (lane 2), human umbilical vein EC (lane 3), bovine pulmonary artery EC (lane 4), and rabbit ear marginal vein EC (lane 5) were fractionated on a 1% agarose formaldehyde gel, transferred on to Hybond N membranes, and hybridized with a 32P-labeled rat NEP cDNA. The sizes of the marker fragments are indicated on the right of the figure.

**DISCUSSION**

The established site for NEP degradation of the circulating vasoactive peptides is the kidney tubular epithelial cells. However, experiments performed on ANP metabolism in binephrectomized rats suggested that there are other sites for ANP degradation by NEP.

This study shows that NEP is found in membranes of cultured EC of arterial or venous origin in various species. The NEP that is bound to the cell membranes of EC appears to be identical to the purified renal enzyme. NEP enzymatic activity was characterized using a natural substrate, bradykinin, and a synthetic substrate derived from Leu6 enkephalin (Tyr-Gly-Gly-Phe-Leu) where the replacement of the glycine in position 2 by D-alanine provides resistance to hydrolysis by aminopeptidases and dipeptidyl aminopeptidases (Pert et al., 1976).

The synthetic substrate was cleaved by NEP between Gly and Phe, and the production of the metabolite Tyr-D-Ala-Gly was measured. This hydrolysis was solely due to NEP, since 10⁻⁷ M of thiorphan, a specific NEP inhibitor, completely abolished Tyr-D-Ala-Gly formation in particulate fractions of kidney, striatum, and EC. That NEP in EC was specifically involved was shown by the IC₅₀ of thiorphan in the nanomolar range, whereas that of ACE, aminopeptidases, or carboxypeptidase inhibitors were 10,000 times less active. This inhibition profile of EC NEP seems similar to that of NEP purified from bovine pituitary or rat kidney (Orlowski and Wilk, 1981; Malfroy and Schwartz, 1982b; Hersh, 1984) and was such that ACE could not have been involved in the assayed activity. This was consistent with the low affinity of ACE for enkephalins (in the millimolar range).

Under these assay conditions, NEP activity in cultured EC was of the same order of magnitude as that of mouse striatum (the brain region with the highest NEP content), but was much lower than that found in mouse or rat kidney (Llorens et al., 1982; De la Baume and Schwartz, 1988). Endothelial cells of either arterial (bovine pulmonary artery EC, human and porcine aorta EC) and venous (human umbilical vein EC, rabbit ear marginal vein EC) origins appear to contain NEP activity. A systematic study of NEP activity among endothelial cells from different regions would define the extent to which endothelial cells are able to express NEP. Interestingly, rabbit-transformed EC, which express NEP relatively strongly, maintain their capacity to express ACE. This suggests that they retain typical differentiation functions such...
as production of ectoenzymes in spite of their immortalization.

NEP has been shown to cleave bradykinin at the Pro¹-Phe⁸ bond into bradykinin 1–7 in vitro (Gafford et al., 1983; Skidgel et al., 1991) and in vivo (Ura et al., 1987). This work shows that bradykinin can be hydrolyzed by a particulate fraction of rabbit EC. This experiment was done in the presence of 10⁻⁷ M captopril to block ACE activity completely. In these conditions, 50% of bradykinin hydrolysis was due to NEP activity, since degradation was 50% inhibited by 10⁻⁷ M thiorphan. The major metabolites of bradykinin were bradykinin 1–7 and bradykinin 1–5. The formation of bradykinin 1–7 was due solely to NEP, since its formation was 50% inhibited by thiorphan. In addition, another minor and nonidentified metabolite which eluted before bradykinins 1–7 and 1–5 was the result (70%) of NEP activity. It could be the consequence of a secondary cleavage of bradykinin 1–7 between Gly¹ and Phe⁸, since NEP selectively cleaves amide bonds comprising the amino group of an hydrophobic amino acid (Schwartz et al., 1981; Erdös and Skidgel 1989) and since previous data have shown that hydrolysis of bradykinin by pure NEP produces bradykinin 1–4 (Gafford et al., 1983).

The presence of NEP was also assessed by immunohistochemistry which showed a diffuse and punctuated immunofluorescence at the plasma membrane of EC. NEP mRNA was found in EC by RT-PCR amplification and Northern blot analysis, showing that NEP is expressed in this cell type. mRNAs of various EC were amplified between primers flanking the zinc binding domain and the 3′-coding region. The transcripts obtained in all tissues and EC studied had the expected size and all hybridized with a rat NEP cDNA fragment. Northern blot analysis of the mRNAs from rat tissues and rabbit EC showed a major band at 3.9 kb, very close to the major transcript of NEP described in rat and rabbit kidneys and in human leukemic cells (Malfroy et al., 1987; Devault et al., 1987; Letarte et al., 1988; D’Adamo et al., 1989). The NEP mRNAs of 5.1 kb in human umbilical vein and of 4.8 kb in bovine pulmonary artery EC could correspond to the use of alternative promoters or of alternative polyadenylation signals. Indeed such alternative transcripts at 2.8, 5.0, 6, and 6.5 kb have also been described in rat tissues and in leukemia cells which express CALLA and probably result in part from the utilization of alternative poly(A) signals (Shipp et al., 1988) and from alternative promoters in the 5′ region (D’Adamo et al., 1989). Another possibility is that these different NEP mRNAs could result from differential splicing, generating different isoform(s) of NEP (Llorens-Cortes et al., 1990).

It is important to compare the NEP mRNA concentration as estimated by RT-PCR amplification or Northern blot analysis with that of the protein concentration. For a similar amount of NEP mRNA, the NEP activity is at least 15 times greater in rat kidney than in rabbit EC. This suggests different turnover rates for either the protein, the mRNA, or both in these cells.

Recent enzymatic studies show that NEP is present in the rat aorta (Soleilhac et al., 1992). Furthermore using an indirect approach consisting of treatment with saponin, they suggest its presence in endothelial cells. But it is likely that NEP is also produced in vascular smooth muscle cells (Dickinson et al., 1991), since the degradation of endothelin by vascular A10 cell membranes is completely blocked by selective NEP inhibitors.

The presence of NEP in EC is of potential importance in evaluating the overall metabolism of circulating vasoactive peptides. ANP, bradykinin, and endothelin have all been shown to be substrates for NEP activity in vitro (Stephenson and Kenny, 1987; Gafford et al., 1983; Vijayaraghavan et al., 1990; Sokоловsky et al., 1990), and they are also possible candidates for in vivo metabolism by endothelial cell NEP.

The importance of NEP for the in vivo metabolism of ANP has led to the design of NEP inhibitors to increase ANP levels and activity in subjects with hypertension or heart failure. Indeed, inhibition of ANP metabolism leads to amplification of the ANP signal and a therapeutically relevant response including reduction in blood pressure in a volume-dependent model of hypertension (Seymour et al., 1983, 1990; Sybertz et al., 1990) and an increase in cardiac output in experimental models of heart failure (Cavero et al., 1990). There is evidence to suggest that NEP inhibitors may affect ANP disposition or protection in extrarenal sites and that these effects may actually be more marked in rats with bilateral nephrectomy (Sybertz et al., 1989; Valentin et al., 1992). These findings should be considered in the light of the present experiments which indicate that NEP is present in endothelial cells. Evaluation of the precise role in ANP metabolism of endothelial NEP compared with renal NEP will require ANP metabolic clearance and degradation studies in various tissues in normal and pathological situations.

The presence of NEP in EC raises other interesting questions. For instance, in addition to its effects on ANP and bradykinin, NEP also has endothelinase activity. Thus, it could play a role in degrading endothelin which is a potent vasoactive peptide which has been reported to be present at increased concentrations in various pathological settings. In these situations use of NEP inhibitors might actually enhance rather than diminish the extent of vasoconstriction. Finally, the characterization of the in vivo interventions between endothelial NEP and these different peptides would help to define the site of action of NEP inhibitors and their role(s) in potentiating the action of endogenous circulating vasoactive hormones.

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