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Characterization of neutral endopeptidase in vascular cells, modulation of vasoactive peptide levels

Walter González [a,*], Jean-Marc Soleilhac [b], Marie-Claude Fournié-Zaluski [b], Bernard P. Roques [b], Jean-Baptiste Michel [a]

[a] *U460 Institut National de la Santé et de la Recherche Médicale (INSERM), Faculté X. Bichat, 16, rue H. Huchard, 75016 Paris, France*
[b] *U266 INSERM-URA D1500 CNRS, UFR des Sciences Pharmaceutiques et Biologiques, 4, rue de l'Observatoire, 75270 Paris Cedex 06, France*

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Abstract

We characterized neutral endopeptidase activity and protein in the three aortic layers and in corresponding cultured primary cells. Neutral endopeptidase was expressed in all three layers of rat aorta with higher protein level and activity in the adventitia than in the media and intimal endothelium. Neutral endopeptidase was also found in primary cultured fibroblasts, smooth muscle and endothelial cells derived from the corresponding layers. Neutral endopeptidase activity and protein were higher in the fibroblasts and smooth muscle cells than in endothelial cells. Neutral endopeptidase inhibition prevented atrial natriuretic peptide (ANP) degradation in endothelial and smooth muscle cells. It potentiated ANP-stimulated cyclic GMP production in these cells. Neutral endopeptidase inhibition also reduced bradykinin degradation and potentiated bradykinin-stimulated release of arachidonic acid in fibroblasts and endothelial cells. Our data demonstrate the presence and functional activity of neutral endopeptidase in all three cell layers of rat aorta as well as in primary cells of the vessel. The data suggest that local concentrations of vasoactive peptides in the vessel wall might be regulated by the neutral endopeptidase cleavage pathway in the immediate vicinity of their target cells. © 1998 Elsevier Science B.V.

Keywords: Neutral endopeptidase; Vascular cell; Vasoactive peptide level

1. Introduction

Neutral endopeptidase (EC 3.4.24.11) is a 94-kDa membrane-bound metalloenzyme that efficiently cleaves potent vasoactive peptides such as substance P, bradykinin, atrial natriuretic peptide (ANP), C-type natriuretic peptide, angiotensins, and endothelins (Roques et al., 1993). Neutral endopeptidase has been found in the epithelial cells of the brush border of the renal proximal tubule where it hydrolyses filtered peptides such as ANP and bradykinin (Ura et al., 1987; Pham et al., 1993, 1996). In this manner, neutral endopeptidase modulates the physiological action of various peptides by degrading them into inactive metabolites or by converting them into active mediators (Roques et al., 1993). However, the kidney seems not to be the only site of ANP catabolism by neutral endopeptidase since previous studies demonstrated that neutral endopeptidase inhibition reduces the clearance of ANP in both intact and binephrectomized rats (Barclay et al., 1991). These observations strongly suggest that there are extra-renal sites of ANP catabolism, including the vascular compartment. Neutral endopeptidase has been detected in cultured endothelial cells (rabbit, mouse, human and pig) (Llorens-Cortes et al., 1992; Graf et al., 1995) and cultured smooth muscle cells originated from rabbit kidney (Dussaule et al., 1992). Soleilhac et al. (1992) demonstrated that neutral endopeptidase activity is present in rabbit aorta. This activity is reduced by $> 95\%$ when the endothelium is removed after saponin treatment (Soleilhac et al., 1992). However, the precise localization of neutral endopeptidase in the vascular wall has not been studied so far. We therefore studied whether neutral endopeptidase is expressed in the intimal endothelium, in the media, and in the adventitia of rat aorta.

We dissected and isolated rat aortic layers (adventitia, media and intimal endothelium), and isolated the derived primary cells, i.e., fibroblasts, smooth muscle and endothelial cells. Neutral endopeptidase was characterized by the

* Corresponding author. Tel.: +33-1-44-85-61-60; fax: +33-1-44-85-61-57.

hydrolysis of a synthetic substrate, by its specific binding with a radioactive inhibitor, and by neutral endopeptidase mRNA detection by polymerase chain reaction.

Cell proliferation during atherogenesis is regulated by several systems including vasoactive peptides. Neutral endopeptidase pathway could modulate cell proliferation in the vessel wall by acting on the availability of vasoactive peptides in front of their targets. The functional role of neutral endopeptidase in the metabolism of ANP and bradykinin was studied in the cultured cells.

Neutral endopeptidase was expressed in the three layers of rat aorta with higher enzyme activity and protein levels in the adventitia than in the media and endothelium. We also found neutral endopeptidase in vascular endothelial, smooth muscle cells and fibroblasts. Inhibition of neutral endopeptidase prevented ANP and bradykinin from degradation and influenced peptide signalling in the cultured cells. Neutral endopeptidase present in the cells of the vessel may regulate vasoactive peptide levels in the vicinity of their target cells.

2. Materials and methods

2.1. Drugs

Retrothiorphan, 1-[(1-mercaptomethyl-2-phenyl) ethyl]amino-1-oxopropionic acid, and RB104, 2-{(3-4-hydroxy)phenylmethyl}-4-*N*-[3-(hydroxyamino-3-*oxo*-1-phenylmethyl)propyl] amino-4-oxobutanoic acid (Fournié-Zaluski et al., 1992) are potent neutral endopeptidase inhibitors (respectively $K_i = 6$ nM and $K_i = 30$ pM) (Roques et al., 1993) and were dissolved in 10% ethanol in Dulbecco's minimum essential medium or in buffer. RB104, retrothiorphan and a converting enzyme inhibitor, captopril, were synthesized in the laboratory of Molecular and Structural Pharmacochemistry, INSERM U266-CNRS D1500. Rat ANP-(1-28), 3[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 3-isobutyl-1-methyl-xantine, amastatin (IBMX), and bestatin were obtained from Sigma (St. Louis, USA). Bradykinin was purchased from Novabiochem (Meudon, France). DL-2-mercaptomethyl-3-guanidinoethyl-thiopropionic acid (MGTA) was purchased from Calbiochem (Frankfurt, Main, Germany).

2.2. Aortic layers microdissection

Male Wistar rats (250–300 g; Iffa-Credo, Labresle, France) were used. The procedure followed in the care and euthanasia of study animals was in accordance with the European Community Standards on the care and use of laboratory animals (Ministére de l'Agriculture, France; authorization No. 00577, 30 April 1989). Rats were killed after deep ether anesthesia and aortas were quickly excised (from the aortic arch to the abdomen limit). The aortas were placed in a petri dish half filled with Hank's balanced salt solution (Sigma) to keep the tissues wet. The fat tissues were removed using fine scissors, and the collateral vessels were cut. Isolation of arterial layers was performed as described previously (Battle et al., 1994). Briefly, the adventitia was removed by gently peeling on the aortic arch above the brachial artery. It was then possible to completely dissociate the adventitia from the media and intima by rotating the aorta in the buffer. This method allowed us to use directly the tissue, after homogenization, or to cultivate cells, after enzymatic treatment of the tissue.

2.2.1. Tissue preparation

The media, together with the intima were longitudinally cut, placed in a dry petri dish (endothelial face up) and the endothelium was removed by gently scraping with a microscaper. The microscaper was immediately plunged in 500 μl of cold Tris–HCl 50 mM pH 7.4 (Tris buffer) with 8 mM CHAPS in order to collect the dislodged endothelial cells. After an incubation of 1 h at 4°C, the preparation was centrifuged at $20\,000 \times g$ for 45 min at 4°C. The supernatant, containing the solubilized proteins, was stored at −80°C until required. Numerous washings of the endothelium-free media were performed in Hank's balanced salt solution to remove the endothelial cell membrane fragments that might still remain adsorbed on the matrix. The whole aorta, adventitia and media were homogenized at 4°C in 500-μl cold Tris buffer using a glass–glass homogenizer. The crude homogenates were immediately centrifuged at $1000 \times g$ for 15 min at 4°C and the resultant pellets were incubated for 1 h at 4°C in cold Tris buffer with 8 mM CHAPS. The preparation was centrifuged at $20\,000 \times g$ for 45 min at 4°C. The supernatants were stored at −80°C until required.

2.2.2. Cell isolation

The cultured endothelial cells, smooth muscle cells and fibroblasts were issued from the respective tunicae as described in detail elsewhere (Battle et al., 1994). Briefly, the adventitia was treated with collagenase (1200 iu ml^{-1}; Eurobio, France) for 1 h at 37°C, releasing fibroblasts that were plated in plastic flasks after sterile filtration. The remaining media plus intima were cut into fine rings and subjected to a slight collagenase digestion (1200 iu ml^{-1}) for 40 min at 37°C. The rings were then flushed in a wide mouth pipette and, following sterile filtration of the rings, the dislodged rat endothelial cells were plated in plastic flasks coated with rat fibronectin. Medial rings were removed from the filter and were more thoroughly digested in a mixture of collagenase (1200 iu ml^{-1}) and elastase (17.5 iu ml^{-1}; Eurobio) for 1 h at 37°C. Smooth muscle cells were plated in plastic flasks coated with 0.1% collagen (Sigma) after sterile filtration. Endothelial cells were cultured in Dulbecco's minimum essential medium (Boehringer Mannheim, France) supplemented with 15% horse

serum (Boehringer), 4% fetal calf serum (Dutscher, Brumath, France), 75 μg ml^{-1} endothelial cell growth supplement (Sigma), 50 iu ml^{-1} penicillin (Sigma), and 50 μg ml^{-1} streptomycin (Sigma). Smooth muscle cells and fibroblasts were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum with 50 iu ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Cell cultures were used from passages 2 to 5.

The purity of the cultures was assessed by morphological and immunohistological criteria. Endothelial cells were stained so as to detect Factor VIII (Dako, Trappes, France) and to detect the specific vascular endothelial antigen (Medac Diagnosika, Hamburg, Germany). Smooth muscle cells were characterized by alpha actin antibody (Dako). Fibroblasts were negative for the previously cited stainings.

All cell types were grown to confluence in 25-cm^2 culture flasks and were made quiescent by deprivation of serum for 48 h. After this period, cells were scraped in 1 ml of phosphate buffer and centrifuged ($800 \times g$; 5 min). The cell pellets were solubilized in 500 μl of cold Tris–HCl buffer with 8 mM CHAPS. After 1 h incubation at 4°C with gentle agitation, the preparation was centrifuged at $20\,000 \times g$ for 45 min, and the supernatants, containing the solubilized proteins, were stored at −80°C until further use.

2.3. Characterization of neutral endopeptidase

2.3.1. Neutral endopeptidase enzymatic activity

Neutral endopeptidase activity was determined by measuring the hydrolysis of 20 nM [^{3}H][D-Ala^2,D-Leu^5]-enkephalin (50 Ci mmol^{-1}), as previously described (Llorens-Cortes et al., 1992), in the presence and in the absence of 10^{-7} M of retrothiorphan ($K_i = 6$ nM for neutral endopeptidase). Briefly, bestatin (10^{-5} M) and captopril (10^{-6} M) were added to prevent both aminopeptidase and angiotensin-converting enzyme activities. Incubations were performed at 37°C under conditions of the measurements of initial velocity and were stopped by the addition of 25 μl 0.3 M HCl after 30–60 min. [^{3}H]-metabolites were isolated from the substrate by chromatography on polystyrene bead columns (Porapak Q, 100–200 mesh, Waters Assoc) and were quantified by liquid scintillation spectrometry. Addition of retrothiorphan (10^{-7} M) before the addition of substrate diminished the hydrolysis of the substrate by 99%. Under these conditions, the results were similar to the reaction blank obtained by adding HCl before incubation.

2.3.2. Inhibitor gel electrophoresis

Non-reducing sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) was carried out to determining neutral endopeptidase content using 100 pM [^{125}I]-RB104 as described in details elsewhere (Fournié-Zaluski et al., 1992). Films were quantified by densitometric analysis (Photoshop software, Agfa studio Scan).

2.3.3. RNA isolation and RT-PCR assay

Total RNA was isolated from quiescent endothelial, smooth muscle cells, fibroblasts and from rat kidney tissue with Trizol® (Gibco BRL, USA) according to the manufacturer's instructions. Total RNA (0.5 μg) was use for reverse transcriptase reaction in the presence of 1 μg of oligo-d(T)$_{12-18}$ and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) in a buffer containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 0.1 mM of dNTP mix, 7.5 mM dithiothreitol and 20 units RNase inhibitor (Boehringer) for 1 h at 37°C. After reverse transcription, samples were heated at 65°C for 10 min so as to denature the reverse transcriptase.

A total of 3 μl of cDNA were amplified in a total volume of 25 μl containing 10 pmol oligonucleotide primers, 0.1 mM of dNTP mix, 1 × PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$ and 1 U AmpliTaq Polymerase (Cetus). The primers, used for amplification of neutral endopeptidase, were determined according to rat neutral endopeptidase sequence (Malfroy et al., 1987): 5′-TGC CAA TGC CAC AAC TAA ACC-3′ (sense, 927–947 bp) and 5′-CAC ATA AAG CCT CCC CAC AGC-3′ (anti sense, 1322–1342 bp). The expected PCR product size was 414 base pairs. PCR was carried out in a DNA thermal cycler 480 (Perkin Elmer) with the sequence of 30 s at 94°C, 1 min at 63°C and 1 min at 72°C for 34 cycles. A total of 10 μl of the PCR was separated by electrophoresis on a 1% agarose gel. The PCR products were visualized by ethidium bromide coloration under UV light.

2.3.4. Protein determination

Protein content was assayed with the commercially available Bio-rad kit® with bovine serum albumin as a standard (Bio-rad Laboratories, Munchen, Germany).

2.3.5. Enzyme purification

Rabbit kidney neutral endopeptidase was purified by affinity chromatography, using a monoclonal antibody, as previously described (Aubry et al., 1987).

2.4. Neutral endopeptidase inhibition in primary vascular cells

2.4.1. Effect of neutral endopeptidase inhibition on ANP-stimulated cyclic GMP production

Confluent cultures in 12-well plates were made quiescent by deprivation of serum for 48 h. In order to determine cyclic GMP generation, the cells were washed with phosphate buffer. Then, serum-free medium, containing 10^{-5} M IBMX and increasing doses of ANP (10^{-12} to 10^{-6} M) was added to the intact cell layers in the presence

of retrothiorphan (neutral endopeptidase inhibitor, 10^{-7} M) or its diluent (control; 10% ethanol in medium with IBMX). After 120 min of incubation at 37°C under CO_2 atmosphere (time known to allow maximum release of cyclic GMP) (Hamet et al., 1989), the cell supernatant was collected in order to determine cyclic GMP. Samples of cell supernatant were briefly centrifuged and the resultant supernatants were quickly frozen in liquid nitrogen. Samples for the measurements of cyclic GMP were stored at −20°C until required. Each assay was performed in triplicate in three independent experiments. Cyclic GMP was measured in cell supernatants by radioimmunoassay (assay range from 50 to 6400 fmol) with commercially available antiserum (Pasteur-Diagnosis, Paris, France) and standard cyclic GMP (Sigma) as previously described (Arnal et al., 1993).

2.4.2. Effect of neutral endopeptidase inhibition on ANP degradation

Confluent cultures in 12-well plates were made quiescent by deprivation of serum for 48 h. In order to determine immunoreactive ANP, the cells were washed with phosphate buffer. Then, serum-free medium with increasing doses of ANP (10^{-12} to 10^{-6} M) was added to the intact cell layers in the presence of retrothiorphan (neutral endopeptidase inhibitor, 10^{-7} M) or its diluent (control; 10% ethanol in medium). After 120 min of incubation at 37°C under CO_2 atmosphere, the cell supernatant was collected. Samples of cell supernatant were briefly centrifuged and the resultant supernatants were quickly frozen in liquid nitrogen. Samples were stored at −80°C until required. Each assay was performed in triplicate in three independent experiments. ANP was measured in cell supernatants by radioimmunoassay using [^{125}I]ANP-(1-28) (assay range from 3 to 200 pg) and rat standard ANP-(1-28) (Bouissou et al., 1989).

2.4.3. [^{3}H]arachidonic acid release

Arachidonic acid release was measured as previously described (Pueyo et al., 1996). Briefly, cells were cultured to confluence in 24-well plates, and then each well was labelled with 0.5 μCi [^{3}H]arachidonic acid (Amersham) in Dulbecco's minimum essential medium for 16–24 h. Cells were then rinsed several times in order to eliminate unincorporated radioactivity. Cells were stimulated with the addition of increasing concentration of bradykinin (10^{-9} M to 10^{-6} M) in the presence or in the absence of a neutral endopeptidase inhibitor, retrothiorphan (10^{-7} M) and/or a converting enzyme inhibitor, captopril (10^{-7} M) for 20 min. After incubations, the medium was collected and radioactivity determined. Each assay was performed in triplicate in four independent experiments.

2.4.4. Degradation of bradykinin

The degradation of bradykinin was measured by using intact cells seeded into six-well plates and grown to confluency. Cells were made quiescent by deprivation of serum for 48 h. Retrothiorphan (10^{-7} M) and/or captopril (10^{-7} M) were added to the incubation medium, 15 min before the addition of bradykinin in serum free-medium (containing amastatin 10^{-5} M and MGTA 10^{-4} M), to a final concentration of 10^{-8} M (~ 10 000 pg ml^{-1}). Samples of 0.3 ml were taken at 1, 3, 5, 8 and 24 h, immediately frozen in liquid nitrogen and stored at −80°C until further use. Each assay was performed in triplicate in four independent experiments. Bradykinin was measured by radioimmunoassay with [^{125}I][Tyr]-bradykinin (assay range 1.6 to 200 pg) (Alhenc-Gelas et al., 1981).

2.5. Statistics

Results are expressed as mean ± S.E. Factorial two-way analysis of variance (ANOVA) for repeated measures was performed to test the interaction of agonist doses and treatment, or time and treatment, on the different variables. Factorial one-way ANOVA (Scheffé's Test) was then performed to test the effects of inhibitors. Neutral endopeptidase protein levels were compared by unpaired *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Characterization of neutral endopeptidase

3.1.1. Aortic layers

The presence of a D-alanine in the substrate and the addition of 10^{-5} M bestatin, an aminopeptidase inhibitor, and 10^{-6} M captopril, a converting enzyme inhibitor, prevented degradation of the substrate by aminopeptidase and angiotensin-converting enzyme. Neutral endopeptidase activity was detected in the supernatants containing the solubilized proteins (whole aorta and three aortic layers) (Fig. 1A). The adventitia contained the highest amount of neutral endopeptidase activity, i.e., 70% of the activity found in the whole aorta. The neutral endopeptidase activity in the endothelium and in the media reached 19% and 20% respectively, corresponding to the values found in the whole aorta. To further establish the presence of neutral endopeptidase in the three wall layers, the solubilized preparations were incubated with [^{125}I]RB104 a specific neutral endopeptidase inhibitor. This molecule is bound to a single protein in the solubilized preparations from the whole aorta and from the three layers, with the same apparent molecular mass (94 kDa) as the pure rabbit kidney NEP (Fig. 1A). This binding was completely inhibited by the addition of 0.1 mM retrothiorphan (data not shown). The amount of NEP present was higher in the adventitia (15.2 ± 2.2 pg NEP/μg protein) than in the media (2.5 ± 0.3 pg NEP/μg protein) and in the endothe-

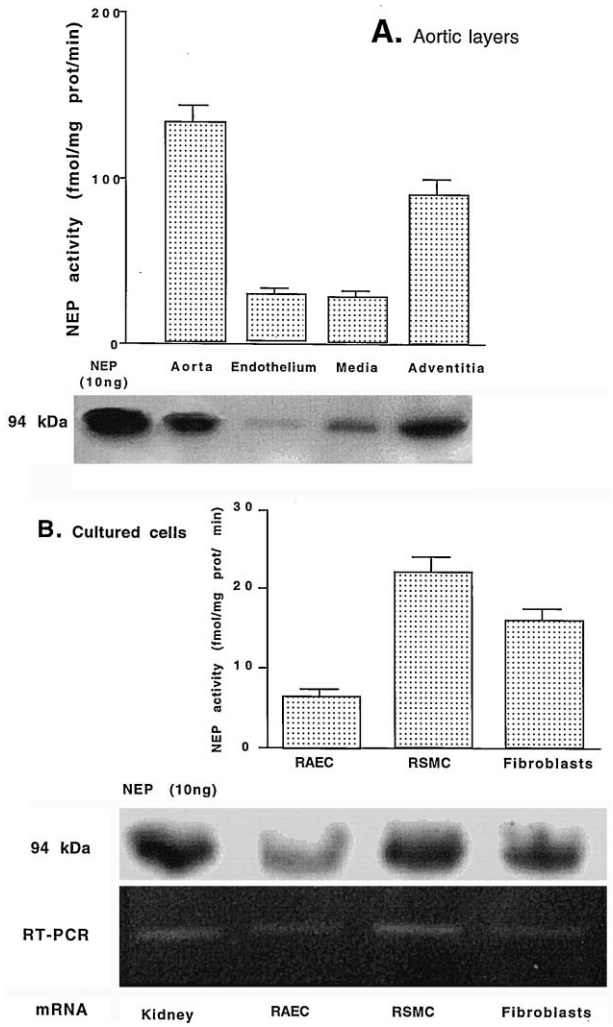

Fig. 1. Characterization of neutral endopeptidase. (A) Characterization of neutral endopeptidase (NEP) in solubilized proteins from the aorta, endothelium, media and adventitia. NEP activity was determined with 20-nM [^{3}H][D-Ala^2,D-Leu^5]-enkephalin, in the presence (blank tubes) or absence of 10^{-7} M retrothiorphan (neutral endopeptidase inhibitor). Data of neutral endopeptidase activity are mean ± S.E. ($n = 6$–8). Representative autoradiogram obtained after separation of pure rabbit kidney NEP solubilized proteins from whole aorta (50 μg) and from the three rat aortic layers (100 μg) by SDS-PAGE. After electrophoresis, the gel was incubated with 100 pM [^{125}I]RB104, as described in Section 2. The experiment was performed three times, with reproductive results. (B) Neutral endopeptidase activity was determined as described above in endothelial (RAEC), smooth muscle cells (RSMC) and fibroblasts. Data of neutral endopeptidase activity are mean ± S.E. ($n = 6$–8). Representative autoradiogram of protein binding assay is as described above. Neutral endopeptidase mRNA after reverse transcription-polymerase chain reaction (RT-PCR) of total RNA from cells (0.5 μg) and rat kidney (*K*; 0.5 μg). Amplified products were electrophoresed and visualized under UV light. PhiX 174 RF DNA-*Hae*III digest was used as size markers. The experiments was performed four times, with reproductive results.

lium (0.4 ± 0.1 pg NEP/μg protein, $P < 0.001$). Quantification of the autoradiograms was obtained by comparing the levels to the counts associated with a definite quantity (10 ng) of pure kidney neutral endopeptidase.

3.1.2. Primary cultures of vascular cells

After the establishment of neutral endopeptidase activity and protein in the three aortic layers, the presence of the peptidase in the three derived primary cell cultures was studied using the same method as described above. Neutral endopeptidase activity was found in quiescent primary rat aortic endothelial cells, smooth muscle cells and fibroblasts (Fig. 1B). Neutral endopeptidase activity was higher in smooth muscle cells and fibroblasts than in endothelial cells ($P < 0.05$). Neutral endopeptidase was also characterized by its binding with [^{125}I]-RB104. Fig. 1B also shows in the three cell types that this molecule is bound to a single protein, with the same apparent molecular mass as the pure rabbit kidney neutral endopeptidase (94 kDa). This binding was completely inhibited by the addition of 0.1 mM retrothiorphan (data not shown). Neutral endopeptidase content was higher in fibroblasts (89.4 ± 10.2 pg NEP/μg protein) and smooth muscle cells (86.8 ± 9.1 pg NEP/μg protein) than in endothelial cells (10.3 ± 0.9 pg NEP/μg protein, $P < 0.01$). Quantification of the autoradiograms was obtained by comparing the levels to the counts associated with a definite quantity (10 ng) of pure kidney neutral endopeptidase.

A specific transcript corresponding to the expected size (414 base pairs) was detected in endothelial, smooth muscle cells and fibroblasts after RT-PCR (Fig. 1B, bottom panel).

3.2. Neutral endopeptidase inhibition potentiated ANP-stimulated cyclic GMP production

Cyclic GMP is known to be the second messenger of ANP and it is released by cells by an active mechanism (Hamet et al., 1989). The addition of IBMX to the medium prevented the degradation of cyclic GMP by phosphodiesterases (Hamet et al., 1989). ANP increased extracellular cyclic GMP in a dose-dependent manner in endothelial (Fig. 2A) and smooth muscle cells (Fig. 2B). Specific neutral endopeptidase inhibition by retrothiorphan potentiated the increase in cyclic GMP production with low doses of ANP in endothelial (10^{-12} to 10^{-9} M; $P < 0.05$ vs. ANP alone) and smooth muscle cells (10^{-12} to 10^{-9}; $P < 0.05$ vs. ANP alone).

There was no response to ANP (cyclic GMP production) in fibroblasts with or without neutral endopeptidase inhibitor at any time or ANP doses under our experimental conditions (data not shown).

Neutral endopeptidase inhibition prevented the degradation of ANP in endothelial (Fig. 3A) and smooth muscle cells (Fig. 3B). In the presence of retrothiorphan (neutral endopeptidase inhibitor), significantly higher immunoreactive ANP concentrations were measured in the supernatant of endothelial and smooth muscle cells compared to ANP alone (10^{-12} to 10^{-9} M).

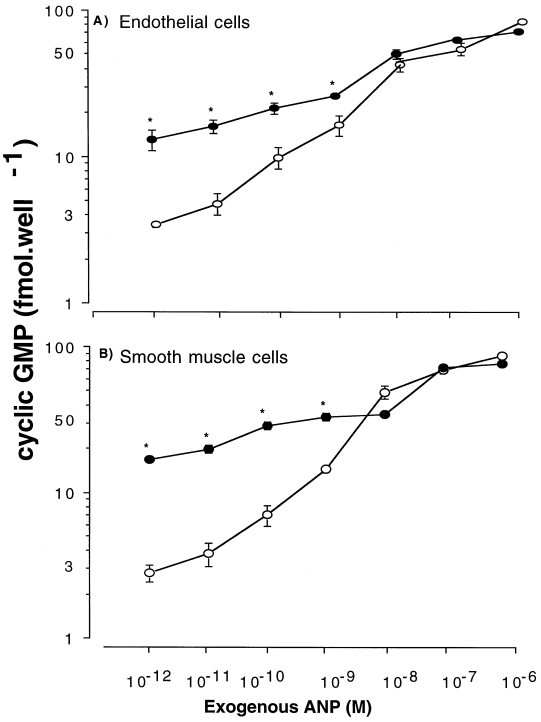

Fig. 2. Neutral endopeptidase inhibition potentiates ANP-stimulated cyclic GMP production in cells. Primary cells made quiescent by 48 h of incubation in serum-free medium. Dose response of exogenous ANP-induced rise in extracellular cyclic GMP levels in the absence (control, empty circle) or presence (plain circle) of retrothiorphan (neutral endopeptidase inhibitor) in rat aortic endothelial cells (panel A) and in rat aortic smooth muscle cells (panel B). Values are expressed as mean ± S.E. of three independent experiments performed in triplicate. * $P < 0.05$ vs. ANP alone at the same concentration.

3.3. Neutral endopeptidase inhibition potentiated bradykinin-induced arachidonic acid release

Phospholipase A_2 hydrolyses a phosphoglyceride producing lysophosphatidylcholine and arachidonic acid (Mombouli and Vanhoutte, 1995). The arachidonic acid release in response to bradykinin was evaluated in cultured endothelial cells and fibroblasts (Fig. 4). Addition of bradykinin to the cultured endothelial cells induced a dose-dependent increase in arachidonic acid release (Fig. 4, top panel). Neutral endopeptidase inhibition potentiated bradykinin effect in endothelial cells ($P < 0.05$). Angiotensin-converting enzyme inhibition also potentiated bradykinin effect on arachidonic acid release ($P < 0.05$). Concomitant inhibition of angiotensin-converting enzyme and neutral endopeptidase induced a better potentiation on arachidonic acid release than neutral endopeptidase inhibition alone at 10^{-7} and at 10^{-6} M bradykinin doses ($P < 0.05$).

Addition of bradykinin to the cultured fibroblasts induced a dose-dependent increase in arachidonic release (Fig. 4, bottom panel). The inhibition of NEP only potentiated bradykinin effect at 10^{-7} and at 10^{-6} M bradykinin doses. The same effect occurred with the inhibition of angiotensin-converting enzyme. The addition of both inhibitors induced a higher arachidonic acid release (10^{-8} to 10^{-6} M of bradykinin) than was seen with either neutral endopeptidase or angiotensin-converting enzyme inhibition alone ($P < 0.05$ vs. both inhibitors; Fig. 4, bottom panel).

The addition of Hoe 140 (antagonist of bradykinin B_2 receptors), before the addition of bradykinin, inhibited the increase in arachidonic acid release in both endothelial cells and fibroblasts (data not shown).

There was no response to bradykinin (arachidonic acid release) in smooth muscle cells with or without neutral endopeptidase inhibitor at any time under our experimental conditions (data not shown).

3.4. Degradation of bradykinin

Neutral endopeptidase activity accounts for the major part of bradykinin degradation in the kidney (Ura et al., 1987; Pham et al., 1996). However, the role of neutral endopeptidase in bradykinin metabolism in vascular cells is not precisely known. The involvement of neutral endopeptidase and angiotensin-converting enzyme in bradykinin degradation was studied in endothelial cells and

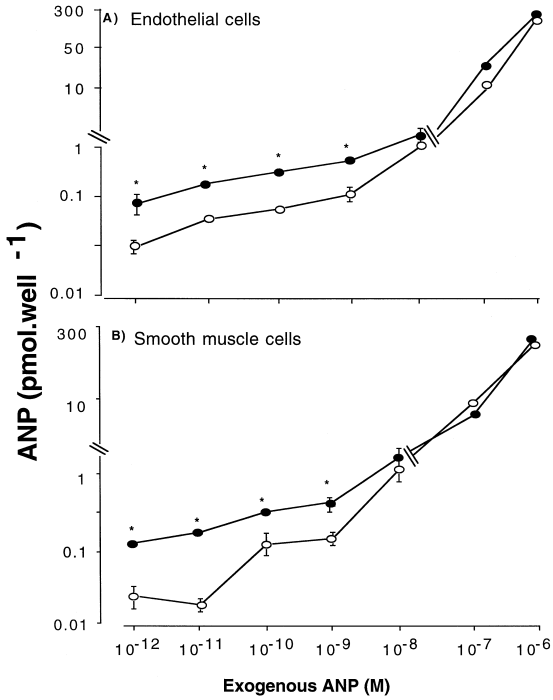

Fig. 3. Neutral endopeptidase inhibition prevents ANP degradation in cell cultures. Primary cells made quiescent by 48 h of incubation in serum-free medium. Determination of immunoreactive ANP in the supernatant of cells 120 min after addition of increasing concentrations of exogenous ANP in the absence (control, empty circle) or presence (plain circle) of retrothiorphan (neutral endopeptidase inhibitor) in cultured rat aortic endothelial (panel A) and in rat aortic smooth muscle cells (panel B). Values are expressed as mean ± S.E. of three independent experiments performed in triplicate. * $P < 0.05$ vs. ANP alone at the same concentration.

fibroblasts using specific inhibitors. The addition of 10^{-5} M amastatin and 10^{-4} M MGTA prevented the degradation of bradykinin by aminopeptidase and carboxypeptidase N, respectively. Fig. 5 (top panel) shows the decrease of 10^{-8} M ($\sim$ 10 000 pg ml^{-1}) exogenous bradykinin in a time-dependent manner in quiescent endothelial cells. Inhibition of neutral endopeptidase by retrothiorphan significantly reduced bradykinin degradation ($P < 0.05$ vs. control) in endothelial cells (Fig. 5, top panel). The angiotensin-converting enzyme inhibitor, captopril, decreased the degradation of bradykinin by intact endothelial monolayers to a greater extent compared to retrothiorphan alone ($P < 0.05$). Incubation in the presence of both retrothiorphan and captopril induced a higher decrease in bradykinin degradation compared to either retrothiorphan or captopril alone ($P < 0.05$ vs. either inhibitor alone).

Bradykinin progressively disappeared in the medium of quiescent fibroblasts (Fig. 5, bottom panel). Inhibition of angiotensin-converting enzyme by captopril significantly reduced bradykinin degradation from 1 h to 8 h ($P < 0.05$ vs. control; Fig. 5, bottom panel). Inhibition of neutral endopeptidase by retrothiorphan significantly reduced bradykinin degradation in cultured fibroblasts ($P < 0.05$ vs. control). Neutral endopeptidase inhibition reduced bradykinin degradation significantly more than angiotensin-converting enzyme inhibition at 8 and 24 h did ($P < 0.05$). The concomitant inhibition of neutral endopeptidase and angiotensin-converting enzyme also significantly prevented bradykinin degradation in fibroblasts, to the same extent as neutral endopeptidase inhibition alone (Fig. 5, bottom panel).

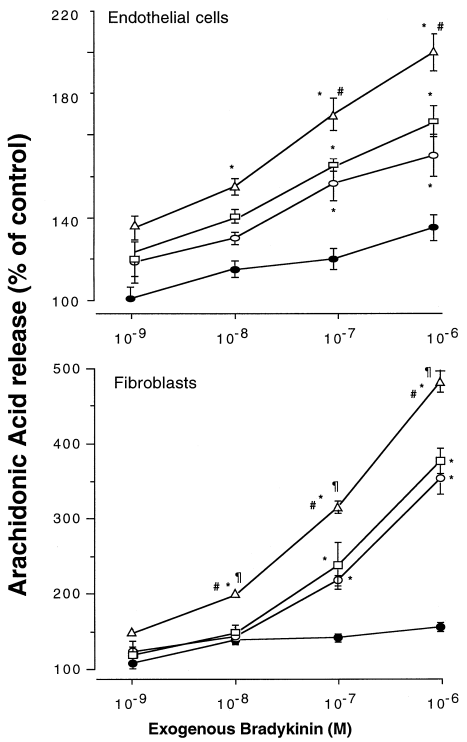

Fig. 4. Bradykinin-stimulated arachidonic acid release in endothelial cells and fibroblasts. Stimulation of [^{3}H]-arachidonic acid release in rat aortic endothelial cells (top panel) and fibroblasts (bottom panel) by bradykinin in the absence (control, plain circle) or the presence of retrothiorphan (empty circle, neutral endopeptidase inhibitor) or captopril (square; angiotensin-converting enzyme inhibitor) or the combination of retrothiorphan and captopril (triangle). Increases in arachidonic acid release are given as the ratio of the values for stimulated and unstimulated cells and represent the mean $\pm$ S.E. of four independent experiments performed in triplicate; $*$ $P < 0.05$ vs. bradykinin alone, $^{\#}P < 0.05$ vs. retrothiorphan, $^{\P}P < 0.05$ vs. captopril.

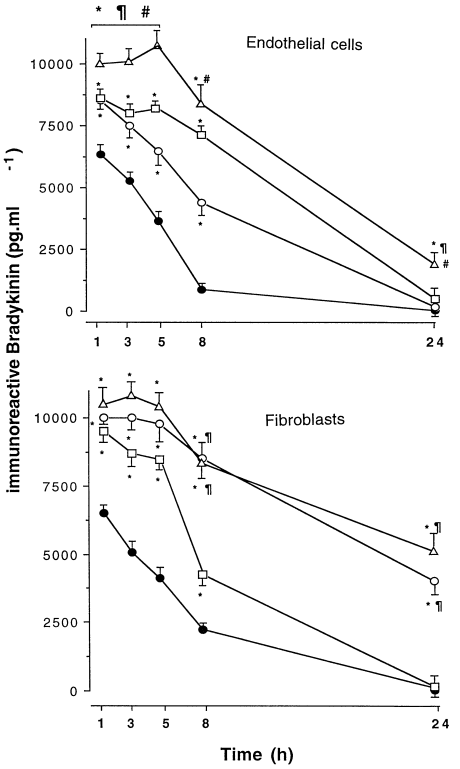

Fig. 5. Metabolism of bradykinin in endothelial cell and fibroblast media. Cells made quiescent by 48 h of incubation in serum-free medium. Time course of degradation of exogenous bradykinin (10^{-8} M $\sim$10,000 pg ml^{-1}) in the absence (control, plain circle) or the presence of retrothiorphan (empty circle; neutral endopeptidase inhibitor) or captopril (square; angiotensin-converting enzyme inhibitor) or the combination of retrothiorphan and captopril (triangle) in the supernatant of rat aortic endothelial cells (top panel) or fibroblasts (bottom panel). Mean $\pm$ S.E. of four independent experiments performed in triplicate; $*$ $P < 0.05$ vs. bradykinin alone, $^{\P}P < 0.05$ vs. captopril, $^{\#}P < 0.05$ vs. retrothiorphan.

4. Discussion

These studies demonstrate the presence of neutral endopeptidase in the three layers of rat aorta and in derived vascular cells. Using enzyme activity and protein binding method, these studies show the higher expression of neutral endopeptidase in the adventitia. Neutral endopeptidase levels in the adventitia were 3- to 4-fold higher than in endothelium by enzymatic assay, whereas they were 30- to

40-fold higher by protein binding assay. This discrepancy can be attributed to the high sensitivity of the protein binding assay. Such a discrepancy for neutral endopeptidase between the results of enzyme assays and protein detections has already been reported (Dussaule et al., 1992; Soleilhac et al., 1992). In cultured cells, derived from the corresponding layer, fibroblasts and smooth muscle cells contained a higher neutral endopeptidase activity and protein levels than endothelial cells. Inhibition of neutral endopeptidase prevented ANP and bradykinin from degradation and potentiated peptide signalling in vascular cells. In endothelial cells, angiotensin-converting enzyme was the main pathway in bradykinin degradation, whereas neutral endopeptidase was the main pathway in fibroblasts.

Tamburini et al. (1989) have previously reported that a peptidase with the characteristics of neutral endopeptidase is present in the homogenate of rat mesenteric artery, while Soleilhac et al. (1992) reported the presence of the mentioned peptidase in rabbit aorta. This study is the first to show the presence of neutral endopeptidase in the all three layers of rat aorta. Moreover, we attempted to characterize neutral endopeptidase quantitatively in the intimal endothelium, the media, and the adventitia. The presence of neutral endopeptidase in the three tunicae suggests the involvement of the peptidase in the endothelial metabolism of circulating neuro or vasoctive peptides and it also suggests the implication of neutral endopeptidase in the local regulation of neuro or vasoactive peptide levels in the vascular wall.

The importance of neutral endopeptidase in the in vivo metabolism of ANP is now well-established. Neutral endopeptidase inhibitors induce natriuresis and vasodilatation in humans and animals by the potentiation of endogenous ANP and bradykinin (Pham et al., 1993; Richards et al., 1993). Protection of ANP by neutral endopeptidase inhibition produces an increase in both plasma and urinary ANP and cyclic GMP (Pham et al., 1993; Richards et al., 1993). The potentiation of bradykinin after neutral endopeptidase inhibition induces an increase in urinary bradykinin, cyclic GMP and prostaglandins (Ura et al., 1987; Pham et al., 1996). In vitro, neutral endopeptidase hydrolyses various inflammatory and vasoactive peptides such as substance P, bradykinin, ANP, C-type natriuretic peptide, angiotensin and endothelin (Roques et al., 1993). However, the implication of neutral endopeptidase in the in vivo metabolism of these peptides has not been clearly established.

The main site for neutral endopeptidase-induced degradation of filtered vasoactive and natriuretic peptides is the kidney. Experiments performed on ANP metabolism in binephrectomized rats suggested that there are sites other than the kidney for ANP degradation (Barclay et al., 1991). Previous studies detected neutral endopeptidase in cultured endothelial cells from the aorta and umbilical veins (Graf et al., 1995; Llorens-Cortes et al., 1992) and in smooth muscle cells originated from rabbit kidney (Dussaule et al., 1992). Our results extend those previous studies showing that the intimal endothelium and derived primary endothelial cells express neutral endopeptidase. In addition, our results show that smooth muscle cells cultured from media and fibroblasts cultured from adventitia also express neutral endopeptidase.

In addition to its natriuretic effect, ANP is a vasoactive peptide able to inhibit smooth muscle cell growth (Abell et al., 1989) depending on its availability to its target cells. Hamet et al. (1989) showed that ANP action was mediated by cyclic GMP production in endothelial and smooth muscle cells. We evaluated the implication of neutral endopeptidase in ANP metabolism in cultured cells. There was no response of fibroblasts to ANP in our experimental preparation. This may be due to the absence of active ANP receptors. We showed that protection of ANP from neutral endopeptidase hydrolysis potentiated cyclic GMP production by endothelial and smooth muscle cells. Circulating ANP may bind to the endothelium, where clearance receptors are present. It may also be metabolized by endothelial neutral endopeptidase, in particular, when ANP levels are increased. Due to the metabolic barrier role of endothelium, the accessibility of ANP to smooth muscle is probably limited under physiological conditions. Even if ANP reaches to smooth muscle cells, the neutral endopeptidase present in these cells may inactivate the peptide and limit its interaction with its receptors. On the other hand, other peptides, such as C-type natriuretic peptide and endothelins, are directly synthesized in the vascular wall and they influence endothelial and smooth muscle cell proliferations. These peptides could act in an autocrine or paracrine way and their local concentrations in the vascular wall may be regulated by the neutral endopeptidase present in smooth muscle cells and fibroblasts.

Neutral endopeptidase degradation is considered a major pathway in renal bradykinin metabolism (Ura et al., 1987; Pham et al., 1996). Bradykinin is also involved in the inflammatory response. There was no response of smooth muscle cells to bradykinin under our experimental conditions. This may be due to the absence of bradykinin receptors as the cells responded to angiotensin II stimulation (data not shown). We showed that bradykinin effect on arachidonic acid release was highly potentiated by neutral endopeptidase and angiotensin-converting enzyme co-inhibition, showing that inhibition of both metallopeptidases may be required for a complete enhancement of bradykinin action in endothelial cells. Our data also demonstrate that neutral endopeptidase is the main enzyme involved in bradykinin degradation in vascular fibroblasts derived from rat adventitia.

In conclusion, the microdissection of rat aorta allowed us to study and compare the levels of neutral endopeptidase both in isolated cells and in cell culture. The present study demonstrates that neutral endopeptidase is expressed in the three layers of rat aorta and in the derived primary cells. Higher expression was seen in the adventitia than in the media and endothelium. In fibroblasts, the peptidase is

the main enzyme involved in the metabolism of bradykinin. The peptidase potentiated ANP action in endothelial and smooth muscle cells. The constitutive expression of neutral endopeptidase in the vascular wall suggests a role of this peptidase in the modulation of local vasoactive peptide levels in the immediate vicinity of their cell targets.

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