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INCREASE OF NEUTRAL ENDOPEPTIDASE-24.11 WITH CELLULAR DENSITY AND ENZYME MODULATION WITH AN INHIBITOR ON HUMAN REH6 CELL LINE

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Abstract—Neutral endopeptidase (EC 3.4.24.11, NEP) is an ectoenzyme, identified as the common acute lymphoblastic leukemia antigen (CALLA, CD10). This enzyme is involved in the inactivation of regulatory peptides such as enkephalins and atrial natriuretic peptide and its expression on the cell surface is therefore essential. NEP levels have been measured under different conditions on leukemic cell lines. NEP activity per cell was found to increase during the cell growth of Reh6 and CEM cells, a cell–cell contact mechanism being suggested by experiments using Transwell cell chambers. The same process was not observed with ICIG-7 fibroblasts. The numbers of enzymatic sites was also found to be selectively modulated by treatment with 0.1 μM *N*-[3-(*R,S*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine (HACBOGly), a potent (K_i = 1.4 nM) and specific inhibitor of NEP. A maximal 13% decrease in sites was observed after 8 hr incubation, this effect disappearing after 12 hr. This weak but specific negative modulation was not observed with a compound, chemically related to HACBOGly, which has a 10,000-fold lower inhibitory potency. The modulation was inhibited by low temperature or monensin treatment and could be brought about by an internalization of the enzyme, compensated for by an increased biosynthesis or by the sequestration of NEP in a non-membranous compartment.

Neutral endopeptidase-24.11 (EC 3.4.24.11, NEP†) is a mammalian ectoenzyme, identified in 1988 as the common acute lymphoblastic leukemia antigen (CALLA, CD10) [1], a differentiation marker of B lymphocytes. This zinc-metallopeptidase, first isolated from rabbit kidney in 1974 [2], is localized in many tissues including the central nervous system [3], neutrophils [4], human fibroblasts [5], melanoma [6], glioma [7] and lymphoid cell lines [8]. NEP inactivates a variety of biologically active peptides, the best documented substrates of the enzyme being the enkephalins [9] and atrial natriuretic peptide [10]. NEP inhibitors have been shown in consequence to act like antinociceptive and antihypertensive drugs (reviewed in Ref. 11).

NEP is essentially localized on the cell surface, but little is known about the modulation of its expression. Several groups have shown a decrease in NEP activity after treatment of cells with phorbol esters [12–14]. A modification of enzymatic activity on ligament fibroblasts has also been observed, which depended on the type of subcellular matrix used to grow the cells [15]. An increase in NEP activity was found both during the growth of NEP-transfected epithelial cells and after budesonide treatment, this glucocorticoid also increasing NEP gene expression [16]. A rapid internalization of NEP after treatment with anti-CALLA monoclonal antibody has also been shown using leukemic cells [17]. More recently, NEP modulation has been described during ontogeny in a large number of organs including the fetal spinal cord [18, 19], suggesting a role for the enzyme in cellular differentiation.

The effects of NEP inhibitors on the membrane expression of the enzyme have been evaluated using Reh6 human leukemic cells. After 8 hr treatment at 37° with *N*-[3(*R,S*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine (HACBOGly) [20], a weak but specific negative modulation was observed which was inhibited by incubation at 15° and treatment with 0.1 μM monensin. With the same cell line, the NEP activity per cell was also found to increase with cell density, probably due to intercellular contact. A similar increase was observed with leukemic CEM cells, but not with ICIG-7 fibroblasts.

MATERIALS AND METHODS

N- [(*R,S*)- 2 -mercaptomethyl- 1- oxo- 3- phenylpropyl]glycine (thiorphan) [21], HACBOGly [20], ((*R*) - *N*[(*R,S*) - 2 - carboxy - 1 - oxoethyl]phenylalanine-*N'*-hydroxyamide) (JFH6) [22] and captopril [23] were synthesized in our laboratory as described previously. RPMI 1640, fetal calf serum and antibiotics were purchased from Gibco (France), chloroquine and monensin were from Sigma (France). [^{3}H]D-Ala-leucine-enkephalin and

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† Abbreviations: CALLA, common acute lymphoblastic leukemia antigen; NEP, neutral endopeptidase-24.11; thiorphan, *N*-[(*R,S*)-2-mercaptomethyl-1-oxo-3-phenylpropyl]glycine; JFH6, (*R*)-*N*[(*R,S*)-2-carboxy-1-oxopropyl]phenylalanine *N'*-hydroxyamide; HACBOGly, *N*-[3(*R,S*)-[(hydroxyamino)carbonyl]-2-benzyl-1-oxopropyl]glycine.

[^{3}H]HACBOGly (45 Ci/mmol) [24] were from the CEA (France) and bestatin was from Bachem (Switzerland). Transwell cell chambers were purchased from Costar (France).

Cell lines. Reh6 and CEM cell lines and ICIG-7 fibroblasts were generous gifts of C. Boucheix (U268 INSERM, France), S. Saragosti (U52 INSERM, France) and B. Azzarone (U268 INSERM, France), respectively. Reh6 [25] is derived from early stages of the B cell lineage and CEM is a T lymphoid cell line [26]. ICIG-7 cells are human embryonic lung fibroblasts [27]. The three cell lines express the NEP/CD10 antigen although it is present at lower levels at the surface of the CEM cells. Reh6 and CEM cell lines were cultured in RPMI medium and ICIG-7 fibroblasts in minimum essential medium, all supplemented with 10% fetal calf serum and antibiotics. Cells were maintained at 37° in a mixture of air and CO_2 (19:1).

Assay for endopeptidase 24.11 *activity.* Cells were centrifuged at 650 *g* for 10 min at 4° and the pellet homogenized in 50 mM Tris–HCl pH 7.4. The homogenates were first preincubated for 10 min with 10 μM bestatin and 1 μM captopril (inhibitors of aminopeptidase and angiotensin-converting enzyme, respectively). One micromolar of the NEP inhibitor thiorphan was included in the controls. NEP activity was measured using 20 nM D-Ala^2[tyrosyl-[^{3}H]-leucine]-enkephalin as substrate in 100 μL of 50 mM Tris–HCl pH 7.4 at 25° for 30 min. The reaction was stopped by addition of 10 μL of 0.5 M HCl. The metabolite [^{3}H]tyrosyl-D-alanyl-glycine was separated from intact substrate using Porapak beads (Waters) as described previously [28], eluted directly into scintillation vials and radioactivity was counted after addition of 15 mL of NEN Biofluor emulsifier cocktail.

Transwell cell chamber utilization. Transwell cell chambers, with a 0.4-μm cut-off polycarbonate membrane, were placed in cluster plates. Reh6 cells were seeded at a density of 1×10^5 cells/mL in RPMI medium in the upper compartment and the lower compartment contained either RPMI medium alone or a high cellular density of Reh6 cells (1×10^6 or 7×10^5 cells/mL for 24 or 72 hr of incubation, respectively). The NEP activity of the cells in the upper compartment was measured as described above.

Modulation of NEP in Reh6 cells by HACBOGly. Cells were seeded in 75 cm^2 flasks at a density of 1×10^5/mL, cultured for 72 hr so as to be in their exponential growth phase and incubated for different times with or without 0.1 μM HACBOGly (IC_{50} = 1.4 nM for NEP). Different incubations were then harvested, rinsed three times by centrifugation (650 *g* for 10 min) with resuspension in RPMI medium and used for binding assays as described below. Reh6 cell membranes were prepared by homogenizing the harvested cells in 50 mM Tris–HCl pH 7.4 and centrifuging at 100,000 *g* for 1 hr at 4°. The pellets were washed twice by homogenizing in Tris buffer and centrifuging as before. The final pellets were homogenized in a strict volume of 50 mM Tris–HCl pH 7.4 to obtain the equivalent of 3.5×10^5 cells/mL.

In some cases, HACBOGly was replaced by its tritiated analogue to ensure that all the inhibitor was removed during the washing procedure. Taking account of the specific activity of [^{3}H]HACBOGly (45 Ci/mmol), it was estimated that about 0.5% of the sites were still occupied after the washing and homogenizing procedure. No observable degradation of HACBOGly occurred during 48 hr (controlled by measuring the IC_{50} of the inhibitor, recovered from the supernatant, for purified NEP). Protein concentration was determined by the method of Bradford [29].

[^{3}H]*HACBOGly binding to neutral endopeptidase* 24.11. Membrane preparations were incubated with 5 and 7 nM [^{3}H]HACBOGly at 35° for 45 min in 1 mL of 50 mM Tris–HCl pH 7.4. Non-specific binding was determined in the presence of 1 μM thiorphan. The incubations were terminated by rapid filtration over Whatman GF/B filters which were washed twice with 5 mL of ice-cold buffer, dried and transferred to scintillation vials. Bound radioactivity was determined after addition of 5 mL Beckman Ready-solvant EP.

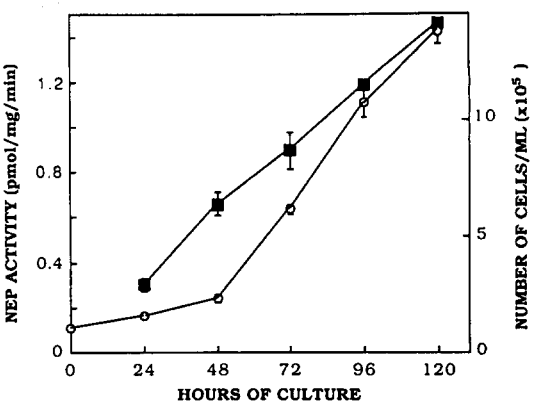

Fig. 1. Relationship between cell growth (unfilled circle) and NEP activity (filled square) of Reh6 cells. Cells were seeded at a density of 1×10^5 cells/mL and cultured for 120 hr. The cells grew slowly for 24 hr and the exponential phase began after 48 hr of culture. Enzymatic activity started to increase at 24 hr. Each point is the average of three independent experiments.

RESULTS

Constitutive modulation of neutral endopeptidase-24.11 activity

The NEP activity of Reh6 cells was investigated during their growth under standard culture conditions. The cells were seeded at a density of 1×10^5/mL and during the exponential growth phase, which started at 48 hr, cellular density doubled every 24 hr. Between 24 and 120 hr, the specific activity of NEP, expressed in pmol/mg protein/min, increased about 4-fold, from 0.3 to 1.3 (Fig. 1). Similar results were obtained if the activity was expressed as pmol/10^6 cells/min. In the culture medium, activity was very low (~1 fmol/mg protein/min) and increased only slightly during cell growth

Table 1. Reh6 cell enzymatic activity in transwell culture chambers

Culture (hr)	Enzymatic activity in upper compartment (pmol/mg protein/min)	
	No cells in lower compartment	High cellular density in lower compartment
24	0.21 ± 0.01	0.23 ± 0.02
72	0.56 ± 0.07	0.51 ± 0.07

Cells were seeded at 1×10^5 cells/mL in the upper compartment. The lower compartment contained either medium alone or a high cellular density of Reh6 cells (1×10^6 cells/mL for 24 hr and 7×10^5 cells/mL for 72 hr). No difference in activity was observed in the upper compartment.

The results are the means of three separate experiments carried out in duplicate.

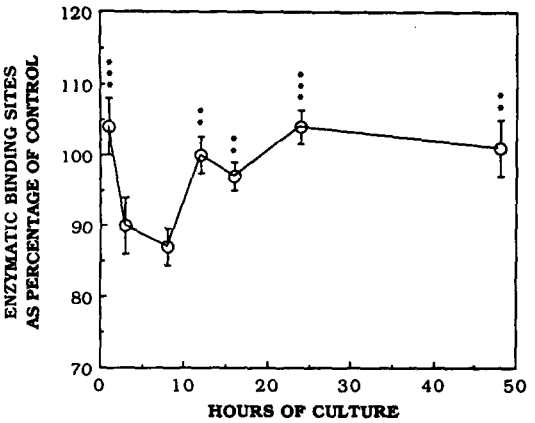

Fig. 2. HACBOGly-induced modulation of NEP on Reh6 cells. Cells were incubated with 0.1 μM HACBOGly at 37° and NEP levels were determined on membranes prepared from these cells by measuring specific binding of [^{3}H]-HACBOGly. Results are expressed as a percentage of enzymatic sites as compared to non-treated cells. Each point represents the mean of three independent experiments (six for the 8 hr of incubation time). *$P < 0.01$, **$P < 0.005$, ***$P < 0.001$ as compared to 8 hr treatment using the Student's *t*-test.

suggesting a low release of NEP in the medium, probably due to membrane shedding. When Reh6 cells were diluted to 10^5/mL after 96–120 hr of culture, specific enzymatic activity decreased to 0.3 pmol/mg/min in 24 hr. CEM lymphocytes grew faster than Reh6 cells, the exponential growth phase being finished after 96 hr of culture. However, a similar increase in NEP activity was obtained between 48 and 120 hr of culture (from 10 to 30 fmol/mg protein/min). No statistically significant increase in activity per mg protein or per cell was registered during the culture of ICIG-7 fibroblasts, each incubation time showing large variations. To ascertain if the increase in NEP activity was in response to a factor/factors released by the cells during the growth phase, transwell cell culture chambers were used as described in Materials and Methods. Enzymatic activity was measured in the upper compartment which was initially seeded with 1×10^5 cells/mL. Under these conditions, no difference in enzymatic activity was noted in the upper compartment, whatever the cellular density in the lower compartment (Table 1). This seems to indicate that no factor diffused through the polycarbonate membrane (pore diameter = 0.4 μm), the increase in enzymatic activity/cell therefore being probably due to a contact phenomenon between cells.

Effects of a NEP inhibitor on NEP cellular level

To determine the effects of an enzyme inhibitor on the NEP levels of Reh6 cells, the maximal number of binding sites (B_{max}) was measured after treatment of the cells with 0.1 μM HACBOGly for various times at 37°. Non-treated cells, cultured under the same conditions, were taken as control. As shown in Fig. 2, a weak (13%), but significant and reproducible negative modulation of enzymatic sites/cell was observed after HACBOGly treatment. The decrease in NEP levels began 3 hr after addition of the inhibitor to the culture medium, was maximal and significant at 8 hr and completely reversed at 12 hr. No further modulation was observed during the incubation with the inhibitor, which lasted for a total of 48 hr. Reh6 cells were also treated under identical conditions with JFH6, an inhibitor with a similar structure to HACBOGly but with a poor affinity for NEP (IC_{50} = 1 μM). Table 2 shows that after 8 hr of incubation with this inhibitor, there was no modulation of NEP activity as compared to HACBOGly ($P < 0.001$ in the Student's *t*-test). To further investigate the process of the negative modulation, the incubations with HACBOGly were carried out under different conditions, using temperature modification or agents which disrupt cellular traffic. No modulation was observed if the temperature was reduced to 15° (Table 2). The effects of lysosomotropic amines were also investigated. Neither chloroquine (30 μM) nor NH_4Cl (10 mM) significantly modulated the effect of HACBOGly on NEP levels (results not shown).

Table 2. Modulation of NEP expression on Reh6 cell line

Cell treatment		Enzymatic sites
NEP inhibitor	T (°C)	(% of control)
HACBOGly	37	87 ± 2.5
JFH6	37	100 ± 4†
HACBOGly	15	99 ± 4.5†
HACBOGly + monensin	37	102.5 ± 9*

Cells were treated with 0.1 μM HACBOGly at 37° or 15°, or with JFH6 at 37°. In some cases, 0.1 μM monensin was added to the culture medium with HACBOGly at 37°.

* $P < 0.01$, † $P < 0.005$, using the Student's *t*-test as compared to 8 hr treatment with HACBOGly at 37°.

However, when carboxylic ionophore monensin (0.1 μM) was included in the incubations, NEP levels did not differ significantly from controls (Table 2) ($P < 0.01$ as compared to HACBOGly treatment in the Student's *t*-test).

DISCUSSION

This study demonstrated that NEP levels on leukemic cell lines (Reh6 and CEM) are dependent on cellular density, the increase in the number of cells per milliliter leading to an increase in enzymatic activity per cell. Experiments with transwell culture plates suggested that the increased enzymatic activity was due to an intercellular contact mechanism rather than the release of factor/factors into the medium. The role of this cell–cell contact increase remains unknown, but cannot be attributed to the fact that the cells were growing in suspension, since the same increase in activity has been demonstrated in transfected human tracheal epithelial cells [16]. There are two possible reasons for this increase: (i) a modification in membrane structure, NEP active sites being masked from substrates when cells are at a low density in the medium [30]; and (ii) a role of NEP in the cleavage of some extracellular peptides or proteins involved in paracrine cell control. The reason why the same phenomenon was not observed using fibroblasts is unknown.

Until now, the only studies on NEP modulation have been carried out using phorbol esters or monoclonal antibodies. The specific interaction of anti-NEP monoclonal antibody with the enzyme often leads to a rapid internalization of the formed complexes. Phorbol esters also provoke a large decrease in NEP levels on cell membranes. This modulation was associated with NEP internalization, but was probably not due to a direct effect of phorbol myristate-13-acetate [12] as phorbol esters are essentially activating agents of protein kinase C.

NEP levels were also found to be modulated by a highly specific and potent inhibitor, HACBOGly. For this study, we used very sensitive binding techniques on crude membrane extracts, since active NEP is exclusively localized on plasma membranes [31]. Incubation of Reh6 cells with HACBOGly at 37° led to a small but significant increase in the number of membrane enzymatic sites (maximal at 8 hr). This phenomenon was shown to be due to a specific action of the inhibitor on NEP, as a chemical analog with a low affinity for the enzyme had no effect. The reversibility of the modulation could correspond to a desensitization of the modulation mechanism of NEP to an inhibitor, which persisted throughout the whole experiment (48 hr).

The negative modulation of NEP was absent at 15°. At this temperature, it has been reported that some post endosomal events are inhibited (reviewed in Ref. 32). Considering the fact that active NEP is essentially localized on plasma membranes, the inhibition of these events is not sufficient to explain the inhibition of NEP modulation. Nevertheless, it is important to note that most of the studies have concerned transport in epithelial cells, little being known about the traffic in lymphoid cells. A decrease in the intracellular transport or membrane fluidity might occur in this type of cell at 15°, explaining the loss of NEP modulation.

Monensin also significantly inhibited NEP modulation (Table 2). This compound is known to modify cellular traffic, its best documented effect being to slow the intracellular transport of newly synthesized secretory proteins, proteoglycans and plasma membrane glycoproteins [33]. It also reduces the rate of endocytosis, endosome recycling and clustering [34]. NEP clustering is unlikely to be involved in the negative modulation, since this membrane phenomenon is not observed with a fluorescent inhibitor (unpublished results). On the other hand, blocking the cellular transport of the peptidase could account for the effects of monensin on NEP modulation if the enzyme is internalized after inhibitor treatment. Two principal mechanisms could be proposed: (i) an inhibitor-evoked sequestration of NEP, analogous to that reported for β-adrenoreceptor incubated with agonists [35], but characterized by a slower rate. NEP would be transiently removed from plasma membrane, the cellular compartment involved in this mechanism remaining unknown. (ii) Internalization and metabolism of NEP complexed with HACBOGly, accelerating the cellular processing of the enzyme and leading to a negative modulation. In this model, the decrease in NEP levels could be eventually compensated for by an increased biosynthesis of the enzyme, a slow process explaining the delay in the recovery of normal levels of enzymatic sites. The blockade of inhibitor-induced reduction of NEP levels by monensin might suggest an initial internalization of the enzyme.

In conclusion, this study has shown the constitutive modulation of cellular NEP on two cell lines and, for the first time, an inhibitor-induced specific modulation of membrane NEP expression. However, considering the weak and transient effects of HACBOGly, the *in vivo* use of inhibitors should not modify dramatically NEP expression on lymphoid cells. Further information on the modulation of NEP should be given by microscopic studies, using a fluorescent inhibitor now in progress in the laboratory.

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