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Ascorbic acid decreases neutral endopeptidase activity in cultured osteoblastic cells

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Abstract

Neprilysin (NEP) is a plasma membrane-bound peptidase with wide expression in kidney, lung, brain and bone. Decreased NEP activity has been linked to increased growth of some cancer cells, but it is unknown whether its activity is related to growth of cells belonging to the osteoblast lineage. We assessed NEP activity in an osteoblastic cell line, PyMS, by cleavage of *N*-Dansyl-D-Ala-Gly-*p*-nitro-Phe-Gly to Dansyl-D-Ala-Gly. NEP activity was completely blocked by 1 μM thiorphan. Most agents affecting growth of these cells (e.g. calcium, insulin-like growth factor I and dexamethasone) did not regulate NEP activity. Ascorbic acid (ASA) increased thymidine incorporation into DNA and potentiated the stimulatory effect of IGF I on DNA synthesis, an effect which was attenuated by echistatin. ASA decreased NEP activity in a dose-dependent manner, and decreased Western-detectable NEP protein in plasma membranes. ASA affects both integrin receptor-mediated signalling and the processing of regulatory peptides.

Keywords: Osteoblasts; NEP; Ascorbic acid; Growth

1. Introduction

Neprilysin (neutral endopeptidase EC 3.4.24.11., NEP) is a cell surface membrane-bound glycoprotein (also designated CD 10), a thermolysin-like zinc-dependent endopeptidase, converting regulatory peptides. NEP is identical to common acute lymphoblastic leukemia antigen (CALLA) [1–3]. The number of more recently identified related enzymes belonging to the same (M13) family has increased, and some substrates and products of the enzyme(s) have been defined [4–7]. NEP is widely expressed throughout most mammalian tissues; it is particularly abundant in kidneys (originally identified as an antigen of renal membranes) and lungs [8,9], but also in brain (characterized as enkephalinase) and bone [10] where it is expressed by cells belonging to the osteoblastic lineage [11–15]. NEP is also expressed by human osteoblast-like cells [16–18]. Since bone is a recognised metastatic site for some common and aggressive cancers (lung, breast, prostate), NEP expression by osteoblasts may not only play a role in bone formation and bone resorption [12] but, in addition, for growth of malignant cells with an avidity for bone [19,20]. The NEP protein is anchored to the plasma membrane, and its active site is extracellular, where it cleaves peptides at the cell surface. NEP plays a role in a variety of biological processes and diseases, such as neurotransmission, inflammation and pain, blood pressure, cell growth, Alzheimer's, cardiovascular and neoplastic diseases. NEP inactivates natriuretic factor in kidney, substance P in the lung, and enkephalins in the brain [21]. NEP plays a role in the control of tumor (lung and prostate cancer) cell growth [22–29] by inactivating mitogenic peptides such as bombesin, bradykinin, calcitonin, endothelin-1, neurotensin and vasoactive intestinal peptide (VIP). However, it is unclear to what extent it may also be related and possibly contribute to the growth of bone cells. We, therefore, sought to study expression and activity of the enzyme in a rat (pre)

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osteoblastic bone cell line to see whether its activity was related to growth. To this end, we tested a series of compounds with known activity on DNA synthesis in this particular cell line, considering the possibility that agents affecting growth of the cells might alter expression of NEP since its substrates could be mitogenic by themselves or transactivate signalling responses of mitogens in the cells [30,31].

2. Materials and methods

2.1. Material, reagents

Fetal calf serum (FCS), cell culture media, gentamicin, glutamine, and trypsin were purchased from Life Technologies (Grand Island, NY). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany) and treated with charcoal. Vitamin C (L-ascorbic acid, ASA), Vitamin E (α-tocopherol succinate), echistatin and $CaCl_2$ were from Sigma, recombinant (rh) insulin-like growth factor I (IGF I) was from Ciba-Geigy, Basel. Bovine parathyroid hormone (PTH, 1–34, from Bachem, Torrance, CA) and vasoactive intestinal peptide (VIP, from Sigma) were dissolved in 0.1 M acetic acid. 1α, 25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$) was a gift from Hoffmann-La Roche, Basel and dissolved in ethanol. Prostaglandin E_2 (PGE_2), retinoic acid (RA) and dexamethasone were all from Sigma and dissolved in absolute ethanol.

N-Dansyl-D-Ala-Gly-*p*-nitro-Phe-Gly (the substrate of the NEP activity assay DAGNPG), Dansyl-D-Ala-Gly (the product of the NEP activity assay DAG) (both dissolved in methanol), *N*-(α-Rhamnopyranosyloxyhydroxyphosphinyl)-L-Leu-L-Trp disodium salt (phosphoramidon) and DL-3-mercapto-2-benzyl propanoylglycine (DL-thiorphan) (NEP inhibitors), captopril (an angiotensin-converting enzyme inhibitor) and EDTA were all from Sigma. CD10 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology. These antibodies were raised against a recombinant protein corresponding to amino acid 230–550 mapping within an internal region of CD10 of human origin.

2.2. Cell culture

PyMS cells, an osteoblastic cell line derived from newborn rat calvaria, were kindly provided by Dr. A. Lichtler, University of Connecticut (Farmington, CT). The cells were passaged in Falcon tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μg/ml), glutamine (2 mmol/l), and FCS (5%) and kept at 37 °C in an atmosphere of 5% CO_2 in air. Cultures between passages 12 and 40 were used.

PyMS cells grown to confluence were detached from the flasks with 0.25% trypsin and replated in multiwell tissue culture plates (Falcon, 35 mm diameter) at a density of 2×10^5 cells per well in DMEM containing 5% FCS. Confluent monolayers formed 3 days after seeding. Cell cultures were rinsed with serum-free medium and kept in serum-free Ham's F12 medium containing gentamicin (50 μg/ml), glutamine (2 mM) and BSA (1 g/l) for the last 24 h. Aliquots of test agents were added directly to the media as indicated.

Rat fibroblasts were obtained by collagenase digestion from the skin of newborn rats [32] and used between passage 5 and 15.

L2 cells, a rat lung cell line derived from type II alveolar epithelial cells [33] were a kind gift from Dr. B Beck-Schimmer, University of Zurich [34]. They were passaged in DMEM supplemented with 10% FCS. Tests were performed with culture media and solutions as described for the PyMS cells.

2.3. Determination of neutral endopeptidase activity (NEP activity assay)

The NEP ("enkephalinase" activity) assay [35,36] is based on the cleavage of a small molecular weight artificial substrate, the fluorogenic peptide *N*-Dansyl-D-alanyl-glycyl-*p*-nitrophenylalanyl-glycine (DAGNPG) where the product can be quantitated by spectrofluorometry. The increase of fluorescence is related to the disappearance of intramolecular quenching of the dansyl fluorescence by the nitrophenyl residue. Intact adherent PyMS cells were cultured in 6-well culture dishes, for the last 24 h in serum-free, BSA-containing media as described and rinsed with 50 mM Tris–HCl, pH 7.4. A volume of 600 μl of DAGNPG (50 μM in Tris–HCl Sigma), a synthetic NEP substrate, was added to each well. Dishes were incubated for 20 min (or, as indicated) at 37 °C. Subsequently, a 500 μl aliquot was transferred to an Eppendorf microcentrifuge tube, heated for 5 min at 100 °C, put on ice and spun for 5 min at 15,000 rpm ($3\times g$). The fluorescence of the supernatant was measured in a spectrophotofluorometer (SLT fluostar U200, Offenburg, Germany) with an excitation at 355 nm (λ_{ex}=355 nm) and emission of 538 nm (λ_{em}=538 nm), the wavelengths which turned out to be optimal under the conditions used. Standard curves were measured in each experiment to determine the amount of product formed. Since the DAGPNG substrate exhibits an intrinsic fluorescence at 538 nm (five times less than the Dansyl-D-Ala-Gly product), a calibration curve with increasing amounts of Dansyl-D-Ala-Gly (product) and decreasing amounts of substrate (total concentration of product and substrate=50 μM) is measured in each experiment. We also tested specific NEP inhibitors [37] such as the Streptomyces product phosphoramidon (considered a more general inhibitor, previously identified as thermolysin inhibitor) or thiorphan (considered to be more selective), and EDTA (an overall metalloprotease inhibitor).

2.4. Western immunoblot analysis for NEP

Protein was extracted either from tissues or from cell membranes and subjected to SDS-PAGE (7.5%), then transferred to a 0.45 μm PVDF membrane (Hybond P, Amersham RPN303F) for Western analysis. Equal loading was confirmed by Ponceau S staining. Filters were incubated overnight at 4 °C for 1 h in primary antibody (anti CD10/H-321, sc-9149, at 1:1000), and were then washed and incubated for 1 h in secondary antibody (goat anti-rabbit HRP, Biorad 170-6515, at 1:3000) at room temperature. After washing, proteins were detected by using the enhanced chemiluminescence (ECL) detection reagents (Amersham 1059250) applied as recommended by the manufacturer (Amersham) and by exposure to light-sensitive film (Kodak 8194540).

2.5. Tissue homogenization, protein extraction, cell fractionation

Tissue pieces of 120 g rats were put into 0.5% Triton-X-100/50 mM HEPES, pH 7.5/140 mM NaCl/1 mM PMSF/3 μg/ml/aprotinin/3 μg/ml leupeptin, mixed to homogeneity, centrifuged at 16,000 × *g*, 4 °C, for 10 min. The supernatant was transferred to an Eppendorf tube and frozen at −80 °C.

To improve sensitivity and specificity for NEP detection by Western in PyMS cells, we used lysis buffer as described above (and also used for kidney, lung and brain), but followed by a sequential differential centrifugation as described by Clark et al. [38] with minor modifications as described by Bostedt [39] in order to obtain the plasma membrane (PM) fraction. For these experiments, cells plated on 10 cm diameter dishes were used, as described below for Northern analysis.

2.6. Determination of protein content and alkaline phosphatase activity

Cells were used from the same (after the NEP activity assay) or from parallel (identically treated) dishes for protein content determination.

After 4 days of culture (24 h in serum-free medium), cells were lysed with 1 ml of 0.1% Triton X-100 for the determination of protein content by the bicinchoninic acid (BCA) method [40] (Pierce, Rockford IL) and alkaline phosphatase activity by cleavage of *p*-nitrophenyl phosphate to *p*-nitrophenol at pH of 10.2 as described elsewhere [41,42].

2.7. [^{3}H]thymidine incorporation into DNA

Cells were plated and grown for 3 days in 5% FCS-containing medium, then washed and exposed to serum-free test medium (containing 1 g/l BSA and test agents as indicated) for 18 h, and pulsed with [methyl-^{3}H]thymidine (Amersham, 80 Ci/mmol; 1 μCi/dish) for 3 h at 37 °C. After rinsing three times with cold PBS, DNA was precipitated and washed three times in situ with 10% trichloroacetic acid. The precipitate was dissolved in 1 N KOH, and incorporated radioactivity was measured in a liquid scintillation counter [41,42].

2.8. Northern analysis

Cells were plated at a density of 10^6 cells/10 cm diameter dish (Falcon) in medium containing 5% FCS and grown for 3 days. Confluent monolayers were kept in serum-free media for 24 h with or without ascorbic acid. Total RNA from PyMS cells, newborn rat calvarial cells [released with bacterial collagenase and grown as described elsewhere [41]], rat fibroblasts, L2 cells, rat calvarial (parietal) bone, rat kidney and lung from newborn (1d) rats, was isolated as described elsewhere [41], [43] electrophoresed and transferred onto gene screen membranes (DuPont-NEN) and hybridized.

The cDNA probe of NEP was PCR-cloned from rat kidney RNA (nucleotides 135—837, GenBank accession no. M15944) as described [44].

The cDNA probes were labelled by random primer extension using a commercial kit (Boehringer Mannheim, Rotkreuz, Switzerland) and [α-^{32}P] deoxy-CTP (3000 Ci/mmol; Amersham) to specific activities of $2-4 \times 10^9$ cpm/μg DNA.

2.9. Statistical analysis

Results were obtained by pooling data from a number of independent experiments in which exactly the same conditions were tested, as indicated. Data are expressed as means ± S.E.M. Statistical significance was assessed by the two-tailed Student's *t*-test. A p-value <0.05 was considered to be statistically significant.

3. Results

3.1. Effects of agents on thymidine incorporation into DNA

When kept in serum-free media, PyMS cells exhibit a slow proliferation rate and become quiescent; the rate of apoptosis is low. In response to the addition of serum or IGF I, there is a marked increase in DNA synthesis. Vitamin C has also a prominent, dose-dependent effect on DNA synthesis of the cells, and it enhanced the stimulatory effect of IGF I (Fig. 1A). 2 mg/l ASA had a significant effect, and maximal stimulation was observed with 200 mg/l, whereas higher concentrations (2 g/l) were less effective (not shown). Combining ASA and IGF I resulted in a more than additive stimulation of DNA synthesis. Echistatin, when added 10 min prior to ASA and IGF I, had no effect on basal, ASA- and IGF I-induced DNA synthesis but significantly attenuated

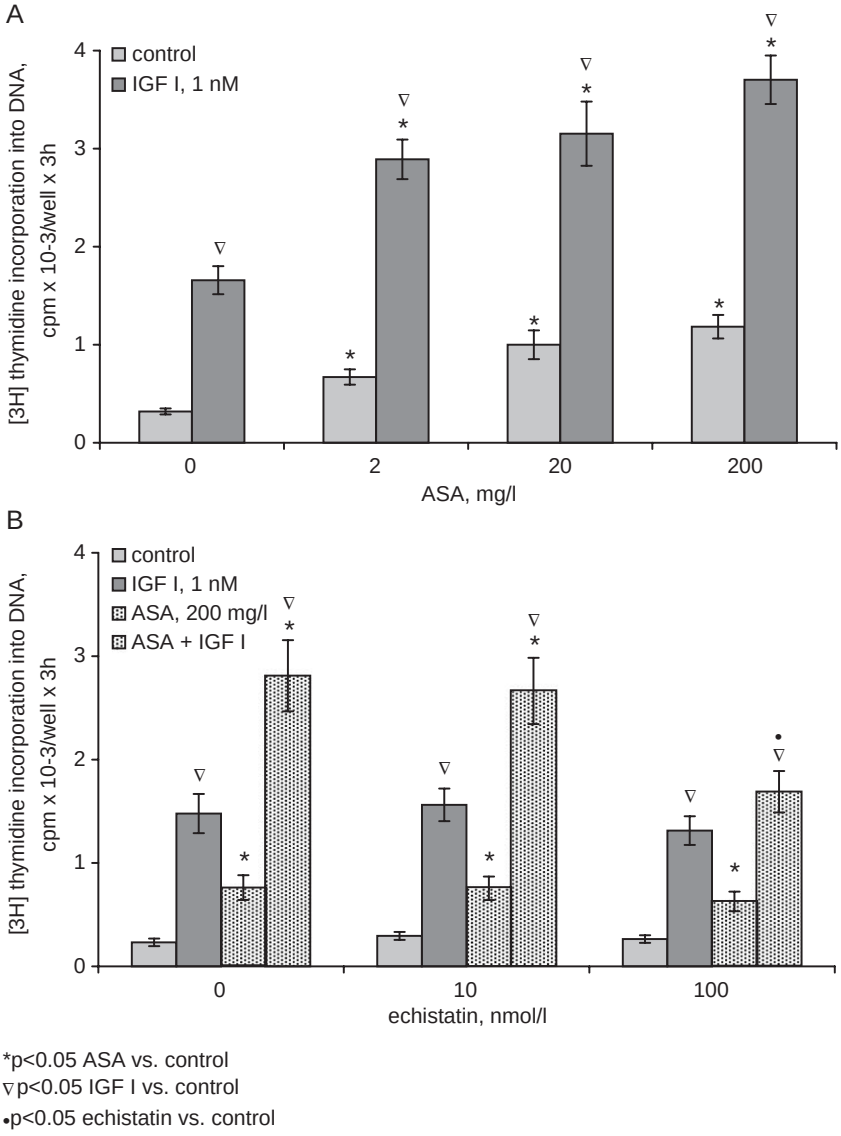

Fig. 1. DNA synthesis in PyMS cells exposed to ascorbic acid and IGF I. A) Dose-dependent effect of ascorbic acid (upper panel). Cells were grown for 3 days in FCS-containing medium, rinsed with serum-free medium and exposed to serum-free test medium containing ASA as indicated ± 1 nM IGF I for 18 h, then pulsed for 3 h with 1 μCi [methyl-^{3}H]thymidine and incorporated radioactivity was measured. Data are given as means ± S.E.M. from 7 independent experiments performed in triplicate. $p < 0.05$ for ASA-treated (all concentrations) versus control (*), and +IGF I-treated versus control (▽). B) Effect of echistatin (lower panel). Cells were grown as above, but exposed to control medium or (10 nM or 100 nM) echistatin 10 min before the addition of ASA and IGF I as indicated. Data are given as means ± S.E.M. from 5 independent experiments performed in triplicate. A significant effect of echistatin ($p < 0.05$ for comparison of ASA-+IGF-+echistatin- versus ASA- +IGF I-treated) was only observed at 100 nM, in cells exposed to both IGF I and ASA (●).

ASA+IGF I-stimulated thymidine incorporation, i.e. ASA no longer enhanced the stimulatory effect of IGF I in the presence of 100 nM echistatin (Fig. 1B). These findings, i.e. an almost synergistic stimulation of DNA synthesis by ASA and IGF I, and an attenuation by echistatin of the stimulation elicited by ASA+IGF I, were qualitatively similar in rat fibroblasts but not in the L2 lung cell line where echistatin increased DNA synthesis (not shown).

In addition, several agents were (some of them, previously) found to stimulate DNA synthesis, such as 1α,25(OH)$_2$ D$_3$ (1 nM), RA (1 μM), PGE$_2$ (1 μM), VIP (10 nM), and calcium (1 mM); in contrast, PTH and dexamethasone are inhibitory (unpublished; Table 1); these findings indicate that this cell line is responsive to all these agents under our experimental conditions.

3.2. NEP activity

Cleavage of *N*-Dansyl-D-Ala-Gly-*p*-nitro-Phe-Gly to Dansyl-D-Ala-Gly was measured in situ and, under the

Table 1
List of tested agents affecting DNA synthesis, but not NEP activity in cultures of PyMS cells

Test compound	Concentration	DNA synthesis, treated/control
Calcium	0.25–**1** mM	4.7±1.0*
IGF I	0.1–**1** nM	8.3±1.4*
PTH	0.1–**10** nM	0.5±0.1*
PGE_2	0.01–**1** μM	3.7±0.8*
VIP	1–**10** nM	1.5±0.1*
$1\alpha,25(OH)_2D_3$	0.1–**1**, 10 nM	2.8±0.3*
Dexamethasone	10–**100** nM	0.4±0.1*
Retinoic acid	0.1–**1** μ**M**	1.9±0.1*
FCS	**5**%	45.2±13.9*

All listed compounds had no effect on NEP activity in 24 h tests (conditions as in Fig. 3), but affected DNA synthesis in cultures of PyMS cells (conditions as in Fig. 1). The concentrations tested and found to be effective are given; the extent of the effect is given for the concentrations given in bold. Data are given as means±S.E.M. from 3–8 independent experiments performed in triplicate.

* Indicates significantly different from control, i.e. $p<0.05$ for the comparison of test compound-treated versus control.

conditions used, found to be linear with regard to time for at least the first 30 min (Fig. 2); cleavage could be completely blocked by 1 μM phosphoramidon (not shown) as well as by 1 μM thiorphan (Fig. 2), a finding which suggests that the activity measured can be attributed in full extent to NEP. Both inhibitors fully blocked enzymatic activity also at 0.1 μM; 1 nM thiorphan was required for half maximal inhibition, as assessed in 3 independent experiments (not shown). Likewise, 5 mM EDTA blocked enzymatic activity, but captopril (tested up to 10 μM) had no effect. Remarkably, cells exposed to 5% FCS or 1 nM IGF I exhibited exactly the same activity as cells exposed to serum-free control media for the last 24 h. The following compounds were also ineffective when tested (all of them, in 2–7 independent experiments) for 24 h: Calcium (1 mM), PTH (10 nM), PGE_2 (1 μM), VIP (10 nM), $1\alpha,25(OH)_2$ D_3 (1 nM), dexamethasone (100 nM), retinoic acid (1 μM) (Table 1), and echistatin. In contrast, ASA had a marked inhibitory effect which was dose-dependent (Fig. 3A); 20 mg/l and 200 mg/l exerted a significant effect, and 2 g/l (not shown) was even more effective. The effect of ASA was time-dependent (Fig. 3B) and could be observed within 2 h, but was more pronounced after 6 h and 24 h. After 24 h of treatment with 200 mg/l ASA, NEP activity per well and per protein was decreased to 35% of the control value (Fig. 3, Table 2a). Calcium, IGF I, PTH and PGE_2 had no effect after 24 h, 6 h and 2 h (not shown). Additional experiments revealed that the effect of ASA was not related to lowering the pH of the media (to 7.39 at 2 mg/l, to 7.36 at 20 mg/l, and to 7.04 at 200 mg/l ASA); acetic acid and HCl (tested at concentrations ranging from 0.01 up to 1 mM; at 1 mM, lowering the pH of the media to 7.05, i.e. to a similar extent as ASA at 200 mg/l) did not affect NEP activity under the same conditions (not shown). We also tested whether the effect of ASA was modified by calcium, IGF I, PTH, PGE_2, dexamethasone, $1\alpha,25(OH)_2$ D_3, RA, i.e. agents which had effects on DNA synthesis, but which were inactive by themselves on NEP activity. The only agent with a reproducible effect in the presence of ASA on NEP activity was calcium which had a small but significant stimulatory effect when added at 1 mM, raising the final concentration from 0.3 to 1.3 mM (not shown).

Vitamin E, another antioxidant, also decreased NEP activity (with slightly lower potency, when compared on a molar basis), but in contrast to vitamin C, decreased DNA synthesis (Table 3). NEP activity was undetectable in L2 (lung) cells and 5-fold lower in (skin) fibroblasts (Table 2b) than in PyMS cells (Table 2a) when measured under the same conditions with the same assay; ASA also decreased NEP activity in the fibroblasts (Table 2b).

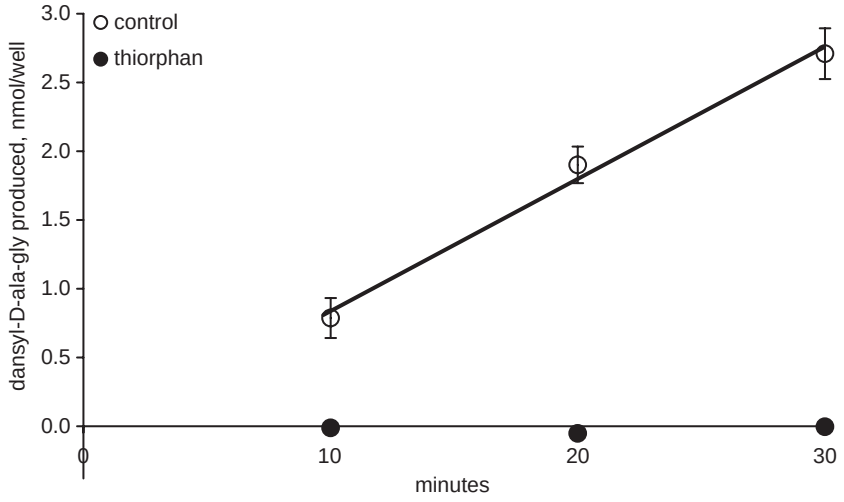

Fig. 2. NEP activity in PyMS cells. Cleavage of DAGNPG/DAG formed as a function of incubation time and inhibition by thiorphan. Open circles, control; closed circles, 1 μM DL-thiorphan. Results are given as means±S.E.M. from 4 independent experiments performed in triplicate.

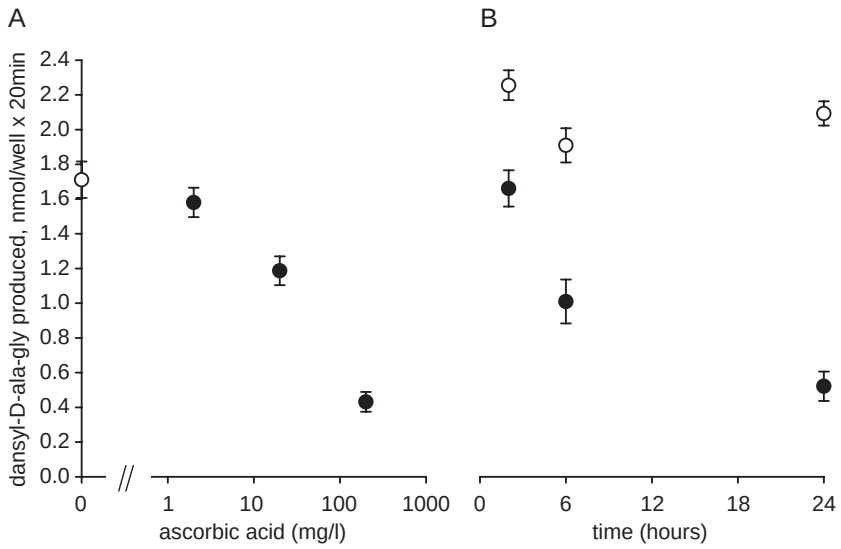

Fig. 3. NEP activity in PyMS cells exposed to serum-free test media for 24 h. A) (Dose-dependency, left panel) Cells were exposed to ASA for 24 h and NEP activity was measured as cleavage of DAGNPG during 20 min. Data are means ± S.E.M. from 7 independent experiments in triplicate. $p < 0.05$ for 20 mg ASA/l and 200 mg ASA/l versus control. B) (Time-dependency, right panel) Cells were exposed to serum-free medium to which control (open circles)- and ASA (closed circles)-aliquots were added (to final concentration of 200 mg/l) for the last 24, 6 h and 2 h, respectively. Data from 4 independent experiments in triplicate. $p < 0.05$ for ASA-treated versus control-treated at 2, 6, and 24 h.

3.3. NEP protein

As assessed by Western analysis, NEP protein was much less abundant in PyMS cells than in kidney. NEP was detected at an apparent molecular weight of about 100 kDa in plasma membrane fraction (PM) of PyMS cells, at the same size as the band in lung and at a slightly higher apparent molecular weight as the band in kidney. NEP was less abundant in PM prepared from cells treated with 200 mg/l ASA for 24 h than from control-treated cells (Fig. 4, 2 experiments shown), a consistent finding in 5 independent experiments. As estimated by densitometry, NEP protein abundance in PM was decreased to 55% of the control value (Table 2a). In contrast, NEP was equally abundant in microsomal fractions from cells with and without ASA treatement (not shown).

NEP could not be detected by Western immunoblot analysis in PM prepared from L2 cells, but a weak signal was detected in PM from rat fibroblasts (Fig. 4).

3.4. Protein, alkaline phosphatase activity, NEP and alkaline phosphatase mRNA

Protein content per dish and alkaline phosphatase activity were not markedly affected by ASA, although there was a slight trend for an increase in the former and a decrease in the latter (Table 2a).

Table 2
Protein content, NEP and alkaline phosphatase activity, NEP abundance in plasma membranes by Western, as assessed by densitometry, in cells exposed to 200 mg/l ASA for 24 h

	Control	ASA, 200 mg/l
a) PyMS cells		
Protein content (mg/well)	0.105 ± 0.002	0.109 ± 0.002
NEP activity(nmol DAG formed/mg protein × 20 min)	28.2 ± 3.2	9.9 ± 1.2*
NEP abundance in plasma membranes by Western (relative to control)	(1)	0.55 ± 0.07*
ALP activity(μmol *p*-nitrophenol formed/mg protein × 20 min)	1.73 ± 0.13	1.57 ± 0.15
b) Fibroblasts		
Protein content (mg/well)	0.102 ± 0.004	0.122 ± 0.006
NEP activity(nmol DAG formed/mg protein × 20 min)	5.81 ± 0.42	3.11 ± 0.28*

Data are from 10 independent experiments in triplicate, except for NEP abundance ($n = 5$), in PyMS cells (a) and from 4 experiments in triplicate in fibroblastic cells (b).

* Indicates significantly different from control, i.e. $p < 0.05$ for the comparison of ASA-treated versus control.

Table 3
DNA synthesis and NEP activity in cultured PyMS cells exposed to ascorbic acid (ASA) and α-tocopherol succinate (vitamin E)

Test compound	DNA synthesis, treated/control	NEP activity, AG formed, nmol/well × 20 min
Control	1 ± 0.1	2.1 ± 0.2
ASA, 20 mg/l	3.1 ± 0.3*	1.4 ± 0.2*
ASA, 200 mg/l	4.4 ± 0.2*	0.9 ± 0.1*
Vitamin E, 100 mg/l	0.5 ± 0.1*	1.3 ± 0.2*

DNA synthesis and NEP activity were assessed and expressed as described in legends to Table 1 and Fig. 3. Data are from 3 experiments performed in triplicate.

* Denotes $p < 0.05$ for the comparison of test compound-treated versus control.

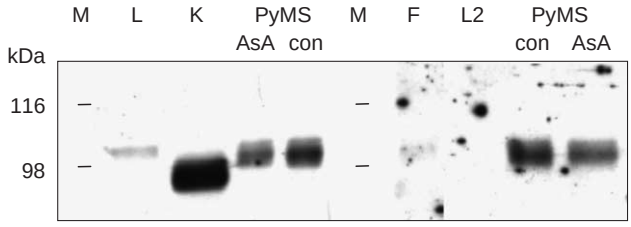

Fig. 4. Western immunoblot analysis for NEP. Supernatant of homogenized tissue pieces from newborn rat lung (L) and kidney (K), and plasma membrane preparations from PyMS cells ± ASA (con, control; AsA, ASA-treated), L2 cells (L2) and rat fibroblasts (F) (the last 24 h in serum-free media, as in Figs. 2 and 3) were subjected to SDS-PAGE, blotting and Western analysis as described in Materials and methods. On the left, an experiment is shown (exposure time 2.5 min) where PM (6 μg protein) from PyMS cells are compared to homogenates (10 μg protein) from lung and kidney; on the right, an experiment (exposure time 20 min) where PM from different cell types (each lane, 2 μg protein) are compared. Bars indicate the position of 2 prestained marker proteins, 116 kDa and 98 kDa, respectively.

In contrast to Western (NEP protein abundance), Northern analysis revealed NEP mRNA expression in PyMS cells at levels comparable to those found in kidney (Fig. 5). NEP mRNA was also detected in primary rat calvaria cells and rat fibroblasts, but at lower levels than in PyMS cells, and undetectable in L2 cells (Fig. 5). In contrast to its effect on NEP protein, ASA had no effect on NEP mRNA expression in PyMS cells (not shown).

4. Discussion

Our data show that vitamin C inhibits NEP activity in the osteoblastic cell line PyMS. This cell line was chosen because it has been well characterized in our laboratory and because it had relatively high expression of NEP mRNA and NEP activity as compared to other cells such as rat fibroblasts. Remarkably high NEP expression by cells belonging to the osteoblast lineage is in agreement with several reports in the literature [11–18]. Moreover, PyMS is a cell line which can survive and be studied under well-defined serum-free conditions. FCS did not affect NEP activity. Our NEP assay appeared to be specific since low concentrations of thiorphan completely prevented substrate degradation, implicating that thiorphan-nonresponsive enzymes did not interfere. Most growth factors and hormones (to which the cells are responsive) did not alter NEP activity (Table 1), but ASA had an inhibitory effect at concentrations which can be considered physiological (the lower concentrations tested) and may well be reached (higher concentrations tested) in the circulation and tissues in rats and humans in vivo. Although an effect of ASA on NEP activity in osteoblasts has not been described before, there have been precedents, potentially related findings in the neuroscience literature: The amyloid peptide may be degraded by NEP in the brain [45]; initial experiments indicated that endopeptidase inhibition by phosphoramidon and thiorphan resulted in enhanced amyloid accumulation [46,47]; remarkably, a subsequent study showed that the vehicle which contained ASA had apparently the same effect [48]. Therefore, the local administration of ascorbic acid appeared to inhibit NEP activity in brain. Our findings in a cell line is in agreement with such a notion; ASA inhibits NEP activity in several cell types, e.g. not only in PyMS cells (Table 2a), but also in fibroblasts (Table 2b). Moreover, vitamin E and C treatment reduced NEP activity in (oxidative) fatty acid-exposed endothelial cells [49], suggesting that antioxidant treatment could reduce increased NEP activity, thereby increasing half-lives of substance P and other peptides which may help wound healing in diabetes [49]. Decreased NEP activity by antioxidant treatment may also lower blood pressure and increase insulin sensitivity, e.g. by reducing bradykinin degradation. Within the short term of our experimental setting (up to 24 h) most agents with marked effects on DNA synthesis have no effect on NEP activity of the cultured cells (Table 1); the ASA effect is therefore quite specific. The inhibitory effect of ASA on NEP activity also appeared to be selective since the activity of alkaline phosphatase, another plasma membrane-bound zinc-dependent enzyme, was not significantly decreased (Table 2a). NEP is located on the cell surface and functions as an ectoenzyme, catalysing peptide hydrolysis at the extracellular face of the plasma membrane. Decreased NEP activity as assessed in situ is associated with a decrease in NEP/CD10 immunoreactivity in enriched isolated plasma membranes (but not in microsomes) of the ASA-treated cells (Fig. 4, Table 2a), but it is not accompanied by a decrease in NEP mRNA. The major band detected by Western immunoblot at an apparent

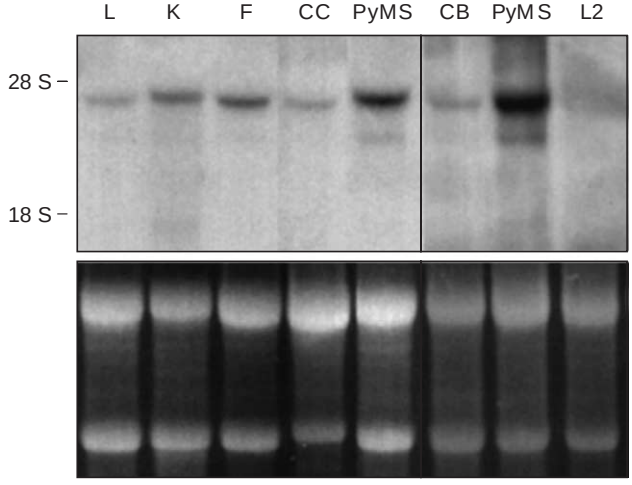

Fig. 5. Northern blot analysis for NEP mRNA. RNA was prepared from newborn rat lung, kidney, (calvarial) bone, cultured calvarial cells, L2 cells, fibroblasts, and PyMS cells, the latter kept for the last 24 h in serum-free culture media. On the left, an experiment is shown where 20 μg total RNA was loaded from lung, kidney, fibroblasts (F), calvaria (CC) and PyMS (PyMS) cells; on the right, a separate experiment with 20 μg RNA from calvarial bone (CB), PyMS and L2 cells. Bars indicate the position of ribosomal RNA (28S and 18S); the latter is shown (ethidium bromide-stained) in the lower panel.

molecular mass of close to 100 kDa is in agreement with reports on NEP in rat intestine and kidney [50,51]. Differences in size of NEP from various tissues can be attributed to differences in glycosylation [52]. The intensity of this band in the PM fraction of the cultured cells (weak signal in PM from fibroblasts, no signal in PM from L2 cells, Fig. 4) fits the NEP activity as assessed by our assay in situ. Consistent with the NEP activity data, L2 (lung) cells and rat fibroblasts exhibit no detectable and low NEP activity, respectively (Fig. 4). Although some, but not all reports on the effects of dexamethasone and glucocorticoids on a human epithelial cell line reported such effects [53,54], NEP activity could not be induced in the L2 lung cell line by dexamethasone.

We recently had the opportunity to check alveolar type 2 cells isolated from adult rat lung in primary cultures. These cells do express NEP activity which is slightly induced by 100 nM dexamethasone and significantly decreased by 200 mg/l ASA (Chengluo Jin, C.G.-K. and C.S., unpublished). Regarding vitamin C, the similar findings in bone cells, fibroblasts and lung cells strongly suggest that the inhibitory effect of ASA on NEP activity is not restricted to bone cells. Because NEP is widely expressed and because ASA lowers NEP activity in many cell types (endothelial and lung alveolar cells, osteoblasts and fibroblasts), the observation of decreased NEP activity following vitamin C exposure may have implications not only with regard to cell growth, but also for the function of brain, kidney, lung and blood vessels, including potentially beneficial and harmful effects.

The vitamin C effect on PyMS cells cannot be explained by the mere presence of ASA in the incubation buffer for the NEP assay (cells were previously washed); the effect was much less pronounced after 2 than after 24 h (Fig. 3B). On the other hand, exposure of PyMS cells to ascorbic acid for 6 h and subsequent washing and removal of vitamin C resulted in an inhibition of NEP activity which persisted (no recovery), comparable to that seen when cells were exposed to ASA for the last 6 h (not shown).

ASA is not only required for hydroxylation of proline residues in collagen and matrix production by connective tissue cells, but also for proliferation and differentiation of osteoblastic cells [55,56]; numerous effects of ASA on osteoblastic cells have been described. In particular, ascorbate is known to affect the response of cells to exogenous growth factors [55,57], including IGF I. Its synergistic effect with IGF I on DNA synthesis of osteoblastic cells (Fig. 1) may be explained, at least in part, by an interaction of integrin and insulin-/IGF 1 receptor-mediated signalling [58]. It has been demonstrated that the blockade of ligand occupancy of the $\alpha V\beta 3$ integrin inhibits IGF I-stimulated biological actions [59,60]. Echistatin is such a blocker ("disintegrin") and has been shown to inhibit IGF I-stimulated smooth muscle cell migration and DNA synthesis [59,60]. Echistatin had no effect on NEP activity; echistatin attenuated ASA+IGF I- but not IGF I-induced stimulation of thymidine incorporation. Such findings are not restricted to bone (PyMS) cells since they were also raised in rat fibroblasts (but not in the rat lung (L2) cell line). They are in agreement with the notion that integrins activate the focal adhesion kinase (FAK) and Shc pathways [61,62].

Our findings indicate a specific effect of ASA on NEP activity in PyMS cells. It remains unclear whether this effect is related to antioxidative properties of ASA, as suggested by others [49]; in our experimental setting, vitamin E (both α-tocopherol succinate and trolox were tested at concentrations of 10 mg/l and 100 mg/l each) was roughly five times less potent (on a weight basis) than ASA, 100 mg/l being roughly equieffective to 20 mg/l ASA (Table 3) with regard to inhibition of NEP activity. In molar terms, this corresponds to a 1.5 fold lower potency of vitamin E as compared to ASA. Comparing the effects of ASA and vitamin E, it is obvious that the effects of these antioxidants are not linked to cell proliferation (Table 3). Overall, there is no relationship between effects of agents on NEP activity and proliferation of this cell line. Concerning ASA, it seems more likely that it has unrelated (or at least not strictly related) effects on DNA synthesis and NEP activity. Since NEP inhibitor treatment resulted in increased FAK phosphorylation on tyrosine (relieving the NEP brakes on FAK signalling) [30] and since NEP substrates transactivate IGF I receptors, resulting in PI3-kinase-mediated Akt phosphorylation [31] in prostate cancer cells, it could be speculated that ASA not only enhances IGF I activity by favoring integrin signalling, but also by favoring cross communication between (an unknown) NEP substrate and IGF I receptor signalling. The NEP enzyme may play an important role by processing regulatory peptides in bone [14]. Apart from their growth rate and bone remodeling activity, osteoblasts may affect the bone milieu (possibly, also favoring hosting) for aggressive tumor cells which could be stimulated by NEP substrates [19,20,30,31].

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