Biochemical and Biophysical Research Communications 446 (2014) 423–427

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Production of soluble Neprilysin by endothelial cells

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ARTICLE INFO

Article history:
Received 8 January 2014
Available online 2 February 2014

Keywords:
ADAM
Endothelial cell
Enzymes
Exosomes
Shedding

ABSTRACT

A non-membrane bound form of Neprilysin (NEP) with catalytic activity has the potential to cleave substrates throughout the circulation, thus leading to systemic effects of NEP. We used the endothelial cell line Ea.hy926 to identify the possible role of exosomes and A Disintegrin and Metalloprotease 17 (ADAM-17) in the production of non-membrane bound NEP. Using a bradykinin based quenched fluorescent substrate (40 μM) assay, we determined the activity of recombinant human NEP (rhNEP; 12 ng), and NEP in the media of endothelial cells (10% v/v; after 24 h incubation with cells) to be 9.35 ± 0.70 and 6.54 ± 0.41 μmols of substrate cleaved over 3 h, respectively. The presence of NEP in the media was also confirmed by Western blotting. At present there are no commercially available inhibitors specific for ADAM-17. We therefore synthesised two inhibitors TPI2155-14 and TPI2155-17, specific for ADAM-17 with IC_{50} values of 5.36 and 4.32 μM, respectively. Treatment of cells with TPI2155-14 (15 μM) and TPI2155-17 (4.3 μM) resulted in a significant decrease in NEP activity in media (62.37 ± 1.43 and 38.30 ± 4.70, respectively as a % of control; $P < 0.0001$), implicating a possible role for ADAM-17 in NEP release. However, centrifuging media (100,000*g* for 1 h at 4 °C) removed all NEP activity from the supernatant indicating the likely role of exosomes in the release of NEP. Our data therefore indicated for the first time that NEP is released from endothelial cells via exosomes, and that this process is dependent on ADAM-17.

1. Introduction

Neprilysin (EC 3.4.24.11) or NEP is known by a variety of other names including Neprilysin, CD10, enkephalinase, common acute lymphoblastic leukemia antigen (CALLA) and endopeptidase-24.11 [1]. After its initial discovery in kidney brush border cells [2], localisation and functional role(s) for NEP have been confirmed in cells of the immune system, human breast tissue, male genital tract, canaliculi of the liver, gastrointestinal tract, brain and cardiovascular system [3]. NEP is a membrane bound enzyme with a large extracellular catalytic domain, a single transmembrane region and a short (27 amino acids) cytoplasmic N-terminal domain [4]. NEP cleaves a wide range of regulatory peptide substrates which have been shown to play key roles in the pathogenesis of Alzheimer's disease, pain transmission, as well as cardiovascular and renal diseases [3]. In the latter context, NEP metabolises bradykinin and atrial natriuretic peptide (ANP) both of which have antihypertensive effects. Cleavage of these substrates by NEP reduces the extracellular concentration available for receptor binding, and thereby regulating their physiological/pathophysiological actions [5].

Previous studies have shown that NEP, like many other membrane bound metalloproteases can be released from the cell surface producing a non-membrane associated form that retains catalytic activity [6,7]. Numerous studies have confirmed the presence of circulating NEP in biological fluids, and also suggesting a potential role as diagnostic/prognostic markers of disease.

Circulating NEP in the systemic circulation can potentially cleave substrates throughout the body leading to a manifestation of systemic effects of NEP. Of note, it has recently been shown that a circulating non-membrane associated form of NEP plays a critical role in lowering brain amyloid beta (Aβ) levels and Aβ deposition [8]. Given the central role of brain Aβ levels in the pathogenesis of Alzheimer's disease [9], these findings indicate that circulating NEP may provide a potential target in the treatment of this debilitating disease [8].

Abbreviations: QFS, quenched fluorescent substrate; NEP, Neprilysin; CALLA, common acute lymphoblastic leukemia antigen; ADAM-17, A Disintegrin and Metalloprotease 17.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.158
0006-291X/

Understanding the mechanisms behind the release of NEP will likely reveal novel targets for the therapeutic manipulation of circulating NEP levels. To date the precise mechanism(s) behind this process are unknown. Previous studies have indicated that cell membrane bound proteins can be released via exosomes or A Disintegrin and Metalloprotease 17 (ADAM-17) mediated cleavage of the extracellular domain. In this study, we examined the possible role of these pathways in the production of a non-membrane associated form of NEP from endothelial cells.

2. Materials and methods

2.1. Cell culture

Immortalised Ea.hy926 cells were grown in T75 flasks using Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% (v/v) foetal bovine serum, 1% penicillin/streptomycin/glutamine and HAT medium (1 vial/500 mL; Sigma) as mentioned previously [10]. Upon reaching 80% confluence, the cells were split into Petrie dishes as required.

When 80% confluent, the cells were washed with phosphate buffered saline three times. The cells were then incubated with serum free media (OPTI-mem) containing the ADAM17 inhibitors TPI2155-14 (0–15 μM) and TPI2155-17 (0–4.3 μM) over 24 h). Media was then harvested and centrifuged (3000*g* × 5 min; 4 °C) to remove cellular debris, concentrated 5-fold using Amicon centrifugal filter devices (MWCO 30 KDa). NEP activity was determined immediately. Where indicated, the concentrated media was subjected to ultracentrifugation (100,000*g*; 1 h at 4 °C) and supernatant alone was used for NEP activity assays.

2.2. NEP assay

NEP activity was measured based on the ability to generate fluorescence following the cleavage of a bradykinin-based quenched fluorescent substrate (QFS; 40 μM, ((7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(2,4-dinitrophenyl), Auspep Pty Ltd). Fluorescence generated was measured using excitation and emission wavelengths of 320 and 405 nm, respectively (Fluostar Optima, BMG). All enzyme assays using concentrated media was conducted in the presence of captopril (0.1 μM). The specificity of cleavage was confirmed using the NEP inhibitor thiorphan (10 μM, Sigma). QFS was added to the reaction mixture pre-incubated for 1 h at 37 °C, containing rhNEP (12 ng) or cell culture media (10% of final volume) and assay buffer (100 mM TrisCl, 150 mM NaCl, pH 6.3). The assay was conducted in a 96 well plate (Nunc, black well plates), and specific enzyme activity was calculated from a standard curve of known fluorophore (7-methoxycoumarin, Sigma) concentrations.

2.3. Synthesis of ADAM-17 inhibitors and enzyme activity assays

TPI2155-14 and -17 was synthesized as using the general protocols described previously [11]. The general scaffold of the TPI series is shown in Fig. 4. These inhibitors were tested for ADAM-10 and 17 inhibition using TNFα-based glycosylated substrate assays [11], while MMP-8 and -14 were tested using commercially available substrate Mca-KPLGL-Dpa-AR-NH_2 [12] following the same general protocol. 5 μL of 3× enzyme solution (30 nM for ADAM10 and 17, and 6 nM for MMP-8 and -14 assays, respectively) in assay buffer (ADAMs: 10 mM Hepes, 0.001% Brij-35, pH 7.5 and MMPs: 50 mM tricine, 50 mM NaCl, 10 mM $CaCl_2$, and 0.005% Brij-35 at pH 7.5, respectively) were added to solid bottom white 384 low volume plates (Nunc cat# 264706). Next, 5 μL of test compounds or pharmacological controls were added to corresponding wells. After 30 min incubation at RT the reactions were started by addition of 5 μL of 3× solutions of respective substrates (30 μM). Fluorescence was measured using the multimode microplate reader Synergy H4 (Biotek Instruments, Winooski, VT) using $\lambda_{excitation}$ = 360 nm and $\lambda_{emission}$ = 460 nm for ADAMs, and $\lambda_{excitation}$ = 324 nm and $\lambda_{emission}$ = 405 nm for MMPs, respectively. Fluorescence values were measured every 30 min for 2 h or every 15 min for 1 h for ADAM and MMP assays, respectively. Rates of hydrolysis were obtained from plots of fluorescence versus time, and inhibition was calculated using rates obtained from wells containing substrates only (100% inhibition) and substrates with enzyme (0% inhibition). Three parameters were calculated on a per-plate basis: (a) the signal-to-background ratio (S/B); (b) the coefficient for variation [CV; CV = (standard deviation/mean) × 100)] for all compound test wells; and (c) the *Z′*-factor (18). The IC_{50} value of the pharmacological control ((N-hydroxy-1-(4-methoxyphenyl)sulfonyl-4-(4-biphenylcarbonyl)piperazine-2-carboxamide, Calbiochem cat#: 444252) was also calculated to ascertain the assay robustness.

2.4. Western blotting

Cell culture media containing 20 μg of total protein was resolved on a 4–12% Bis–Tris gel (NuPage), and transferred to a PVDF membrane. After blocking non-specific binding with 5% skim milk, the membrane was incubated over night with anti-rhNEP antibodies as per manufacturer's instructions (R & D systems). Immunoreactive bands were detected using appropriate secondary antibodies and ECL chemiluminescence reagent.

2.5. Data analysis

NEP activity in the media of Ea.hy926 cells was taken as the difference in the enzyme activities observed in the presence and absence of thiorphan. In cells treated with TPI2155-14 or TPI2155-17, the NEP activity was calculated as μmols of substrate cleaved per min and expressed as a % of control. Where indicated statistical significance was determined using an unpaired *t*-test or one-way ANOVA.

3. Results

3.1. Measurement of NEP activity

The activity of rhNEP (12 ng) was confirmed by the cleavage of 17.79 ± 1.31 μmols of substrate over 6 h. Significant inhibition of enzyme activity (3.31 ± 0.35 μmols of substrate cleaved after 6 h; $P = 0.004$; unpaired *t*-test; $n = 3$) occurred in the presence of the NEP inhibitor thiorphan (10 μM).

All enzyme assays using concentrated media was conducted in the presence of captopril (0.1 μM). The specificity of NEP mediated cleavage was confirmed using the NEP inhibitor thiorphan (10 μM). The NEP activity in media was taken as the difference between total enzyme activity, and that observed in the presence of thiorphan (Fig. 1A). Soluble NEP in cell culture media demonstrated a constant reaction rate (i.e. zero order reaction) over the first 2 h, after which the substrate concentration becomes rate limiting (i.e. first order reaction). Soluble NEP in the media cleaved 6.54 ± 0.38 μmols of substrate over 3 h (Fig. 1A).

3.2. Western blotting

Proteins in media of control cells or those treated with TPI2155-14 were analysed by Western blotting using anti-NEP antibodies. The data demonstrating the presence of a single protein band

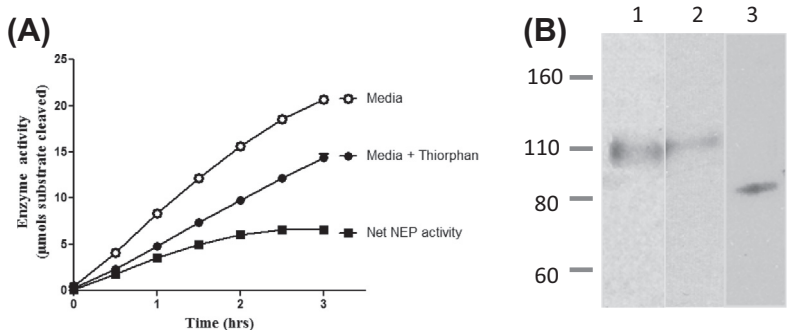

Fig. 1. NEP activity in media. (A) Media was treated with captopril, and total enzyme activity was measured in the presence and absence of NEP inhibitor thiorphan. Difference in activities observed was taken as the net NEP activity. (B) A representative Western blot indicating NEP in the media of (1) control or (2) TPI2155-14 treated cells, and rhNEP. Molecular weight markers are indicated on the left.

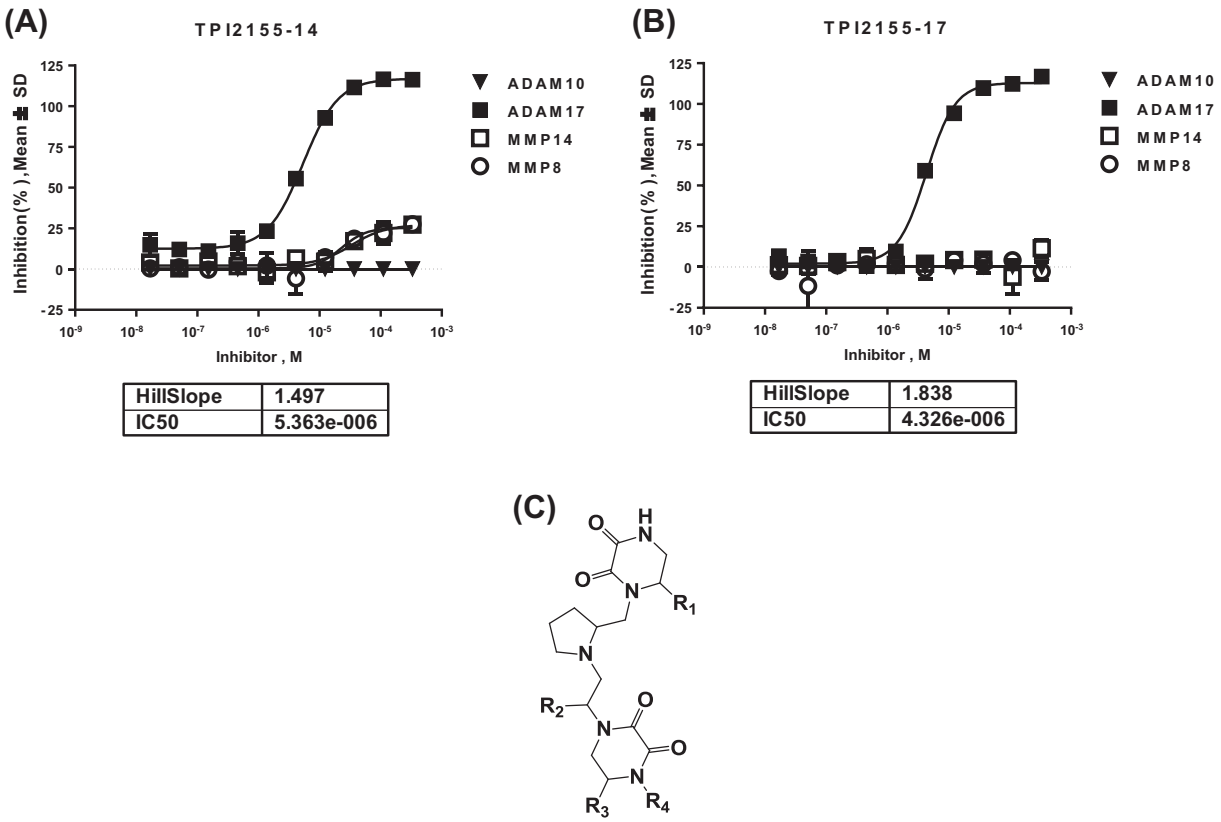

Fig. 2. ADAM-17 inhibitors. The inhibition of ADAM10, ADAM17, MMP-14 and MMP-8 using (A) TPI2155-14, (B) TPI2155-17, and (C) the common scaffold for the TPI2155 series. Glycosylated substrates were used to test ADAM inhibition while Mca-KPLGL-Dpa-AR-NH_2 was used for MMP inhibition; $n = 3$.

corresponding to an approximate molecular mass of 100 KDa, which is indicative of full-length NEP (Fig. 1B). rhNEP which only consists of the extracellular catalytically active domain was detected as a single band with an approximate molecular mass of 80 KDa.

3.3. ADAM-17 inhibition and NEP release

The inhibitors TPI2155-14 and -17 were tested for the inhibition of ADAM-10, ADAM-17, MMP-14 and MMP-8. TPI2155-14 and TPI2155-17 displayed an IC_{50} of 5.3 and 4.32 μM, respectively against ADAM-17 (Fig. 2). At the IC_{50}, both compounds displayed a high level of selectivity for ADAM-17 with no significant inhibition in the activity of other proteases tested. Although TPI2155-14 induced a 25% inhibition of MMP-14 and MMP-8 activity at concentrations above 100 μM, TPI2155-17 appeared highly specific at these concentrations (Fig. 2).

Neither TPI2155-17 (10 μM) or TPI2155-14 (15 μM) had any significant effects on the activity of rhNEP (103.26 ± 21.44 and 100.56 ± 20.13 as % of control). However, the incubation of cells with TPI2155-14 or TPI2155-17 over 24 h resulted in a significant decrease in NEP activity in the media (Fig. 3). Although the effect of TPI2155-14 was concentration dependant (Fig. 3), toxic effects in endothelial cells precluded the testing of TPI2155-17 at concentrations above its IC_{50}.

3.4. Ultracentrifugation and NEP activity

The supernatant of media from Ea.hy926 cells subjected to ultracentrifugation had no detectable NEP activity over 6 h

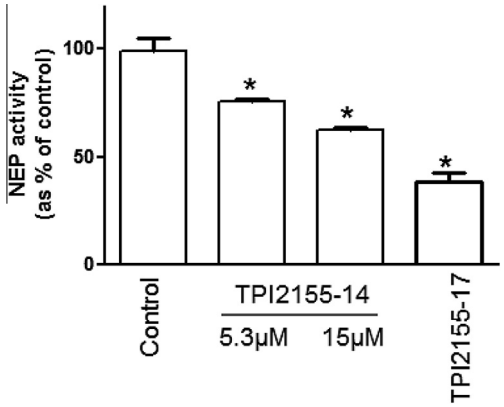

Fig. 3. ADAM-17 inhibition and NEP release. The effect of ADAM-17 inhibition by TPI2155-14 and TPI2155-17 on the levels of catalytically active NEP in media of Ea.hy926 cells; * Significantly different compared to control; $P < 0.0001$; one-way ANOVA; $n = 3–4$.

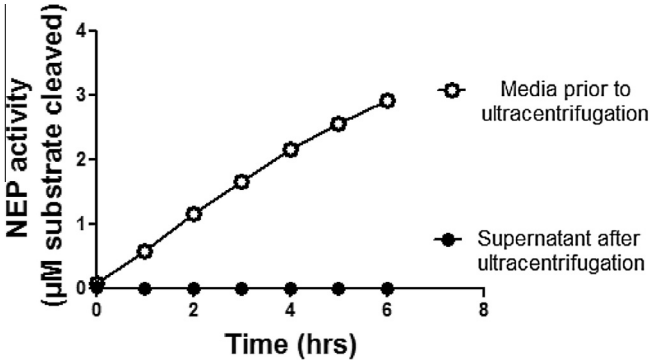

Fig. 4. Ultracentrifugation and NEP activity. Effect of ultracentrifugation on the specific NEP activity of media in Ea.hy926 cells.

suggesting exosome-mediated release of NEP from endothelial cells as opposed to classical shedding from the cell surface (Fig. 4).

4. Discussion

Here we report on the development and application of an assay to measure the activity of NEP in conditioned media. The assay is based on the ability of NEP to cleave a bradykinin based quenched fluorescent substrate. Using this assay we determined the activity of non-membrane bound NEP in the media of Ea.hy926 cells, which appears to be released from the cell surface via exosomes. Furthermore, this is the first report to indicate that the process of exosome mediated release of NEP is also dependant on the activity of ADAM-17.

The production of soluble/non-membrane associated counterparts of membrane-bound proteins has been extensively studied and is known to occur as a consequence of: (1) ecotodomain shedding, which involves the proteolytic cleavage of the extracellular domain; or (2) release of non-membrane associated enzyme from cells *via* exosomes. Members of the ADAM family of proteins in particular ADAM-17, have been implicated in the process of ectodomain shedding, however, this has been thought to be *via* a classical ectodomain shedding process. Exosomes are small microvesicles released from the endosomal compartment of cultured cells. These vesicles have also been found in various bodily fluids including blood, urine and amniotic fluid [13]. Exosomes can however be removed from biological fluids and cell culture media through ultracentrifugation [14].

Bradykinin is one of the natural substrates for NEP [15]. Previous studies indicate that a quenched fluorescent substrate based on the sequence of bradykinin but with minor modifications can be cleaved by both Endothelin Converting Enzyme-1 (ECE-1) and NEP [16]. Quenched fluorescent substrates have no inherent fluorescence in its intact form. However, enzyme-mediated cleavage of the substrate results in the separation of the fluorophore from the quenching molecule which in turn leads to an increase in fluorescence. We therefore used this substrate to monitor NEP activity in the media of endothelial cells. Centrifugation and concentrating of media after harvesting removed all cellular debris leaving only non-membrane associated NEP for analysis. The amount of fluorescence specifically due to soluble NEP was calculated by taking the difference in respective fluorescence values generated in the presence and absence of thiorphan.

Despite the relative specificity of thiorphan for NEP with an IC_{50} in the low nanomolar range [17], some studies have shown its ability to inhibit ACE [18], an enzyme which can also cleave bradykinin [15]. Given that the sequence of our QFS substrate is based on bradykinin, all enzyme assays were conducted in the presence of ACE inhibitor captopril in order to accurately determine NEP activity.

Previous studies have clearly demonstrated a role for ADAM-17 in the shedding of membrane-bound proteins and specifically proteases, using siRNA knockdowns [19,20] and broad specificity ADAM-17 inhibitors [20]. These inhibitors, including TAPI-1 also have non-specific effects inhibiting closely related ADAM-10 and MMPs. In addition, to a degree they can directly inhibit NEP activity (unpublished observation). At present there are no specific inhibitors of ADAM-17 which are commercially available. We therefore used two narrow specificity ADAM-17 inhibitors synthesised in house, to test the hypothesis that NEP, like many other cell membrane-bound proteins is cleaved by ADAM-17 to produce a soluble form. These inhibitors displayed a high level of selectivity and failed to show any inhibitory effect on ADAM-10, MMP-8 or MMP-14. Ea.hy926 cells were incubated for 24 h in the presence of TPI2155-14 and TPI2155-17. Both compounds had no effect on the activity of rhNEP at the concentrations tested. However, a significant decrease in the NEP activity in media was detected in response to both inhibitors. Although TPI2155-14 (5.3–15 μM) induced a concentration dependant decrease in NEP activity in media, TPI2155-17 could only be tested at its IC_{50} of 4.3 μM reflecting its toxic effects on endothelial cells. Inhibition of NEP activity in the media by both compounds implicates a possible role for ADAM-17 in the production of non-membrane associated NEP.

Catalytically active NEP levels have been detected both in the media of lymphoblastoid cell lines [7], and in the serum of coal miners exposed to coal dust [21] even after ultracentrifugation. This suggests the production of "soluble" NEP detected in these studies was likely mediated by ectodomain cleavage as opposed to an exosome-mediated release. Similarly to the data reported in this study, the molecular mass of NEP detected was approximately 100 KDa which is inconsistent with the mass expected following ADAM-17 mediated ectodomain cleavage. However, it is possible that the size exclusion chromatography and Western blotting used in these studies were not sufficiently sensitive enough to detect the relatively small difference (~5 KDa) in the molecular mass between the shed and full-length forms of the enzyme. In contrast to the results from studies mentioned above, in our study, ultracentrifugation of media removed all NEP activity from the supernatant suggesting the possibility that at least in the case of endothelial cells, the release of NEP into the media occurs *via* an exosomal route. This study was limited to Ea.hy926 cells which are known to retain many of the characteristics of primary endothelial cells [22]. A different mechanism of NEP release may occur in other cell types. Determining the N-terminal sequence of NEP in media and or access to specific antibodies that recognize the

cytoplasmic domain of NEP would perhaps help clarify the precise molecular form of the released enzyme. However, to our knowledge such antibodies are not available nor could sufficient quantities be purified from culture media to allow definitive N-terminal sequence determination.

In conclusion, we have measured and characterised NEP activity in the media of endothelial cells using a QFS based assay. Western blotting confirmed the presence of NEP in media, which was completely removed following ultracentrifugation. Furthermore, a reduction of NEP activity in media was observed following exposure to specific ADAM-17 inhibitors. Our results thus suggest that the release of NEP from endothelial cells occur *via* exosomes, a process dependent on ADAM-17. To the best of our knowledge this is the first report to shed light on the role played by ADAM-17 in the exosome mediated release of NEP from endothelial cells. Given that soluble NEP can reduce the deposition of brain amyloid beta [8] and is perhaps involved in other disease pathologies, an understanding of these mechanism(s) behind NEP release from cell membranes sheds light on specific targets for the therapeutic manipulation of circulating NEP levels.

Acknowledgments

This work was supported by funding from the National Health and Medical Research Council of Australia (Program Grant ID 490990), as well as the National Heart Foundation of Australia (NHF PF09M4668).

References

[1] N.N. Nalivaeva, C. Beckett, N.D. Belyaev, A.J. Turner, Are amyloid-degrading enzymes viable therapeutic targets in Alzheimer's disease?, J Neurochem. 120 (Suppl. 1) (2012) 167–185.
[2] E.G. Erdos, R.A. Skidgel, Neutral endopeptidase 24.11 (enkephalinase) and related regulators of peptide hormones, FASEB J. 3 (1989) 145–151.
[3] V. Maguer-Satta, R. Besancon, E. Bachelard-Cascales, Concise review: neutral endopeptidase (CD10): a multifaceted environment actor in stem cells, physiological mechanisms, and cancer, Stem Cells 29 (2011) 389–396.
[4] P. Crine, N. Dion, G. Boileau, Endopeptidase-24.11, in: A.J. Kenny, C.M. Boustead (Eds.), Cell Surface Peptidase in Health and Disease, Bios Scientific Publishers, Oxford, 1997, pp. 79–98.
[5] B. Malfroy, W.J. Kuang, P.H. Seeburg, A.J. Mason, P.R. Schofield, Molecular cloning and amino acid sequence of human enkephalinase (neutral endopeptidase), FEBS Lett. 229 (1988) 206–210.
[6] Y. Komada, H. Ochiai, K. Shimizu, E. Azuma, H. Kamiya, M. Sakurai, Shedding of leukemia-associated P24 antigen by lymphoblastoid cell lines, Jpn. J. Clin. Oncol. 17 (1987) 333–342.
[7] Y. Komada, S. Peiper, B. Tarnowski, S. Melvin, H. Kamiya, M. Sakurai, Shedding of the common acute lymphoblastic leukemia antigen (CALLA) by lymphoblastoid cell lines, Leuk. Res. 10 (1986) 665–670.
[8] Y. Liu, C. Studzinski, T. Beckett, M.P. Murphy, R.L. Klein, L.B. Hersh, Circulating neprilysin clears brain amyloid, Mol. Cell. Neurosci. 45 (2010) 101–107.
[9] K. Honjo, S.E. Black, N.P. Verhoeff, Alzheimer's disease, cerebrovascular disease, and the beta-amyloid cascade, Can. J. Neurol. Sci. 39 (2012) 712–728.
[10] M.U. Norman, S.B. Reeve, V. Dive, A.I. Smith, R.A. Lew, Endopeptidases 3.4.24.15 and 24.16 in endothelial cells: potential role in vasoactive peptide metabolism, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H1978–H1984.
[11] D. Minond, M. Cudic, N. Bionda, M. Giulianotti, L. Maida, R.A. Houghten, G.B. Fields, Discovery of novel inhibitors of a disintegrin and metalloprotease 17 (ADAM17) using glycosylated and non-glycosylated substrates, J. Biol. Chem. 287 (2012) 36473–36487.
[12] U. Neumann, H. Kubota, K. Frei, V. Ganu, D. Leppert, Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme, Anal. Biochem. 328 (2004) 166–173.
[13] S. Keller, M.P. Sanderson, A. Stoeck, P. Altevogt, Exosomes: from biogenesis and secretion to biological function, Immunol. Lett. 107 (2006) 102–108.
[14] C. Lasser, M. Eldh, J. Lotvall, Isolation and characterization of RNA-containing exosomes, J. Vis. Exp. (2012) e3037.
[15] A. Kuoppala, K.A. Lindstedt, J. Saarinen, P.T. Kovanen, J.O. Kokkonen, Inactivation of bradykinin by angiotensin-converting enzyme and by carboxypeptidase N in human plasma, Am. J. Physiol. Heart Circ. Physiol. 278 (2000) H1069–H1074.
[16] G.D. Johnson, K. Ahn, Development of an internally quenched fluorescent substrate selective for endothelin-converting enzyme-1, Anal. Biochem. 286 (2000) 112–118.
[17] K.E. Dickinson, A.A. Tymiak, R.B. Cohen, E.C. Liu, M.L. Webb, A. Hedberg, Vascular A10 cell membranes contain an endothelin metabolizing neutral endopeptidase, Biochem. Biophys. Res. Commun. 176 (1991) 423–430.
[18] B.P. Roques, E. Lucas-Soroca, P. Chaillet, J. Costentin, M.C. Fournie-Zaluski, Complete differentiation between enkephalinase and angiotensin-converting enzyme inhibition by retro-thiorphan, Proc. Natl. Acad. Sci. USA 80 (1983) 3178–3182.
[19] D.W. Lambert, M. Yarski, F.J. Warner, P. Thornhill, E.T. Parkin, A.I. Smith, N.M. Hooper, A.J. Turner, Tumor necrosis factor-alpha convertase (ADAM17) mediates regulated ectodomain shedding of the severe-acute respiratory syndrome-coronavirus (SARS-CoV) receptor, angiotensin-converting enzyme-2 (ACE2), J. Biol. Chem. 280 (2005) 30113–30119.
[20] M. Peng, S. Guo, N. Yin, J. Xue, L. Shen, Q. Zhao, W. Zhang, Ectodomain shedding of Fcalpha receptor is mediated by ADAM10 and ADAM17, Immunology 130 (2010) 83–91.
[21] J. Sinisalo, F. Fyhrquist, M. Syrjala, M.S. Nieminen, Stimulated release of endothelin-converting enzyme is simultaneous with tissue-type plasminogen activator and decrease in coronary heart disease, Scand. Cardiovasc. J. 36 (2002) 100–104.
[22] K. Ahn, S. Pan, K. Beningo, D. Hupe, A permanent human cell line (EA.hy926) preserves the characteristics of endothelin converting enzyme from primary human umbilical vein endothelial cells, Life Sci. 56 (1995) 2331–2341.