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Production of soluble Neprilysin by endothelial cells

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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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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