Biochimie 146 (2018) 73–78

Contents lists available at ScienceDirect

Biochimie

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

Research paper

Degradation of tropoelastin and skin elastin by neprilysin

Angela C. Mora Huertas [a], Christian E.H. Schmelzer [a, b], Chiara Luise [a], Wolfgang Sippl [a], Markus Pietzsch [a], Wolfgang Hoehenwarter [c], Andrea Heinz [a, d, *]

[a] Institute of Pharmacy, Faculty of Natural Sciences I, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany
[b] Fraunhofer Institute for Microstructure of Materials and Systems IMWS, Halle (Saale), Germany
[c] Proteome Analytics, Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany
[d] Department of Pharmacy, University of Copenhagen, Copenhagen, Denmark

ARTICLE INFO

Article history:
Received 17 October 2017
Accepted 25 November 2017

Keywords:
Skin fibroblast-derived elastase
Mass spectrometry
Skin aging
Matrikines

ABSTRACT

Neprilysin is also known as skin fibroblast-derived elastase, and its up-regulation during aging is associated with impairments of the elastic fiber network, loss of skin elasticity and wrinkle formation. However, information on its elastase activity is still limited. The aim of this study was to investigate the degradation of fibrillar skin elastin by neprilysin and the influence of the donor's age on the degradation process using mass spectrometry and bioinformatics approaches. The results showed that cleavage by neprilysin is dependent on previous damage of elastin. While neprilysin does not cleave young and intact skin elastin well, it degrades elastin fibers from older donors, which may further promote aging processes. With regards to the cleavage behavior of neprilysin, a strong preference for Gly at P1 was found, while Gly, Ala and Val were well accepted at P1′ upon cleavage of tropoelastin and skin elastin. The results of the study indicate that the progressive release of bioactive elastin peptides by neprilysin upon skin aging may enhance local tissue damage and accelerate extracellular matrix aging processes.

1. Introduction

Elastin is the main component of elastic fibers and confers elasticity and resilience to tissues such as skin, arteries, lungs and ligaments. The structure of its precursor tropoelastin (TE) is characterized by the presence of alternating hydrophobic and hydrophilic domains. The hydrophilic regions are referred to as KA-type and KP-type cross-linking domains and contain Lys residues separated by either two/three Ala residues (KA) or at least one Pro residue (KP). The hydrophobic domains are rich in repeated motifs containing Gly, Val and Pro, which constitute about 55% of the elastin sequence. The hydrophobic and cross-linked nature of mature elastin makes it durable and resistant to enzymatic proteolysis [1].

Elastin degradation by fibroblast- (e.g. skin fibroblast elastase, matrix metalloproteinases (MMPs)) or inflammatory cell-derived (e.g. leukocyte elastase (HLE), cathepsin G (CG)) elastases is linked to the progression of several diseases affecting multiple organs and tissues. Elastolysis interferes with tissue homeostasis through the generation of elastin-derived peptides. Such peptides, in particular those containing the GXXPG motif, may influence processes such as chemotaxis, proliferation, protease activation and apoptosis [2]. Although the characteristics and elastolytic activity of many elastases are well-recognized, not much is known about neprilysin (NEP). NEP (EC 3.4.24.11, CD10, enkephalinase and CALLA) is a type-II integral membrane glycoprotein and member of the M13 subgroup of zinc-dependent endopeptidases [3]. It is also known as skin fibroblast-derived elastase [4] and is widely expressed throughout mammalian tissues, including lungs, male genital tract, various epithelia and neural synapses [3]. It is mainly involved in extracellular catabolism of bioactive peptides such as enkephalins and atrial natriuretic peptide [5], but also degrades components of the elastic fiber system [4,6].

The up-regulated expression and activity of NEP during mouse skin aging and exposure to UV radiation are associated with

Abbreviations: AE, elastin from skin samples of adults; CALLA, common acute lymphoblastic leukemia antigen; CD10, cluster of differentiation 10; CE, elastin from skin samples of children; CG, cathepsin G; HPLC, high performance liquid chromatography; MMP, matrix metalloproteinase; MS, mass spectrometry; NEP, recombinant human neprilysin; TE, tropoelastin; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol.

* Corresponding author. Department of Pharmacy, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.
E-mail address: andrea.heinz@sund.ku.dk (A. Heinz).

https://doi.org/10.1016/j.biochi.2017.11.018
0300-9084/

impairment of the elastic fiber network and subsequent loss of skin elasticity. It has been postulated that NEP may play a pivotal role in wrinkle formation through degrading elastic fibers surrounding fibroblasts [4,7]. The effect of NEP on mature elastin including the influence of the donor's age on cleavage by NEP have never been characterized. However, this would contribute to a better understanding of the role of NEP during skin aging. Hence, the current study attempts to comprehensively describe the effect of NEP on young and aged skin elastin and on elastin's precursor TE.

2. Materials and methods

2.1. Materials

Human tropoelastin (TE) isoform 2 was produced in an *E. coli* expression system based on previous work [8] with some modifications with respect to the purification procedure. Three foreskin samples (8 mm × 8 mm) were collected from children aged ten years, and three skin biopsies (5 mm in diameter) from individuals aged 75 were acquired postoperatively from the tumor-free border of excised skin cancer tissue from the upper lip. The study was approved by the ethics committee of the Medical Faculty of the Martin Luther University Halle-Wittenberg (Germany) and carried out in compliance with the Helsinki Declaration. Each participant or each subject's legally authorized representative provided full written consent. Each skin sample was treated following the method described previously to obtain intact elastin fibers free from contamination with other extracellular matrix components [9]. The dried samples were stored at −26 °C prior to analysis. Recombinant human neprilysin (CHO cell-derived; >95% purity) was purchased from R&D Systems (Minneapolis, MN, USA), and analytical grade 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and formic acid were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile (ACN; VWR Prolabo, Leuven, Belgium) was used. Trifluoroacetic acid was purchased from Sigma-Aldrich. All other chemicals used were of analytical grade.

2.2. Proteolysis of tropoelastin and human elastin

TE was dissolved in 50 mM Tris-HCl buffer, pH 7.5, at a concentration of 1 mg mL^{-1}. Human skin elastin samples from children (CE) and adults (AE) were weighed and dispersed in 50 mM Tris-HCl buffer, pH 7.5, at a concentration of 1 mg mL^{-1}, respectively. All samples were incubated with NEP for 48 h at 37 °C. The enzyme-to-substrate ratio was 1:100 (w/w). All digestions were stopped by addition of trifluoroacetic acid to a final concentration of 0.5% (v/v). The digested samples were stored at −26 °C until further analysis.

2.3. Mass spectrometric analysis

Prior to their analysis, all digested samples were desalted using ZipTip C18 (EMD Millipore, USA) pipette tips following the procedure from the supplier. An EASY-nLC 1000 nano UPLC system (Thermo Fisher Scientific, Idstein, Germany) coupled online to an Orbitrap Q Exactive plus mass spectrometer (Thermo Fisher) was used. Each sample was loaded onto an EASY-Spray column, 50 cm × 75 μm ID, PepMap C18, 2 μm ES803 (Thermo Fisher) and eluted using a binary system of solvent (0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B)). A linear binary gradient was applied: 5–40% B in 120 min, to 90% B in the next 5 min, followed by maintenance at 90% B for 5 min, and then from 90% to 10% in 5 min. The precursor ion detection was done in an *m/z*-range from 400 to 2000, the acquisition range for fragment ions was *m/z* 120 to 1800, and the fragmentation mode was HCD with 28% normalized energy. The ten most abundant signals were used for MS/MS experiments.

2.4. Peptide sequencing

The raw data was imported into the software Peaks Studio (version 8.0; Bioinformatics Solutions, Waterloo, Canada) [10], in which data processing and automated *de novo* sequencing of tandem mass spectra followed by database matching were performed. An error tolerance of 6.0 ppm (parent mass) and 0.015 Da (fragment mass) were used and the database was taxonomically restricted to *Homo sapiens*. Enzyme specificity was set to none. Hydroxyproline was set as a variable modification (only for the analysis of the human elastin samples). A peptide false discovery rate (FDR) threshold of 5% was used.

2.5. Docking studies

Six peptides with Pro at P2 or P3′ (GVPGAIPG, GGPGFGPG, PVPGGLAG, LVPGGVAD, VKPGKVPG and PGVGVLPG) present in the NEP digests of CE or AE were docked into the NEP substrate binding pocket using GOLD docking program version 5.2 [11] to investigate the preference of S2 and S3′ for Pro. The crystal structure of NEP in complex with the inhibitor MCB3937 (PDB ID 2QPJ) solved at 2.05 Å [12] was retrieved from the Protein Data Bank [13]. Heteroatoms and water molecules were removed and hydrogen atoms were added to the protein by using the Molecular Operating Environment (MOE) modeling package version 2012.10 (Montreal, Canada). The protein structure was generated at physiological pH using the protonate 3D protocol implemented in MOE. The protein structure was subsequently energy-minimized by applying AMBER12_EHT force field and the GB/SA solvation model using default settings. The peptide substrates were prepared using the Protein Builder tool in MOE. A sphere of 8 Å around the co-crystallized inhibitor was defined as the binding site and Chemscore was chosen as fitness function. The search efficiency was set at 100%, a maximum of 100 genetic algorithm (GA) runs for each peptide was allowed and early termination of docking was switched off. To reduce the conformational sampling, hydrogen bond constraints were applied that were observed in the crystal structures of NEP bound to peptide-like inhibitors: side chain NH of Asn542, backbone CO of Ala543, backbone NH of Tyr545, side chain NH of His711. Except for the hydrogen bond constraints, the same protocol was used for re-docking the co-crystallized inhibitor MCB3937. The chosen setup was able to reproduce the crystal structure of the NEP inhibitor MCB3937 with an RMSD value of 1.97 Å (top-scored docking pose). In case of the docked peptides under study the top-ranked docking pose (Chemscore) of each compound was selected and visually analyzed.

3. Results and discussion

The TE and elastin samples were incubated with NEP for 48 h at 37 °C, and the resultant peptide mixtures were subsequently analyzed by LC-MS/MS. As confirmed by MS, TE was comprehensively degraded, while elastin samples from children (CE) and adults (AE) remained partly undigested in the reaction tubes after 48 h of incubation with NEP. MS measurement of the peptide digests resulted in almost complete sequence coverage of TE (Fig. 1). Cleavage occurred throughout the TE molecule, and a total number of 606 peptides and 289 cleavage sites were identified (Supplementary Table S1). Elastin samples were not cleaved as comprehensively, and substantial differences were found between CE and AE. While only 43 cleavage sites (25% sequence coverage) were identified for CE from 27 identified peptides (Supplementary Table S2), 164 cleavage sites (65% sequence coverage) were