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# Neutral endopeptidase (CD10) is abundantly expressed in the epididymis and localized to a distinct population of epithelial cells – Its relevance for CNP degradation

Arief Thong, Dieter Müller, Caroline Feuerstacke, Andrea Mietens, Angelika Stammler, Ralf Middendorff *

*Institute of Anatomy and Cell Biology, Justus-Liebig-University Giessen, 35385 Giessen, Germany*

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ABSTRACT

Neutral endopeptidase (NEP, metallo-endopeptidase EC 3.4.24.11; enkephalinase, neprilysin, CD10, CALLA) represents a major regulator of bioactivity of natriuretic peptides. C-type natriuretic peptide (CNP) is present in high levels in epididymis and seminal plasma. However, detailed expression pattern and CNP-related function of NEP in the epididymis are unknown.

Comparison of NEP protein levels in various organs revealed an extremely high expression in human and mouse epididymis. NEP was localized exclusively to apical (luminal) parts of epithelial cells. In man, strong NEP-immunoreactivity was associated with epithelia of efferent ducts and the epididymal duct including stereocilia. Segment-by-segment analysis in mouse revealed a distinct distribution along the epididymal duct. We also found the CNP receptor guanylyl cyclase B (GC-B) in epithelial cells of the epididymal duct. Two different NEP inhibitors decreased CNP degradation and increased CNP/GC-B-induced cGMP production by epididymal membranes, suggesting a functional involvement of NEP.

Data indicate an important, previously neglected, role of NEP for regulation of luminal factors in the epididymis and suggest a novel role for CNP/GC-B in the epididymal epithelium, presumably in context of local water balance.



## 1. Introduction

Neutral endopeptidase (NEP, also known as metallo-endopeptidase EC 3.4.24.11; enkephalinase, neprilysin, CD10, CALLA) is a plasma membrane protein containing a short N-terminal cytoplasmic domain, a single hydrophobic transmembrane domain and a large C-terminal extracellular domain with metallopeptidase activity (Terawaki et al., 2007). NEP cleaves and inactivates a variety of biologically active peptides such as enkephalins, bradykinin, endothelin-1, neurotensin and natriuretic peptides (Corti et al., 2001). By this, NEP regulates the local concentrations of these peptides at the cell surface and affects their receptor-mediated cellular activities (Turner et al., 2001). For example, NEP inhibits prostate cancer cell proliferation and migration via inactivation of certain neuropeptide substrates (Papandreou et al., 1998). In brain, NEP is implicated in the degradation of amyloid β protein (Aβ), and decreases in endogenous NEP levels lead to enhanced Aβ concentrations, amyloid plaque formation and Aβ-associated neuropathology (Farris et al., 2007). Cellular activities of NEP can also be induced by its cytoplasmic domain. Binding of this domain to ezrin/radixin/moesin (ERM) proteins, which interact with the cytoskeleton, can affect cell adhesion and migration (Iwase et al., 2004; Terawaki et al., 2007).

Due to tissue-specific differences in glycosylation, NEP native molecular masses range from 90 to 110 kDa (Hemming et al., 2007; Müller et al., 2010b).The presence of multiple androgen response elements in the NEP gene and transcriptional regulation of NEP by androgen in prostate cancer cells (Zheng et al., 2006) suggest particular roles for NEP in androgen-responsive tissues. Expression of NEP has been reported for various tissues, including prostate, kidney, intestine, brain and lung (Zheng et al., 2010). In the male genital tract, high local NEP enzyme activity (Erdös et al., 1985) and immunoreactivity localized to epithelial cells of vas deference and epididymis (Cerilli et al., 2003; Ordi et al., 2001; Sasaki et al., 2009) was described. The epididymis is responsible for maturation, transport and storage of mammalian spermatozoa after leaving the testis. During their transit through the epididymal duct, the spermatozoa are exposed to different luminal

*Abbreviations:* ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; cGMP, cyclic guanosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; GC-B, guanylyl cyclase type B; neutral endopeptidase, NEP; SMA, smooth muscle actin.

* Corresponding author. Address: Institute of Anatomy and Cell Biology, Justus Liebig University Giessen, Aulweg 123, 35385 Giessen, Germany. Tel.: +49 64199 47160; fax: +49 64199 47169.

*E-mail address:* Ralf.Middendorff@anatomie.med.uni-giessen.de (R. Middendorff).

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environments, which allow the germ cells to acquire progressive motility and the ability to fertilize an egg (Robaire et al., 2006).

Region-specific differences in cellular activities of the epididymal epithelium, resulting in the secretion (and re-absorption) of various compounds, provide the basis for the distinct local luminal environments required for proper maturation and storage of the sperm cells (Kirchhoff, 1999; Shum et al., 2011). A second important organ function is localized to the peritubular smooth muscle cell layer, since contractions of the epididymal duct facilitate controlled propulsion of the spermatozoa through the epididymis (Mewe et al., 2006b; Mietens et al., 2012).

C-type natriuretic peptide (CNP) is one well established *in vitro* substrate of NEP (Pankow et al., 2009; Potter, 2011). Interestingly, this peptide is highly abundant in seminal plasma (Chrisman et al., 1993), and epididymal epithelial cells were found to produce huge amounts of CNP (Nielsen et al., 2008). The epididymis is also a major expression site of the CNP receptor, guanylyl cyclase B (GC-B), and CNP binding to epididymal membrane preparations elicits marked accumulation of cyclic GMP (cGMP), representing the second messenger of this signaling pathway. This data suggests a previously unrecognized role for CNP/GC-B signaling in the epididymis with activities exceeding those involved in regulation of contractility (Mewe et al., 2006a).

To address this issue, we aimed to characterize the cellular localization of NEP and GC-B in mouse and human epididymis and to evaluate potential functional implications. Our findings suggest a novel role for CNP/GC-B in the epididymal epithelium and a function of NEP in regulating luminal CNP levels.

## 2. Materials and methods

### 2.1. Materials

Human epididymis tissue was available from a local tissue bank from 84 patients aged 35 to 89 years. Most of them were undergoing orchiectomy as the primary treatment of prostatic carcinoma. Collection and use of human tissue was approved by the ethics commission of the Ärztekammer Hamburg, Germany. One to 2 h after surgery, pieces of chilled human epididymis were cut and tissue samples were either frozen in liquid nitrogen (for subsequent protein preparation or laser capture microdissection) or fixed in Bouin's fluid (for immunohistochemical analyses). Whole mouse epididymides were dissected after decapitation of the animals, and either used for paraffin embedding or immediately frozen in liquid nitrogen and stored at −80 °C until protein preparations. The animals (C57/Bl6 mice) were used according to government principles regarding the care and use of animals with permission (G8151/591-00.33) of the local regulatory authority.

$^{125}$I-[Tyr$^{0}$]-CNP (2200 Ci/mmol) was obtained from Biotrend (Cologne, Germany). CNP (CNP-22, Code H-1296) was from Bachem (Bubendorf, Switzerland), thiorphan from Biomol (Hamburg, Germany) and phosphoramidon from Sigma–Aldrich (St. Louis, MO, USA).

### 2.2. Preparation of membrane protein fractions

Whole epididymides (mouse) or pieces derived from different parts of the epididymis as specified in Fig. 1 (human), both without treatments to remove spermatozoa, were used. Membrane protein fractions were generated from the frozen tissues as described before (Müller et al., 2010b). Protein concentrations were determined by using a dye-binding assay (#500-0006, Bio-Rad, Munich, Germany) according to the manufacturer's protocol with bovine serum albumin (fraction V, Sigma–Aldrich) as standard.

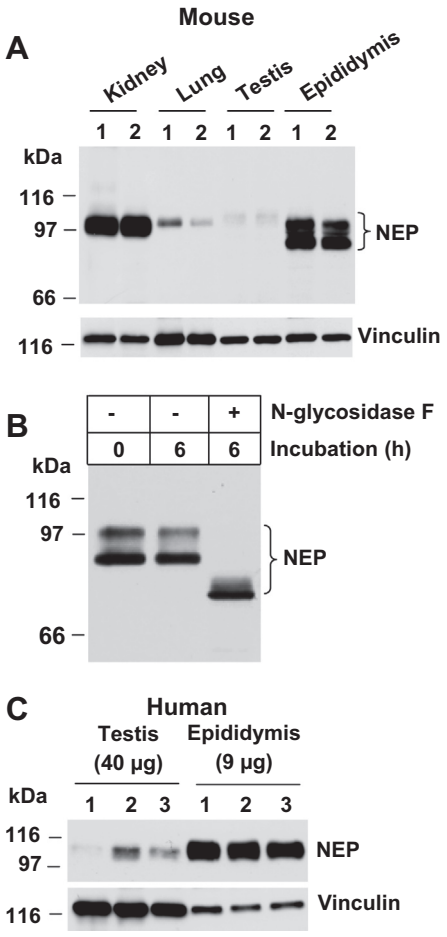


**Fig. 1.** Protein expression of NEP in mouse and human epididymis. (A): Equal amounts (25 µg protein) of membranes from the mouse tissues indicated were characterized for NEP protein levels by immunoblotting. Samples from two animals (1 and 2) each were analyzed. Detection of vinculin served as loading control. The migration of reference proteins (in kDa) is indicated. (B): Enzymatic deglycosylation of mouse epididymal NEP. Equal amounts of membrane protein were analyzed by NEP immunoblotting after incubations for 6 h at 37 °C in either the absence (−) or presence (+) of N-glycosidase F. An untreated sample (0) served as reference. The size range of NEP immunoreactive bands is indicated to the right. (C): NEP immunoblot of human testis (40 µg of protein) and epididymis (9 µg) membranes, derived from three (1 to 3) individuals each. Epididymal tissue was from corpus (1 and 3) and cauda (2), respectively. After stripping, the blot was analyzed for vinculin levels. The migration of reference proteins (in kDa) is indicated to the left.

### 2.3. Immunoblot analyses

Proteins were resolved by SDS–PAGE under reducing conditions and transferred to nitrocellulose membranes. After staining with Ponceau S (Sigma–Aldrich) to visualize the positions of co-migrated reference proteins (S8320, Sigma–Aldrich) and pre-treatment with blocking solution (#1096176; Roche, Mannheim, Germany), blots were exposed to mouse monoclonal anti-NEP (anti-CD10, Menarini Diagnostics, Berlin, Germany, product number NCL-CD-10-270, host: mouse, 1:1500). Goat anti-mouse IgG (#31432, Pierce, Rockford, IL, USA), linked to peroxidase, served as secondary antibody, and signals were detected as described (Müller et al., 2010b). After stripping (Müller et al., 2010b), blots were re-exposed to anti-vinculin (Sigma–Aldrich, St. Louis, MO, USA, product number V9264, host: mouse, 1:6,000).

### 2.4. Immunostaining and azan staining

For immunohistochemistry and azan staining, 5 µm thick depa-raffinized human and mouse epididymis sections from Bouin-fixed