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MINIREVIEW

The immuneregulator role of neprilysin (NEP) in invertebrates

E Ottaviani[1], D Malagoli[1], A Grimaldi[2], M de Eguileor[2]

[1]*Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/D,41125 Modena, Italy*
[2]*Department of Biotechnology and Life Science, University of Insubria, Via J. H. Dunant 3, 21100 Varese, Italy*

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Abstract

Neprilysin (NEP) represents an important enzyme in both vertebrates and invertebrates. In the present report we have focused our attention to invertebrates. In particular, a structure related to CD10/NEP as well as its activity in different tissues, such as immunocytes, nervous tissue and muscle of various species were detected. Moreover, the role played by the enzyme in the interactions between host and parasite has also been reported. The findings indicate that NEP immunoregulation is a well-balanced process that, with appropriate physiological and homeostatic responses to challenges, allows the survival and well-being of the species.

Key Words: Neprilysin (NEP); invertebrates; immunoregulation

Introduction

Neutral endopeptidase, NEP (EC 3.4.24.11) now referred to as neprilysin is a type II integral membrane protein with a MW of 93 Kda, consists of a short NH_2-terminal cytoplasmic domain of 27 amino acids, a trasmembrane region of 22 hydrophobic residues, and a large extracellular domain of about 700 residues that contains zinc in the active center (Fig. 1). It cleaves substrates on the amino side of hydrophobic amino acids (Kerr *et al.*, 1974a, b; Gafford *et al.*, 1983; Turner and Tanzawa, 1997; Turner *et al.,* 2001). The *NEP* gene exists in a single copy, extents more than 80 kb, is composed of 24 exons and is highly conserved in mammalian species (D'Adamio *et al.*, 1989). Furthermore, NEP was shown to be identical to CD10, a tumor-associated cluster differentiation antigen expressed on the surfce of neutrophils and some lymphoid progenitors, and also known as the common acute lymphoblastic leukemia antigen (CALLA) (Letarte *et al.*, 1988).

Neprilysin (NEP)

The NEP has been reported in vertebrates and invertebrates. With regards vertebrates, the majority of the data refer mammals. In particular, from the literature it emerges that this enzyme is mainly localized in the human, rat, rabbit and pig kidney (Kerr and Kenny, 1974a, b; Mumford *et al.,* 1981; Gafford *et al.*, 1983; Edwards *et al.*, 1999), but also in human fibroblasts (Lorkowski *et al.*, 1987; Kletsas *et al.*, 1998), human genital tract (Erdös and Skidgel, 1989), rat brain (Back and Gorenstein, 1989), human blood cells (Connelly *et al.*,1993), rat nerve ending membranes (Vandenbulcke *et al*., 1994), mammalian membrane (Turner and Tanzawa, 1997), mouse mesangial cells (Ebihara *et al*., 2003), and so on.

As far as invertebrates are concerned, a structure related to CD10/NEP as well as its activity were detected in the molluscan immunocytes of *Mytilus edulis* (Shipp *et al.*, 1990), *Planorbarius corneus*, *Viviparus ater* (Ottaviani and Caselgrandi, 1997) and *Mytilus galloprovincialis* (Caselgrandi *et al.*, 2000), and in the central nervous system of *Aplysia californica* (Zappulla *et al.*, 1999). With refer other groups, data were reported on the hemocytes of the insect *Heliothis virescens* (Grimaldi *et al*., 2012), neural membranes from the locust *Schistocerca gregaria* (Isaac, 1988), from the nematode *Ascaris suum* muscle (Sajid and Isaac, 1995) and from the head parts of the leech *Theromyzon tessulatum* (Laurent and Salzet, 1995).

Genomic studies revealed that *Caenorhabditis elegans* and *Drosophila melanogaster* contain 22 and 24 NEP-like genes (Turner et al., 2001). Moreover, further investigations in *D. melanogaster* have reported that the *Nep2* gene codes for a secreted endopeptidase with a highly restricted

Corresponding author:
Enzo Ottaviani
Department of Biotechnology and Life Sciences
University of Modena and Reggio Emilia
Via Campi 213/D, 41125 Modena, Italy
E-mail: enzo.ottaviani@unimore.it

pattern of expression, and this protein for its localization seems involved in renal function and in spermatogenesis (Thomas *et al*., 2005).

We detected the presence of NEP and its activity by using different approaches. In the first case, the cytofluorimetric analysis of *M. galloprovincialis* immunocytes revealed that the cells were positive to CD10 in a range from 8 to 10 % (Fig. 2) (Caselgrandi *et al.*, 2000), while in granulocytes of the insect *H. virescens* the presence of the enzyme was detected by immunocytochemical (Fig. 3) and Western Blot analysis (Grimaldi *et al.*, 2012). For the determination of the NEP activity, we used a spectrofluorimetric procedure (Ottaviani and Caselgrandi, 1997) and the Shape Factor (SF) protocol (Ottaviani *et al.*, 1997; Sassi *et al.*, 1998; Caselgrandi *et al.*, 2000). This last procedure is based on the capacity of NEP to cleave biological peptides (Duvaux-Miret *et al.*, 1992; Otttaviani and Ceselgrandi, 1997) and cytokines (Pierart *et al.*, 1988; Casey *et al.*, 1993; Caselgrandi *et al.*, 2000) molecules that, in turn, activate the cell motility of immunocytes (Scharrer and Stefano, 1994; Sassi *et al.*, 1998; Caselgrandi *et al.*, 2000; Ottaviani *et al.*, 2004). The enzymatic activity of NEP was confirmed by phosphoramidon, a potent inhibitor of NEP (Fulcher *et al.,* 1982).

The induced changes in cell shape, from a round form (inactive) to an ameboid form (active), were recorded by measuring the cellular area and the perimeter allowing the evaluataion of the SF (Fig. 4). The SF formula of the American Innovision Analysis System software package (San Diego, CA) was used to express changes mathematically, as described in detail elsewhere (Schön *et al.*, 1991). The evaluation of the SF has been described previously (Sassi *et al.*, 1998). Briefly, this method is based on the use of a computer-assisted microscopic image analysis. A vaseline ring on a microscopy slide delimited a chamber within which 100 μl of hemolymph were placed (Fig. 5), as described in detail in a previous paper (Ottaviani *et al.*, 1997).

Functions of Neprilysin (NEP)

This endopeptidase plays a regulatory role on the peptides that are involved in the physiological mechanisms of mammalian nervous, cardiovascular and immune systems (Turner *et al.*, 2001).

In the present paper we will focus our attention on its role in the invertebrate immune system. At a glance observation of literature it emerges that the enzymatic degradation of NEP induces a downregulation according to the following scheme: a first stimulus (for instance biopeptides, cytokines) activating an immunocyte upregulates NEP. Consequently immunocyte response to a second stimulus, that serves as NEP substrate, is downregulated (Fricchione and Stefano, 1994).

The activation of invertebrate immunocytes was found to be suppressed by adrenocorticotropin hormone (ACTH) and alpha-melanocyte-stimulating hormone (α-MSH). The effect of ACTH is largely due to its conversion to α-MSH by immunocyte-

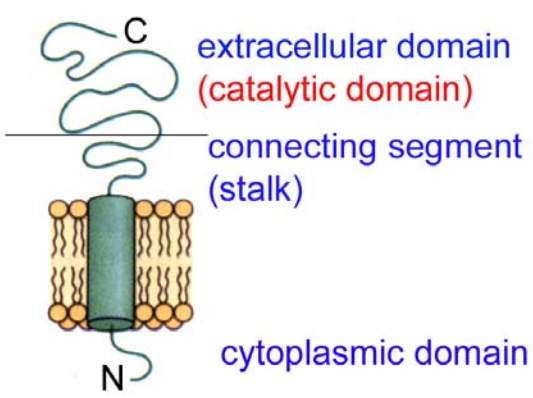

Fig. 1 Schematic representation of NEP.

associated NEP. This is the topic point, since α-MSH inhibits adherence and locomotory activity of polymorphonuclear leukocytes (PMN), monocytes and invertebrate immunocytes. It should be underlined that while α-MSH acts rapidly (min) on the cells, ACTH requires much more time (h) in order to act, and this is due its processing into α-MSH (Smith *et al.*, 1992).

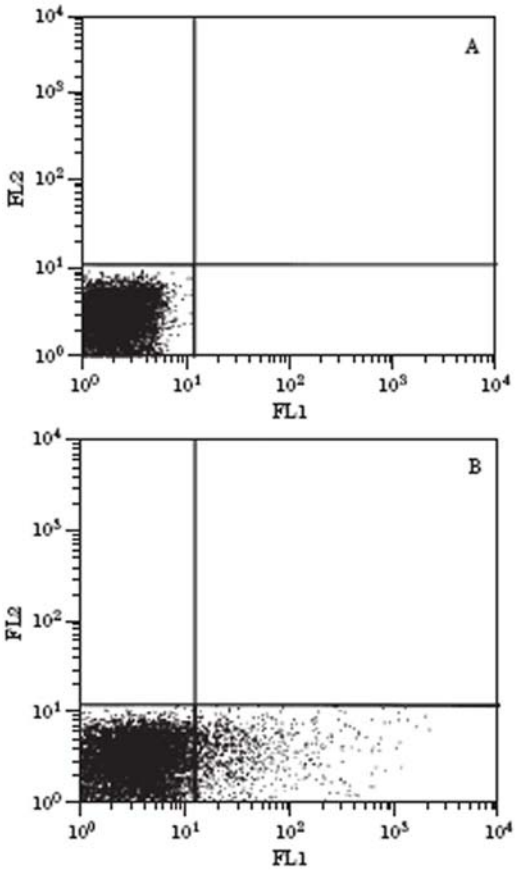

Fig. 2 Cytofluorimetric analysis of *M. galloprovincialis* immunocytes. (A) control, (B) staning with anti-CD10 mAb. FL1 = fluorescence 1 channel; FL2 = fluorescence 2 channel. From Caselgrandi *et al.*, 2000 (reprinted with permission).

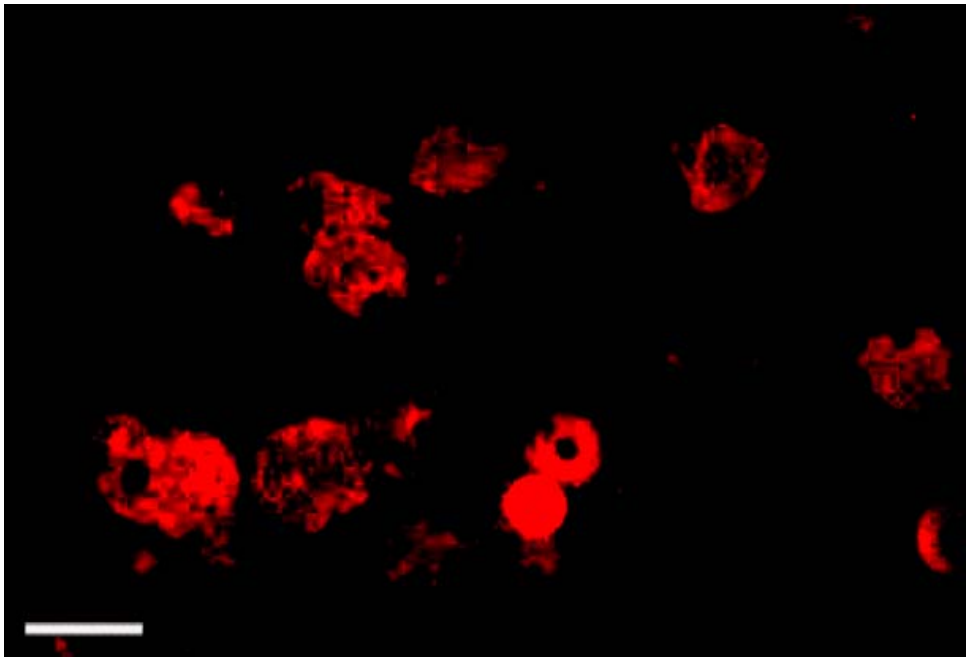

Fig. 3 Immunocytochemical evidence of NEP in the in larva hemocytes of the insect *H. virescens.* Bar = 15 μm.

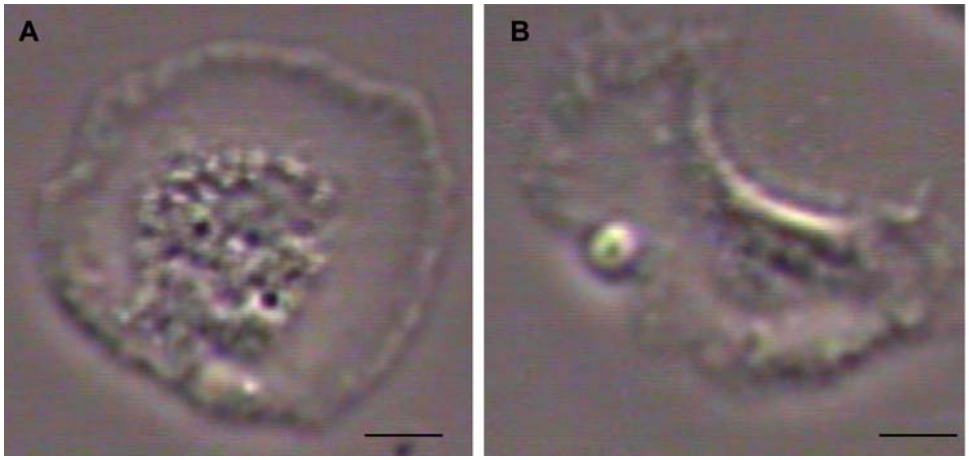

Fig. 4 Phase-contrast photographs of *M. galloprovincialis* immunocytes. (A) control, (B) activated immunocytes 5 min after the addition of 10^{-8} M ACTH (1-24). Bar = 10 μm.

Parasites use a similar mechanism for immunosuppresion involving the proopiomelanocortin-derived peptides released from the parasite *Schistosoma mansoni* (Duvaux-Miret *et al.*, 1992). *S. mansoni* may escape immune reactions from its vertebrate (man) by using signal molecules common to both host and parasite. In the experiments of coincubation of adult worms with human PMN or snail immunocytes has been detected the presence of α-MSH in the medium suggesting that α-MSH results from the conversion of the parasite ACTH by NEP.

NEP has also been detected in the *T. nigriceps/H. virescens* parasitic model. During the parasitization of the insect *H. virescens* larva by another insect *Toxoneuron nigriceps*, the host activates a series of humoral and cellular defenses in which plasmatocytes and granulocytes are the circulating immunocytes (Grimaldi *et al.*, 2012). The granulocytes activated by parasitization produce

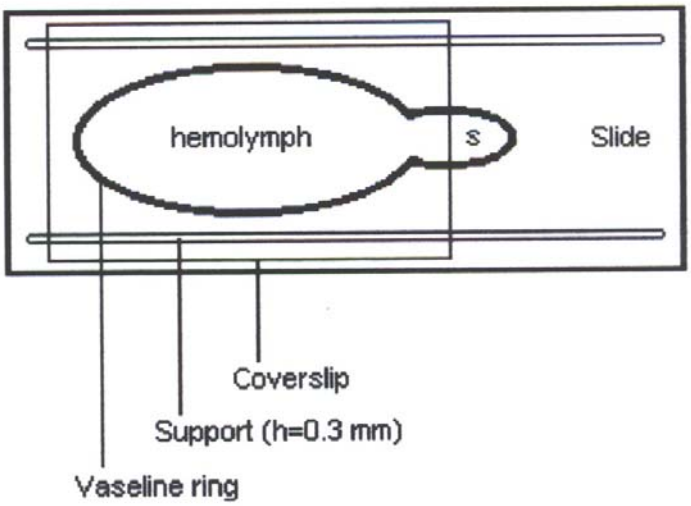

Fig. 5 Microscopy slide for cell activity assay. S = substance to test.

large amount of amyloid fibrils to package melanin. The stimulation in response to parasitic attack involves a cross-talk between the immune and neuroendocrine systems with the activation of stress-sensoring circuits to produce and release molecules, such as ACTH (responsible of the autocrine/paracrine activation of cells), α-MSH (resulting in activation of melanin production), NEP and the overproduction of reactive oxygen species. In this contex NEP present on the cell surface plays an important role in controlling the ACTH/α-MSH loop modulation. The same enzyme after exocitosis of amyloid fibrils massively hydrolyzes amyloid fibrils poured in circulating fluid and this cleavage prevents the unnecessary accumulation in hemolymph of amyloid resistant material.

Conclusive remaks

The majority of the findings reported in the present paper on NEP have been observed in parallel also in man (Duvaux-Miret *et al.*, 1992; Smith *et al.*,1992; Scharrer and Stefano, 1994) suggesting that:

1) NEP activity is present on blood cells, immunocytes, peripheral fluids and hemolymph;
2) NEP is a highly important factor in controlling the response of immunocytes in invertebrates and blood cells in man to the influence of biologically active substances;
3) the presence and the activity of NEP in invertebrates and man substantiate the importance of this intercellular regulatory mechanism of communication.

In summary the NEP immunoregulation is a well-balanced process that, with appropriate physiological and homeostatic responses to challenges, allows the survival and well-being of the species.

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