REVIEW

The hidden life of NAD$^+$-consuming ectoenzymes in the endocrine system

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Abstract

Ectoenzymes are a family of cell surface molecules whose catalytic domain lies in the extracellular region. A subset of this family, nucleotide-metabolizing ectoenzymes, are key components in the regulation of the extracellular balance between nucleotides (e.g. NAD$^+$ or ATP) and nucleosides (e.g. adenosine). Their substrates and products are signalling molecules that act by binding to specific receptors, triggering signals that regulate a variety of functions, ranging from the migration of immune cells, to synaptic transmission in the brain, to hormone/receptor interactions in the glands. Almost two decades of accumulated data indicate that these regulatory processes significantly affect the endocrine system, a tightly controlled information signal complex with clear evidence of fine regulation. Functional models discussed in this review include insulin secretion, bone modelling and the association between hormones and behaviour. The emerging pattern is one of a system operating as a scale-free network that hinges around hubs of key molecules, such as NAD$^+$ or ATP. The underlying natural link between nucleotides, ectoenzymes and the endocrine system is far from being clearly demonstrated. However, the body of evidence supporting the existence of such connection is growing exponentially. This review will try to read the available evidence in a hypothesis-oriented perspective, starting from the description of NAD$^+$ and of ecto- and endoenzymes involved in its metabolism.

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Introduction

Nucleotides are the building blocks of RNA and DNA. Within the cell, they play a central role in metabolism by serving as sources of chemical energy and by functioning as cofactors of enzymatic reactions. A relatively recent observation is that they are also deeply involved in cell signalling.

Nucleotides can be released or leaked into the extracellular milieu by virtually every cell in the body. Once outside the cell, they either serve as signalling molecules by binding specific type 2 purinergic receptors (P2X or P2Y) or are degraded to the related nucleoside. Nucleosides, mainly adenosine, can then bind different types of P1 purinergic receptors (Abbracchio & Burnstock 1994, Burnstock & Knight 2004). Nucleotide/nucleoside conversion is performed by special molecules located on the outer surface of the cell membrane and characterized by the presence of an enzymatic domain in the extracellular region. For this reason, they are called ectoenzymes (Goding & Howard 1998). Recent evidence indicates that the different ectoenzymes work in concert to dismantle extracellular nucleotides. The balance between nucleotides and nucleosides is conditional upon the expression and function of such enzymes.

Nucleotide-metabolizing ectoenzymes may thus represent a scavenging system for recycling nucleotides released from the cells in response to different events or stresses. This schematic view is augmented by strong evidence indicating that i) nucleotides and nucleosides can act on their own as signalling molecules, and that ii) the network of extracellular nucleotides/nucleosides, enzymes involved in their metabolism and purinergic receptors serves multiple functions in a balanced and finely tuned fashion (North 2002, Salmi & Jalkanen 2005, Malavasi *et al*. 2008, Burnstock 2009). The biological relevance of the ectoenzyme connection

is also sustained by the fact that most ectoenzymes share common evolutionary steps: indeed, their ancestors were soluble enzymes, which later reached the surface cell membrane. Therefore, they acquired new abilities and became adhesion molecules/receptors with role(s) in the social life of a cell (Deaglio & Malavasi 2006; Fig. 1).

It is tempting to speculate that this complex network represents a universal model selected during phylogeny with the final outcome of finely tuning different extracellular signals (Burnstock & Verkhratsky 2009). These signals could be involved in a variety of functions from migration of immune cells (Trautmann 2009) to synaptic transmission in the brain (Burnstock 2008), or to hormone/receptor interactions in the glands (Petit *et al.* 2009, Stojilkovic 2009).

Like the nervous and the immune systems, the endocrine system is an information signal complex, tightly controlled at various levels and with clear evidence of fine regulation. The hypothesis of a functional link among nucleotides ubiquitously present in biological fluids in enzymatic structures involved in their extracellular metabolism and in the endocrine system may appear difficult to demonstrate. A reasonable assumption is that the endocrine system makes use of compounds and signals that have been tested and conserved over millions of years to sustain redox

reactions and generate energy. The scenario is thus quite simple and fits the requisites for being an ancient and delicately calibrated design of nature.

Nature's underlying design-linking nucleotides, ectoenzymes and endocrine system are far from being clearly demonstrated. However, the body of evidence supporting the existence of such connection is growing exponentially. This review will try to read the available evidence in a hypothesis-oriented perspective, starting from the description of NAD^+ and of ecto- and endoenzymes involved in its metabolism.

NAD^+

NAD^+, identified a century ago as a cofactor and coenzyme, has attracted attention for its versatile function in relation to energy metabolism. The biosynthesis of NAD^+ occurs through a *de novo* pathway starting from tryptophan, and from three distinct salvage pathways originating from nicotinic acid, nicotinamide and nicotinamide riboside (Grahnert *et al.* 2010, Houtkooper *et al.* 2010).

Extracellular levels of NAD^+ result from the balance between active release/secretion and the chained actions of NAD^+-consuming proteins operating outside the cells (i.e. CD38 and ADP-ribosyl transferase (ART)).
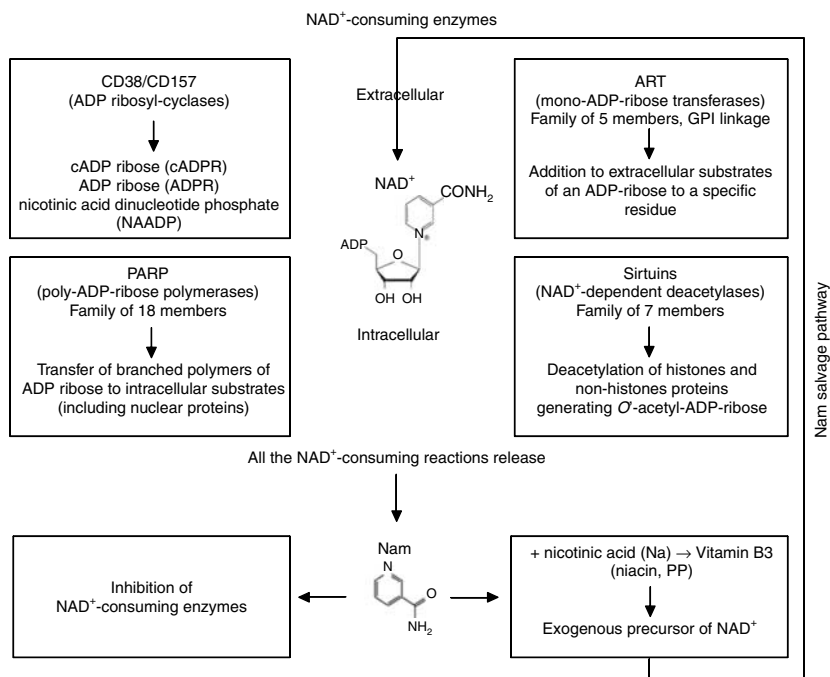
Figure 1 Schematic representation of the four classes of NAD^+-metabolizing enzymes. CD38/CD157 and ARTs are ectoenzymes, while PARPS and sirtuins are endoenzymes. All of these reactions generate nicotinamide (Nam), which in turn is a non-competitive inhibitor of the reactions. Nicotinamide can be converted into NAD^+ through a salvage pathway.

NAD^+ also acts outside the cell, where the molecule can signal directly by binding specific purinergic receptors (Moreschi *et al.* 2006) or – indirectly – by serving as the substrate for selected enzymes. For these reasons, NAD^+ is considered a signalling molecule that guides a series of events linked to cell life and death.

NAD^+-consuming enzymes

NAD^+-consuming ectoenzymes are primarily represented by the CD38/CD157 system (Malavasi *et al.* 2008) and by the mono-ARTs (Koch-Nolte *et al.* 2008). On the contrary, poly-ADP-ribose-polymerases (PARPs; Kim *et al.* 2005) and sirtuins (NAD^+-dependent protein deacetylases; Michan & Sinclair 2007) operate within the cell.

CD38/CD157 gene family

The *CD38* gene codes for a type II transmembrane molecule with a widespread cell distribution. The other member of the family is *CD157*, which differs in structure and tissue distribution (Malavasi *et al.* 2008).

The identification of a sequence similarity between the human lymphocyte antigen CD38 (and later CD157) and the *Aplysia* ADP-ribosyl cyclase (States *et al.* 1992) was the starting point of ongoing investigations into their enzymatic properties and their role in human physiology and pathology. The catabolism of NAD^+ and NAD(P) mediated by CD38 leads to the generation of potent intracellular Ca^{2+}-mobilizing compounds, including cADPR, NAADP and ADP-ribose (Lee 2004). In addition to binding the TRPM2 membrane Ca^{2+} channels (Perraud *et al.* 2001), ADPR, the main product of the reaction, can be covalently attached to proteins by ARTs. This post-translational modification of target proteins can have dramatic effects on their functions. The relevance of these enzymatic products and pathways was tested initially in the immune system (Howard *et al.* 1993, Malavasi *et al.* 1994), and then extended to different organs and tissues, e.g. pancreas, uterus, bronchi and kidney. Several conceptual and technical issues remain unsolved. The most intriguing novel observation concerns the relation(s) between the molecule's enzymatic and receptorial functions. Evolutionary studies confirm that the enzymatic function precedes the receptorial one and that the dual behaviour of the molecule is likely a reflection of environmental pressure (Deaglio & Malavasi 2006). If one accepts that evolution has played such a role in CD38, then the results obtained in animal models can be transferred to the human system only with specific caveats. Another intrinsic limit of the evidence collected to date is that the receptorial functions are limited to surface CD38, which in turn is prevalently found in immune cells. New clues have been provided by the recent demonstration that the molecule is also present in exosomes (Zumaquero *et al.* 2010).

Mono-ADP-ribosyl transferases

The process of ADP-ribosylation was originally identified by studying the pathogenic effects of bacterial toxins (including diphtheria, cholera, pertussis and clostridial toxins). Once inside the cell, these toxins act by modifying specific host cell proteins, such as small GTPases and monomeric actin (Koch-Nolte *et al.* 2008).

Mono-ADP-ribosylation of proteins is a covalent post-translational modification, and causes the transfer of a single ADP-ribose moiety of NAD^+ to a specific amino acid residue of an acceptor protein, with the creation of an N- or S-glycosidic linkage and the release of nicotinamide. The results are generally paralleled by functional modifications of the acceptor protein (Koch-Nolte *et al.* 2008). The most intriguing action of ART is ADP-ribosylation of the P_2X_7 purinergic receptor. P_2X_7 activation by micromolar concentrations of ATP induces T-cell death. The same effects are triggered by NAD^+ at micromolar concentrations through the ADP-ribosylation of P_2X_7, known as NAD^+-induced cell death. These events contribute to a dynamic regulation of T-cell homoeostasis (Seman *et al.* 2003).

Although the ecto-ARTs are the only well-characterized family, mono-ADP-ribosylation has also been demonstrated for intracellular proteins involved in cell signalling and metabolism. These endo-ARTs ADP-ribosylate a set of intracellular proteins, which includes the endoplasmic reticulum-resident chaperone GRP78/BiP, the β-subunit of heterotrimeric G-proteins and the mitochondrial glutamate dehydrogenase (reviewed in Grahnert *et al.* (2010)).

Poly-ADP-ribose polymerases

Poly-ADP-ribosylation is the covalent addition of multiple ADP-ribose groups to proteins. This post-translational modification plays a role in a wide range of biological processes, including DNA repair, transcriptional regulation, trafficking of endosomal vesicles, apoptosis and necrosis (Rouleau *et al.* 2010).

Poly-ADP-ribosylation is catalysed by the family of PARPs, which in humans includes at least 18 different genes. The most widely studied member is PARP1, a 116 kDa protein. Further members of the family are PARP5 (tankyrase-1) and PARP6 (tankyrase-2). Their enzymatic activities regulate telomere length.

Genes similar to PARPs have also been identified in low eukaryotes, eubacteria and archaebacteria (reviewed in Grahnert *et al.* (2010)).

Sirtuins

Sirtuins encompass a family of NAD^+-dependent deacetylases, which operate prevalently in the nucleus. This reaction leads to the transfer of acetyl groups from lysine residues of the target protein to the ADPR moiety of NAD^+, generating nicotinamide and *O*-acetyl-ADP-ribose. The latter compound induces the activation of the cytoplasmic domain of the TRPM2 channel, a non-selective cation channel whose prolonged activation leads to cell death (Perraud *et al.* 2001).

At least, seven sirtuins are described in mammalian cells. SIRT1, the most widely studied, deacetylates different histone and non-histone proteins, among which FOXO, p53 and NF-κB are some of them. Deacetylation of the target proteins modulates their function, influencing apoptosis, senescence and tumour transformation. A common trait shared with the other ecto- and endoenzymes consuming NAD^+ is that the sirtuins are a phylogenetically old family, conserved from archaea to humans (Haigis & Sinclair 2010).

An integrated view: how the network operates in the endocrine system

The potential link between NAD^+-consuming ectoenzymes and the endocrine system has been tested in selected models.

Pancreas

CD38

The effects induced by signals set off by NAD^+ were initially addressed by H Okamoto (Osaka, Japan), who proposed a model of insulin release and pancreatic β-cell damage based on a complex interplay between NAD^+, PARP, CD38 and cADPR (Okamoto & Takasawa 2002). Mice overexpressing CD38 showed higher insulin levels than controls in glucose-tolerance tests, suggesting enhanced release (Kato *et al.* 1995). The inference was that Ca^{2+} release from intracellular cADPR-sensitive stores and Ca^{2+} influx from extracellular sources play important roles in insulin secretion (Kato *et al.* 1995). Later experiments performed in CD38 knockout (KO) mice showed impaired glucose tolerance, with lower serum insulin levels than wild-type controls. The pathological phenotype was rescued by β-cell-specific expression of CD38 cDNA (Kato *et al.* 1999).

Further interest was sparked by the observation that CD38 KO islets are significantly more susceptible to apoptosis than islets isolated from littermate controls, suggesting a role in novel anti-apoptotic signalling pathways (Johnson *et al.* 2006).

The observations inferred from animal models were assessed in spontaneous autoimmune type 1 diabetes in the NOD strain. The onset of diabetes is significantly anticipated in the CD38 KO NOD mice due to an impairment of the regulatory T-cell compartment and the invariant NKT cells. The molecular mechanisms remain partially unknown, although an interplay between CD38 and ART2 has been hypothesized (Chen *et al.* 2006*a,b*).

SIRT1

SIRT1 regulates glucose or lipid metabolism through its deacetylase activity on over 20 substrates and is involved – directly or indirectly – in insulin signalling. For these reasons, it regulates lifespan under calorie restriction (Cohen *et al.* 2004). SIRT1 stimulates glucose-dependent insulin secretion from pancreatic β-cells with a direct action on insulin signalling pathways. SIRT1 also influences adiponectin secretion, inflammatory responses and gluconeogenesis, as well as the levels of reactive oxygen species, all of which contribute to the development of insulin resistance. Indirect confirmation comes from the observation that overexpression of SIRT1 (as well as several SIRT1 activators) has beneficial effects on glucose homoeostasis and insulin sensitivity in obese mouse models (Liang *et al.* 2009).

PARP

The role of these enzymes in diabetes has not been fully addressed as yet.

Brain and behaviour

CD38

A role for the CD38/cADPR system in regulating hormone secretions was recently proposed by H Higashida (Kanazawa, Japan). Careful observation of adult male and female CD38 KO mice led to the identification of marked defects in maternal nurturing and social behaviour. Detailed examination of the mice revealed that oxytocin (OXT), synthesized and detectable in the neurohypophysis, was not released in biological fluids. Vasopressin was surprisingly unaffected in the model. The impaired release of OXT could be reverted by re-establishing CD38 expression in neurohypophysis (Jin *et al.* 2007), clearly linking CD38 to OXT secretion (Fig. 2).

The work concluded that CD38 KO mice are characterized by a deficit in short-term social memory. OXT levels and clinical phenotype could be rescued by

Figure 2 Schematic representation of the CD38/OXT axis in neurohypophysis. CD38 is expressed on the surface of selected OXT-secreting neurons, where it plays a non-redundant role in the secretion of the hormone. The mechanism is likely mediated through the generation of Ca^{2+}-mobilizing compounds. Secreted OXT can be uptaken by OXTR, a G-protein-coupled specific receptor, whose function is tightly regulated by intrinsic and extrinsic factors.

correcting the genetic defect of the mice. In light of these findings, CD38 stands to become an important element in the diagnosis and study of neurodevelopmental disorders also in human pathology (Munesue *et al.* 2010, Salmina *et al.* 2010).

SIRT1

The link between SIRT1 and brain function derives from the observation that the enzyme plays a neuroprotective role, accentuated during calorie restriction. Furthermore, it has been demonstrated that Alzheimer's and Huntington's disease neurons are rescued by overexpression of SIRT1, induced by either calorie restriction or administration of resveratrol, a potential activator of this enzyme (Pallas *et al.* 2008).

PARP

Owing to its role in DNA repair and regulation of inflammatory transcription, PARP activation has been detected in acute and chronic neurodegenerative disorders (Kauppinen 2007, Kauppinen & Swanson 2007). PARP1 activation as well as accumulation of poly-ADP-ribose has been shown to be a constitutive element marking brain damage in Alzheimer patients. Notwithstanding the availability of at least eight different PARP inhibitors, no clinical trial has as yet tested their activity (Peralta-Leal *et al.* 2009).

Bone tissue

CD38

M Zaidi (New York, NY, USA) conducted extensive studies on the bone considered as a closed environment where endocrine factors and NAD^+-consuming ectoenzymes may interact. The first finding was that CD38 is expressed by osteoblasts and osteoclasts. CD38 activation in the osteoclasts triggers Ca^{2+} release and interleukin 6 production while inhibiting bone resorption (Adebanjo *et al.* 1999, Sun *et al.* 1999). These observations were confirmed in CD38 KO mice. Haematopoietic stem cells isolated *ex vivo* from the same animals showed a

significant increase in osteoclast formation (Sun *et al.* 2003). Furthermore, cADPR or exogenous addition of ADP-ribosyl cyclase stimulated osteoclast formation, which was in turn inhibited after blocking cADPR actions. These effects were predominantly attributed to the NADase activity of CD38 (Iqbal & Zaidi 2006).

Further clues were provided by A Zallone (Bari, Italy), who reported that OXT is indeed a direct regulator of bone mass. Deletion of OXT or the OXT receptor (OXTR) in male or female mice causes osteoporosis resulting from reduced bone formation. The proposed role of CD38 as a regulator of OXT secretion opens possibilities – yet to be explored in full – linking the two pathways (Imam *et al.* 2009, Tamma *et al.* 2009).

SIRT and PARP

The role of these enzymes in bone metabolism has not been fully addressed as yet.

The authors' perspective

Endocrinology was recently proposed as a model to be read as a whole complex network system (Koshiyama *et al.* 2010). According to this view, the endocrine system operates as a scale-free protein network hinging around hubs of key molecules (such as NAD^+, ATP, adenosine, cADP-ribose or acetyl CoA) that change as a function of the power of their metabolic degree of interaction or protein mass concentration (Clauset & Redner 2009). The system might easily support simple mutations, while it would be more sensitive to complex diseases targeting the hubs. Network systems with similar characteristics

are present in cells and proteins or in transcription factors. Similar connections may also be retrieved at supracellular levels in social networks as well as in disease (Barabasi & Oltvai 2004, Barabasi 2007).

Shifting the focus from the complexity of the general picture to clues revealed by closer inspection of CD38, perhaps the most intriguing novel observation concerns the role of the enzyme in OXT secretion. We extended this information to our home field, trying to answer whether i) OXT has a role in the lymphoid system, and whether ii) the OXT/OXTR axis might be influenced by nucleotides and their receptors. Attention was addressed to OXTR in B lymphocytes using CD38 as a reference ectoenzyme in which to test the influence of NAD^+ and of non-substrate ligands.

Analysed during a discrete step of B lymphocyte differentiation, OXTR was detected on the surface of human plasma cells, which also host CD38 at high epitope density. Considering that plasma cells live most of their life in bone and bone niches, this observation may be instrumental in the definition of an unexplored circuit in which to analyse the influence of OXT. In that microenvironment, plasma cells and bone tissue interact dynamically through adhesion receptors and soluble factors.

Owing to the limited accessibility of human plasma cells, we stabilized a line from a patient with plasma cell leukaemia. The line obtained (referred to as DL06) maintains a phenotype and IgA secretion identical to that of the patient, even after 1 year.

Out of the panel of molecules characterizing plasma cell phenotype, the DL06 line expresses CD38 (as expected), transferrin receptor 2 (TfR2) and OXTR: the latter findings are original observations, confirmed
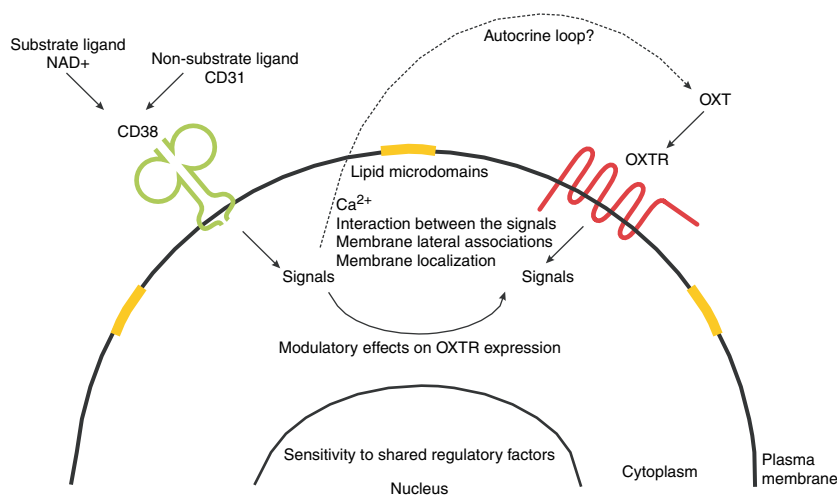
Figure 3 Hypothetical model linking CD38/OXT and OXTR in cells of the immune system. According to this hypothesis, plasma cells would secrete OXT (an event controlled by CD38), which would function as an autocrine growth factor. The relationship between CD38/OXT/OXTR and bone remodelling is discussed.

also in normal and tumoral plasma cells. The OXTR molecule was identified by means of a monoclonal antibody produced *ad hoc* in the laboratory (Cassoni *et al.* 2000). In functional terms, TfR2 remains an elusive molecule (Deaglio *et al.* 2002).

The limited number of surface molecules conserved is evidence of the functional priorities set by the plasma cells and is related to survival and Ig synthesis. Some are adhesion molecules, which drive plasma cells to bone niches favourable to their generally ephemeral life.

The expression of OXTR suggests a regulatory role for this receptor/ligand system in plasma cells. OXTR is a G-protein-coupled receptor, whose engagement by the ligand leads to positive or negative signals. The outcome depends on several variables, such as the presence of divalent ions, cholesterol and other components in the environment and in the plasma membrane. Also critical is the location of OXTR inside or outside the lipid microdomains, leading to opposing outcomes (Zingg & Laporte 2003; Fig. 2).

In light of these considerations, plasma cells and bone niches appear to be good testing grounds for the working hypothesis of a connection between ectoenzymes and neuropeptides. The system is closed, and nucleosides may represent additional signals to those provided by cytokines, chemokines and other conventional regulators. The multifunctional nucleotides ATP and NAD^+ operating *in situ* may complement the physiological regulatory system of the plasma cells.

The initial results obtained indicate that OXT at physiological concentrations influences the distribution of surface CD38 and increases the growth rate of plasma cells, at least in the DL06 model. As a consequence, it seems reasonable to propose the existence of a CD38/OXT/OXTR axis in plasma cells and potentially in other components of the lymphoid tissue.

In conclusion, these early findings support the view that CD38 is the master coordinator of a process that leads plasma cell to secrete OXT, which in turn is uptaken by the OXTR. This circuit has all the characteristics of an autocrine pathway. Furthermore, the link between brain, NAD^+-consuming ectoenzymes and OXT may also explain some deficits observed in the immune response of patients with disorders of the autistic spectrum (Munesue *et al.* 2010; Fig. 3).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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