

Emerging Functions of Extracellular Pyridine Nucleotides

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In addition to the well-known metabolic functions of NAD and NADP, it is rapidly emerging that these 2 pyridine nucleotides and their derivatives also play important roles in cell signaling. Surprisingly, a number of NAD(P) metabolizing enzymes and NAD(P) targets have been found on the outer surface of the plasma membrane and the presence of NAD has been confirmed in extracellular fluids. These findings have opened the door to a new field of research aimed at elucidating the contribution of extracellular pyridine nucleotides in physiological signaling pathways and pathological conditions.

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INTRODUCTION

The important role of NAD(P) in the regulation of intracellular processes is undebated. In addition to the well-known metabolic functions of pyridine nucleotides, several signaling pathways which require NAD(P) or their derivatives have been discovered (1). These include ADP-ribosylation, release of calcium from intracellular stores by NAD(P) derivatives, regulation of Ca²⁺ influx by ADPR/TRPM2, and NAD-dependent protein deacetylation. It is not surprising that NAD(P) and their derivatives have roles unrelated to basic metabolism, but what is surprising is that an ever increasing number of these functions require extracellular NAD(P). The present review, written by speakers at the "CD38 Ectoenzyme Family: Advances in Basic Science and Clinical Practice" conference, brings together some of the recent knowledge on extracellular pyridine nucleotides, illustrating the potential importance of this field.

IS NAD(P) PRESENT EXTRACELLULARLY?

The presence of extracellular NAD, and its possible contribution to physiology, is not new in nature. It is interesting to note that a number of pathogenic bacteria also exhibit a rather selective interest in, in fact, a requirement for NAD. For example, the need of *Haemophilus influenzae* for 2 growth factors (V and X) was established more than eighty years ago (2). Factor V was subsequently identified as NAD (while Factor X turned out to be protoporphyrin) (3). Indeed, growth of NAD-dependent strains can be strongly inhibited under conditions of NAD deprivation.

Recent reports suggest that the concentration of NAD in mammalian serum is around 100 nM (4,5). The redox state of this NAD and the levels of NADP(H) have, so far, not been determined. The origin of extracellular NAD is still unclear and, while cell death and inflammation (6) will release NAD, it has also been shown that cells can efficiently re-

lease NAD (7,8). For example, it has been suggested that in neurons, activation of the pre-synaptic terminal can lead to the release of vesicular NAD (7). Furthermore, in a number of cell models, it has been shown that NAD can also translocate both across the plasma membrane and into subcellular vesicles through hexameric connexin 43 (Cx43) hemichannels by means of an equilibrative mechanism (8). As with vesicle release, this process is also controlled, as phosphorylation of Cx43 protein by PKC results in the block of the hemichannels, thereby preventing the potentially detrimental consequences of otherwise unrestricted NAD fluxes (9).

WHAT ARE THE TARGETS OF EXTRACELLULAR NAD(P)?

Rather surprisingly, there is an arsenal of NAD(P)-metabolizing enzymes and an ever increasing number of targets for both extracellular NAD(P) itself or its metabolites (Figure 1). The 2 principal families of NAD(P)-utilizing enzymes are CD38/CD157 and the mono(ADP-ribosyl)transferases (ARTs).

CD38 is a multifunctional enzyme able to generate a wide range of biologically active compounds using NAD(P)

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ing proteins (6,18–20). ADP-ribosylation inhibits the enzyme activity of CD38 (19). On the other hand, ADP-ribosylation activates P2X₇, causing an influx of calcium and efflux of potassium, shedding of L-selectin (CD62L), exposure of phosphatidylserine, activation of caspases, and apoptosis [6]. This apoptotic pathway has been dubbed NICD (NAD-induced cell death) (6).

CD38 has been shown to play a crucial role in controlling the activity of cell surface ARTs by limiting the availability of the substrate NAD; i.e. ADP-ribosylation of T cell membrane proteins is limited by hydrolysis of NAD via CD38 (21). Consequently, ADP-ribosylation of cell surface proteins is particularly prominent on T cells of CD38KO mice, and T cells of CD38KO mice are highly sensitive to NICD (21). Interestingly, CD38 deficiency has been shown to accelerate autoimmune disease in an ART2-dependent manner in the NOD mouse model for autoimmune type I diabetes (22). CD38KO mice develop a more aggressive and rapid disease progression than their wild-type NOD counterparts. This, evidently, depends on ART2, because disease progression is greatly retarded in ART2/CD38 double KO mice (22).

In addition to enzyme targets, extracellular NAD⁺ has been shown to act directly on plasma membrane receptors/channels. A number of reports have shown NAD-induced Ca²⁺ influx via unidentified mechanisms that may relate to the TRPM2 Ca²⁺-influx channel (23). This channel possesses an intracellular nucleotide binding domain, but whether NAD/ADPR can activate the channel directly from the outside or whether the nucleotides must be internalized remains unclear. NAD⁺ (both α and β) has also been shown to be an agonist of the P2Y₁₁ purinoceptor in human granulocytes (24). In these cells, binding of NAD⁺ to P2Y₁₁ elicits: i) activation of adenylate cyclase and overproduction of cAMP; ii) PKA-mediated activation of CD38; iii) overproduction of cADPR; iv) increase of [Ca²⁺]_i, mostly via Ca²⁺ influx; v) functional activation of granulocytes,

with increased O₂⁻ and NO production and enhanced chemotaxis toward 0.1–10 μ M NAD⁺. Thus, NAD⁺ can act as a pro-inflammatory cytokine targeting human granulocytes through the CD38/cADPR system (24,25).

In addition to the effects of extracellular NAD, a report has shown contraction of mouse aorta in response to extracellular NADP(H) (26). Contraction in response to NADPH was observed both in the presence and absence of endothelium but, strikingly, was not blocked by inhibition of NADPH oxidase. On the other hand, contractions were markedly attenuated by pharmacological inhibition of P2X receptors, suggesting that NADP(H) may also be able to act directly on purinergic receptors (26). Furthermore, 2 different reports have proposed a role of extracellular NAADP in the activation of purinergic receptors (27,28), although it has also been shown that NAADP and cADPR can be transported into cells (see below).

CAN CD38-SYNTHEZIZED MESSENGERS REACH THEIR INTRACELLULAR TARGETS?

CD38 can generate a plethora of active compounds, including cADPR and NAADP. The widely accepted targets for these molecules are intracellular Ca²⁺-channels located on intracellular organelles (see other reviews in this issue). The predominantly extracellular or intravesicular location of CD38 raises the question of whether extracellularly produced cADPR and NAADP can cross the plasma membrane (29). Surprisingly, it has been shown that both extracellular cADPR and NAADP enter cells and raise intracellular Ca²⁺ concentrations (9,27,30).

The entry of cADPR into cells is mediated by CD38 itself as well as by members of both the equilibrative (ENT2) and the concentrative nucleoside transporter (CNT2, CNT3, and especially *cs-csg*) families, as determined by both pharmacology and molecular biology (9). While the equilibrative transporters are likely to be of little physiological significance due to

the concentration gradient, the CNTs are able to efficiently concentrate nanomolar cADPR. The transporter(s) responsible for NAADP uptake have not yet been identified although pharmacological characterization of the transport of both molecules in the same cell line suggests that the protein(s) involved in NAADP transport are distinct from those responsible for cADPR transport (30). Uptake of NAADP occurs even at picomolar concentrations in the extracellular medium.

These reports raise the possibility that cADPR and NAADP can play an autocrine/paracrine function (9). Indeed, experiments using transwell co-cultures of CD38⁺ and CD38⁻ cells have shown that cADPR produced by CD38⁺ cells can diffuse and is taken up by CD38⁻ cells (31). These findings suggest that cADPR and NAADP could potentially act, not just on the cell that produces the messenger, but also on other cells in a hormone-like manner.

It is becoming clear that the pyridine nucleotides, once thought of as metabolic workhorses, have a role in more “prestigious” cellular processes from both sides of the plasma membrane. Much still remains to be clarified regarding the physiology and pathology of such NAD(P)-mediated pathways and it is likely that novel roles of extracellular NAD(P) remain to be discovered. Central to all of these pathways seems to be CD38 which, in addition to the wide variety of active compounds it is able to produce from NAD(P), may play a crucial role in regulating the availability of NAD for such pathways. The role of extracellular pyridine nucleotides in diseases where CD38 or ARTs have been implicated will need to be carefully assessed with a mind to novel therapeutic strategies. For example, the interconnected roles of CD38 and ART2 in the regulation of immune cell functions pose interesting targets for the development of novel immune-modulating drugs. To this end, single-domain antibodies derived from llamas and other camelids (32) hold promise as specific, reversible inhibitors of leukocyte cell surface en-

zymes in vivo (Koch-Nolte et al., unpublished). Furthermore, the presence of a cell surface CD38-like enzyme in *Schistosoma* (33), capable of degrading NAD and producing second messengers, may open new avenues in other therapies.

REFERENCES

- Berger F, Ramirez-Hernandez MH, Ziegler M. (2004) The new life of a centenarian: signaling functions of NAD(P). *Trends Biochem. Sci.* 29:111–8.
- Thjötta T, Avery OT. (1921) Studies on bacterial nutrition: II. Growth accessory substances in the cultivation of hemophilic bacilli. *J. Exp. Med.* 34:97–114.
- Lwoff A, Lwoff M. (1937) Studies on codehydrogenases. 1. Nature of growth factor 'V'. *Proc. Roy. Soc., London, series B.* 122:352–9.
- Zocchi E et al. (1999) Ligand-induced internalization of CD38 results in intracellular Ca²⁺ mobilization: role of NAD transport across cell membranes. *Faseb. J.* 13:273–83.
- O'Reilly T, Niven DF. (2003) Levels of nicotinamide adenine dinucleotide in extracellular body fluids of pigs may be growth-limiting for *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*. *Can. J. Vet. Res.* 67:229–31.
- Seman M, Adriouch S, Scheuplein F et al. (2003) NAD-induced T cell death: ADP-ribosylation of cell surface proteins by ART2 activates the cytolytic P2 / times / 7 purinoceptor. *Immunity.* 19:571–82.
- Smyth LM, Bobalova J, Mendoza MG, Lew C, Mutafova-Yambolieva VN. (2004) Release of beta-nicotinamide adenine dinucleotide upon stimulation of postganglionic nerve terminals in blood vessels and urinary bladder. *J. Biol. Chem.* 279:48893–903.
- Bruzzozone S, Guida L, Zocchi E, Franco L, De Flora A. (2001) Connexin 43 hemi channels mediate Ca²⁺-regulated transmembrane NAD⁺ fluxes in intact cells. *FASEB. J.* 15:10–2.
- De Flora A, Zocchi E, Guida L, Franco L, Bruzzozone S. (2004) Autocrine and paracrine calcium signaling by the CD38/NAD⁺/cyclic ADP-ribose system. *Ann. N.Y. Acad. Sci.* 1028:176–91.
- Ziegler M, Niere M. (2004) NAD⁺ surfaces again. *Biochem. J.* 382:e5–6.
- Aksoy P, White TA, Thompson M, Chini EN. (2006) Regulation of intracellular levels of NAD: a novel role for CD38. *Biochem. Biophys. Res. Commun.* 345:1386–92.
- Young GS, Choleris E, Lund FE, Kirkland JB. (2006) Decreased cADPR and increased NAD⁺ in the Cd38-/- mouse. *Biochem. Biophys. Res. Commun.* 346:188–92.
- Koch-Nolte F et al. (2006) ADP-ribosylation of membrane proteins: unveiling the secrets of a crucial regulatory mechanism in mammalian cells. *Ann. Med.* 38:188–99.
- Aktories K, Just I. (2000) Bacterial Protein Toxins. Springer Verlag, Berlin.
- Okazaki JJ, Moss J. (1998) Glycosylphosphatidylinositol-anchored and secretory isoforms of mono-ADP-ribosyltransferases. *J. Biol. Chem.* 273:23617–20.
- Glowacki G et al. (2002) The family of toxin-related ecto-ADP-ribosyltransferases in humans and the mouse. *Protein Sci.* 11:1657–70.
- Haag F, Koch-Nolte F, Kuhl M, Lorenzen S, Thiele HG. (1994) Premature stop codons inactivate the RT6 genes of the human and chimpanzee species. *J. Mol. Biol.* 243:537–46.
- Bannas P et al. (2005) Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts. *Blood.* 105:3663–70.
- Han MK, Cho YS, Kim YS, Yim CY, Kim UH. (2000) Interaction of two classes of ADP-ribose transfer reactions in immune signaling. *J. Biol. Chem.* 275:20799–805.
- Nemoto E, Yu Y, Dennert G. (1996) Cell surface ADP-ribosyltransferase regulates lymphocyte function-associated molecule-1 (LFA-1) function in T cells. *J. Immunol.* 157:3341–9.
- Krebs C et al. (2005) CD38 controls ADP-ribosyltransferase-2-catalyzed ADP-ribosylation of T cell surface proteins. *J. Immunol.* 174:3298–305.
- Chen J et al. (2006) Targeted disruption of CD38 accelerates autoimmune diabetes in NOD/Lt mice by enhancing autoimmunity in an ART2-dependent fashion. *J. Immunol.* in press.
- Sano Y et al. (2001) Immunocyte Ca²⁺ influx system mediated by LTRPC2. *Science.* 293:1327–30.
- Moreschi I et al. (2006) Extracellular NAD⁺ is an agonist of the human P2Y11 purinergic receptor in human granulocytes. *J. Biol. Chem.* 281:31419–29.
- Bruzzozone S et al. (2006) Extracellular NAD⁺ regulates intracellular calcium levels and induces activation of human granulocytes. *Biochem. J.* 393:697–704.
- Judkins CP et al. (2006) NADPH-induced contractions of mouse aorta do not involve NADPH oxidase: a role for P2X receptors. *J. Pharmacol. Exp. Ther.* 317:644–50.
- Heidemann AC, Schipke CG, Kettenmann H. (2005) Extracellular application of nicotinic acid adenine dinucleotide phosphate induces Ca²⁺ signaling in astrocytes in situ. *J. Biol. Chem.* 280:35630–40.
- Singaravelu K, Deitmer JW. (2006) Calcium mobilization by nicotinic acid adenine dinucleotide phosphate (NAADP) in rat astrocytes. *Cell Calcium.* 39:143–53.
- De Flora A, Guida L, Franco L, Zocchi E. (1997) The CD38/Cyclic ADP-ribose system: A topological paradox. *E. Int. J. Biochem. Cell Biol.* 29:1149–66.
- Billington RA et al. (2006) A transport mechanism for NAADP in a rat basophilic cell line. *FASEB. J.* 20:521–3.30.
- Franco L et al. (2001) Paracrine roles of NAD⁺ and Cyclic ADP-ribose in increasing intracellular calcium and enhancing cell proliferation of 3T3 fibroblasts. *J. Biol. Chem.* 276:21642–8.
- Lauwereys M et al. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. *EMBO. J.* 17:3512–20.
- Goodrich SP et al. (2005) Production of calcium-mobilizing metabolites by a novel member of the ADP-ribosyl cyclase family expressed in *Schistosoma mansoni*. *Biochemistry.* 44:11082–97.