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Detection and pharmacological modulation of nicotinamide mononucleotide (NMN) *in vitro* and *in vivo*

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ABSTRACT

The emerging key role of NAD-consuming enzymes in cell biology has renewed the interest in NAD resynthesis through the rescue pathways. The first step of the nicotinamide-dependent NAD-rescue pathway is operated by nicotinamide phosphoribosyl transferase (NaPRT) forming nicotinamide mononucleotide (NMN). Because of the difficulties in measuring NMN, numerous open questions exist about the pathophysiological relevance of NaPRT and NMN itself. Here, we describe a new method of fluorimetric NMN detection upon derivatization of its alkylpyridinium group with acetophenone. By adopting this method, we analyzed the kinetics of nicotinamide-dependent NAD recycling in HeLa and U937 cells. Measurement of NMN contents in subcellular fractions revealed that the nucleotide is highly enriched in mitochondria, suggesting intramitochondrial NAD synthesis. NMN increases in cells undergoing hyperactivation of the NAD-consuming enzyme poly(ADP-ribose) polymerase (PARP)-1, or exposed to gallotannin, a putative inhibitor of NMN-adenylyl transferases. Evidence that the inhibitor of NAD resynthesis FK866 selectively inhibits NaPRT having no effect on NMNAT activity is also provided. Importantly, NMN reduces NAD and ATP depletion in cells undergoing PARP-1 hyperactivation, significantly delaying cell death. Finally, we show that a single injection of FK866 in the mouse induces long-lasting (up to 16 h) but mild (~20%) reduction of NMN contents in different organs, suggesting slow rate of basal NAD consumption *in vivo*. Data provide new information on the biochemistry and pharmacology of NAD biosynthesis, allowing a better understanding of pyridine nucleotide metabolism.

1. Introduction

Pyridine nucleotides have long been considered hydride ion carriers exclusively involved in oxidoreduction reactions. However, the recent identification of different enzyme families having nicotinamide adenine dinucleotide (NAD) as substrate has significantly renewed the interest in the biochemistry and pharmacology of pyridine nucleotides [1–3]. In particular, being these enzyme families responsible for the irreversible transformation of NAD into different products, attention has been focused on the mechanisms of NAD resynthesis into eukaryotic cells. Classically, two metabolic pathways regulate the formation of NAD in mammalian cells; i.e. the *de novo* (also called the "kynurenine") pathway, leading to NAD from tryptophan, and the rescue pathway using the nicotinamide moiety produced as a by-product by the NAD-consuming enzymes. The Preiss–Handler route also contributes to pyridine nucleotide formation, transforming nicotinic acid absorbed from the gut into NAD [1,4,5]. Notably, a recently identified metabolic route leading to NAD formation from nicotinamide riboside also exists [6,7]. Conversely, vertebrates are unable to convert nicotinamide into nicotinic acid [5].

A central metabolite in NAD resynthesis is nicotinamide mononucleotide (NMN, Fig. 1). It is produced by nicotinamide phosphoribosyl transferase (NaPRT) and by nicotinamide riboside kinase (NRK). NMN is then converted into NAD by three isoforms of NMN-adenylyl transferase (NMNAT). Recently, NaPRT received much attention by the scientific community because of its pleiotypic functions. Specifically, besides being an intracellular enzyme involved in NAD resynthesis, the protein has been also identified as a released factor behaving as an adipokine and as an inflammatory cytokine [8]. Adipokines are fat tissue-derived hormones with central roles in metabolism and disease pathogenesis. A protein with the same amino acid structure of NaPRT has been identified as a 52-kDa release product of visceral fat and therefore called visfatin [9,10]. Controversies exist as for the endocrine properties of visfatin/NaPRT. Although originally identified as an insulin receptor-interacting protein able to improve glucose-stimulated insulin secretion, the protein is now thought to be devoid of insulin receptor-interacting properties but

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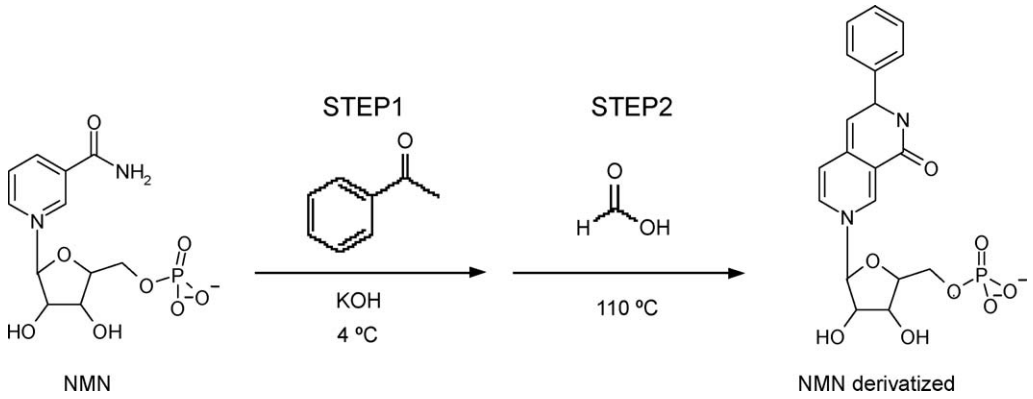

Fig. 1. Derivatization of NMN. NMN is converted into a fluorescent compound upon reaction with acetophenone and formic acid.

still able to regulate glucose homeostasis by promoting pancreatic islet insulin secretion. Of note, these properties are entirely dependent on NMN production by secreted visfatin/NaPRT [11]. The secreted protein can also behave as a cytokine called Pre-B cell colony-enhancing factor (PBEF). NaPRT/visfatin/PBEF is released by various immune cells, promotes TNFα, IL1β and IL-6 production, and is increased in the sera of patients affected by sepsis, acute lung injury, myocardial infarction, rheumatoid arthritis and inflammatory bowel disease. The protein is also able to inhibit neutrophil apoptosis, thereby promoting the immune response [12]. Whether NMN production contributes to the immunoregulatory functions of NaPRT/visfatin/PBEF is still unknown but a specific receptor has not been identified. Also, the permeability of NMN through the plasma membrane is not clear and, again, a possible NMN-interacting receptor waits to be identified.

Given the potential relevance of NaPRT/visfatin/PBEF to pathophysiology, several ELISA kits able to detect the protein have been recently made commercially available. Yet, these kits have different sensitivities and are heterogeneous in nature. These inconsistencies are probably responsible for the apparent contrasting properties of secreted NaPRT/visfatin/PBEF present in the literature. Also, since the functional properties of NaPRT/visfatin/PBEF may in part depend on its enzymatic activity [11,13], evaluation of intra as well as extracellular NMN production can certainly help understanding the pathophysiological role of the protein. Unfortunately, analytical determination of NMN is difficult because of its physicochemical features. Indeed, very few studies have measured NaPRT activity and/or NMN concentrations in biological fluids or tissue extracts using radioactive or complex HPLC/MS techniques [11,13,14]. In the present study we provide new information on NMN metabolism and pharmacological modulation by using an original method of NMN measurement as well as the newly identified inhibitor of NaPRT (E)-N-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3-yl) acrilamide (FK866) [15,16].

2. Materials and methods

2.1. Cell culture and treatment

Unless otherwise stated, all chemicals and cell culture products were from Sigma (Milan, Italy). HeLa or U937 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 10% fetal bovine serum and antibiotics. Cultures were brought to 50–70% confluence and exposed to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG, 100 μM), nicotinamide 10–1000 μM, NMN 10–1000 μM, NAD 0.1–1 mM. (E)-N-[4-(1-benzoylpiperidin-4-yl) butyl]-3-(pyridin-3-yl) acrilamide (FK866, 1-100 μM) was obtained from NIH, Bethesda, USA. Cell viability was evaluated by measuring lactate dehydrogenase (LDH) release in the incubating media or reduction of methylthiazolyl tetrazolium (MTT) and described [17]. An inverted Nikon TE-2000U microscope equipped with a CDD camera was used for cell visualization.

2.2. Cell fractionation

As previously described [17], cells were disrupted using a glass/glass homogenizer in 500 μl of buffer A (Tris HCl 50 mM, pH 7.4, mannitol 225 mM, saccarose 75 mM, 1 mM PMSF, 10 μl of protease inhibitor cocktail), and centrifuged at 600 × *g* to obtain the nuclear pellet. Supernatants were centrifuged at 12,000 × *g* to obtain the mitochondrial pellet and the cytosolic fraction. Cell fractions were then processed for NMN determination as described above.

2.3. NMN, NAD and ATP measurement

Ultra pure NMN standards (Sigma, Milan, Italy) were dissolved in water and solutions analyzed by HPLC with UV or fluorimetric detection. For UV detection, cells grown in a 48-well plate were scraped with 100 μl of HCl 0.6. The cell extract was centrifuged at 14,000 × *g*/5 min and 25 μl of the supernatant injected in an HPLC system consisting in a mobile phase of 0.1 M buffer phosphate pH 6.5, 1% acetonitrile, 10 mM tetra butyl-ammonium bromide (TBAB), a Supelco 25 cm column (5 μm) and an UV detector (PerkinElmer) set at 260 nm. For fluorimetric detection, we modified a method previously described [18]. Briefly, cells grown in a 48-well plate were lysed with 100 μl of $HClO_4$ 1N, whereas mouse organs were sonicated in $HCLO_4$ 1N (1:4, w/v). Then, 100 μl of the extract were neutralized with KOH 1N and, after 5 min, additional 100 μl of 0.1 M bicine pH 7.4 were added. The cell extract was centrifuged at 14,000 × *g*/5 min and 10 μl of the supernatant were mixed with 100 μl of KOH 1N and 50 μl of acetophenone. The solution was incubated for 15 min at 4 °C, then 100 μl formic acid were added and the solution incubated 5 min at 100 °C. By means of this derivatization procedure, NMN is converted into a highly fluorescent compound as shown in Fig. 1. Excitation and emission spectra of the fluorescent compound were determined by means of a spectro fluorophotometer RF5000 (Shimadzu, Milan, Italy). Samples were injected into the HPLC system consisting in a mobile phase of 0.1 M buffer phosphate pH 6.5, 10% acetonitrile, a Supelco 25 cm column (5 μm) and a fluorimetric detector (PerkinElmer) with excitation and emission wavelength of 332 and 454 nm, respectively.

NAD contents were quantified by means of an enzymatic cycling procedure as described [19]. ATP was measured by the ATPlight kit (PerkinElmer, Milan).