Minireview

Pathophysiological relevance of mitochondria in NAD^+ metabolism

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Abstract Pyridine nucleotides are mostly stored within mitochondria where they are involved in different functions ranging from energy metabolism to cellular signaling. Here we discuss the mechanisms of mitochondrial NAD^+ metabolism and release that may contribute to the crucial roles played by these organelles as triggers or amplifiers of physiological and pathological events.

Key words: Permeability transition; Myocardial ischemia; Cell death; Poly(ADP-ribose) polymerase; ADP-ribosylation; Cyclic ADP-ribose; Oxyradical

1. Introduction

The pyridine nucleotides serve two important functions in the cell. They are coenzymes in the majority of metabolic redox reactions and thus are essential for energy transduction. On the other hand, in a number of signaling pathways $NAD(P)^+$ is used to form potent calcium-mobilizing agents or as substrate for covalent protein modification, ADP-ribosylation [1]. This dual function in energy and signal transduction is also characteristic for ATP. It is not surprising then that in cells exposed to pathophysiological conditions (e.g. DNA-damaging agents, 'oxidative stress' etc.) the pyridine nucleotides play an essential role in the mechanisms counteracting the potential damage.

A significant proportion of the cellular NAD^+ pool is compartmentalized within the mitochondria [2,3]. Several lines of evidence suggest that NAD^+-dependent signaling events may occur in mitochondria. This review will summarize these observations and also focus on the pathophysiological implications of these processes, specifically in the heart.

2. Pathways of NAD^+ metabolism

As pointed out above, the synthetic routes of $NAD(P)^+$ lead to the formation of an essential energy transducing molecule and precursors of signaling pathways. Therefore, it should be expected that $NAD(P)^+$ synthesis reactions are well controlled. Surprisingly, little is known about the regulation of $NAD(P)^+$ formation [4]. Moreover, although the pathways of $NAD(P)^+$ generation have been established a long time ago, several enzymes have not been characterized on a molecular level. For example, the amino acid sequence of the human nicotinamide mononucleotide adenylyltransferase (NMNAT) has been established only very recently [5,6] as the first primary structure from higher eukaryotes. No sequence information is available for NAD^+ kinase from any organism. NMNAT and NAD^+ kinase appear to be essential, because they catalyze the only known anabolic reactions in which NAD^+ and $NADP^+$, respectively, are formed.

The sources of mitochondrial NAD^+ and $NADP^+$ are unknown. The inner membrane of mammalian mitochondria is normally impermeable to pyridine nucleotides. The only feasible possibility to enter the mitochondria would be through the permeability transition pore (PTP, see below). Alternatively, the final steps of NAD^+ and $NADP^+$ synthesis should be catalyzed within the organelles. However, NMNAT was localized exclusively to the nucleus [6], although a mitochondrial isoform may exist [7]. A mitochondrial NAD^+ kinase activity has not been reported, whereas it is well known that NADH and NADPH are linked through the activity of the energy-linked transhydrogenase [8].

The degradation of $NAD(P)^+$ has been a major field of investigation over the last few years, because several routes are now known to carry signaling functions (Fig. 1). These reactions have in common the cleavage of the glycosidic bond between the nicotinamide moiety and the terminal ribose of NAD^+. In its simplest form this would be hydrolysis of NAD^+ to ADP-ribose and nicotinamide catalyzed by NAD^+ glycohydrolases (NADases). These enzymes have been known for long and were initially regarded as purely catabolic. However, it has been found that a second product of the reaction is cyclic ADP-ribose (cADPR) in which a bond is formed between the terminal ribose and the N^1 of the adenine ring, again under release of nicotinamide [9]. Therefore, NADases have also been termed ADP-ribosyl cyclases. It is

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Abbreviations: ART, ADP-ribosyl transferase; $\Delta\psi_m$, mitochondrial membrane potential; $[Ca^{2+}]_i$, intracellular Ca^{2+}; cADPR, cyclic ADP-ribose; CsA, cyclosporin A; IMM, inner mitochondrial membrane; $InsP_3$, inositol-1,4,5-trisphosphate; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; PTP, permeability transition pore; NAADP, nicotinic acid adenine dinucleotide phosphate; PARP, poly(ADP-ribose) polymerase

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now well established that cADPR represents an important intracellular messenger which releases calcium from the ER.

ADP-ribosyltransferases use NAD^+ to attach the ADP-ribose moiety to proteins. This protein modification is reversed by hydrolases leading to the liberation of ADP-ribose. Therefore, ADP-ribose appears to be the major catabolite of NAD^+. ADP-ribose itself does not appear to exert any biological activity. Presumably, these pathways of NAD^+ degradation are tightly controlled owing to their signaling functions. In addition, the resynthesis of NAD^+ from ADP-ribose and nicotinamide requires as many as four molecules of ATP [10].

NAD^+ may also be cleaved by pyrophosphatase(s), e.g. phosphodiesterase I, to AMP and NMN. The physiological significance of this pathway is unknown. Moreover, the reverse reaction of NMNAT ($NAD^+ + PPi \rightarrow ATP + NMN$) may be regarded as a catabolic route which would permit a direct conversion of NAD^+ into ATP. In the nucleus an analogous pathway has been described that makes use of ADP-ribose (and pyrophosphate) instead of NAD^+ to form ATP and ribose phosphate [11]. This reaction appears to be an important source of ATP required for the ligation step in DNA repair [11].

3. $NAD(P)^+$-dependent signaling pathways

The known mechanisms of NAD-dependent signaling appear to be widely conserved in living organisms. Enzymes that catalyze the synthesis of cADPR or monoADP-ribosylation have been found in prokaryotes and eukaryotes (reviewed in [1]). However, the biological roles of these reactions are still far from being understood. Poly(ADP-ribosyl)ation appears to be restricted to the nucleus and is, therefore, limited to eukaryotes. It is important to point out that all NAD^+-mediated signaling pathways use only the oxidized form of the pyridine nucleotides, $NAD(P)^+$.

3.1. Mono- and poly(ADP-ribosyl)ation

Over the past years the cloning of several monoADP-ribosyl transferases (ART) as well as the corresponding hydrolases from mammalian and avian tissues [12] has clearly supported the potential importance of this posttranslational protein modification. However, owing to the diversity of identified putative acceptor proteins it is still difficult to recognize a general principle that underlies regulation by monoADP-ribosylation. In addition, the majority of transferases and hydrolases identified have been localized to the outer surface of the plasma membrane.

The role of poly(ADP-ribosyl)ation has been established to be related to vital cellular events such as DNA repair, transcription, and apoptosis, [13–15] but also to the regulation of telomere length [16] and of sister chromatid exchange [17]. The major enzyme catalyzing the reaction is strongly activated by DNA single strand breaks. Recent evidence suggests that poly(ADP-ribose) may serve as the source of ATP for the ligation step in base excision DNA repair [11]. Moreover, previous reports have documented that poly(ADP-ribosyl)-ation may consume virtually all cellular NAD^+, if induced by genotoxic treatment [18].

3.2. Calcium-mobilizing derivatives of $NAD(P)^+$

As pointed out above, NADases are capable of forming cADPR. In a large number of investigations cADPR has been documented to trigger calcium release from the endoplasmic reticulum (reviewed in [19]). The calcium is apparently released via ryanodine receptors. Evidence has been presented that cADPR- and inositol-1,4,5-trisphosphate ($InsP_3$)-dependent calcium release pathways are redundant [20], but also that they may be activated during different phases of intracellular calcium signaling pathways [21].

The NADases have also been shown to carry out a transglycosidation reaction leading to the replacement of nicotinamide by nicotinic acid in $NADP^+$ [22]. The resulting molecule, nicotinic acid adenine dinucleotide phosphate (NAADP), also represents a highly potent calcium-mobilizing agent (reviewed in [19]). Interestingly, NAADP appears to activate calcium stores that are different from those activated by cADPR or $InsP_3$ [19].

NADase/ADP-ribosyl cyclase activities have been reported in heart and liver mitochondria [3,23–26], nuclei [27] and plasma membrane [19]. However, in higher eukaryotes including mammals, no intracellular NADase/ADP-ribosyl cyclase has been cloned so far. The only identified mammalian enzymes of this type (CD38 and BST-1) are localized to the outer surface of the plasma membrane [19]. The recent identification of connexin-43 as a bidirectional transport system for pyridine nucleotides across the plasma membrane [28] indicates a potential pathway permitting both the access of intracellular NAD^+ to the ectoenzyme and the cellular uptake of cADPR.

4. NAD^+ and mitochondria

A substantial fraction of the cellular NAD^+ pool (about 75% in heart muscle) is compartmentalized within the mitochondria [2,3]. It is now well established that in isolated mitochondria NAD^+ can be utilized for monoADP-ribosylation and the synthesis of cADPR [1]. Importantly, the reduced form [NAD(P)H] is not a substrate for any of the $NAD(P)^+$-dependent signaling pathways. Accordingly, ADP-ribosylation and synthesis of cADPR are triggered under oxidizing conditions resembling 'oxidative stress' [26].

The precise location of the enzymes catalyzing the production or the consumption of pyridine nucleotides, that is, whether the various activities are present within the matrix or outside the inner membrane, is still in debate.

Scarce information is available about the mechanism(s) that supply mitochondria with $NAD(P)^+$. NMNAT activity has been reported for the matrix of liver mitochondria [7] and an NADH kinase activity has been detected in yeast mitochondria [29].

Under physiological conditions, in both isolated mitochondria and intact cells the matrix content of NAD^+ is stable [3,30,31] and is not influenced by changes in ATP concentrations which affect the cytosolic levels of NAD^+, probably by modulating the activity of NADase [31]. On the other hand, results from a classic study on isolated mitochondria demonstrate that NAD^+ content becomes rapidly depleted upon Ca^{2+} addition [30]. Such a process has been recently ascribed to the opening of the PTP [3], a high conductance channel located in the inner mitochondrial membrane (IMM) [32]. The opening of PTP causes a Ca^{2+}-dependent increase of mitochondrial permeability to ions and solutes with molecular weights up to 1500 Da, matrix swelling and collapse of mitochondrial membrane potential ($\Delta\psi_m$). Cyclosporin A (CsA), a

high affinity inhibitor of PTP, completely prevents both the swelling and the subsequent hydrolysis of NAD^+ in rat heart and liver mitochondria added with Ca^{2+} [3]. In the same study it was shown that the NADase activity of isolated mitochondria is not modified by CsA or Triton X-100. On the other hand, the activity was eliminated by treatment with Nagarse, a serine protease commonly used to extract heart mitochondria [33] which does not affect the intactness of the IMM. These indirect lines of evidence support the localization of NADase in the outer mitochondrial membrane. Thus, a model emerges whereby upon PTP opening NAD may be released from the mitochondrial matrix to the intermembrane space becoming a substrate of NADase (Figs. 1 and 2). Besides Ca^{2+}, such a process could be favored by oxyradicals that promote PTP opening [32,34] and stimulate the activity of NADase [26]. In addition, NAD^+ hydrolysis could be further enhanced by a more oxidized state of pyridine nucleotides caused by the uncoupling of oxidative phosphorylation as a consequence of the collapse of the membrane potential.

Previous reports, which firstly highlighted the relationship between oxidative stress and NAD^+ hydrolysis, suggested that ADP-ribose produced by a CsA-inhibitable mitochondrial glycohydrolase would be utilized for the non-enzymatic monoADP-ribosylation of an intrinsic protein of the inner membrane [35–37]. Such a mechanism, which would eventually result in the stimulation of a specific efflux pathway for Ca^{2+}, has been challenged by recent reports [3,24]. Indeed, besides the lack of glycohydrolase inhibition by CsA, PTP opening, and thus Ca^{2+} efflux, precedes and is necessary for NAD^+ hydrolysis. Moreover, mitochondrial ADP-ribosylation has been demonstrated to be enzyme-catalyzed, that is, independent of the activity of an NADase [38,39].

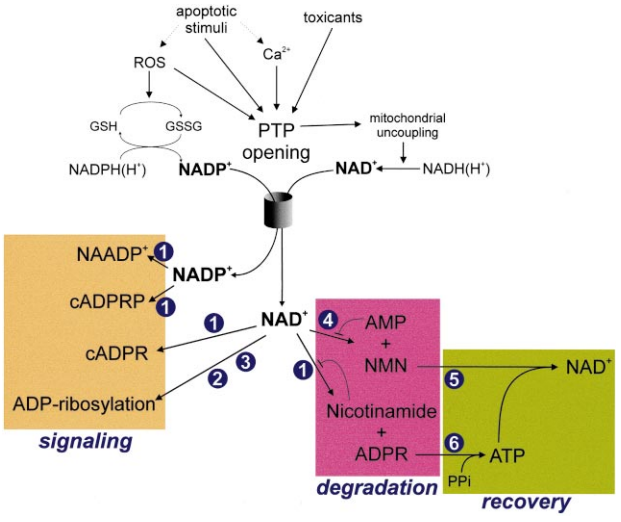

Fig. 1. Possible metabolic pathways of NAD^+ released from mitochondria. Under physiological conditions a major fraction of the cellular NAD^+ is stored within the matrix space. The opening of the mitochondrial PTP, a large channel located within IMM, induces a profound decrease of mitochondrial NAD^+. The degradation pathway and the generation of cyclic nucleotides are likely to occur outside of the matrix space which, on the other hand, might be a site for ADP-ribosylation and reactions resulting in the recovery of both ATP and NAD^+. The numbers indicate the following enzymes: 1, NADase/ADP-ribosyl cyclase; 2, ART; 3, PARP; 4, nucleotide pyrophosphatase; 5, NMNAT; 6, ART. cADPRP, cADPR phosphate; GSH, reduced glutathione; GSSG, oxidized glutathione, NMN, nicotinamide mononucleotide; PPi, pyrophosphate; PARP, poly(ADP-ribosyl) polymerase; ROS, reactive oxygen species.

The fate of NAD^+ after PTP opening is determined by the action of many enzymes that can be grouped into three major pathways (Fig. 1). Far from being mutually exclusive, these modes of NAD^+ utilization are closely related, so that the prevalence of one pathway over the others will result from other cellular events that in part will be discussed in Section 5. Under conditions of ATP depletion the formation of ADP-ribose and nicotinamide or AMP can be considered a degradative pathway, since these metabolites cannot be used directly for NAD^+ resynthesis. Moreover, ADP-ribose may be further degraded to AMP and ribose phosphate by a matrix pyrophosphatase [40]. In the recovery pathway ATP might be generated from ADP-ribose by means of a reaction similar to that described above for the nucleus. Finally, the signaling pathways comprise the cyclase reactions and the covalent modification of proteins by means of ADP-ribosylation.

Beside the pathological relevance of PTP in NAD metabolism discussed below, the opening of PTP might mediate bidirectional fluxes of NAD^+ through the IMM which was observed in digitonin-treated cell lines [41]. The passage of NAD^+ through IMM contrasts with the accepted notion of the lack of permeability of this membrane for pyridine nucleotides. The transient and reversible opening of PTP [42], which has been proposed to act physiologically as a Ca^{2+} release channel [43], could allow NAD^+ trafficking and turnover within the cell. It should be noted, however, that despite the numerous observations compatible with the occurrence of the PTP, the isolation and characterization of the constituents of the PTP are still required to elucidate the biochemical mechanisms conclusively.

5. Mitochondrial NAD^+ in injured cells

The relevance of PTP in NAD^+ metabolism and fate is highlighted by post-ischemic reperfusion of myocardium [3]. In fact, mitochondrial NAD^+ content, which is hardly affected during ischemia, becomes almost depleted when coronary flow is restored after a prolonged period of ischemia. The inhibition of mitochondrial NAD^+ depletion exerted by CsA suggests that upon reperfusion the rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$), along with the recovery of neutral pH and the boosting of oxyradical generation [44], promotes PTP opening causing the release of intramitochondrial NAD^+ and its subsequent hydrolysis. Not only is the decrease of mitochondrial NAD^+ prevented when PTP is inhibited, but also tissue viability is significantly protected, as documented in different experimental models [3,45–47]. The protection afforded by CsA is mimicked by *N*-methylvaline-4-cyclosporin, a cyclosporin derivative that binds cyclophilins and inhibits the PTP but does not affect calcineurin activity [3,48]. Thus, a direct action on mitochondria results in myocyte protection indicating that PTP opening represents a key step in the rapid sequence of events that eventually results in the loss of sarcolemma integrity.

Besides affecting energy metabolism, the mitochondrial release of NAD^+ is likely to modify several intracellular processes triggered by ischemia or other pathological conditions. Indeed, once released out of the mitochondrial matrix, NAD^+ could be transformed into cADPR which promoting Ca^{2+} release from intracellular stores may amplify and extend the

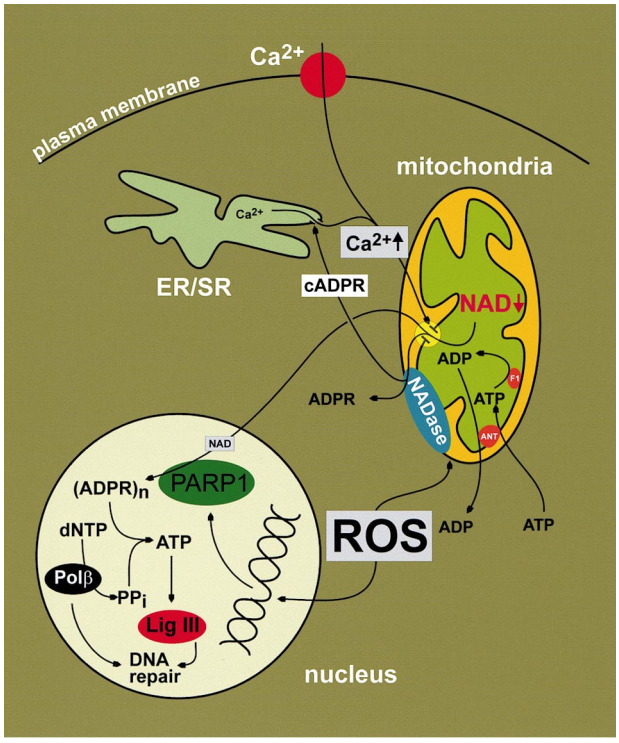

Fig. 2. Intracellular utilization of mitochondrial NAD^+ following the opening of the mitochondrial PTP. Conditions, such as the increase in cytosolic Ca^{2+} and the generation of reactive oxygen species (ROS) occurring upon post-ischemic reperfusion in the heart, favor PTP opening. Besides resulting in $\Delta\psi_m$ collapse and ATP hydrolysis, PTP opening allows the release of mitochondrial NAD^+ which could enter into several metabolic pathways. Along with its hydrolysis into ADPR and nicotinamide, NAD^+ can be utilized by other cellular sites or generate molecules which affect the cellular responses to injury. For instance, the formation of cyclic nucleotides could further enhance the cytosolic $[Ca^{2+}]$ by promoting Ca^{2+} release from intracellular sites. On the other hand, depending on the severity of damage potential repair reactions could be supported by NAD^+-dependent mechanisms. This concept is likely to apply to PARP when nuclear DNA damage is concomitant with ATP depletion. As discussed in the text, under these circumstances NAD^+ could be converted to ATP via ADP-ribosylation in an attempt to counteract the fall in ATP levels. ADPR, ADP-ribose; ANT, adenine nucleotide translocase; ES/SR, endoplasmic/sarcoplasmic reticulum; F1, F_oF_1 ATPase; Lig III, DNA ligase III; Polβ, DNA polymerase β; PPi, pyrophosphate.

effects exerted by an initial rise in $[Ca^{2+}]_i$ (Fig. 2). Thus, the release of NAD^+ from mitochondria and its subsequent utilization within other cell compartments could be part of the mechanisms through which mitochondria transduce and amplify an initial trigger provided by reperfusion [49].

That is, the relationship between NAD^+ and ATP is not limited to mitochondria, but also concerns other cellular sites (Fig. 2). For instance in the ischemic heart, as well as in other pathological states, NAD^+ has been reported to be actively utilized within the nucleus as a consequence of poly(ADP-ribose) polymerase (PARP) activation [18,50]. It has been proposed that subsequent NAD^+ resynthesis causes the depletion of ATP [18]. According to this suggestion (i) the depletion of NAD^+ should precede that of ATP; (ii) the depletion of ATP would not require mitochondrial derangements; (iii) PARP should have access to the entire cellular NAD^+. It appears, however, that mitochondrial contributions have to be taken into account when considering the depletion of NAD^+ or ATP.

The assumption that PARP activity alone is sufficient to drain the entire content of cellular NAD^+ implies the consumption of the mitochondrial pool which is the largest of the cell. The utilization of NAD^+ by PARP within the nucleus clearly requires the mobilization of mitochondrial NAD^+, which in turn would depend on a release pathway. Transient openings of PTP are perfectly reversible and might be important for mitochondrial ionic homeostasis and intracellular signaling [42,47,51]. Conversely, a prolonged opening of PTP results in $\Delta\psi_m$ collapse and ATP hydrolysis, conditions which have been consistently documented in experimental and clinical settings of myocardial ischemia [49,52]. Thus, NAD^+ and ATP are not likely to be depleted without a concomitant, perhaps causative, alteration of mitochondrial function. Accordingly, the depletion of ATP does not require a preceding fall in NAD^+ content. Mitochondrial derangements are likely to exert a substantially greater impact on ATP depletion than that of the presumed requirement for NAD^+ resynthesis. In fact, under conditions leading to ATP depletion NAD^+ might be used as an emergency source for ATP synthesis. It has been observed that in the nucleus poly(ADP-ribose) (synthesized from NAD^+) may serve to generate ATP which is specifically used for DNA repair [11]. Conversely, high levels of ATP prevent poly(ADP-ribosyl)ation and are sufficient for efficient DNA repair. This implies that poly(ADP-ribosyl)ation is triggered only after ATP depletion which most likely results from mitochondrial derangements.

In conclusion, the relationship between mitochondria and pyridine nucleotides extends far beyond the role of anaerobic dehydrogenases in oxidative metabolism. Besides being the major reservoir of the cellular $NAD(P)^+$, mitochondria also represent a site of active pyridine nucleotide metabolism as indicated by the generation of signaling molecules and protein ADP-ribosylation. The molecular characterization of mitochondrial enzymes and transporters involved in pyridine nucleotide metabolism will further define the relevance of the mitochondrial contribution to the overall $NAD(P)^+$ turnover in the cell and will facilitate the development of novel therapeutic approaches.

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