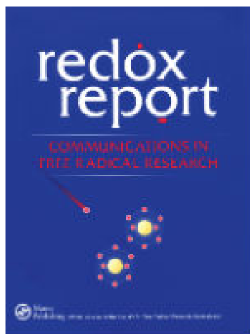

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Review

The phosphate makes a difference: cellular functions of NADP

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Recent research has unraveled a number of unexpected functions of the pyridine nucleotides. In this review, we will highlight the variety of known physiological roles of NADP. In its reduced form (NADPH), this molecule represents a universal electron donor, not only to drive biosynthetic pathways. Perhaps even more importantly, NADPH is the unique provider of reducing equivalents to maintain or regenerate the cellular detoxifying and antioxidative defense systems. The roles of NADPH in redox sensing and as substrate for NADPH oxidases to generate reactive oxygen species further extend its scope of functions. $NADP^+$, on the other hand, has acquired signaling functions. Its conversion to second messengers in calcium signaling may have critical impact on important cellular processes. The generation of NADP by NAD kinases is a key determinant of the cellular NADP concentration. The regulation of these enzymes may, therefore, be critical to feed the diversity of NADP-dependent processes adequately. The increasing recognition of the multiple roles of NADP has thus led to exciting new insights in this expanding field.

Keywords: NADP, NADPH, redox sensing, reactive oxygen species, electron donor, second messenger, calcium signaling

Introduction

Nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADP) play an essential role in the metabolism of all organisms. They were originally discovered for their universal roles as co-enzymes in cellular electron transfer reactions. The similarity of the redox potentials of the redox pairs $NADH/NAD^+$ and $NADPH/NADP^+$ raises the question of why there is a requirement for two such pairs. Indeed, several NAD(P)-dependent dehydrogenases can use either co-enzyme. However, the separation of two pools of redox carriers with a similar, high redox potential is apparently essential to life. All organisms so far investigated can synthesize NADP from NAD, with the one exception of intracellular parasites that make use of the host cell's metabolism. Moreover, deletion of the enzyme catalyzing this conversion, NAD kinase, is lethal even in lower prokaryotes.[1] Obviously, the two different pyridine nucleotides have adopted specific roles early in evolution. NADPH represents a universal pool of reducing equivalents. Therefore, it holds a key position in all detoxifying and oxidative defense mechanisms of the cell and provides electrons to feed reductive biosyntheses.

Besides their vital functions as electron carriers, the pyridine nucleotides are also important signaling molecules. Expression of several genes is sensitive to the redox state of pyridine nucleotides, based on their differential binding to some transcription factors, also referred to as redox sensors.[2,3] NAD^+ mediates post-translational protein modifications such as ADP-ribosylation and NAD-dependent protein deacetylation.[4,5] NAD^+ is also a direct precursor of second messenger molecules such as ADP-ribose, cyclic ADP-ribose and *O*-acetyl-ADP-ribose, which are all involved in intracellular Ca^{2+} mobilization.[5–7]

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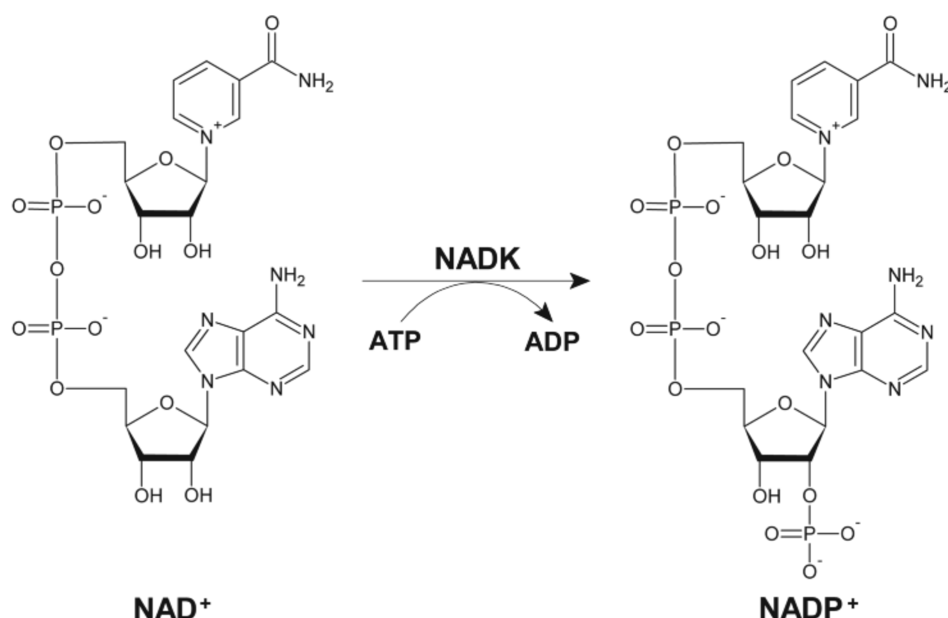

Figure 1 Reaction catalyzed by NAD kinase (NADK). NADH can also be utilized as substrate, yielding NADPH instead of NADP+

Importantly, NADP+, too, is a precursor of potent intracellular calcium-releasing molecules.[8]

In this review, we will first summarize the current knowledge about functions and regulation of NAD kinases, the enzymes that catalyze the synthesis of NADP. Then, we will focus on the physiological roles of NADP both as electron carrier and signaling molecule.

Generation of NADP by NAD kinase

The generation of NADP is catalyzed by NAD kinases, which transfer a phosphate group, most often from ATP, onto the 2′-hydroxyl group of the adenosine ribose moiety of NAD (Fig. 1). Thus, NADP synthesis critically depends on the availability of NAD and may be regarded as another important NAD-consuming process, in addition to the NAD-dependent signaling reactions. In 1950, NAD kinase (NADK) activity was enriched in extracts from *Saccharomyces cerevisiae* for the first time[9] and has been studied in various organisms since. However, it was not until 50 years later that the first genes encoding two bacterial NADKs were identified,[10] which eventually enabled studies of this enzyme at the molecular level.

The ability to generate NADP from NAD was a very early requirement in evolution, because at least one gene coding for NADK is present in all sequenced genomes, except for the intracellular parasite *Chlamydia trachomatis*.[11] Three NADK isoforms were identified in the yeast *S. cerevisiae*, two of which were localized to the cytosol (Utr1p, Yef1p) and the third (Pos5p) to mitochondria.[12] Of the three NADKs found in *Arabidopsis thaliana*, NADK1 is a cytosolic enzyme,[13,14] whereas NADK2 resides in chloroplasts.[14,15] The localization of NADK3 is less clear. The enzyme was suggested to be directed to the mitochondria via an N-terminal targeting sequence,[16] but was recently detected in peroxisomes.[14] In contrast to this complexity, only a single mammalian NADK has so far been identified.[17] NADK activity is essential for cell survival. Disruption of the NADK gene in organisms carrying a single gene is lethal.[1,18]

NADKs are homo-oligomers of 2–8 subunits. Most of the known primary structures give rise to polypeptides of 30–60 kDa in molecular mass. They contain a conserved C-terminal catalytic domain and a more variable N-terminal region, which is particularly extended in the eukaryotic enzymes. According to site-directed mutagenesis, a GGDG motif and a glycine-rich motif are highly conserved and essential for catalysis. The GGDG motif is also present in enzymes that phosphorylate targets other than NAD, which suggests NADK to be a member of a kinase superfamily.[19]

The three-dimensional structures of NADKs have been resolved for several prokaryotic enzymes. The N-terminal domains contain a Rossmann fold, a classical dinucleotide binding site, but oligomeric assembly of NADKs is required for substrate binding, because inter-subunit contacts create the binding site for NAD.

ATP is the preferred nucleoside triphosphate used as phosphoryl donor for NADKs, but some bacterial enzymes can also utilize the ancient energy carrier polyphosphate. With the exception of the enzymes from Gram-negative bacteria, NADKs accept the reduced form of NAD, NADH, as substrate. However, the preferred substrate for most NADKs is NAD^+.[20] Interestingly, the mitochondrial NADK isoform from yeast, and NADK1 and NADK3 from *A. thaliana*, prefer NADH over NAD^+. Gene knockout of the mitochondrial NADK in yeast showed that this isoform is an important, but not indispensable, generator of NADPH. The cells displayed a high sensitivity towards oxidative stress, which particularly affected mitochondrial function. Deletion of any of the two cytosolic isoforms resulted in a comparably mild phenotype that neither displayed severe growth defects nor a higher sensitivity to increased oxygen concentrations. However, a double mutant lacking both mitochondrial Pos5p and cytosolic Utr1p caused a synthetically lethal phenotype, which could be rescued by overexpression of any of the yeast NADKs or human NADK.[21] These studies indicate that cytosolic NADK isoforms might partially supply NADP across the border of cellular compartments. However, in yeast, a direct exchange of pyridine nucleotides between mitochondria and the cytosol was shown only for NAD^+.[22] Thus, there seems to be a requirement for the maintenance of cytosolic and mitochondrial NADP pools by compartment-specific NADK isoforms. In this regard, the presence of a single mammalian NADK remains elusive.

A calcium/calmodulin (Ca^{2+}/CaM)-dependent increase in NADK activity leading to an elevated NADPH level is known to precede the hardening of the egg membrane immediately after fertilization of sea urchin eggs.[23] The increase of NADPH concentration is probably required to supply the substrate for NADPH oxidase[24] to generate $O_2^{\bullet-}$ for the respiratory burst required for cross-linking the protective surface envelopes of the fertilized egg. A Ca^{2+}/CaM-dependent regulation was also reported for NADKs from plants.[25] However, a direct catalytic stimulation of the plant enzymes was not detected when expressed from recombinant DNA. The same is true for the human enzyme.[17] Thus, the molecular mechanism of the Ca^{2+}/CaM-dependent activation of NADKs might be indirect or depend on additional, yet to be identified, factors.

The role of the mammalian NADK in maintaining the cellular NADP pool was investigated by modulating its expression levels in engineered cells.[26] Changes in NADK expression did not significantly alter the cellular $NADP^+$ concentration, but strongly affected the level of NADPH. An almost 200-fold higher expression level of human NADK led to a 4–5-fold increase in the NADPH concentration. Since the human enzyme prefers NAD^+ as substrate, the $NADP^+$ generated by NADK seems to be immediately reduced to NADPH. The increase in NADPH improved cell viability in response to oxidative damage, but not to the extent the increase in NADPH would have suggested. However, a higher sensitivity towards hydrogen peroxide was observed when NADK expression levels were reduced to about 30% by constitutive expression of a small interfering short hairpin RNA. This reduction of NADK expression resulted in a significant (~70%) decrease of the cellular NADPH concentration. These experiments also underscored the critical role of $NADP^+$-dependent dehydrogenases for the maintenance of the cellular NADPH pool. These observations suggested that, in humans, the rate of NADPH regeneration is at least as important as the actual concentration of NADP formed by NADK for protection against oxidative stress.

Functions of NADP

A major role of NADP is its role as co-enzyme in cellular electron transfer reactions. Moreover, the cell spends a significant amount of energy to keep NADP in its reduced form, thereby maintaining a readily available pool of electrons to reduce oxidized compounds. NADPH is the universal electron donor in reductive biosyntheses and detoxification of the cell; it also holds a key position in oxidative defense systems (Fig. 2). However, it is also able to cause oxidative damage through the activity of NADPH oxidases.

Reductive biosyntheses

Generally, anabolic reactions are reducing processes and NADPH is used as the main reductant. For example, fatty acid synthesis is a reductive process that requires ATP and NADPH. Reduced NADP is necessary to produce triacylglycerols, phospholipids and steroids, such as cholesterol, bile acids and steroid hormones. Biosynthesis of some amino acids (*e.g.* glutamate and proline) is also dependent on NADPH. Amino acid synthesis, in turn, is not only critical for protein synthesis, but also to provide building blocks for other molecules including nucleotides. NADPH is also essential for the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase and, thereby, indirectly involved in DNA synthesis.

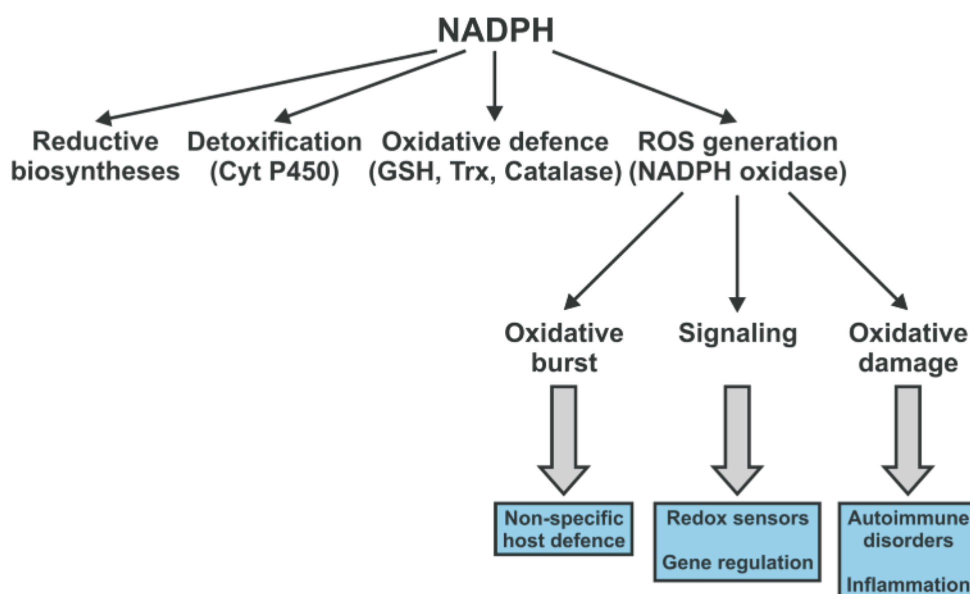

Figure 2 Functions of NADPH. The major function of NADPH is to donate electrons to reduce oxidized compounds. All reactions shown here are redox reactions yielding NADP$^+$, which can be re-reduced to NADPH or degraded. Cyt P450, cytochrome P-450; GSH, glutathione; TRX, thioredoxin

Detoxification

The liver detoxifies xenobiotics such as drugs, toxins, preservatives and other possibly harmful agents. Detoxification often involves the microsomal mono-oxygenase system. Cytochrome P-450-dependent hydroxylation converts relatively insoluble organic compounds to hydrophilic ones, thereby facilitating their breakdown and secretion. Cytochrome P-450 (CYP) enzymes are conserved in all organisms, from bacteria to humans.[27] There are 57 genes in the CYP family in humans.[28] The regeneration of CYPs requires continuous re-reduction which is accomplished by NADPH-dependent cytochrome P450 reductases (CPRs). They transfer electrons from NADPH to their FAD-FMN electron transport chain before eventually donating them to the heme moiety of CYPs.[29] There is only one gene coding for CPR in humans, and knockout of the gene in mice is embryonic lethal.[29] CPR can also transfer electrons to heme oxygenases, enzymes involved in the degradation of heme molecules to bilirubin.[30]

Besides the mono-oxygenase system, glutathione *S*-transferases play a major role in the biotransformation and detoxification of xenobiotics.[31] However, they also have an important function in the restoration of oxidatively damaged macromolecules.

Oxidative defense

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the oxidative defense systems. ROS and their toxic products can accumulate by a variety of mechanisms. Accordingly, there are various mechanisms that contribute to the prevention and removal of oxidative damage, including glutathione, thioredoxins, catalase, superoxide dismutase and glutathione *S*-transferase.[32] Importantly, the maintenance and regeneration of all the known anti-oxidative defense systems ultimately requires NADPH as reducing agent. For example, NADPH is used to regenerate GSH from GSSG trough the action of glutathione reductase. GSH, in turn, is essential for the functions of several antioxidation enzymes including glutathione peroxidases and glutathione *S*-transferases. In some cell types, NADPH is required for the reactivation of catalase, when the enzyme is inactivated by H_2O_2.

NADPH oxidase

In addition to being an essential antioxidant, NADPH can also contribute to ROS generation through the activity of NADPH oxidases (NOX). NOX proteins are a family of multicomponent enzymes that produce ROS in various cellular compartments. Members of the mammalian NOX family, including NOX1–5, generate superoxide anions ($O_2^{\bullet-}$) which can be rapidly converted to other ROS. A related family, Dual oxidase (DUOX1 and DUOX2), have a peroxidase domain to catalyze O_2 conversion to other ROS. NOX and DUOX members are differentially expressed in different tissues and cell types.[33]

NADPH oxidase was first discovered in neutrophils and other phagocytes, where it is involved in unspecific host defense against bacteria and fungi.[34]

Stimulation leads to a rapid release of ROS to the extracellular or phagosomal space, often called an oxidative burst.[34] More recently, NOX enzymes have also been discovered in other, non-phagocytic cells. Under physiological conditions, constitutively active non-phagocytic NOX produces low levels of superoxide anions, which are removed by cellular antioxidants.[35] This non-phagocytic superoxide generation is intracellular and levels are lower than in neutrophils, even after stimulation.[35] The activities of NOX enzymes thus contribute to ROS signaling, which is involved in diverse processes such as cell growth, apoptosis, migration and extracellular matrix remodelling.[33,34]

Maintenance of the reduced NADP pool

An increased sensitivity of cells to oxidative stress is often associated with a dysfunction of $NADP^+$-dependent dehydrogenases that provide reducing equivalents in the form of NADPH. Among them, glucose-6-phosphate dehydrogenase (G6PD), which catalyzes the rate-limiting step of the pentose phosphate pathway (PPP), is a prominent representative. The deficiency of this enzyme strongly impairs the viability of erythrocytes.[36] Owing to their role in oxygen transport, these cells are highly dependent on the availability of NADPH for recycling oxidized glutathione (GSSG). In fact, cells exposed to oxidative stress react with a rapid increase of both G6PD expression and activity.[37] Conversely, inhibition of G6PD dehydrogenase renders cells more susceptible to oxidative damage,[38] whereas overexpression of the enzyme is protective.[38,39] The generation of $G6PD^{-/-}$ mice failed because of lethality during embryogenesis.[40] However, the gene encoding this enzyme is dispensable for cultured mouse embryonic stem cells.[41] The G6PD deficiency in these cells did not even cause significant changes in the ratios of reduced and oxidized NADP and glutathione, as long as they were grown in the absence of oxidative stress.[42] Thus, the activity of the PPP is important for the immediate regeneration of NADPH under conditions of oxidative stress. However, the spatial separation of the PPP-derived cytosolic NADPH from the NADPH pool in mitochondria, where most $O_2^{\bullet-}$ is produced, suggests there are alternative pathways to maintain a high NADPH level.

Indeed, other NADP-dependent enzyme isoforms have been identified that have a critical impact under conditions of oxidative stress. These include isocitrate dehydrogenase (IDP), malic enzymes (ME) and aldehyde dehydrogenase (ALDH), which in mammals all have cytosolic and mitochondrial isoforms (Fig. 3). IDP converts isocitrate to α-ketoglutarate by oxidative decarboxylation. In rat liver, its activity was shown to be 16–18-fold higher with regard to NADPH production compared to G6PD.[43] Alterations of the cellular IDP activity correlate with the susceptibility to various stressors.[44–46] Malic enzymes catalyze the oxidative decarboxylation of malate to pyruvate. NADPH generated from the cytosolic ME is used for the biosynthesis of long-chain fatty acids and might

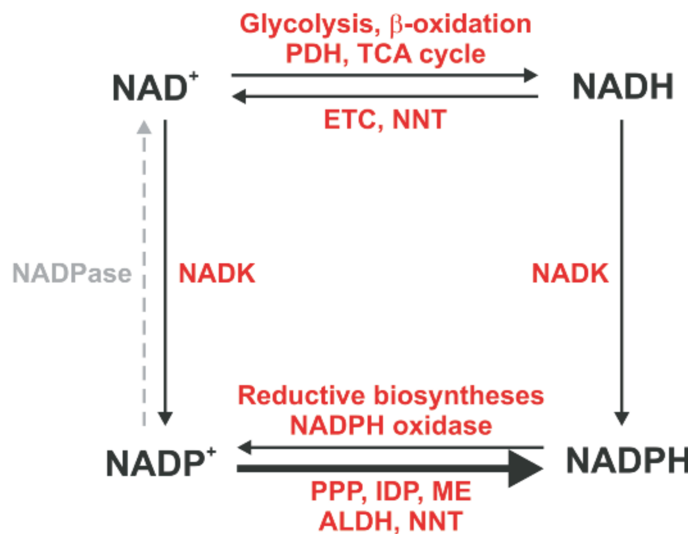

Figure 3 Metabolism of NAD(P) in eukaryotic cells. NADPase (grey) activity has been detected in various organisms. However, no protein with this activity has been isolated so far. PDH, pyruvate dehydrogenase; TCA cycle, tricarboxylic acid cycle; ETC, electron transport chain; NNT, nicotinamide nucleotide transhydrogenase; NADK, NAD kinase; PPP, pentose phosphate pathway; IDP, isocitrate dehydrogenase; ME, malic enzyme; ALDH, aldehyde dehydrogenase

provide reducing power for detoxification reactions initiated upon the development of chronic inflammation of the liver.[47] The pyruvate–malate shuttle appears to be particularly important for the protection of pancreatic islets, since this system provided far more NADPH than the cytosolic PPP.[48] The mitochondrial enzyme is most active in brain, where it is required for the maintenance of reduced glutathione (GSH).[49] Relatively little is known about the role of ALDHs in maintaining the cellular NADPH pool. ALDHs detoxify aldehyde substrates via oxidation to the corresponding carboxylic acids. Defects in ALDH genes, whose expression is up-regulated after exposing cells to oxidative stress, are associated with a higher susceptibility to cancer and Alzheimer's disease.[50]

In addition to these NADPH producing activities, in the membranes of bacteria and mammalian mitochondria another enzyme activity appears to be critically involved in the supply of NADPH (Fig. 3). Driven by the proton-motive force, nicotinamide nucleotide transhydrogenase (NNT) transfers a hydride ion directly from NADH to $NADP^+$ to produce NADPH. Gram-negative bacteria, which express NADK that is highly selective for $NADP^+$, have been proposed to generate up to almost half of their NADPH pool through this reaction.[51] Importantly, a similar contribution has been considered for mammalian mitochondria, in which NADPH is indeed accumulated. Lysates from *Caenorhabditis elegans* lacking a functional NNT gene showed a considerably lowered GSH/GSSG ratio, which caused severe growth defects after exposure of the

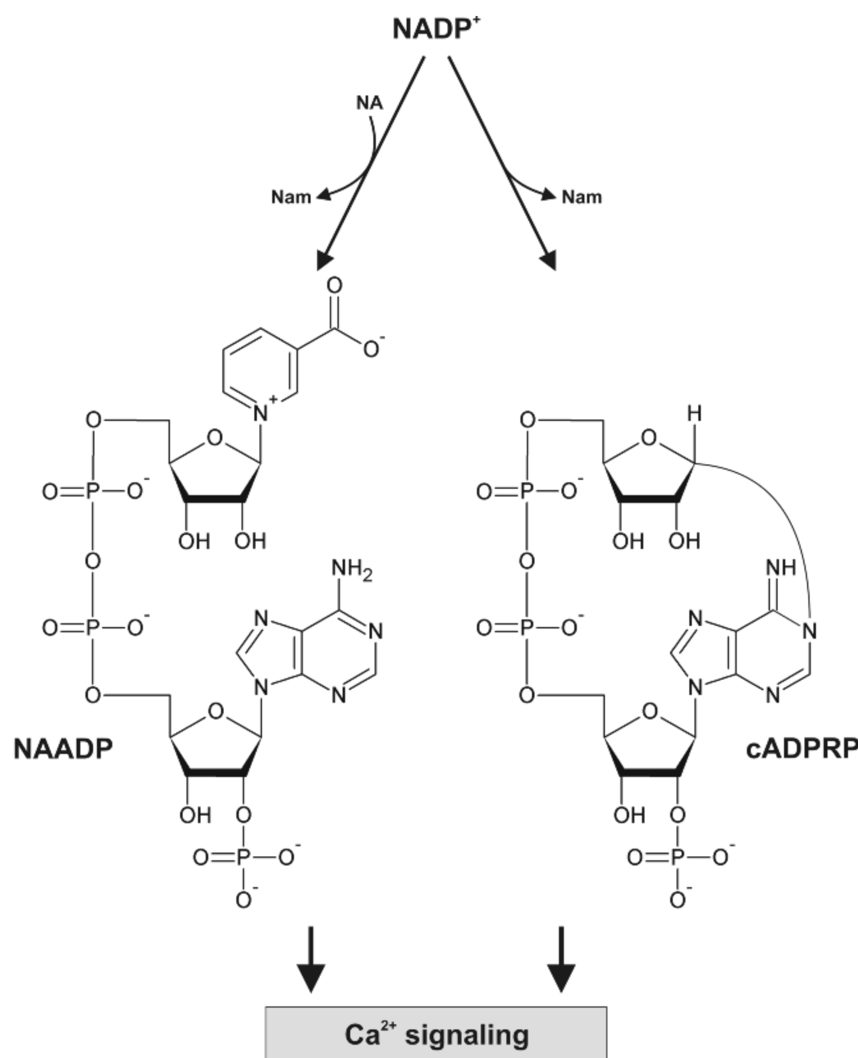

Figure 4 Signaling by $NADP^+$ derivatives. $NADP^+$ can be transformed into nicotinic acid adenine dinucleotide phosphate (NAADP) by a base exchange, replacing nicotinamide (Nam) by nicotinic acid (NA). It can also be converted into cyclic ADP-ribose phosphate (cADPRP). Both reactions are catalyzed by NAD glycohydrolase (NADase). NAADP and cADPRP are both involved in Ca^{2+} signaling

nematodes to oxidative stress.[52] Mutations in the NNT gene of C57BL/6J mice led to the accumulation of reactive oxygen species in pancreatic islets. This gene defect might, therefore, cause plasma glucose intolerance resembling human type 2 diabetes.[53,54] In yeast, no mitochondrial membrane NNT has been identified.

The differential localizations along with the individual contributions of the NADPH regenerating enzymes thus seem important to provide sufficient capacity to counteract oxidative damage. They might also reflect the importance of a higher preference for NADH as substrate for the mitochondrial NADK found in yeast and plants. Cytosolic IDP, rather than the mitochondrial isoform, was found to be of major importance in plants.[55] In yeast, neither IDP (mitochondrial or cytosolic) nor the mitochondrial malic enzyme is essential, even in the context of G6PD deletion.[56]

Regulatory role of NADP

Several proteins bind NAD or NADP not as co-enzymes, but at regulatory sites. These proteins are referred to as redox sensors, when the binding distinguishes between the reduced and oxidized states of the nucleotides. Thereby, they sense the cellular redox state which influences their activity, linking cellular metabolism to regulation of protein activity, protein localization or gene expression.[57,58]

Voltage-dependent potassium channels of the Shaker family (Kv channels) are important for repolarizing the cell membrane of excitable cells, such as neurons and muscle cells, at the end of the action potential.[59] Acute changes in channel current have been observed when the cellular redox state is altered (*e.g.* under oxidative stress). The β-subunit (Kvβ) of the Kv channel (found in plants, insects and mammals) has been suggested as a redox sensor, leading to channel inactivation as NADPH is oxidized.[60]

NADPH has recently been found to be an allosteric regulator of HSCARG (also known as short-chain dehydrogenase/reductase family 48A, member 1, SDR48A1).[61] HSCARG has apparently an important role in redox sensing. It binds NADPH under normal conditions, whereas a decreased NADPH/$NADP^+$ ratio causes release of NADPH and subsequent alterations in protein structure and subcellular distribution.[3,62] These changes promote an interaction between HSCARG and argininosuccinate synthetase (AS), a rate-limiting enzyme in nitric oxide synthesis. As a consequence, AS is inhibited and NO production decreased.[3,61,62] Translocation of HSCARG from the cytoplasm to the nucleus has also been shown to terminate activation of transcription factor NF-κB,[63] thereby establishing a close link between the cellular redox state and gene regulation.

NAD(P) has also been implicated in regulation of circadian rhythms. The heterodimeric transcription factors Clock:BMAL1 and NPAS2:BMAL1 regulate the expression of genes encoding other components of the circadian clock. Both heterodimers have been shown to bind NAD(P). The reduced forms induce, while the oxidized forms inhibit, DNA binding activity.[2]

Degradation of NADP – signaling by $NADP^+$ derivatives

While NADPH, the reduced form of NADP, is vital as an electron reservoir and donor, the oxidized form, $NADP^+$, is normally far less abundant in cells due to its immediate reduction to NADPH. However, only $NADP^+$ is subject to further conversion. Degradation products of NADPH are not known, whereas $NADP^+$ can be degraded into several derivatives.

NADP phosphatase (NADPase), catalyzing the removal of phosphate from $NADP^+$, has been observed in dormant seeds of *Avena sativa* L.[64] and rat liver.[65] However, so far, neither has an enzyme been purified nor a corresponding gene identified.

$NADP^+$ can be transformed to nicotinic acid adenine dinucleotide phosphate (NAADP) by a base-exchange catalyzed by NAD^+ glycohydrolase (NADase) at acidic pH (Fig. 4).[8] In mammals, two NADases have been identified, the lymphocyte antigenic markers CD38 and CD157.[7] NAADP represents the most potent calcium-mobilizing agent known so far. It participates in Ca^{2+} signaling in a variety of biological systems and acts independently of the other Ca^{2+}-releasing second messengers, cyclic ADP-ribose and inositol 1,4,5-triphosphate (IP_3).[8] Mammalian and other NADases are also referred to as ADP-ribose cyclases (ADPRCs), because they catalyze not only the conversion of $NAD(P)^+$ to ADP-ribose (phosphate), but also a cyclized form of this molecule.[6,66,67] As for the related NAD-derived cyclic ADP-ribose, the 2′-phosphorylated form (cADPRP; Fig. 4) is a potent inducer of Ca^{2+} release from intracellular stores.[67] It was shown that cADPRP is an endogenous metabolite in mammals, although present at a lower level than cADPR. Cyclic ADPRP might link oxidative stress with intracellular calcium release.[68] Since, under normal metabolic conditions, the NADP pool is maintained in a highly reduced state, $NADP^+$, the direct precursor of the signaling

molecules, is kept at a rather low level. This limiting concentration is in fact very suitable for a signaling molecule.

Conclusions

The variety of physiological roles played by the pyridine nucleotides has long been underestimated. While NAD^+-mediated signaling processes have now moved into focus, there might still be much to learn about the functions of NADP as well. NADPH is critically in all known mechanisms counteracting toxic, in particular oxidative, assaults. However, both NADPH and $NADP^+$ also appear to carry important regulatory functions, as for example in redox and calcium signaling. NAD kinase is a key determinant of the cellular concentration and may thus have a strong impact on these multiple pathways.

Acknowledgements

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