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Comprehensive Invited Review

NAD^+/NADH and $NADP^+$/NADPH in Cellular Functions and Cell Death: Regulation and Biological Consequences

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ABSTRACT

Accumulating evidence has suggested that NAD (including NAD^+ and NADH) and NADP (including $NADP^+$ and NADPH) could belong to the fundamental common mediators of various biological processes, including energy metabolism, mitochondrial functions, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death: First, it is established that NAD mediates energy metabolism and mitochondrial functions; second, NADPH is a key component in cellular antioxidation systems; and NADH-dependent reactive oxygen species (ROS) generation from mitochondria and NADPH oxidase-dependent ROS generation are two critical mechanisms of ROS generation; third, cyclic ADP-ribose and several other molecules that are generated from NAD and NADP could mediate calcium homeostasis; fourth, NAD and NADP modulate multiple key factors in cell death, such as mitochondrial permeability transition, energy state, poly(ADP-ribose) polymerase-1, and apoptosis-inducing factor; and fifth, NAD and NADP profoundly affect aging-influencing factors such as oxidative stress and mitochondrial activities, and NAD-dependent sirtuins also mediate the aging process. Moreover, many recent studies have suggested novel paradigms of NAD and NADP metabolism. Future investigation into the metabolism and biological functions of NAD and NADP may expose fundamental properties of life, and suggest new strategies for treating diseases and slowing the aging process. *Antioxid. Redox Signal.* 10, 179–206.

I. INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+), reduced nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) have been known as classic molecules involving in energy metabolism, reductive biosynthesis, and antioxidation (27, 29, 233). Structurally $NADP^+$ is identical to NAD^+ except for the additional 2' phosphate on the adenosine ribose moieties of $NADP^+$. However, NAD (including NAD^+ and NADH) are mainly used by the enzymes that catalyze substrate oxidation, while NADP (including $NADP^+$ and NADPH) are mainly used by the enzymes that catalyze substrate reduction (233).

Increasing evidence has suggested that the pyridine nucleotides NAD and NADP have far more extensive biological functions than their classical functions (27, 29, 233, 328, 329). The following findings may be of particular interest: a) Recent studies have indicated pivotal roles of NAD^+-dependent histone deacetylases (*i.e.*, sirtuins) in aging (35); b) poly(ADP-ribose) polymerase-1 (PARP-1)—-a major NAD^+-consuming enzyme—-appears to mediate oxidative cell death under many conditions (296, 328); c) cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP)—-two endogeneous molecules generated from NAD^+—-are key signaling molecules mobilizing intracellular calcium stores (159, 160); and d) NADPH oxidase is a major generator of reactive oxygen species (ROS) in both immunological reactions and multiple diseases (26). These seemingly diverse topics of biomedical research are fundamentally linked by NAD and NADP. In recent years there have also been a number of novel findings regarding the metabolism of NAD and NADP. For examples, three isoforms of nicotinamide mononucleotide adenylyltransferases (NMNATs)—-the key NAD^+-synthesizing enzymes—-have been found in various subcellular organelles (30, 182, 235); and novel pathways by which NADH and NADPH are generated have also been found (30, 102, 221). Collectively, these findings have strongly suggested the necessity to search for novel paradigms about the metabolism and biological functions of NAD and NADP, which may be required for exposing the fundamental mechanisms in biology as well as the essential relationships among various biological and pathological processes.

II. METABOLISM OF NAD AND NADP

A. General information about NAD and NADP

Intracellular levels of NAD are significantly higher than those of NADP under physiological conditions (233). Because it is generally belived that mitochondrial membranes are impermeable to NAD and NADP (82, 83, 164, 233), there are two major pools of NAD and NADP in cells: the cytosolic pool and the mitochondrial pool. However, cytosolic reducing equivalents of NADH can be shuttled into mitochondria by the NADH shuttles, which maintain the NADH homeostasis in cytosol (195). The mitochondria pool of NAD^+ represents a significant portion of the total NAD^+ pool in myocytes (82). However, there is no sufficient information about the percentage of mitochondrial NAD^+ in the total pools of NAD^+ in other cell types. It has been reported that mitochondrial permeability transition (MPT) pore opening in myocytes can lead to mitochondrial NAD^+ release and subsequent hydrolysis of NAD^+ by NAD^+ glycohydrolase (82). A recent study has also suggested that MPT mediates the PARP-1 activation-induced mitochondrial NAD^+ loss of mouse neurons and astrocytes (7), which may significantly contribute to metabolic dysfunction (271). Due to the critical roles of NAD and NADP in cellular functions and cell death, it is of great interest to further determine the relationships between cytosolic NAD/NADP and mitochondrial NAD/NADP.

Under physiological conditions, the ratio of cytosolic free NAD^+/NADH is $\sim$ 700 to 1 (276, 294, 342), while the mitochondrial NAD^+/NADH ratio has been reported to be 7–8 (276, 294). In contrast, the levels of NADPH are much higher than those of $NADP^+$ (122, 233). Cumulating evidence has suggested that cytosolic free NAD^+/NADH ratios are altered under various pathological conditions. For example, in diabetic

tissues there are sorbitol pathway-mediated decreases in NAD^+/NADH ratios (122, 207), which may play a crucial role in the pathogenesis of diabetic complications (122). Because the ratios of NAD^+/NADH and $NADP^+$/NADPH can affect numerous enzymatic activities and MPT (352) which play important roles in cell death under many conditions (69, 150, 165), it is warranted to further determine the changes of these ratios under both physiological and pathological conditions.

B. NAD synthesis

NAD^+ biosynthesis plays a central role in the metabolism of NAD and NADP, because NAD^+ is necessary for the generation of NADH, $NADP^+$, and NADPH. Two known NAD^+ biosynthesis pathways are the *de novo* pathway and the salvage pathway (181, 182). Nicotinamide and nicotinic acid are the NAD^+ precursors in the salvage pathway (30, 181, 182), which are first transferred onto phosphoribosyl pyrophosphate by phosphoribosyl transferases to form nicotinamide mononucleotide (NMN) or nicotinic acid mononucleotide (NaMN), respectively. Subequently NMN and NaMN are converted by NMNATs to NAD^+ and NaAD, respectively. NaAD is then amidated by NAD^+ synthase to generate NAD^+. There are distinct differences between the salvage pathway of mammals and that of yeast and invertebrates (238). Mammals use nicotinamide instead of nicotinic acid as the main precursor for NAD^+ synthesis: Nicotinamide is directly converted by nicotinamide phosphoribosyltransferase (Nampt) to NMN that is subsequently used by NMNATs for NAD^+ generation (238). In contrast, in yeast and invertebrates nicotinamide can not be used directly for NAD^+ synthesis before its conversion to nicotinic acid (238).

Quinolinic acid is the NAD^+ precursor in the *de novo* pathway, which is generated from either L-tryptophan in animals and some bacteria, or L-aspartate in some bacteria and plants (135). Quinolinic acid is converted by quinolinic acid phosphoribosyltransferase to NaMN, which is subsequently converted to NAD^+ by NMNATs and NAD^+ synthase (233).

The nuclear enzyme NMNAT-1 is a key enzyme in both the *de novo* pathway and the salvage pathway of NAD^+ synthesis (29), which had been the only known NMNAT until recently. It has been reported that the loss of NMNAT-1 in *Drosophila* leads to rapid and severe neurodegeneration that can be ameliorated by blockage of neuronal activity (340). A latest study has also found that NMNAT-1 can bind the poly(ADP-ribose) (PAR) on activated PARP-1 and promote poly(ADP-ribosyl)ation (31). Protein kinase C-mediated phosphorylation of NMNAT-1 can lead to decreased binding of NMNAT-1 to PAR (31). This close interaction between a key NAD^+-synthesizing enzyme and a key NAD^+-consuming enzyme is intriguing, since it implicates potential coordination between NAD^+ generation and NAD^+ consumption in the nucleus. It has also been found that the gene product of human homolog of NMNAT-1 constitutes a major portion of the chimeric protein that mediates the delay in Wallerian neurodegeneration of Wld(S) mice (14, 182), suggesting a potential role of NMNAT-1 in axonal degeneration (14, 302). Recent studies have indicated the presence of three isoforms of human NMNATs—-NMNAT-1, NMNAT-2, and NMNAT-3 (30, 182, 235), which are located in the nucleus, the Golgi complex, and mitochondria, respectively (30, 182, 235). These findings, together with the observations indicating the presence of tankyrase in Golgi complex (54) and NAD^+-consuming enzymes in mitochondria (82, 85, 172), suggest that there are relatively independent machineries of NAD metabolism in the nucleus, the Golgi complex, and mitochondria.

Recent studies have indicated multiple novel and interesting properties of Nampt—-another key enzyme in NAD^+ synthesis: it has been demonstrated that three seemingly different proteins—-Nampt, the presumptive cytokine pre-B-cell colony-enhancing factor (PBEF), and a new visceral fat-derived hormone visfatin—-are actually the same protein (95, 188, 237, 241). The presence of Nampt/PBEF/visfatin in plasma raises an intriguing possibility that this protein might generate NMN extracellularly using the nicotinamide in plasma as a substrate, which may be subsequently transported into cells for NMNAT-catalyzed NAD^+ synthesis (238). While many future studies are needed to demonstrate this hypothesis, our understanding regarding NAD^+ synthesis could be significantly revised if this hypothesis were demonstrated: The processes of NAD^+ synthesis may not only occur intracellularly in the nucleus and other subcellular organelles, but also occur extracellularly. It has also been proposed that the cytokine-like functions of PBEF and the insulin-mimetic functions of visfatin may be accounted for by the NAD^+-synthesizing functions of Nampt (238). Demonstration of this hypothesis would further deepen our understanding about the biological functions of the extracellular metabolic intermediates in NAD^+ synthesis. Figure 1 provides diagrammatic presentation of the NAD^+ metabolic machineries in cells.

It is noteworthy that the kynurenine pathway leads to generation of several neuroactive intermediates, including quinolinic acid, kynurenic acid, and 3-hydroxykynurenine (206, 249, 255). Thus, the kynurenine pathway has been a target for treatment of multiple neurological diseases (206, 249, 255). Increasing evidence has also suggested significant biological activities of nicotinamide and nicotinic acid—-two important components in NAD^+ metabolism: Nicotinic acid can significantly affect brain functions by such pathways as inducing glutamate release (301); and nicotinamide can also enhance energy metabolism, inhibit PARPs and sirtuins, and activate Akt (145, 168, 183, 312). A number of studies have also suggested therapeutic potential of nicotinamide for multiple diseases such as cerebral ischemia (19, 145, 183, 191). Recently it has been found that nicotinamide riboside—-a novel NAD^+ precursor in eukaryotes—-can significantly extend the replicative lifespan of yeast (28).

A recent study has suggested a novel pathway for NADH generation: NADH may be directly generated from reduced form of NMN and ATP by NMNAT-2 and NMNAT-3, but not NMNAT-1 (30). It is warranted to further determine the physiological significance of this pathway.

C. NADP synthesis

There are two major mechanisms by which $NADP^+$ can be formed: $NADP^+$ can be generated *de novo* from NAD^+ through the action of NAD^+ kinases (NADKs) (166); and $NADP^+$ can also be formed from NADPH by multiple NADPH-dependent enzymes such as glutathione reductase (233). There are also two major mechanisms by which NADPH can be formed: The

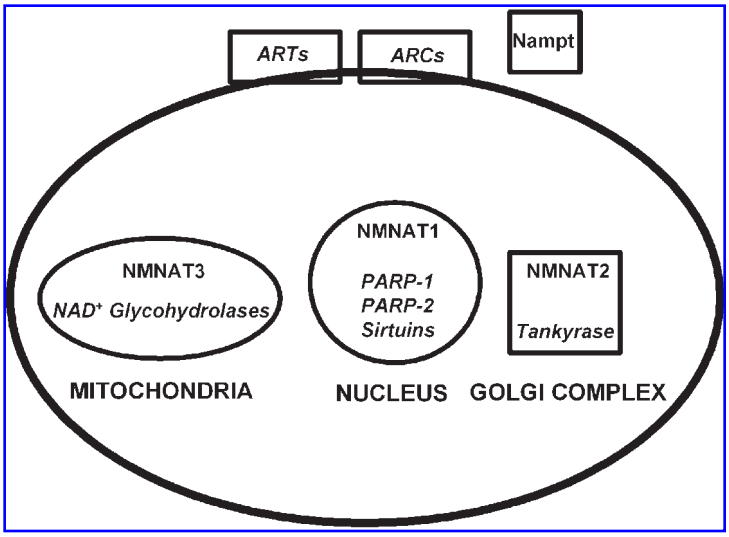

FIG. 1. NAD$^+$ metabolism in cells. NAD$^+$ metabolism occurs both intracellularly in various subcellular organelles and extracellularly. The key NAD$^+$ synthesizing enzymes NMNAT-1, NMNAT-2, and NMNAT3 are located at the nucleus, the Golgi complex, and mitochondria, respectively. There are NAD$^+$-consuming enzymes in these organelles, including poly(ADP-ribose) polymerase-1 (PARP-1), PARP-2, and certain sirtuins in the nucleus, tankyrase in the Golgi complex, and NAD$^+$ glycohydrolases in mitochondria. On plasma membranes, mono(ADP-ribosyl) transferases (ARTs) and ADP-ribosyl cyclases (ARCs) produce mono(ADP-ribosyl)ation on target proteins and generate cyclic ADP-ribose, respectively. Nicotinamide phosphoribosyltransferase (Nampt) may exist extracellularly and produce its biological effects by generating nicotinamide mononucleotide from nicotinamide.

first is that NADPH is generated from NADH and NADP$^+$ by mitochondrial transhydrogenase; and the second is that NADPH is generated from NADP$^+$ by multiple NADP$^+$-dependent enzymes.

NADKs play a critical role in determining the levels of NADP, since the enzymes are the sole enzymes that can *de novo* generate NADP$^+$. Thus, elucidation of the mechanisms underlying the regulation of NADKs is critical for understanding the regulation of NADP levels. Essential roles of NADKs in various biological activities of both prokaryotes and eukaryotes have been reported (105, 233, 264). Whereas three isoforms of NADKs have been found in yeast, there is only one known NADK in mammals (233).

There are four known groups of enzymes that catalyze NADPH formation from NADP$^+$ in cells: First, glucose-6-phosphate dehydrogenase and 6-gluconate phosphate dehydrogenase—-two enzymes in the pentose phosphate pathway (also called 'hexose monophosphate shunt'); second, cytosolic and mitochondrial NADP$^+$-dependent isocitrate dehydrogenases (IDPc and IDPm) (201); third, cytosolic and mitochondrial NADP$^+$-dependent malic enzymes (MEPc and MEPm); and fourth, mitochondrial transhydrogenase (246).

In cytosol, NADPH can be generated by glucose-6-phosphate dehydrogenase, 6-gluconate phosphate dehydrogenase, IDPc, or MEPc (162, 180, 315). It has been reported that in yeast cytosolic acetaldehyde dehydrogenase also mediates NADPH generation from NADP$^+$ (102). In mitochondria NADPH can be generated from NADP$^+$ by IDPm, MEPm, or transhydrogenase (127). Figure 2 shows the pathways by which NADPH is generated in cytosol and mitochondria.

Glucose-6-phosphate dehydrogenase is a key enzyme for NADPH generation (146). While it is long thought as a "housekeeping" enzyme present in all cell types, the enzyme can also undergo tissue-specific regulation by various factors, including oxidative stress, hormones, and nutrients (146). Although glucose-6-phosphate dehydrogenase is the most well-studied enzyme for NADPH synthesis, some studies have also suggested significant contributions of IDPc and MEPc to cytosolic NADPH synthesis and cellular antioxidation capacity (162, 180).

Mitochondrial transhydrogenase is located in the inner membranes of animal mitochondria, which couples the translocation of protons across mitochondrial membranes to the transfer of reducing equivalents between NAD(H) and NADP(H) (127). Under most physiological conditions, the enzyme is driven toward the reduction of NADP$^+$ by NADH via utilization of mitochondrial transmembrane electrochemical gradient of proton (246). Recent studies using transhydrogenase-knockout mice

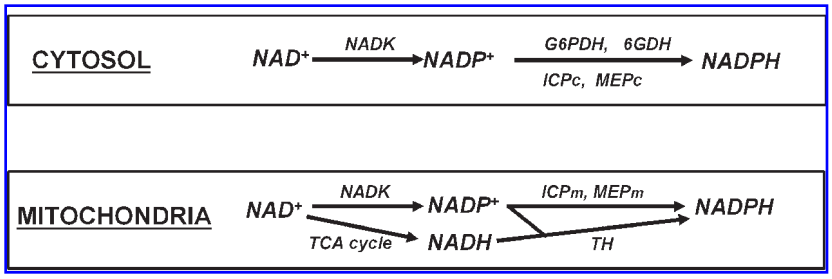

FIG. 2. Pathways by which NADPH is generated in cytosol and mitochondria. In cytosol NADP$^+$ is generated from NAD$^+$ by NAD$^+$ kinase (NADK). NADPH can be generated from NADP$^+$ by glucose-6-phosphate-6-phosphate dehydrogenase (G6PDH), 6-glyconate phosphate dehydrogenase (6GPDH), cytosolic NADP$^+$-dependent isocitrate dehydrogenase (IDPc), or cytosolic NADP$^+$-dependent malic enzymes (MEPc). In mitochondria NADP$^+$ is generated from NAD$^+$ by NAD$^+$ kinase, and NADPH can be generated from NADP$^+$ by mitochondrial NADP$^+$-dependent isocitrate dehydrogenase (IDPm), mitochondrial NADP$^+$-dependent malic enzymes (MEPm), or mitochondrial transhydrogenase (TH).

have indicated that deletion of the gene can lead to type II diabetes (94), which raises the question why the transhydrogenase deletion selectively impairs insulin secretion.

Whereas multiple enzymes can generate NADPH, the relative contribution of these enzymes to the general NADPH production in cells can be variable in different cell types or under different conditions. It has been suggested that at least for brain mitochondria, all of the three mitochondrial enzymes that can generate NADPH contribute to the mitochondrial NADPH generation and the reduction of oxidized glutathione in mitochondria (297). A significant role of IDPm in cellular antioxidation capacity has also been shown in NIH3T3 cells (162). Recent studies have suggested that at least in certain mammalian cells IDPm is a major source of mitochondrial NADPH, which can be inhibited by lipid peroxidation products (313) and regulated by Ca^{2+} (275). It has also been found that ROS can induce IDPm expression (130).

In yeast, Outten *et al*. found a novel NADPH-generating pathway—-the NADH kinase-dependent pathway for NADPH generation (221): the POS5 gene product Pos5p has NADH kinase activity that generates NADPH by catalyzing phosphorylation of NADH, which appears to be a major NADPH-generating enzyme in yeast (221). Disruption of *POS5* led to a 50-fold increase in the mitochondrial mutation rate in yeast (264). However, it is unclear if similar mechanisms also exist in high eukaryotes.

D. Catabolism of NAD and NADP

NAD^+ can be consumed by multiple families of enzymes, including PARPs, sirtuins, ADP-ribosyl cyclases, and mono(ADP-ribosyl) cyclases, leading to generation of nicotinamide and other products containing ADP-ribose as the core structural component. The reactions catalyzed by these NAD^+-dependent enzymes can profoundly affect various biological processes. The major NAD^+-consuming enzymes include:

First, PARPs are a family of enzymes that consume NAD^+ to produce PAR on target proteins (296). PARP-1 has been the most intensively studied member of PARP family, which appears to play important roles in regulation of various cellular and subcellular processes, including DNA repair, gene expression, genomic stability, cell cycle, and cell death (254, 296, 328). PARP-1 has also been shown to mediate multiple biological functions of tissues and organs, such as inflammation and learning and memory (44,296). Excessive PARP-1 activation has been found to mediate ischemic injuries of various organs, diabetes, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism, traumatic brain injury, hypoglycemic brain damage, and shock (296, 328).

Second, bifunctional ADP-ribosyl cyclases/cyclic ADP-ribose hydrolases can consume NAD^+ to generate cyclic ADP-ribose as well as hydrolyze cyclic ADP-ribose into free ADP-ribose (347). The mammalian ADP-ribosyl cyclase CD38 is of particular interest: This enzyme could be a major regulator of intracellular NAD^+ concentrations under physiological conditions (4); and CD38-generated cyclic ADP-ribose could play critical roles in many biological processes (159, 160, 329).

Third, the NAD^+-dependent histone deacetylases (or called Sir2 family proteins or sirtuins) produce deacetylation of both histones and nonhistone proteins by consuming NAD^+. This process could profoundly affect aging, carcinogenesis, and cell death (79, 250).

Fourth, mono(ADP-ribosyl)transferases (ARTs) are a family of enzymes that consume NAD^+ to produce mono(ADP-ribosyl)ation of proteins (64, 81). Recent studies have suggested that ART1-5 are expressed in various cell types (63, 261). Notably, the ecto-enzyme ART2 on the plasma membranes of Treg cells—-a subset of T cells that mediates immunological activities—-can produce mono(ADP-ribosyl)ation of $P2X_7$ receptors resulting in apoptosis of Treg cells (17, 147).

There is increasing evidence suggesting that CD38 could mediate intracellular NAD^+ levels under physiological conditions, while PARP-1 could mediate intracellular NAD^+ levels when significant DNA damage occurs: A recent study reported that there are significant increases in the the tissue levels of NAD^+ in CD38 knockout mice compared with wild-type mice (4). The extent of the increases is tissue-dependent, ranging from 2-fold (kidney) to 25-fold (heart). These results suggest that CD38 is a fundamental regulator of NAD^+ levels under physiological conditions (4). It has also been established that PARP-1 mediates NAD^+ depletion when significant DNA damage occurs (74, 329), while there is no significant difference between the NAD^+ levels in the brains of PARP-1 knockout mice and the brains of wild-type mice under physiological conditions (88).

The major known $NADP^+$-degrading enzyme is $NAD(P)^+$ nucleosidase that degrades $NADP^+$ to ADP-ribose(2'-phosphate) and nicotinamide (182). $NADP^+$ could also be converted to NAADP—-an important regulator of intracellular calcium stores (96, 159, 160). While increasing evidence has indicated that NAD^+ can be consumed through many pathways, thus producing various major biological effects, it remains unknown if $NADP^+$ may also be catabolized through multiple pathways.

E. Relationships between NAD and NADP

Two enzymes, NADKs and mitochondrial transdehydrogenase, are essential for regulating the conversion between NAD(H) and NADP(H): NADKs are the sole enzymes catalyzing the generation of $NADP^+$ from NAD^+, while it can not catalyze the conversion between NADH and NADPH (194); in contrast, transhydrogenase catalyzes the generation of ($NADPH + NAD^+$) from ($NADP^+ + NADH$). Due to the critical biological functions of NAD and NADP, NADKs and transhydrogenase could produce profound effects on cellular functions through its effects on the balance between NAD pool and NADP pool.

It has been reported that NADKs can be regulated by multiple factors: NADKs can be inhibited by such factors as NADH and NADPH (339), and activated by such factors as calcium/calmodulin (9,65). It is particularly interesting that oxidative stress (41, 105) and calcium/calmodulin can activate NADKs (9, 65), considering the established critical roles of oxidative stress and calcium/calmodulin in numerous biological and pathological processes (113, 190, 291, 318, 321, 322). Future studies are certainly warranted to further determine the regulation of these enzymes, and to determine the biological consequences of the regulation.

There are studies suggesting that the total levels of (NAD + NADP) could be increased under certain conditions: It was found that fasting led to increased NAD^+ levels by 33% in liver,

which returned to control levels by refeeding (240); serum withdrawl also induced increased PBEF expression in smooth muscle cells, which can lead to increased intracellular NAD^+ levels (292); and pyridine nucleotide synthesis was also inducted by mitogens (32). Moreover, it was reported that the total NADP levels were significantly increased in phorbol myristic acetate-treated human neutrophils (306).

F. NAD transport across mitochondrial membranes

It is generally believed that mitochondrial inner membranes are not permeable to NAD (286). However, this belief has been challenged by several studies: Two mitochondrial NAD^+ transporters, named Ndt1p and Ndt2p, have been found to import NAD^+ into the mitochondria of *Saccharomyces cerevisiae* (282); intact plant mitochondria can uptake NAD^+ in a concentration-dependent and temperature-dependent manner (215, 281); and there was NAD^+ influx into the mitochondrial matrix of cultured human cells harvested under quiescent conditions, when external NAD^+ was added to the digitonin-permeabilized cells (244).

Whereas mitochondrial inner membranes could be impermeable to NADH, the reducing equivalents of cytosolic NADH can be shuttled into mitochondria by NADH shuttles, including the malate—aspartate shuttle and the glycerol-3-phosphate shuttle (195). The major components of the malate—aspartate shuttle include cytosolic malate dehydrogenase, aspartate transaminase, mitochondrial aspartate—glutamate carrier, and mitochondrial malate dehydrogenase. The glycerol-3-phosphate shuttle is composed of cytosolic glycerol-3-phosphate dehydrogenase and mitochondrial glycerol-3-phosphate dehydrogenase.

The levels of cytosolic NADH can be regulated by not only the NADH shuttles, but also the lactate dehydrogenase-catalyzed pyruvate—lactate conversion and other dehydrogenase-catalyzed reactions (195). Due to the critical roles of these pathways in energy metabolism and other biological functions, the NADH shuttles may profoundly affect cellular functions due to its impact on cytosolic NADH. It is also conceivable that alterations of the NADH shuttles may produce major pathological consequences.

Several recent studies have used the malate—aspartate shuttle- or the glycerol-3-phosphate shuttle-deficient mice to determine the biological functions of the NADH shuttles. A recent study provided critical information regarding the regulation of NADH shuttles in neurons (224): Because ARALAR—-the neuronal Ca^{2+}-binding mitochondrial aspartate—glutamate carrier—-plays a role in the malate—aspartate shuttle and has Ca^{2+} binding domains facing the extramitochondrial space, the researchers determined the effects of Ca^{2+} signals on the NADH shuttling activity. The study indicated a novel mechanism by which small Ca^{2+} signals that are below the levels to activate Ca^{2+} uniporters affect mitochondrial NADH levels: the small Ca^{2+} signals can enhance NADH shuttling from cytosol to mitochondria by activating ARALAR. It is of interest to further determine the post-translational regulation of the NADH shuttles under both physiological and pathological conditions.

G. NAD transport across the plasma membranes of cells

It had been generally thought that NAD^+ and NADH can not be transported across the plasma membranes of any cell types. However, recent studies have suggested that NAD^+ and NADH can be transported across the plasma membranes of at least certain types of cells: Connexin 43 hemichannels could allow gradient-dependent NAD^+ flux across fibroblast plasma membranes (42); and the studies by us and other researchers have also suggested that NAD^+ can be transported across the plasma membranes of astrocytes (5, 295, 326).

Our latest study has provided the first evidence suggesting that NADH can be transported across the plasma membranes of astrocytes, which is mediated by $P2X_7$ receptors (177): we found that treatment of astrocytes with 10 μM—10 mM NADH significantly increased intracellular NADH and NAD^+. Three lines of evidence have suggested that the NADH transport is mediated by purinergic $P2X_7$ receptors: the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid abolished the NADH transport; RNA silencing-produced reductions of $P2X_7$ receptors also decreased the NADH transport; and transfection of $P2X_7$ receptor-deficient HEK293 cells with mouse $P2X_7$ receptor cDNA increased the NADH transport in the cells. Collectively, our study provides the first direct evidence demonstrating that NADH can be transported across the plasma membranes of certain cell types by a $P2X_7$ receptor-mediated mechanism. Our study also suggests a new approach for manipulating intracellular NADH and NAD^+ levels. Future studies are needed to determine if NADH can also be transported across the plasma membranes of other cell types, and if pathological conditions can alter the NADH transport.

III. BIOLOGICAL FUNCTIONS OF NAD AND NADP

A. General information about the biological functions of NAD and NADP

While it has been long thought that the major biological functions of NAD are modulating cellular energy metabolism, increasing evidence has suggested that NAD also mediates cell death (5, 296, 326) and various major biological activities such as calcium homeostasis (29, 347) and gene expression (245, 342). Growing evidence has further indicated significant roles of NAD in such important biological processes as aging, carcinogenesis, and immunological functions (29, 35).

The major biological functions of NADPH are three fold: the first is to act as a key component in cellular antioxidation systems; the second is to act as an electron source for reductive synthesis of fatty acids, steroids, and DNA (233); and the third is to act as the substrate for NADPH oxidase that plays key roles in many biological and pathological processes by generating ROS.

Recent studies have suggested distinct biological functions of the multiple NADPH-generating machineries: While both IDPc and IDPm play a significant role in defending oxidative damage (130), IDPc also mediates lipid metabolism (148). The

sources of NADPH generation may determine the biological effects of NADPH: The NADPH generated by the mitochondrial enzymes could mainly contribute to mitochondrial antioxidation and biosynthesis, while the NADPH generated by the cytosolic enzymes may also contribute to NADPH oxidase-dependent ROS generation when NADPH oxidase is activated.

The major known biological function of $NADP^+$ is acting as the precursor for NADPH formation. $NADP^+$ could also be a precursor for generation of NAADP—-an endogenous molecule that can mobilize acidic intracellular calcium stores (96, 159, 160). NAADP and cyclic ADP-ribose—-two molecules generated from $NADP^+$ and NAD^+—-have emerged as important regulators of calcium homeostasis.

B. NAD and NADP in antioxidation and oxidative stress

PARP-1 plays a key role in oxidative cell death under many conditions (296, 328). Increasing evidence has suggested that NAD^+ depletion mediates PARP-1-induced cell death (5, 326–329). NAD may also affect antioxidation and generation of oxidative stress through several pathways: First, the NADH/NAD^+ ratio is an index of cellular reducing potential, since the redox couple plays key roles in numerous redox reactions and has one of the most negative reduction potential (-0.32 V) in cells; second, NAD^+ can be converted by NADKs to $NADP^+$—-the precursor for NADPH formation (194); third, some studies have suggested direct antioxidation effects of NADH (144, 192, 220); and fourth, NAD^+ can inhibit ROS generation from α-ketoglutarate dehydrogenase and pyruvate dehydrogenase as well as permeablized rat brain mitochondria (274). Seemingly paradoxically, excessive intracellular NADH can produce 'reductive stress', which may result from its capacity to induce release of ferrous iron from ferritin (128), or from the capacity of xanthine oxidase/xanthine dehydrogenase to generate ROS by oxidizing NADH (344).

NADPH is one of the most important factors in cellular antioxidation through the following pathways: first, NADPH is required for regeneration of GSH from GSSG through the action of glutathione reductase. GSH is essential for the functions of several key antioxidation enzymes including glutathione peroxidase and glutathione *S*-transferases (309). Second, at least in some cell types, a large portion of NADPH binds the important H_2O_2-disposing enzyme catalase (143), which reactivates catalase when catalase is inactivated by H_2O_2. Third, NADPH is also an essential component in another important antioxidation system—-the thioredoxin system (15).

A crucial role of the pentose phosphate pathway in defending oxidative stress has been reported by multiple studies (223, 269). For example, in a study that used male mouse embryonic stem cells with genetic inhibition of glucose-6-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase appears to be essential in defending oxidative stress but is dispensable for pentose synthesis (223). Whereas it is widely accepted that NADPH mediates cellular antioxidation mainly through its effects on GSH regeneration, it has been reported that in red blood cells, NADPH plays a significantly more important role than GSH in defending oxidative insults (258), possibly due to the capacity of NADPH in reactivating catalase. A key role of glucose-6-phosphate dehydrogenase in NADPH synthesis and antioxidation has been further indicated by the findings that the red cells from the patients of glucose-6-phosphate dehydrogenase deficiency have increased sensitivity to oxidative stress (268). Recent studies have also established important roles of IDPm in defending oxidative stress: overexpression and decreased expression of the enzyme leads to decreased or increased sensitivity of mitochondria to oxidative stress, respectively (130). It has also been reported that IDPc plays a significant role in cellular antioxidation capacity (163). Figure 3 shows the pathways by which NADPH can decrease oxidative stress in cells.

Because mitochondrial transhydrogenase mediates the coupling of the H^+ translocation across mitochondrial membranes to the transfer of reducing equivalents between NAD(H) and NADP(H), it is tempting to propose that this enzyme may coordinate the activity of the tricarboxylic acid (TCA) cycle and the reducing potential of mitochondria: Increased TCA cycle activity can lead to increased NADH generation, which could both increase the H^+ gradient across mitochondrial membranes and potentiate ROS generation from the electron transport chain. Through transhydrogenase, an elevated H^+ gradient could lead to increased NADPH generation and increased antioxidation capacity of mitochondria. It is expected that inacti-

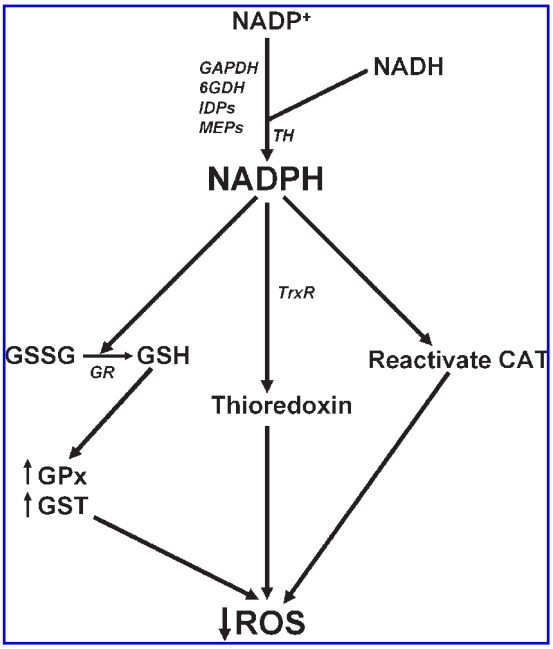

FIG. 3. Effects of NADPH on cellular antioxidation capacity. NADPH can be generated by glucose-6-phosphate dehydrogenase (G6PDH), 6-glyconate phosphate dehydrogenase (6GPDH), $NADP^+$-dependent isocitrate dehydrogenases (IDPs), $NADP^+$-dependent malic enzymes (MEPs), and transhydrogenase (TH). NADPH can increase cellular antioxidation capacity by acting as a substrate for glutathione reductase (GR) to reduce GSSG to GSH that is required for the activities of the antioxidation enzymes glutathione peroxidase (GPx) and glutathione-S-transferases (GST). NADPH can also increase antioxidation capacity by reactivating H_2O_2-inactiated catalase (CAT), and by promoting thioredoxin reductase (TrxR)-mediated regeneration of thioredoxin.

vation of this enzyme under certain pathological conditions may exacerbate oxidative damage to mitochondria, due to the uncoupling between mitochondrial NADPH generation and the NADH-dependent generation of oxidative stress from the electron transport chain.

Seemingly paradoxically, increasing evidence has suggested that NADPH could also significantly contribute to generation of oxidative stress through the activity of NADPH oxidase. NADPH oxidase is an enzyme that catalyzes the generation of superoxide from oxygen and NADPH. Increasing evidence has indicated that NADPH oxidase activity is present not only in phagocytes, but also in various tissues and cell types (26, 288). Seven members of NOX family of NADPH oxidase, including phagocyte NADPH oxidase itself (NOX2/gp91(phox)) and six homologs of the cytochrome subunit of the phagocyte NADPH oxidase (*i.e.*, NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) have been found (26). These enzymes share the capacity to transport electrons across the plasma membrane, leading to superoxide generation, which could be mediated by such factors as small guanosine triphosphatase Rac, protein kinase C, and Ca^{2+} (26, 124, 280, 288). There are significant tissue-dependent differences in the distribution of the various members of the NOX family (26, 101, 123).

Due to the pivotal roles of ROS in redox-based regulation of various biological functions (263), it is not surprising that NADPH oxidase appears to play important roles in not only host defense, but also a large variety of biological processes, including redox signaling (86), regulation of gene expression and cell differentiation, and post-translational modifications of proteins (26, 123). Of particular interest, many studies have indicated key roles of the NADPH oxidase-generated ROS in a variety of diseases, such as brain ischemia (26, 154, 266, 303), diabetic nephropathy (67), and cardiac hypertrophy (211).

It was found that NOX4 is preferentially localized to the nucleus of human umbilical vein endothelial cells, which appears to regulate gene expression by generating ROS in the nucleus (153). A recent study showed that ischemia induced NOX2 expression mainly at the nucleus of cardiomyocytes, which appears to mediate ischemia-induced apoptosis (196). It has also been found that the NADPH oxidase NOX2 is recruited to the early phagosomes of dendritic cells, which causes alkalinization of the phagosomal lumen by generating ROS (251). These studies have indicated that NADPH oxidase is localized not only on plasma membranes, but also in such subcellular organelles as the nucleus. It is expected that future studies regarding the NADPH oxidases that are localized in subcellular organelles would provide novel information about the roles of NADPH oxidase in biological functions and cell death. Figure 4 provides diagrammatic presentation of the pathways by which NAD and NADP affect antioxidation and ROS generation.

It is worthy to note that GSH/GSSG ratios may also affect the overall redox potential of cells due to the effects of GSH/GSSG on NAD/NADP metabolism: Decreased GSH/GSSG ratios may lead to decreased NADPH/$NADP^+$ ratios, due to the glutathione reductase-catalyzed regeneration of GSH from GSSG with consumption of NADPH; and the decreased NADPH/$NADP^+$ ratios may also affect NADH/NAD^+ ratios due to the modulating capacity of NADKs on the equilibrium between NADPH/$NADP^+$ ratios and NADH/NAD^+ ratios.

C. NAD and NADP in calcium homeostasis

Mounting evidence has suggested that NAD^+ can mediate calcium homeostasis through multiple pathways: a) ADP-ribosyl cyclases can generate cyclic ADP-ribose from NAD^+, which is a potent endogenous agonist of ryanodine receptor-mediated calcium channels (108); b) NAD^+ can also modulate calcium metabolism by promoting mono-ADP-ribosylation of $P2X_7$ receptors, which has been shown to increase $P2X_7$ receptor opening (17), thus leading to Ca^{2+} influx (216); c) ADP-ribose, a molecule that can be generated from NAD^+ by NAD glycohydrolases or PARPs/poly(ADP-ribose) glycohydrolase (PARG), can activate TRPM2 receptors leading to Ca^{2+} influx (98, 151); and d) Sir2 family proteins can generate O-acetyl-ADP-ribose that can directly bind to the cytoplasmic domain of the TRPM2 channels and produce TRPM2 channel opening, resulting in Ca^{2+} influx (107).

There is evidence suggesting that NADH can also directly modulate calcium homeostasis: Under hypoxic conditions, NADH can directly increase Ca^{2+} release from inositol 1,4,5-triphosphate (IP_3)-gated Ca^{2+} channels on ER membranes of cerebellar Purkinje cells and nerve growth factor-differentiated PC12 cells (134). It has been further found that the GAPDH that is associated with IP_3-gated calcium channels can locally generate NADH to promote the Ca^{2+} channel opening (228). NADH was also shown to inhibit ryanodine receptors of cardiac muscle, but not skeletal muscle (348, 349), which could be mediated by the NADH oxidase activity in cardiac sarcoplasmic reticulum (53).

$NADP^+$ is the major substrate for generation of NAADP that can mobilize intracellular Ca^{2+} stores (96). It has also been reported that NAADP regulates TRPM2 channels in T lympho-

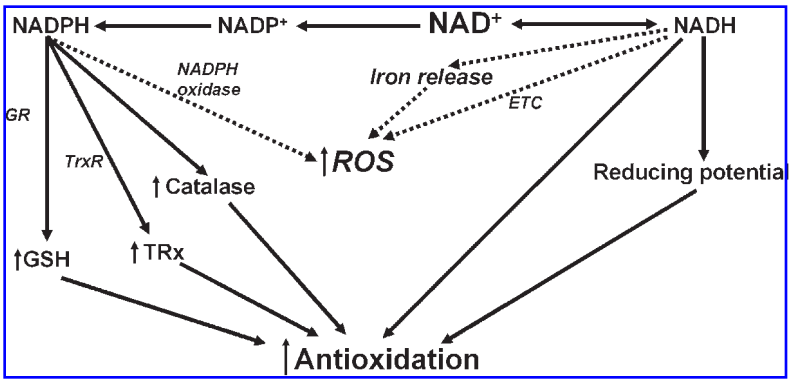

FIG. 4. Pathways by which NAD and NADP affect cellular antioxidation capacity and ROS generation. NADH contributes to cellular reducing potential, and can also increase ROS generation by the electron transport chain (ETC) or by inducing iron release from ferritin. NADPH plays a key role in cellular antioxidation capacity by promoting glutathione reductase (GR)-mediated regeneration of GSH, reactivating catalase, and contributing to thioredoxin reductase (TrxR)-mediated generation of thioredoxin (TRx). NADPH can also be used by NADPH oxidase to generate superoxide.

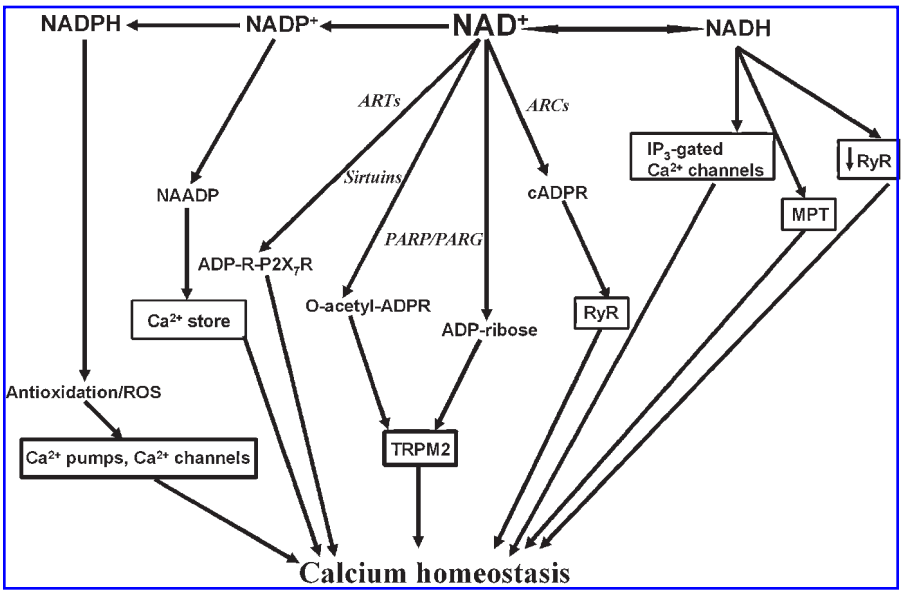

FIG. 5. Pathways by which NAD and NADP can affect calcium homeostasis. ADP-ribosyl cyclases (ARCs), poly(ADP-ribose) polymerases (PARPs)/poly(ADP-ribose) glycohydrolase (PARG), and sirtuins use NAD^+ as a substrate to generate several Ca^{2+}-mobilizing second messengers, including cyclic ADP-ribose (cADPR), ADP-ribose, and O-acetyl-ADP-ribose (O-acetyl-ADPR), which can activate TRPM2 receptors and ryanodine receptors (RyR). NAD^+-dependent mono(ADP-ribosyl)transferases (ARTs) can also affect calcium homeostasis by producing mono-ADP-ribosylation of $P2X_7$ receptors (ADP-R-$P2X_7$R). NADH could modulate calcium homeostasis by affecting IP_3-gated calcium channels, mitochondrial permeability transition (MPT) and RyR. NAADP generated from NADP can also mobilize intracellular NAADP-dependent Ca^{2+} stores. NADPH may affect calcium homeostasis by its major effects on antioxidation and ROS generation, which can affect Ca^{2+} pumps and Ca^{2+} channels.

cytes (25). Increasing evidence has suggested that NAADP is one of the important endogenous factors mobilizing intracellular calcium stores (89, 159). It is of interest to note that $NADP^+$ and NAD^+, which are essentially linked by NADKs, are the precursors for generating NAADP and cyclic ADP-ribose, respectively. Thus, it would be of interest to determine the roles of NADKs in the cyclic ADP-ribose and NAADP signaling pathways. Diagrammatic presentation of the pathways by which NAD and NADP affect calcium homeostasis is shown in Fig. 5.

D. NAD and NADP in energy metabolism and mitochondrial functions

NAD plays key roles in nearly all major aspects of energy metabolism (29, 328). NAD can mediate cytosolic energy metabolism through several pathways: NAD mediates glycolysis by acting as the co-factors for the glycolytic enzyme GAPDH; and NAD also modulates other important energy metabolism-related reactions in cytosol, such as the lactate dehydrogenase-catalyzed lactate—pyruvate conversions. In addition, cytosolic NADH can also affect mitochondrial oxidative phosphorylation due to the NADH shuttling from cytosol to mitochondria.

There are multiple mechanisms by which NAD can mediate mitochondrial energy metabolism: a) NADH is one of the major electron donors for the electron transport chain; b) NAD^+ is the coenzyme for the three rate-limiting enzymes in TCA cycle (193); c) AIF is a NADH oxidase that plays an important role in the mitochondrial complex I activity (202); d) NADH could directly interact with and inhibit voltage-dependent anion channels, that is a component of MPT pores and controls the transport of small molecules across mitochondrial membranes (104); e) recent studies have demonstrated that NAD^+-dependent sirtuins can deacetylate the active lysine residues of acetyl-CoA synthetases, thus activating the enzymes, which could mediate the conversion of free acetate to acetyl-CoA (114, 257, 273). In mammalian cells it appears that SIRT1 can deacetylate and activate acetyl-CoA synthetase 1 in cytosol (114), while SIRT3 can deacetylate and activate acetyl-CoA synthetase 2 in mitochondria (114, 257); and f) the NADH/NAD^+ ratio is one of the modulators of MPT pore opening (352), which can significantly influence mitochondrial memebrane potential. It has been suggested that maintainance of mitochondrial pyridine nucleotides in reduced redox state mediates the capacity of bcl-2 overexpression to block oxidative stress-induced MPT (132).

Studies suggest that under certain conditions the reducing equivalents of cytosolic NADH could be directly transferred to the oxygen in the mitochondria with the generation of electrochemical membrane potential, which is mediated by cytosolic cytochrome c and mitochondrial cytochrome oxidase (156, 189). In the model proposed by the researchers, the high energy electron from NADH is transferred to cytoslic cytochrome c by the NADH-cytochrome b_5 oxido-reductase complex on external mitochondrial membrane; and the cytochrome c transfers the electron to mitochondrial complex IV (cytochrome oxidase) at the respiratory contact sites. Subsequently, the molecular oxygen is reduced with generation of electrochemical membrane potential. This process may occur at the early stage of apoptosis when significant amount of cytochrome c is released into cytosol, which may lead to additional energy generation for apoptosis. Moreover, this process may also occur under physiological conditions, since there may be constitutive release of cytochrome c from mitochondria to the cytosol (155). This process may not only contribute to removal of excessive cytosolic NADH, but also promote cell survival when the first three respiratory complexes are impaired.

Because transhydrogenase catalyzes the formation of ($NADPH + NAD^+$) from ($NADP^+ + NADH$) by utilizing the mitochondrial transmembrane electrochemical H^+ gradient

(127, 246), mitochondrial NADPH generation could be linked with oxidative phosphorylation via transhydrogenase. It is possible that reversal of the transhydrogenase-catalyzed reactions may utilize NADPH to produce an increase in the proton gradient across the mitochondrial membranes. However, the relatively low free energy available for the reversal reaction suggests that the contribution of the reaction to the proton gradient is transient and insignificant (246). It was also hypothesized that IDPm may operate in reverse mode [*i.e.*, in the mode of generating isocitrate and $NADP^+$ from α-ketoglutarate and NADPH, which may contribute to fine regulation of TCA cycle activity (253)]. This hypothesis has been supported by the studies demonstrating the reversal of the IDPm in liver (80) and heart (61), but not by a study applying a specific inhibitor of IDPm (253). Future studies are warranted to elucidate the relationships among transhydrogenase, mitochondrial NADPH generation, TCA cycle activity and mitochondrial oxidative phosphorylation. The potential significance of the studies has been highlighted by the findings suggesting that transhydrogenase deficiency could mediate the impairments of glucose-induced insulin release in C57BL/6J mice (284).

A recent study has indicated that IDPc mediates glucose-induced increases in pyruvate cycling and insulin secretion in primary rat islets (242), suggesting significant effects of IDPc on pyruvate-related energy metabolism. The finding that disruption of the NADH kinase POS5 in yeast dramatically increased the mitochondrial mutation rate (264) suggests a critical role of NADPH in protecting the integrity of mitochondrial DNA.

E. Effects of NAD and NADP on gene expression

NAD may affect gene expression through several pathways. NADH mediates the activity of the corepressor carboxyl-terminal binding protein—-a transcriptional factor important for cell cycle regulation, development, and transformation (342); and NADH also modulates the activities of Clock:BMAL1 and NPAS2:BMAL1 that are heterodimeric transcription factors controlling circadian clock-associated gene expression (245).

A number of studies have indicated important roles of PARP-1 in gene expression. For example, it has been found that both DNA topoisomerase IIa-dependent, transient double-strand DNA breakage and subsequent PARP-1 activation is required for signal-dependent activation of gene expression by nuclear receptors and multiple other DNA-binding transcriptional factors (131, 175). Increasing evidence has suggested that PARP-1 could mediate gene expression through a number of mechanisms: first, PARP-1 can profoundly affect multiple transcriptional factors, including AP-1, AP-2, NFκB, p53, cAMP-responsive element-binding protein, Sry, and HIF1 (13, 111, 141, 170, 171, 186); second, PARP-1 binding on necleosomes can reversibly modulate chromatin structure in a NAD^+-dependent manner: PARP-1 binding on necleosomes can promote the formation of transcriptionally repressed, compact chromatin structure, while PARP-1 auto-poly(ADP-ribosyl)ation in the presence of NAD^+ produces dissociation of PARP-1 from chromatin, leading to the formation of transcriptionally active, decondensed chromatin structure (140, 141); third, PARP-1-produced poly(ADP-ribosyl)ation of histone H1 could also produce chromatin de-condensation (74, 232); fourth, PARP-1 can NAD^+-dependently silence RNA polymerase II-dependent transcription (197, 217, 218); fifth, PARP-1 itself can directly affect gene expression by binding the promoters of certain genes such as *iNOS* and *CXC ligand1* (8, 334); sixth, PARP-1 could affect gene expression by modulating DNA methylation (236, 335–337); and seventh, PARP-1-dependent NAD^+ consumption could affect gene expression by influencing the NAD^+-dependent sirtuins that can modulate the activities of multiple transcriptional factors (200). The complexity of the mechanisms underlying the effects of PARP-1 on gene expression has been further indicated by the findings that PARP-1 can affect transcriptional factors through multiple mechanisms, such as direct protein—protein interactions (51, 118), modulations of the expression of transcriptional factors (111, 187), and poly(ADP-ribosyl)ation of transcriptional factors (170, 217).

Cumulative evidence has indicated increasingly extensive and important roles of PARP-1-mediated gene expression in various biological and pathological processes, such as inflammation and carcinogenesis (8, 111, 118, 141, 187). For example, a recent study has suggested that PARP-1 mediates nitric oxide-dependent negative feedback regulation of the expression of iNOS gene (334): PARP-1 appears to be a novel *trans*-activator of the iNOS promoter; and NO can inhibit iNOS expression by nitrosylating PARP-1. Figure 6 provides diagrammatic presentation of the pathways by which PARP-1 affects gene expression.

A number of studies have suggested that sirtuins can also mediate gene expression through multiple pathways: First, both yeast Sir2 and mammalian SIRT1 can produce histone hypoacetylation and gene repression by promoting the formation of heterochromatin—-a tightly packed form of chromatin (200); second, SIRT1-produced deacetylation of multiple transcriptional factors, including p53 (179), FOXO transcriptional factors (209, 214), NFκB (317), p73 (75), and Tat (222), can mediate the transcriptional activities of these factors; third, SIRT7 is an activator of RNA polymerase I-mediated transcription (92); and fourth, SIRT1 has been found to inhibit RNA polymerase I-mediated transcription by deacylating TAF_I68 (212). A rapidly growing body of evidence has indicated that the effects of sirtuins on gene expression can significantly affect various biological processes, including aging, cell death, carcinogenesis, and stress resistence (200).

Since ROS can mediate gene expression by modulating intracellular redox state (262), it is conceivable that NADPH could affect gene expression by its profound effects on both cellular antioxidation and ROS generation. An interesting study reported that NOX4 is localized in the nucleus of human umbilical vein endothelial cells, which appears to regulate gene expression by generating superoxide (153). This finding provides a novel mechanism by which NADPH can affect gene expression: The NADPH oxidase in the cell nucleus may modulate gene expression by initiating redox signaling. It has also been found that the endothelial NADPH oxidase can be activated by angiogenic factors such as VEGF (289). The NADPH oxidase-generated ROS can activate various redox signaling pathways, resulting in angiogenesis-related gene expression, which may mediate postnatal angiogenesis *in vivo* (289).

F. NAD and NADP in immunological functions

It has been found that CD38-produced cyclic ADP-ribose plays a critical role in inflammation and innate immune re-

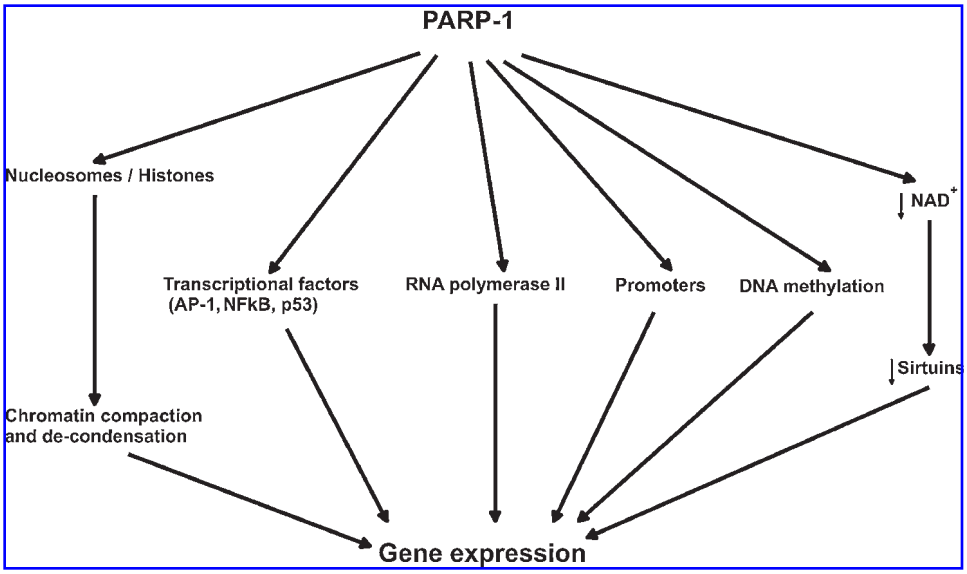

FIG. 6. Pathways by which PARP-1 affects gene expression. PARP-1 can affect gene expression by influencing a number of transcriptional factors, or by direct binding to the promoters of certain genes. PARP-1 can also affect gene expression by producing chromatin de-condensation or compaction. The enzyme can further influence gene expression by silencing RNA polymerase II-dependent transcription, or by modulating DNA methylation. Moreover, PARP-1-dependent NAD^+ consumption could affect gene expression by influencing the NAD^+-dependent sirtuins that can modulate the activities of several transcriptional factors.

sponses by mediating neutrophil chemotaxis to bacteria chemoattractants (225, 226). A recent study also indicated cyclic ADP-ribose as a second messenger mediating the lipopolysaccharide-induced proliferation of human peripheral blood mononuclear cells (43). Many studies have further demonstrated important roles of PARP-1 in inflammatory responses, due to its major effects on NFκB (296). It has also been reported that NADH can dose-dependently induce IL-6 release from human peripheral blood leukocytes (213). Recent studies have suggested that the ecto-ARTs can produce mono-ADP-ribosylation of $P2X_7$ receptors by consuming extracellular NAD^+, leading to opening of $P2X_7$ receptors. The $P2X_7$ receptor opening can produce death of Treg cells—-the cells that can inhibit the activation of other types of T cells (17).

PBEF was isolated as a presumptive cytokine that can enhance the maturation of B-cell precursors in the presence of stem cell factor and IL-7 (248). It has been demonstrated that PBEF is the same protein as Nampt—-a key enzyme in the mammalian salvage pathway for NAD^+ synthesis (241), suggesting that a key NAD^+-synthesizing enzyme can produce cytokine-like effects when acting extracellularly. It is of interest to further determine if the cytokine-like activity of PBEF might be accounted for by the nicotinamide mononucleotide-synthesizing capacity of Nampt (238).

The NADPH oxidase in phagocytes plays critical roles in innate immunity by generating microbicidal ROS (167). Increasing evidence has suggested that several other members of the Nox family of oxidases are also involved in host defense (167). Important roles of NADPH oxidase in the inflammation under several pathological conditions have been indicated by the findings that inhibition of NADPH oxidase can block inflammatory processess (157, 239). The interactions between NAPDH oxidase and inducible NOS (iNOS) play key roles in inflammation-induced cytotoxicity: iNOS is a Ca^{2+}-independent and transcriptionally regulated isoform of NOS. Activated iNOS can generate large toxic amount of NO in a sustained manner (109). The NADPH oxidase-generated superoxide can rapidly interact with the iNOS-generated NO to produce peroxynitrite that mediates the toxicity of NO by producing DNA damage, inhibiting mitochondrial respiration and activating PARPs (110, 125). Multiple studies have indicated that NADPH oxidase and iNOS can produce synergistic effects in inducing death of several types of cells (38, 169).

A recent study has demonstrated that the NADPH oxidase NOX2 is recruited to the early phagosomes of dendritic cells, which produces alkalinization of the phagosomal lumen by generating low levels of ROS (251). Through this process NOX2 confers dendritic cells the ability to function as specialized phagocytes for processing antigens rather than killing pathogens (251). It has also been found that the absence of one of the components of NADPH oxidase is causative to chronic granulomatous disease—-an inherited immune deficiency disease (21). Diagrammatic presentation of the mechanisms by which NAD and NADP affect immunological functions is shown in Fig. 7.

G. NAD and NADP in vascular activity

Angiotensin II plays key roles in regulating vascular activity, which produces multiple vascular effects through NADPH oxidase-derived ROS (120). Recent studies have suggested that NADH/NAD^+ ratios and NADPH oxidase play critical roles in two of the important models for the oxygen sensing in hypoxic pulmonary vasoconstriction (304). There are also studies suggesting that NADPH mediates the differential responses of pulmonary artery and coronary artery to hypoxia (307): The hy-

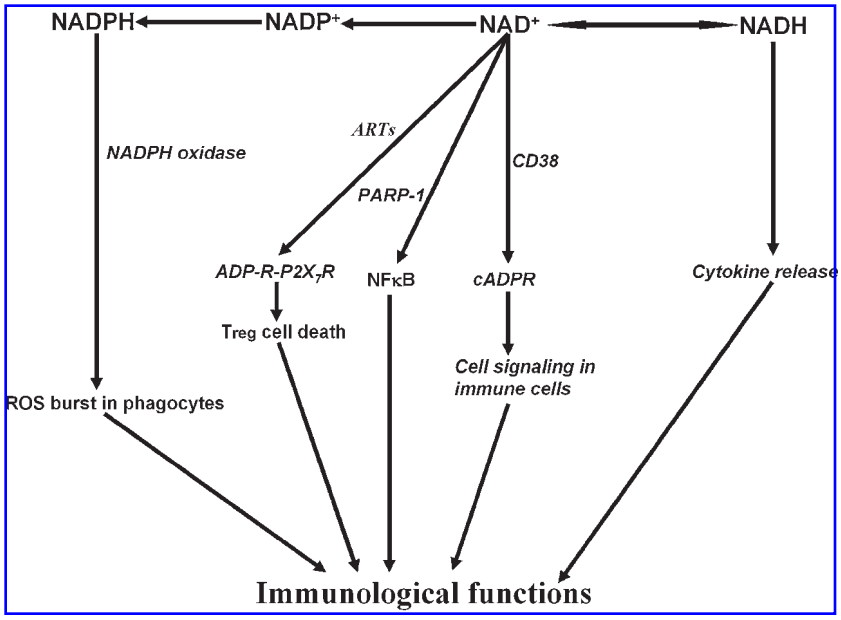

FIG. 7. Pathways by which NAD and NADP could affect immunological functions. NAD^+-dependent poly (ADP-ribose) polymerase-1 (PARP-1) plays a significant role in immunological functions due to its close relationships with NFκB. NAD^+-dependent generation of cyclic ADP-ribose (cADPR) by CD38 can also mediate the cell signaling in immune cells. Ecto-mono-ADP-ribosyl transferases (ARTs) can induce Treg cell death by using NAD^+ to produce mono-ADP-ribosylation of $P2X_7$ receptors (ADP-R-$P2X_7$R). NADPH oxidase is responsible for the ROS burst in phagocytes. NADH may also affect the immune system by inducing cytokine release from peripheral leukocytes.

poxia-induced pulmonary artery contraction could be mediated by a mechanism associated with a decrease in the basal levels of NADPH oxidase-generated peroxide; while hypoxia-induced coronary artery dilation could be mediated by a decrease in the basal levels of NADPH.

Mounting evidence has suggested that NAD and NADP metabolism also plays significant roles in vascular damage under various pathological conditions. For example, a PARP inhibitor was found to decrease cardiopulmonary bypass-induced mesenteric vascular dysfunction by improving hemodynamics, decreasing neutrophil adhesion, and restoring nitric oxide production (12); and the C242T CYBA polymorphism of NADPH oxidase was found to be associated with essential hypertension (205).

H. NAD and NADP in carcinogenesis and cancer treatment

Selective inhibition of NAD^+ synthesis has been shown to induce apoptosis of tumor cells (117). Because PARP-1 plays critical roles in regulating DNA repair, genomic stability, and cell cycle progression (296), many studies have been conducted to determine the roles of PARP-1 in carcinogenesis (103). It has been found that PARP inhibitors can restore the sensitivity of resistant tumors to topoisomerase I inhibitors or methylating agents (103). Because telomerases and telomere play significant roles in carcinogenesis (112), NAD^+-dependent tankyrases—-the enzymes that regulate telomerase activity (259, 260)—-may affect carcinogenesis by influencing telomere.

Increasing evidence has indicated that sirtuins may be involved in carcinogenesis and cancer treatment. A recent study suggests that cancer cells, but not noncancerous cells, may require SIRT1 for survival (93): decreased levels of SIRT1 by RNA silencing selectively induced apoptosis and/or growth arrest in human epithelial cells, while the RNA silencing did not affect normal human epithelial cells. Another recent study also indicated that SIRT1 inhibition by tumor suppressor HIC1 in human MCF-7 cancer cells mediates DNA damage-induced apoptosis by both producing p53 acetylation and suppressing the antiapoptotic factor bcl-2 (52).

It has been indicated that the thioredoxin system, that consists of NADPH, thioredoxin, and thioredoxin reductase, plays an important role in carcinogenesis and invasive phenotype of cancer (16). Because NAD and NADP can profoundly affect cell death and various biological processes including gene expression and signal transduction, future studies may further elucidate important roles of NAD and NADP in carcinogenesis and cancer treatment.

I. NAD and NADP in aging

Accumulating evidence has suggested that NAD could be a crucial factor in the aging process by regulating sirtuins, PARP-1, tankyrases, and oxidative stress. It has been indicated that Sir2 is a key enzyme mediating the life span of yeast and C-*elegans* (35). The study of Lin *et al*. suggested that calorie restriction could modulate Sir2 activity, thus extending the lifespan of yeast by decreasing NADH levels (173). However, Anderson *et al*. (10) have suggested an intriguing alternative mechanism by which calorie restriction and Sir2 mediate the lifespan of yeast: *PNC1* (pyrazinamidase/nicotinamidase 1) encodes an enzyme that converts nicotinamide to nicotinic acid, which can lead to Sir2 activation by depleting the Sir2 inhibitor nicotinamide. It was shown that *PNC1* is a novel longevity gene that is both necessary and sufficient for the lifespan extension by calorie restriction, which appears to be mediated by *PNC1*-dependent activation of Sir2. It has also been found that deficiency of SIRT6, a human homolog of Sir2, produces aging-like phenotype and genomic instability in mice (208). A recent study has suggested that the gene encoding the key NAD^+-synthesizing enzyme Nampt is a novel longevity gene, which can significantly extend the replicative lifespan of human cells by increasing SIRT1 activity, leading to inhibition of age-dependent p53 expression and increased p53 degradation (293).

Telomere and telomerases have been indicated as mediators of cellular aging (36). NAD^+ may also affect the aging processes through NAD^+-dependent tankyrases, because tankyrases mediate telomerase activity (270). It has also been reported that PARP activities of mononuclear blood cells are strongly correlated with the longevity of thirteen mammalian species, which may be accounted for by the DNA repair function of PARP-1 (45, 106). A role of PARP-1 in aging has been further suggested by the finding that PARP-1 inhibits the catalytic activities of the protein of Werner syndrome, a human disease of premature aging (298, 299). Moreover, due to the significant roles of mitochondrial impairments in the aging processes (47, 265), it is conceivable that NAD may further influence aging by its profound effects on mitochondrial activities.

Due to the important role of oxidative stress in aging (113, 272, 321), it is conceivable that NADPH may play significant roles in aging due to the effects of NADPH on both antioxidation and ROS generation. It has been reported that IDPc regulates replicative senescence (139). There are also increased levels of glycated IDP in IMR-90 cells and rat kidney during normal aging (138). The glycation-mediated damage to IDP may increase oxidative stress and contribute to aging-related alterations (138).

Seemingly paradoxically, the patients of glucose-6-phosphate dehydrogenase deficiency could have longer lifespan (256), which might be accounted for by the decreased NAPDH oxidase-dependent ROS generation resulting from decreased NADPH generation. Future studies are needed to further elucidate the roles of NADPH in both chronological and replicative senescence, and to search for the potential strategies to increase longevity by manipulating NADPH metabolism. Figure 8 provides diagrammatic presentation of the pathways by which NAD and NADP could affect the aging process.

IV. NAD AND NADP IN CELL DEATH

A. PARP-1 and NAD in cell death

Oxidative stress has been indicated as a key mediator of ischemic brain damage (49, 50), Parkinson's disease (PD) (24, 308, 321), Alzheimer's disease (AD) (137, 199, 204, 319, 346) and many other diseases (113). Excessive PARP-1 activation appears to mediate cell death induced by oxidative stress under many conditions (87, 341). There has also been compelling evidence indicating that PARP-1 activation plays a key role in ischemic brain injury: both pharmacological and genetic inhibition of PARP-1 can profoundly decrease infarct formation in animal models of brain ischemia (87, 88), and increased PARP activities have been found in animal models of cerebral ischemia (88, 283) and in human brains after cardiac arrest (176).

Evidence suggests that PARP-1 may also mediate the neuronal injury in PD and AD: PARP-1 activation has been shown to mediate the neuronal death induced by MPTP, a model toxin for PD, both *in vitro* (66, 185) and *in vivo* (126, 184, 234). Increased nuclear PARP activity has also been found in the brains of AD patients (48, 126). A recent study reported that PARP-1 activation mediates the β-amyloid-induced neuronal death, which is an *in vitro* model of AD (91, 119). Cumulative evidence has further indicated that PARP-1 activation is an important pathological factor in traumatic brain injury (158), hy-

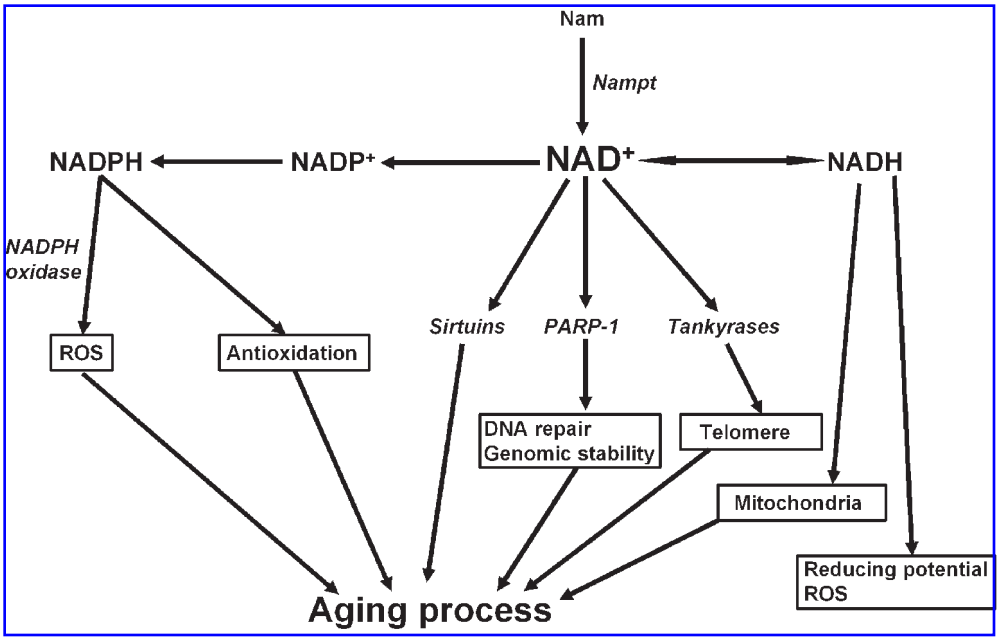

FIG. 8. Potential pathways by which NAD and NADP can affect the aging process. NAD^+ could affect aging process through the NAD^+-dependent enzymes sirtuins, poly(ADP-ribose) polymerase-1 (PARP-1), and tankyrases. NADPH may affect aging by its major effects on cellular antioxidation capacity and ROS generation. NADH may affect aging by its effects on mitochondrial activities, ROS generation, and cellular reducing potential. Nicotinamide phosphoribosyltransferase (Nampt) may slow aging by promoting NAD^+ generation from nicotinamide.

poglycemic brain injury (277), diabetes (229), and shock and inflammation (279, 296). PARP-1 has become a valuable therapeutic target for multiple diseases (230, 279, 296).

Our studies have provided direct evidence demonstrating that NAD^+ depletion is a key step mediating PARP-1-induced cell death (5, 326). Several studies have also indicated that MPT (5) and apoptosis-inducing factor (AIF) translocation (332) link NAD^+ depletion to cell death. Our studies have further suggested that NAD^+ depletion could induce mitochondrial impairments by producing glycolytic inhibition (326), which would reduce pyruvate supply to TCA cycle (325). This suggestion has been supported by the findings of us and other researchers, which show that pyruvate treatment after PARP-1 activation can profoundly decrease PARP-1-induced impairments of energy metabolism and cell death in cell cultures, brain slices, and animal models of diseases (278, 325, 338).

Several recent studies have suggested novel mechanisms underlying PARP-1 cytotoxicity. It was reported that SIRT1 is a key link between NAD^+ depletion and cell death (231). A latest study has further indicated interactions between PARP-1 and SIRT1: SIRT1 deficiency produces significant increases in PARP-1 activity, leading to AIF-mediated cell death (149). Several studies have also indicated that the ADP-ribose generated by PARP-1/PARG can produce TRPM2 opening, leading to increased intracellular calcium concentrations and cell death (90, 91, 314). However, two recent studies suggested that PAR, instead ADP-ribose monomers, mediates PARP-1-induced AIF translocation and cell death (11, 333).

Increasing evidence has indicated multiple protein kinases, including extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs), could contribute to PARP-1-mediated cell death (59, 136, 310, 343). Xu *et al*. reported that JNK, particularly JNK1, is required for PARP-1-induced mitochondrial impairments and subsequent cell death (6, 310). A recent study has further indicated that JNK1 could activate PARP-1 by directly phosphorylating the enzyme (343). It has also been indicated that ERK1/2 induces PARP-1 activation by directly phosphorylating the enzyme (136). The latest study by Cohen—Armon *et al*. reported that phosphorylated ERK2 can induce DNA damage-independent PARP-1 activation by directly interacting with the enzyme (59). Figure 9 provides diagrammatic presentation of the mechanisms for PARP-1-mediated cell death.

There are several possibilities underlying the seeming diversity of the mechanisms underlying PARP-1 toxicity. First, while the reported mechanisms for PARP-1 toxicity seem diverse, future studies may expose a common pathway linking these mechanisms. Second, multiple factors may form a detrimental network that leads to PARP-1-initited cell death, thus inhibition of any one of the major components of the network may block PARP-1 toxicity. Third, there may be differential PARP-1-mediated cell death cascades that are selectively activated depending on cell types and intensities of insults. Fourth, we have demonstrated that inclusion of such nutritional factors as pyruvate or α-ketoglutarate can profoundly affect PARP-1 toxicity (325, 338). Thus, the differential nutritional compositions of the experimental media in different studies may contribute to the variability of the experimental outcomes (327).

Recent studies have suggested that other PARPs could also mediate cell injury. It has been found that overexpression of tankyrase 2 can produce rapid cell death (182). Tankyrase 1 has also been shown to interact with both Mcl-1L (myeloid cell leukemia-1 long) and Mcl-1S (myeloid cell leukemia-1 short) proteins, which are anti-apoptotic and pro-apoptotic bcl-2 family proteins, respectively (22). Tankyrase 1 overexpression can antagonize both Mcl-1L-mediated cell survival and Mcl-1S-induced cell death (22). Intriguingly, PARP-2 was detrimental in focal brain ischemia, while it was beneficial in a model of global ischemia (181). A recent study has further suggested that PARP-2 mediates the survival of $CD4^+CD8^+$ double-positive T cells during thymopoiesis (316). It is warranted to further determine the interactions among PARPs under both physiological and pathological

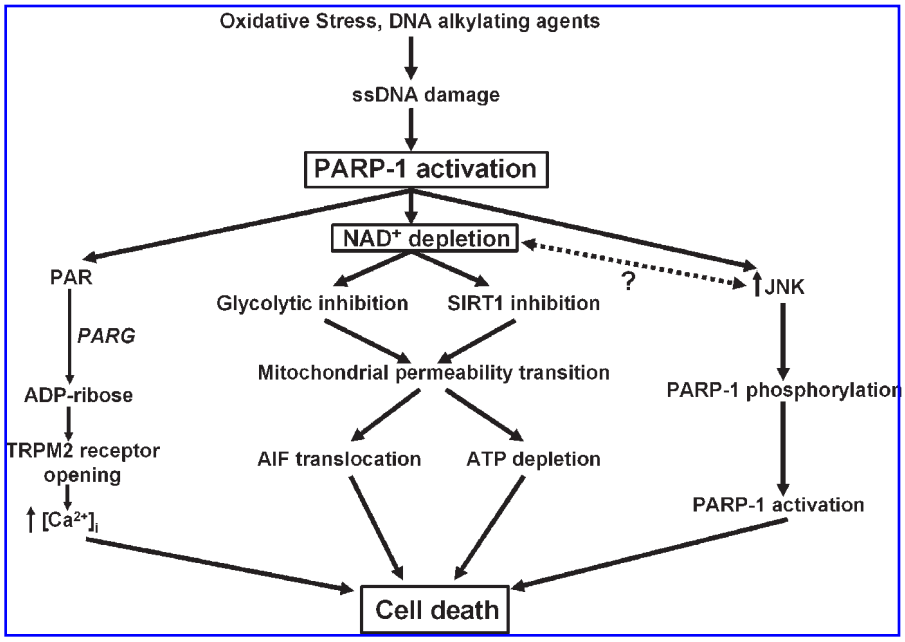

FIG. 9. Mechanisms of poly(ADP-ribose) polymerase-1 (PARP-1) cytotoxicity. Excessive PARP-1 activation triggered by single-strand DNA (ssDNA) can induce NAD^+ depletion, leading to glycolytic inhibition and SIRT1 inhibition, which could lead to mitochondrial permeability transition, resulting in apoptosis-inducing factor (AIF) translocation, ATP depletion, and subsequent cell death. ADP-ribose can be generated by poly(ADP-ribose) glycohydrolase (PARG) from poly(ADP-ribose) (PAR), which can induce TRPM2 receptor opening, leading to increased intracellular calcium concentration and cell death. PARP-1 activation can also activate c-Jun N-terminal kinase (JNK) that can produce mitochondrial impairments and cell death.

conditions, and to elucidate the relationships between NAD and PARPs in cell survival.

B. PARG in cell death

PARG mediates PAR catabolism in cells (77), which degrades PAR into ADP-ribose (243). PARG is an endo-exoglycosidase that exists in low abundance in cells, which appears to play significant roles in regulation of gene expression, cell cycle, and cell differentiation (84, 219, 287). A recent study reported that in *Drosophila* PARG mediates Sir2-dependent silencing and chromatin structure (285).

Cumulative evidence has implicated that PARG inhibition may prevent PARP-1-mediated cell death by several mechanisms (323, 324): First, PARP-1 can auto-poly(ADP-ribosyl)ate itself, leading to PARP-1 auto-inhibition (74). Therefore, PARG inhibition prevents removal of PAR from PARP-1, thus indirectly inhibiting PARP-1 activation. Second, PARG inhibition could block the rapid PAR turnover, thus preventing NAD^+ depletion. Third, Ca^{2+}-Mg^{2+}-dependent endonucleases (CME) mediate DNA fragmentation in certain apoptotic cascades (39). It has been found that CME can be poly(ADP-ribosy)lated, thus being inhibited under physiological conditions (39, 311). Thus, PARG inhibition can prevent removal of PAR from CME, leading to sustained CME inhibition. Fourth, several studies have suggested that PARP-1/PARG activities can generate ADP-ribose by hydrolyzing PAR, leading to activation of TRPM2 receptors and consequent cell death (90, 91, 314). Therefore, PARG inhibition could also decrease cell death by blocking ADP-ribose generation from PAR. Figure 10 provides diagrammatic presentation of the potential mechanisms underlying the protective effects of PARG inhibition.

All of the *in vitro* and *in vivo* studies using various structurally different PARG inhibitors, including GPI 16552 [*N*-bis-(3-phenylpropyl)9-oxo-fluorene-2,7-diamide] (178), GPI 18214 (100), gallotannin, and nobotanin B (23, 121, 142, 323, 324), have supported the hypothesis that PARG may be a new target for decreasing oxidative cell death (71, 328): The PARG inhibitor gallotannin and nobotanin B can decrease cell death induced by various PARP activators *in vitro* (23, 121, 142, 323, 324); our latest study has shown that intranasal gallotannin administration can inhibit PARG and profoundly decrease ischemic brain injury and AIF translocation *in vivo* (305); the PARG inhibitor GPI 16552 can also markedly reduce ischemic brain injury (178) and spinal cord injury (72); and it was reported that the PARG inhibitor GPI 18214 is beneficial for septic shock-like syndrome (100) and inflammatory bowel disease (73).

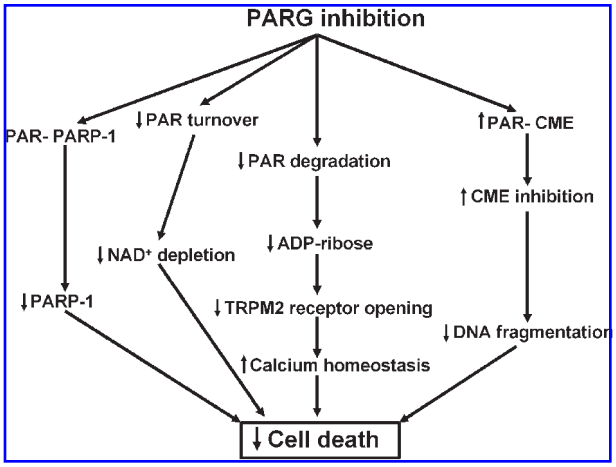

FIG. 10. Potential mechanisms by which PARG inhibition may decrease PARP-1-mediated cell death. PARG inhibition may be cytoprotective by prolonging auto-poly(ADP-ribosylation)-produced PARP-1 inhibition, by slowing poly(ADP-ribose) (PAR) turnover, by blocking ADP-ribose generation, or by maintaining poly(ADP-ribosylation)-produced inhibition of Ca^{2+}-Mg^{2+}-dependent endonucleases (CME).

In the cell culture studies in which PARG activity was decreased by PARG antisense oligonucleotides or PARG small interference RNA, it was also found that PARG inhibition is cytoprotective: Decreases of PARG levels by PARG antisense oligonucleotides (46) or RNA silencing (37) led to reduced PARP-1-mediated cell death. Of particular interest, a recent study using a cell culture model that has increased PARG activity has further suggested that PARG inhibition could be protective: The increased PARG activity was found to accelerate NAD^+ depletion and increase cell death (97).

However, the studies using PARG knockout mice have generated variable results: the mice that have genetic deletion of the 110 kDa PARG isoform have significantly decreased spinal cord injury (72) and ischemic damage of intestine (70) and kidney (227) compared with wild-type mice. However, there are also studies suggesting that genetic deletion of PARG leads to detrimental effects (11, 68). Since increasing evidence has indicated that PARG significantly affects gene expression and other biological properties (84, 219, 287) and that the *PARG* gene is closely associated with *inner mitochondrial membrane translocase 23* (*TIM23*) gene (198), attention should be paid to the potential genetic alterations in the PARG knockout mice. Indeed, it has been reported that in PARG knockout mice, there are marked changes in the gene expression of cyclooxygenase 2 and heat shock protein 70 (68)—-two important proteins in cellular functions and cell survival.

Based on the considerations of the available observations about PARG in cell death, it is tempting to conclude that partial PARG inhibition can be cytoprotective, whereas complete PARG inhibition can be detrimental. Future studies using conditional PARG knockout mice or more selective PARG inhibitors are warranted to further elucidate the role of PARG in cell death.

C. NAD in apoptosis

In contrast to the extensiveness of the studies on PARP-1, a mediator of programmed necrosis (350, 351), there have been only insufficient number of studies on the roles of NAD in apoptosis. Several studies have suggested that NAD may be involved in apoptosis: it was reported that selective inhibitors of NAD^+ synthesis can induce apoptosis (117), and that NADH/NADPH depletion is an early event in apoptosis (99). NAD may affect apoptosis through several potential mechanisms: First, NAD mediates cellular energy metabolism that is a critical factor determining cell death modes; second, the NADH/NAD^+ ratio is a major index of cellular reducing power that affects MPT—-a mediator of apoptosis under many conditions (352); third, NAD^+ levels mediate the activity of caspase-dependent en-

donuclease DFF40–-an executioner of DNA fragmentation in certain apoptotic cascades (128); and fourth, NAD^+-dependent sirtuins may mediate apoptosis (317). Future studies on this topic are critical for our comprehensive understanding about the roles of NAD in cell death.

D. NAD in axonal degeneration

Axonal degeneration is one of the major pathological changes in many neurodegenerative diseases (60). The Wallerian degeneration slow [Wld(S)] mouse model has been valuable for investigating the mechanisms underlying axonal degeneration (60). The mutation of Wld(S) mice leads to overexpression of a chimeric protein—-Wld(S) protein consisting of the key NAD^+-synthesizing enzyme NMNAT-1 and the ubiquitin assembly protein Ufd2a, which can lead to delay of injury-induced axonal degeneration. Recent studies have suggested that the NMNAT-1 in the Wld(S) protein could mediate the protective effects of the Wallerian mutation (14, 302): one study suggested that the increased NMNAT-1 expression produces its effects by affecting SIRT1, a member of sirtuins (14). However, another study reported that NMNAT-1 may affect axonal degeneration by preventing NAD^+ loss in degenerating axons (302). A later study has further suggested that NMNAT-1 activity is required for the protective effects of Wld(S) protein (129). However, there are also studies suggesting that NMNAT-1 itself may be insufficient to account for the protective effects of the Wld(S) protein (62, 340).

E. AIF and GAPDH in cell death

In addition to PARP-1, the other two NAD-dependent proteins—-AIF (202) and GAPDH (58, 115)—-are also important mediators of cell death. GAPDH has been established as a mediator of apoptosis under many conditions: GAPDH binds Siah which is then translocated into nucleus to mediate apoptosis (58, 115, 116, 252).

AIF is a NADH oxidase, which appears to be both an important pro-death factor and an important pro-survival factor (76, 174, 202). Translocation of AIF from mitochondria to the nucleus has been indicated as mediators of caspase-independent apoptosis (202) and PARP-1 cytotoxicity (332). However, AIF also plays a significant role in mitochondrial complex I activity (290). Genetic deletion of AIF has been shown to produce skeleton muscle atrophy, dilated cardiomyopathy, and neurodegeneration (202). There has been evidence indicating that prevention of NAD^+ depletion can block PARP-1-mediated nuclear translocation of AIF (5). This finding, together with the fact that AIF is a NADH oxidase, suggests that NAD could be an important regulator of AIF. Our latest study has shown that aurintricarboxylic acid—-a CME inhibitor—-can nearly abolish DNA alkylating agent-induced nuclear condensation in astrocytes, despite nuclear translocation of AIF (unpublished finding). This result suggests that, at least under certain conditions, nuclear AIF translocation itself may be insufficient to induce chromatin condensation.

F. NADP in cell death

There are studies suggesting the protective roles of IDPm in defending against cell apoptosis induced by various insults: IDPm is involved in cell defense against cadmium-induced apoptosis (139); administration with oxalomalate—-a competitive inhibitor of IDPm—-leads to increased ionizing radiation-induced apoptosis in mice (161); and modulation of IDPm activity in HEK293 cells also significantly affects high glucose-induced apoptosis (267).

Of particular interest, a large number of studies have indicated that NADPH oxidase plays a key role in cell death under both *in vitro* and *in vivo* conditions (2). For example, it was reported that the NADPH oxidase activation in astrocytes mediates β-amyloid-induced neuronal death (1); NADPH oxidase also plays a key role in the ROS generation in the neurons that are exposed to oxygen—glucose deprivation—-an *in vitro* model for brain ischemia (3); and genetic or pharmacological inhibition of NADPH oxidase is protective against ischemic brain injury (300). A recent study also reported that ischemia induced NOX2 expression mainly at the nucleus of cardiomyocytes, which appears to mediate ischemia-induced apoptosis (196). Due to the critical roles of oxidative stress in cell death (247), it is expected that there would be an increasing number of studies indicating significant roles of NADPH in cell death.

V. THERAPEUTIC POTENTIAL OF NAD AND NADP

A. Therapeutic potential of NAD^+ precursors

A number of *in vitro* studies have shown that nicotinamide can produce cytoprotective effects against various insults, including oxidative stress and oxygen—glucose deprivation (57, 168). Nicotinamide administration has also been shown to decrease tissue injury in several animal models of diseases, including cerebral ischemia (19, 20, 203, 312), spinal cord injury (40), PD (210), and multiple sclerosis (133). Nicotinamide could produce cytoprotective effects by multiple mechanisms, including inhibition of PARP-1 (145, 296), restoration of NAD^+ levels (145, 312), activation of Akt1 (56), and blockage of mitochondrial permeability transition and mitochondrial depolarization (55, 168). However, since it is also an inhibitor of sirtuins (18), nicotinamide may produce detrimental effects on cell survival and longevity.

A recent study reported that nicotinamide riboside—-a newly discovered NAD^+ precursor in eukaryotes—-can promote Sir2-dependent gene silencing and markedly extend the replicative lifespan of yeast without calorie restriction (28). It was further found that the beneficial effects of nicotinamide riboside are mediated by the capacity of nicotinamide riboside to increase NAD^+ synthesis (28). This study suggests a novel approach to increase NAD^+ synthesis and extend life span of cells.

B. Therapeutic potential of NAD^+

The recent studies by our research group have provided the first evidence indicating that treatment with NAD^+ can abolish PARP-1-induced astrocyte death (5, 326, 327, 345). It was also shown that NAD^+ treatment can decrease PARP-1-induced myocyte death (231). These results raise the possibility that NAD^+ may be used *in vivo* to decrease PARP-1-mediated tis-

sue injury. This possibility has been further enhanced by the observations that NAD^+ levels are significantly decreased by a PARP-mediated mechanism in the brains that underwent ischemia/reperfusion (77).

Based on this information, we have used a rat model of transient focal ischemia to test our hypothesis that administration with NAD^+ can reduce ischemic brain damage (277): we found that intranasal delivery of NAD^+ at 2 h after ischemic onset decreased infarct formation by up to 87% and significantly attenuated ischemia/reperfusion-induced neurological deficits (Fig. 11). In contrast, intranasal delivery of nicotinamide at the same dose did not reduce ischemic brain damage. These results provide the first *in vivo* evidence that NAD^+ metabolism is a new target for treating cerebral ischemia, and that NAD^+ administration may be a novel strategy for decreasing ischemic brain injury.

There is evidence implicating that NAD^+ administration may decrease brain injury in not only cerebral ischemia, but also multiple other diseases (331): pathological roles of PARP-1 have also been implicated in many diseases such as diabetes, PD, and AD (329). Since NAD^+ treatment provides the most profound protection against PARP-1-mediated cell injury in cell culture studies, NAD^+ administration might reduce the cell injury in these diseases at least partially by decreasing PARP-1 toxicity. Our latest study has suggested that intranasal NAD^+ administration might also decrease traumatic brain injury (330), which supports our proposal that NAD^+ may be used to treat multiple diseases (331).

NAD^+ may have distinctive merits as a cytoprotective agent. *In vitro* studies have shown that NAD^+ can produce greatest protective effects against PARP-1 cytotoxicity (5, 326), and NAD^+ is also protective even when applied at 3–4 h after PARP-1 activation, suggesting that NAD^+ administration may have a long window of opportunity in decreasing tissue injury. In addition, NAD^+ may further decrease cell death by other pathways, such as enhancing sirtuin activities and energy metabolism.

C. Therapeutic potential of NADH

Several studies have reported beneficial effects of NADH administration in treating PD (34, 152), which may be partially explained by the capacity of NADH to increase bioavailability of plasma levodopa. NADH administration can also improve cognitive functions (78), suggesting the potential of NADH for treating AD patients. Our recent studies have provided direct evidence that NADH treatment can significantly decrease PARP-1-mediated cell death (345), which further raises the possibility that NADH may be used to treat PARP-1-associated illnesses.

D. Therapeutic potential of modulations of NADPH oxidase

Because NADPH can act as either a 'good guy' or a 'bad guy' in cellular antioxidation systems, it could be important for cell survival to maintain NADPH as a 'good guy'. Particularly since NADPH oxidase can play critical pathological roles in multiple diseases, it is of importance to modulate NADPH-related properties to decrease the detrimental effects of NADPH in the diseases. There could been several strategies for mitigating NADPH oxidase activity: First, to directly inhibit NADPH oxidase by using NADPH oxidase inhibitors; second, to indirectly inhibit NADPH oxidase by manipulating the intracellular modulators of the enzyme, such as the small guanosine triphosphatase Rac and protein kinase C (26, 124, 288); third, to inhibit excessive generation of NADPH by modulating the multiple NADPH-generating reactions; and fourth, to maintain the activities of NADPH-consuming enzymes such as glutathione reductase so as to ensure efficient NADPH flux through these pathways, thus preventing excessive NADPH supply to NADPH oxidase.

VI. CONCLUSIONS

Based on the above discussion, it appears that the classical paradigm regarding the biological functions of NAD and NADP is too narrow to generalize the growing functions of these molecules. It is tempting to propose that a novel paradigm about the biological functions of NAD and NADP may be emerging: NAD and NADP could be the fundamental common mediators of nearly all major biological activities, including mitochondrial function, energy metabolism, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death. Selected from the information reviewed above regarding the biological functions of NAD and NADP, the following pieces of information could serve as the highlights for supporting the emerging new paradigm: In addition to the established pivotal roles of NAD in mitochondrial functions and energy metabolism, cyclic ADP-ribose and multiple other molecules that are generated from NAD and NADP could be the essential regulators of calcium homeostasis. NAD and NADP also play key roles in both antioxidation and ROS generation: NADPH is an essential component in cellular antioxidation systems; and the NADH-dependent ROS generation from the electron transport chain and the ROS generation by NADPH oxidase are two key mechanisms of ROS generation. NAD and NADP appear to mediate cell death by modulating several key factors in cell death,

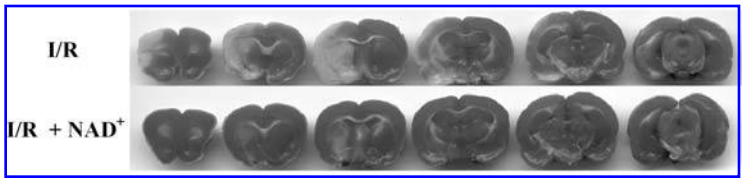

FIG. 11. Intranasal NAD^+ administration can profoundly decrease ischemic brain injury in a rat model of brain ischemia. The infarct size of the rats that underwent ischemia/reperfusion (I/R) (*upper panel*) was significantly larger than that of the rats that underwent I/R and received intranasal administration with NAD^+ (*lower panel*). As shown in the photographs, the *white-colored* tissues are the infarcted tissues.

such as MPT, energy state, and the activities of the NAD- and NADP-dependent enzymes, including PARP-1, GAPDH, AIF, and NADPH oxidase. Furthermore, in addition to the major effects of NAD and NADP on several factors that could play key roles in senescence, including oxidative stress, mitochondrial functions, and telomere metabolism, NAD-dependent sirtuins have emerged as a mediator of the aging process; and the genes encoding the enzymes involving in NAD^+ metabolism, including *PNC1* (10) and the gene encoding Nampt (293), appear to be novel longevity genes.

Growing evidence has also suggested a novel paradigm for the metabolism of NAD and NADP, which consists of the following major concepts: first, NAD and NADP can be metabolized by many enzymes to generate multiple bioactive molecules, such as cyclic ADP-ribose, ADP-ribose, poly (ADP-ribose), NAADP, and O-acetyl-ADP-ribose; second, there are NAD^+-synthesizing and NAD^+-catabolizing enzymes in not only the nucleus, but also in other subcellular organelles including the Golgi complex and mitochondria; third, extracellular NAD^+ can be metabolized by such ecto-enzymes as ecto-ARTs or CD38 to produce biological effects (33); and NAD^+-synthesizing processes might also be catalyzed extracellularly by Nampt (238); fourth, NAD can be transported across the plasama membranes of certain cell types; fifth, there are close interactions among the key enzymes in NAD and NADP metabolism, such as the interactions between NMNAT-1 and PARP-1; and sixth, at least in yeast there are such novel metabolic pathways of NAD and NADP as the pathways mediated by NADH kinase and acetaldehyde dehydrogenase. Figure 12 provides a diagrammatic overview about the metabolism and the biological functions of NAD and NADP.

As proposed in previous articles (328, 329), NAD, together with ATP and Ca^{2+}, may be the most fundamental components in life which mediate nearly all of the key biological processes (328, 329). The close interactions among these components may constitute a 'Central Regulatory Network' in life (329). The highly extensive functions of these seemingly simple molecules and ions may be important factors underlying the exquisite regulation and profound potential of life.

Much theoretical investigation is still needed to improve the 'Central Regulatory Network Hypothsis of Life.' The interactions between ROS and the 'Central Regulatory Network' could be of particular importance. As discussed in the articles about 'deleterious network hypothesis' of neurodegenerative diseases, aging, and cell death (318–322), there are close interactions among ROS, calcium homeostasis, and energy metabolism. Profound interactions between ROS and NAD/NADP have also been generalized in the current review. Because ROS plays important roles in many biological functions, it is proposed that ROS is an important factor that closely interacts with all of the three major components of the 'Central Regulatory Network.' Certain environmental and genetic factors may cause excessive

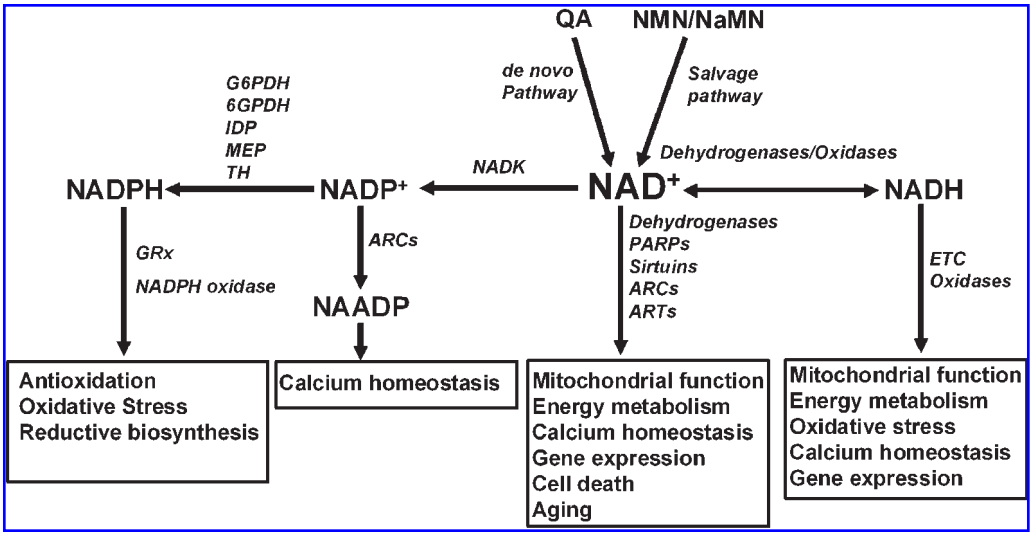

FIG. 12. Metabolism and biological activities of NAD and NADP. NAD^+ can be generated from the salvage pathway using nicotinic acid mononucleotide (NaMN) or nicotinamide mononucleotide (NMN) as precursors, or from the *de novo* pathway using quinolinic acid (QA) as the precursor. Through NAD^+-dependent dehydrogenases, poly(ADP-ribose) polymerases (PARPs), sirtuins, ADP-ribosyl cyclases (ARCs), and mono(ADP-ribosyl)transferases (ARTs), NAD^+ can significantly affect mitochondrial function, energy metabolism, calcium homeostasis, gene expression, aging, and cell death. NADH can be generated from NAD^+ by NAD-dependent dehydrogenases, which is used by the electron transport chain (ETC) or NADH oxidases. NADH can significantly affect mitochondrial function, energy metabolism, oxidative stress, calcium homeostasis, and gene expression. $NADP^+$ can be generated from NAD^+ by NAD^+ kinases (NADK), which can be used for NADPH generation through glucose-6-phosphate dehydrogenase (G6PDH), 6-glyconate phosphate dehydrogenase (6GPDH), $NADP^+$-dependent isocitrate dehydrogenases (IDPs), $NADP^+$-dependent malic enzymes (MEPs), and transhydrogenase (TH). NADPH can be used by glutathione reductase, NADPH oxidase, and other NADPH-dependent enzymes to mediate antioxidation, ROS generation, and reductive synthesis. $NADP^+$ could also be used by ADP-ribosyl cyclases (ARCs) to generate NAADP that can mobilize intracellular Ca^{2+} stores.

ROS generation, thus disrupting the 'Central Regulatory Network', leading to aging and numerous diseases. Future studies into the interactions between oxidative stress and the 'Centrual Regulatory Network' may further elucidate fundamental biological mechanisms.

Increasing evidence has suggested that NAD^+ is the central molecule in the metabolism and biological functions of NAD^+, NADH, $NADP^+$, and NADPH: out of these four molecules, NAD^+ could be the only one that can be *de novo* synthesized, while the generation of NADH, $NADP^+$, and NADPH essentially requires NAD^+ as the original precursor. NAD^+ also appears to have particularly extensive biological functions compared with the other pyridine nucleotides. Therefore, it is tempting to propose that NAD^+ is the pivotal molecule in these pyridine nucleotides.

Whereas there have been numerous significant findings about the metabolism and biological functions of NAD and NADP during the last 20 years, these findings have also raised many questions that need to be answered by future studies. The following research directions may be of particular interest:

First, recent studies have suggested the presence of new NAD/NADP metabolic machineries in various subcellular organelles as well as extracellular space. It is of significance to further elucidate the regulation and biological significance of these machineries.

Second, NADKs play key roles in the balance between the NAD pool and the NADP pool. It is warranted to determine the roles of NADKs in modulating NAD-dependent and NADP-dependent biological activities under both physiological and pathological conditions.

Third, several studies have suggested close interactions among NAD-generating enzymes and NAD-dependent enzymes, such as that between PARP-1 and NMNAT-1. Future studies are needed to further determine the interactions among these proteins, and to determine the effects of these interactions on cell death, aging, and diseases.

Fourth, increasing evidence has indicated that multiple metabolic products of NAD^+, including cyclic ADP-ribose, NAADP, ADP-ribose, and O-acetyl-ADP-ribose, are regulators of calcium homeostasis. It is warranted to determine the interactions among these regulators of calcium homeostasis.

Fifth, it is of great interest to determine the regulation of NADH shuttling and NAD transport across the plasma membranes of cells under physiological and pathological conditions.

Sixth, it is expected that future studies regarding the roles of NADPH oxidase in biological and pathological processes would yield ample information for understanding both basic biology and pathogenesis of many diseases.

Seventh, it is warranted to further determine the therapeutic potential of NAD^+ precursors, NAD and NADP for various diseases.

ACKNOWLEDGMENTS

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ABBREVIATIONS

AD, Alzheimer's disease; AIF, apoptosis-inducing factor; ART, mono(ADP-ribosyl)transferases; IP_3: inositol 1,4,5-triphosphate; IDPc, cytosolic $NADP^+$-dependent isocitrate dehydrogenases; IDPm, $NADP^+$-dependent isocitrate dehydrogenases; MAS; MEPc, cytosolic $NADP^+$-dependent malic enzyme; MEPm, mitochondrial $NADP^+$-dependent malic enzyme; MPT, mitochondrial permeability transition; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Nampt, nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase; PARG, poly(ADP-ribose) glycohydrolase; PARP-1, poly(ADP-ribose) polymerase-1; PD, Parkinson's disease; ROS, reactive oxygen species; TCA cycle, tricarboxylic acid cycle; TH, transhydrogenase; Wld(S), Wallerian degeneration slow.

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