NAD$^+$ and NADH in cellular functions and cell death

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1. ABSTRACT

Increasing evidence has indicated that NAD$^+$ and NADH play critical roles not only in energy metabolism, but also in cell death and various cellular functions including regulation of calcium homeostasis and gene expression. It has also been indicated that NAD$^+$ and NADH are mediators of multiple major biological processes including aging. NAD$^+$ and NADH produce the biological effects by regulating numerous NAD$^+$/NADH-dependent enzymes, including dehydrogenases, poly(ADP-ribose) polymerases, Sir2 family proteins (sirtuins), mono(ADP-ribosyl)transferases, and ADP-ribosyl cyclases. Of particular interest, NAD$^+$-dependent generation of ADP-ribose, cyclic ADP-ribose and O-acetyl-ADP-ribose can mediate calcium homeostasis by affecting TRPM2 receptors and ryanodine receptors; and sirtuins and PARPs appear to play key roles in aging, cell death and a variety of cellular functions.

It has also been indicated that NADH and NAD$^+$ can be transported across plasma membranes of cells, and that extracellular NAD$^+$ may be a new signaling molecule. Our latest studies have shown that intranasal NAD$^+$ administration can profoundly decrease ischemic brain damage. These new pieces of information have fundamentally changed our understanding about NAD$^+$ and NADH, suggesting novel paradigms about the metabolism and biological activities of NAD$^+$ and NADH. Based on this information, it is tempted to hypothesize that NAD$^+$ and NADH, together with ATP and Ca^{2+}, may be four most fundamental components in life, which can significantly affect nearly all major biological processes. Future studies on NAD$^+$ and NADH may not only elucidate some fundamental mysteries in biology, but also provide novel insights for interfering aging and many disease processes.

2. INTRODUCTION

NAD$^+$ and NADH have been long known as key co-factors in numerous dehydrogenase-mediated reactions (1). It has also been established that there are two pathways by which NAD$^+$ is synthesized: The salvage pathway and the *de novo* pathway (1). However, recent studies have provided numerous pieces of novel information about the metabolism and biological functions of NAD$^+$ and NADH, which are generalized in this review. This generalization may suggest new paradigms regarding the metabolism and the biological functions of NAD$^+$ and NADH, which could be of both theoretical and clinical significance.

Four major aspects about NAD$^+$ and NADH will be reviewed in this article: 1) NAD$^+$ and NADH metabolism; 2) biological functions of NAD$^+$ and NADH; 3) NAD$^+$ and NADH in cell death; and 4) therapeutic potential of NAD$^+$. There are multiple new findings that are of particular significance. In the research field about NAD$^+$ and NADH metabolism, recent studies have suggested the existence of three isoforms of nicotinamide mononucleotide adenylyltransferases (NMNATs) --- the key enzymes for NAD$^+$ synthesis --- in cells with specific subcellular compartmentation. It has also been reported that NAD$^+$ and NADH can be transported across the plasma membranes of certain cell types by connexin43 hemichannels- or P2X$_7$ receptors-mediated mechanisms. The most exciting information regarding the biological functions of NAD$^+$ and NADH includes the findings indicating profound effects of NAD$^+$-dependent histone acetylases, i.e., the Sir2 family proteins (sirtuins), on aging and gene expression. In the field about NAD$^+$ and NADH in cell death, significantly improved understanding about the roles of NAD$^+$ in poly(ADP-ribose) polymerase-1 (PARP-1)-mediated cell death has been achieved by recent studies; and multiple studies have also suggested new pathways by which NAD$^+$ and NADH can determine cell survival. Regarding the therapeutic potential of NAD$^+$, the finding about the protective effects of NAD$^+$ administration on ischemic brain injury has raised the possibility that NAD$^+$ is a new agent for treating multiple PARP-1-associated diseases.

3. NAD$^+$ AND NADH METABOLISM IN CELLS

3.1. General information about NAD$^+$ and NADH in cells

The major biological activities of NAD$^+$ and NADH can be classified into four categories: First, in numerous redox reactions NAD$^+$ and NADH act as coenzymes (1). In these reactions NAD$^+$ and NADH is converted into each other, while the total pool of (NAD$^+$ + NADH) remains unchanged; second, NAD(P)H oxidase oxidizes NADH to NAD$^+$ with generation of reactive oxygen species (ROS), without changes of total pool of (NAD$^+$ + NADH); third, NAD$^+$ is catabolized into other nicotinamide-containing molecules through the actions of multiple NAD$^+$-consuming enzymes such as PARPs; and fourth, NAD$^+$ is converted to NADP$^+$ by the action of NAD$^+$ kinase.

Under physiological conditions the ratio of cytosolic free NAD$^+$ / NADH is approximately 700 to 1 (1-4), while the ratio of mitochondrial NAD$^+$ / NADH is 7-8 to 1 (2, 3). Physiological intracellular NADH concentrations are approximately 1 – 10 μM (2). It is notable that mitochondria contain a major portion of intracellular NAD$^+$ (5-7). While nuclear membranes could be freely permeable to NAD$^+$ and NADH, mitochondrial membranes are impermeable to NAD$^+$ and NADH. It has been suggested that mitochondrial permeability pore (MPT) opening can lead to mitochondrial NAD$^+$ release and subsequent hydrolysis of NAD$^+$ by NAD$^+$ glycohydrolases (5). However, in many cell types including neurons and astrocytes the percentage of mitochondrial NAD$^+$ in total cellular NAD$^+$ pool has not been identified. In light of the findings that NAD$^+$ and NADH metabolism plays important roles in various cellular functions and cell death, it is warranted to further determine the subcellular NAD$^+$ and NADH distribution in various cell types.

3.2. NAD$^+$ and NADH synthesis in cells

As extensively reviewed by previous articles (8, 9), two well established NAD$^+$ biosynthesis pathways are the *de novo* pathway and the salvage pathway. A recent study suggests the presence of a new pathway: NADH can be directly generated from reduced form of nicotinamide mononucleotide (NMNH) and ATP by the actions of NMNATs (10), while the physiological significance of this new pathway has not been established.

The *de novo* pathway is required for NAD$^+$ generation when niacin availability is restricted (8, 9), through which mammals can synthesize nicotinamide-containing nucleotides through the kynurenine pathway: L-Tryptophan is converted to L-kynurenine that is then converted to quinolinic acid. Quinolinic acid is subsequently converted to nicotinate mononucleotide that is used for NAD$^+$ synthesis. In contrast, in the salvage pathway as well as the newly found pathway for NADH generation, NAD$^+$ and NADH are generated directly from nicotinamide-containing molecules (8-10). All of these three pathways converge to the common reversible reactions catalyzed by NMNATs, which generate NAD(H) plus PPi from NMN(H) and ATP (8-10). Because these reactions are fully reversible, NAD$^+$ can also be reconverted to ATP. This reversibility may have important biological implications: ATP may be consumed to replete NAD$^+$ when NAD$^+$ is selectively depleted by such enzymes as PARP-1; vice versa, NAD$^+$ may also be consumed to regenerate ATP under the pathological conditions when ATP is selectively depleted.

While the nuclear enzyme NMNAT1 had been the only known NMNAT until recently, current studies have indicated presence of three isoforms of human NMNATs --- NMNAT1 – 3 (9-11). In contrast to NMNAT1 that is a known nuclear enzyme, NMNAT2 and NMNAT3 may be localized in mitochondria and Golgi complex, respectively (9-11). The subcellular

compartmentation of the three NMNATs suggests that there could be relatively independent NAD$^+$ synthesis machineries in these subcellular organelles (10). These findings could be critical for understanding the NAD$^+$ and NADH metabolism in the subcellular organelles (10), considering the reports suggesting the presence of tankyrase, a telomere-regulating PARP, in Golgi complex (12) and PARP activities in mitochondria (13).

In vitro studies have indicated that NMNAT1 could bind PARP-1 and inhibit PARP-1 activity (14). If similar observations can be found *in situ* in cells and tissues, the NMNAT1-PARP-1 interactions could be of great significance for understanding NAD$^+$ metabolism. Considering that PARP-1 is the key NAD$^+$-consuming enzyme (15) while the NMNAT1 is the pivotal NAD$^+$ synthesizing enzyme, it is tempted to propose that the NMNAT-PARP-1 complex may form a NAD$^+$ Metabolism Core. The NAD$^+$ Metabolism Cores might be the key centers in cells which sense the intracellular levels of NAD$^+$ thus modulating NAD$^+$ synthesis. Because NAD$^+$ synthesis is an energy expensive process and NAD$^+$ plays critical roles in multiple cellular functions, future studies into the relationship between NMNAT1 and PARP-1 are warranted. The interest regarding NMNAT1 is further increased by the finding that the gene product of human homolog of NMNAT1 constitutes a major portion of the chimeric protein that mediates the delay in Wallerian neurodegeneration of Wlds mouse (9, 16).

3.3. NAD$^+$ and NADH catabolism in cells

While NAD$^+$ and NADH are used as coenzymes in numerous dehydrogenase-mediated reactions, multiple families of enzymes catalyze various reactions by consuming NAD$^+$. These reactions result in degradation of NAD$^+$ into nicotinamide and other products containing ADP-ribose as the core structural component. As discussed later, these reactions can significantly affect multiple biological functions. The major NAD$^+$-consuming enzymes include: (1) Poly(ADP-ribose) polymerases (PARPs), that consume NAD$^+$ to produce nicotinamide and poly(ADP-ribose) (PAR) on target proteins (15, 17, 18); (2) mono(ADP-ribosyl)transferases (ARTs) --- A family of enzymes that use NAD$^+$ as a substrate to produce nicotinamide and mono-ADP-ribosylation of proteins (19, 20); (3) NAD$^+$-dependent histone deacytalyses, i.e., the Sir2 family proteins, that deacylate histones by consuming NAD$^+$, leading to generation of O-acetyl-ADP-ribose and nicotinamide (21); and (4) bifunctional ADP-ribosyl cyclases / cyclic ADP-ribose hydrolases, that can consume NAD$^+$ to both generate cyclic ADP-ribose (cADPR) and hydrolyze cADPR into free ADP-ribose (22). Based on current knowledge PARP-1 is the most potent NAD$^+$-consuming enzyme (15): Activation of PARP-1 by DNA alkylating agents can lead to a decrease in intracellular NAD$^+$ by nearly 70% with 30 minutes (15, 18, 23, 24).

4. NAD$^+$ AND NADH TRANSPORT ACROSS MITOCHONDRIAL MEMBRANES AND PLASMA MEMBRANES OF CELLS

4.1. NAD$^+$ and NADH transport across mitochondrial membranes of cells

It is established that mitochondrial inner membranes are not permeable to NADH and NAD$^+$ (1). The reducing equivalents of cytosolic NADH are shuttled into mitochondria by malate-aspartate shuttle and / or glycerol 3-phosphate shuttle (1, 25). It is found that malate-asparate shuttle is the predominant NADH shuttle in neurons, while astrocytes have minimal malate-asparate shuttle activity and appear to have glycerol 3-phosphate shuttle activity (25, 26). Because NADH in cytosol and mitochondria can mediate multiple biological functions, understanding of the NADH shuttle activities under biological and pathological conditions may be of great significance. However, due to a lack of molecular approaches and selective pharmacological agents for modulating the NADH shuttles (25, 26), there is insufficient information regarding this topic.

4.2. NAD$^+$ and NADH transport across plasma membranes of cells

Not until recently it was generally thought that NAD$^+$ and NADH can not be transported across the plasma membranes of any cell types. However, recent studies suggest that Connexin 43 hemichannels allow NAD$^+$ gradient-dependent NAD$^+$ flux across fibroblast plasma membranes (27). Both the study by Varderio et al. and the study by our research group have suggested that NAD$^+$ can also be transported across the plasma membranes of murine astrocytes (24, 28, 29).

Our recent study has provided first evidence suggesting that NADH may be transported across plasma membranes into astrocytes: Treatment with 5 mM NADH significantly increased intracellular NAD$^+$ levels; and treatment with 10 μM – 5 mM NADH after washout of the PARP activator and DNA alkylating agent MNNG significantly decreased astrocyte death (30). Our latest studies have used both molecular and pharmacological approaches to demonstrate that as low as 10 μM NADH can be transported into astrocytes through P2X$_7$ receptor (P2X$_7$R)-mediated mechanisms (31). We found that incubation of murine astrocytes with 10 μM – 10 mM of NADH significantly increased intracellular NAD$^+$. Treatment with the purinergic (P2) receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) prevented the NADH-produced increases in intracellular NAD$^+$, suggesting that P2 receptors mediate the NADH transport. A critical role of P2X$_7$R in the NADH transport is indicated by the findings that a reduction of P2X$_7$R by RNA silencing led to decreased NADH transport, and transfection of P2X$_7$R-deficient HEK293 cells with mouse P2X$_7$R cDNA increased NADH transport. These observations collectively provide the first direct evidence demonstrating that NADH can be transported across the plasma membranes of cells. The observation that micromolar concentrations of extracellular NADH can be

transported into astrocytes also suggests that the NADH transport may be involved in cell-cell signaling in brains. Future studies are warranted to determine if NADH can also be transported across the plasma membranes of other types of cells under both *in vitro* and *in vivo* conditions, and if the NADH transport is altered under various pathological conditions such as brain ischemia.

5. BIOLOGICAL FUNCTIONS OF NAD$^+$ AND NADH

While it has been long thought that the major cellular functions of NAD$^+$ and NADH are modulating cellular energy metabolism, increasing evidence has suggested that NAD$^+$ and NADH also play key roles in cell death (17, 23, 24) and various major cellular functions such as calcium homeostasis (22, 32) and gene expression (4, 33). Accumulating evidence has further indicated significant roles of NAD$^+$ and NADH in such important biological processes as aging, carcinogenesis and immunological functions (21, 32).

5.1. NAD$^+$ and NADH in energy metabolism

NAD$^+$ and NADH levels significantly affect cellular energy metabolism via several pathways (1, 32): NAD$^+$ and NADH regulate glycolysis by acting as the co-factors for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase; and NAD$^+$ and NADH mediate other important energy metabolism-related reactions occurring in cytosol, such as the lactate dehydrogenase-catalyzed lactate-pyruvate conversions. NAD$^+$ and NADH are also pivotal mediators of mitochondrial oxidative phosphorylation, because they are the co-factors for the three rate-limiting enzymes in mitochondrial tricarboxylic acid (TCA) cycle, and NADH is one of the major electron donors for electron transport chain (1). Recent studies have suggested novel mechanisms by which NAD$^+$ and NADH may modulate energy metabolism, e.g., NAD$^+$ may affect energy metabolism by regulating Sir2 family proteins, which can modulate acetyl-CoA synthetase and glycolysis / glycogenesis (34, 35).

5.2. NAD$^+$ and NADH in mitochondrial functions

In addition to the well established roles of NAD$^+$ and NADH in the functioning of mitochondrial TCA cycle and electron transport chain, cumulative evidence suggests that NAD$^+$ and NADH can profoundly affect mitochondrial activities through other pathways: First, NAD$^+$ / NADH ratio is an important regulator of mitochondrial permeability transition (MPT) (36). Second, NADH can directly interact with and inhibit voltage-dependent anion channels (VDAC), that controls the transport of small molecules across mitochondrial membranes and is a component of MPT pore (37). The NADH-produced inhibition of VDAC opening suggests that abnormally increased cytosolic NADH may limit mitochondrial activity by blocking VDAC. Third, cytosolic NADH could be directly oxidized by cytochrome oxidase to increase mitochondrial membrane potential (38). NAD$^+$ and NADH may also affect mitochondrial functions indirectly through other

mechanisms, e.g., by modulating calcium homeostasis that is known to profoundly affect mitochondrial activities (39); and by regulating Sir2 family proteins that may affect Bax expression via modulating p53 activity (40, 41).

5.3. NAD$^+$ and NADH mediate calcium homeostasis

Increasing evidence has suggested that both NAD$^+$ and NADH can mediate calcium homeostasis. NAD$^+$ may affect calcium homeostasis through the following pathways: First, ADP-ribosyl cyclases can generate cADPR from NAD$^+$, which is a most potent endogenous agonist of ryanodine receptor-mediated calcium channels (42). Second, recent studies have suggested that ADP-ribose, which could be generated from NAD$^+$ by NAD glycohydrolases or PARPs / poly(ADP-ribose) glycohydrolase (PARG), is an activator of TRPM2 receptors (43, 44). Third, Sir2 family proteins can generate O-acetyl-ADP-ribose that could be a novel signaling molecule: O-acetyl-ADP-ribose was found to directly bind the cytoplasmic domain of the TRPM2 channels and activate TRPM2 channels (45). NAD$^+$ can further modulate calcium metabolism by promoting mono-ADP-ribosylation of P2X$_7$R or through its conversion to NADP$^+$ by NAD$^+$ kinase: The ecto-ARTs-mediated mono-ADP-ribosylation of P2X$_7$R has been shown to promote opening of P2X$_7$R (46), which can lead to Ca^{2+} influx (47); and NAADP generated from NADP$^+$ can also potently mobilize intracellular NAADP-dependent Ca^{2+} stores (22, 48, 49).

The ecto-enzyme ADP-ribosyl cyclases or unidentified ADP-ribosyl cyclases located within the cytosol generate cADPR from NAD$^+$ in many cell types, which is a potent Ca^{2+} mobilizing second messenger via activating type 2 and 3 ryanodine receptors (42, 49). cADPR may activate ryanodine receptors by direct binding onto the receptors or by interacting with separate cADPR-binding proteins. Recent studies have suggested that cADPR can also affect calcium homeostasis by gating TRPM2 receptors (50). Due to the important roles of ryanodine receptors and TRPM2 receptors in calcium homeostasis, it is expected the cADPR generation by ADP-ribosyl cyclases may profoundly modulate cellular functions and cell survival by mediating cellular calcium metabolism.

Cumulating evidence has demonstrated that transient receptor potential (TRP) family proteins play crucial roles in a variety of cellular functions (51, 52). It has been indicated that TRPM7 receptors mediate NMDA-induced neurotoxicity (53, 54); and intracellular ADP-ribose appears to be an endogenous activator of TRPM2 (43, 44). The ADP-ribose / TRPM2 receptor pathways could mediate oxidative cell death by the following mechanisms (55-57): Oxidative damage causes PARP-1-induced PAR formation, leading to PARG-mediated generation of ADP-ribose that can produce opening of TRPM2 receptors, Ca^{2+} influx and consequent cell death.
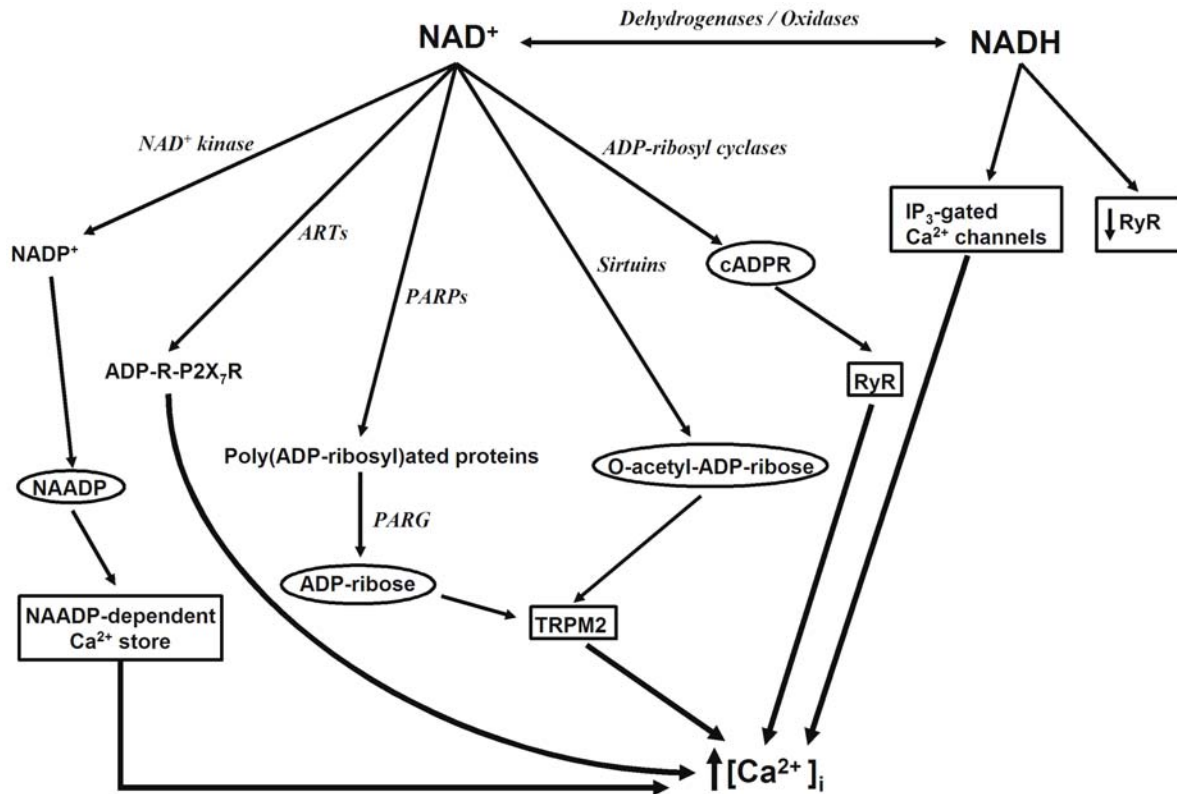
Figure 1. Diagrammatic presentation of pathways by which NAD$^+$ and NADH affect calcium homeostasis. ADP-ribosyl cyclases, poly(ADP-ribose) polymerases (PARPs) and sirtuins use NAD$^+$ as a substrate for generation of several Ca^{2+}- mobilizing second messengers, including cyclic ADP-ribose (cADPR), ADP-ribose and O-acetyl-ADP-ribose. These messengers can produce opening of 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). NAD$^+$ can further modulate calcium metabolism after being converted to NADH and NADP$^+$ by NAD$^+$/ NADH-dependent dehydrogenases and NAD$^+$ kinase, respectively: NADH can lead to opening of the IP3-gated Ca^{2+} channels and inhibit ryanodine receptors (RyR); and NAADP generated from NADP$^+$ can potently mobilize intracellular NAADP-dependent Ca^{2+} stores. The molecules in open ovals are calcium-mobilizing second messengers; and the cellular components in open rectangles are the targets of these second messengers.

NADH can also modulate calcium homeostasis mainly by affecting intracellular Ca^{2+} channels. It was found that increased NADH under hypoxic conditions 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 (58). The recent study by Patterson et al. has further suggested that the GAPDH associated with IP$_3$-gated calcium channels can locally generate NADH to increase the Ca^{2+} channel opening (59). It was also shown that NADH can inhibit ryanodine receptors of cardiac muscle, but not skeletal muscle (60, 61), which could be mediated by the NADH oxidase activity of cardiac sarcoplasmic reticulum (62). Figure 1 is a diagrammatic presentation showing the pathways by which NAD$^+$ and NADH affect calcium homeostasis.

5.4. Effects of NAD$^+$ and NADH on gene expression

NAD$^+$ may affect gene expression through several pathways: 1) The NAD$^+$-consuming enzyme PARP-1 can affect several transcriptional factors such as p53, AP-1 and NF-κB (15, 63). 2) NAD$^+$ is required for the activities of other PARPs such as tankyrases, which could also affect gene expression and genomic structure (64). 3) Yeast Sir2 can silence transcription at telomeres and ribosomal DNA (65); sirtuins can also mediate the activities of such transcriptional factors as p53 and forkhead transcription factors (66); and mammalian Sir2 homolog SIRT7 was found to be an activator of RNA polymerase I transcription (67); and 4) NADH is a potent regulator of the activity of the corepressor carboxyl-terminal binding protein --- a transcriptional factor important for cell cycle regulation, development and transformation (4); and NADH also regulates the activities of Clock:BMAL1 and NPAS2:BMAL1, that are heterodimeric transcription factors controlling gene expression as a function of circadian clock (33).

5.5. NAD$^+$ and NADH in oxidative stress

NAD$^+$ and NADH might affect the processes of aging and oxidative damage-mediated diseases due to its effect on cellular antioxidation capacity: NADH / NAD$^+$

ratio is an index of cellular reducing potential; and NAD$^+$ can be converted by NAD$^+$ kinase to NADP$^+$ --- the precursor for synthesizing the major reducing molecule NADPH (1). Seemingly paradoxically, excessive intracellular NADH can produce 'reductive stress', which may result from its capacity to induce release of ferrous iron from ferritin resulting in increased oxidative damage (68). The 'reductive stress' may also result from the capacity of xanthine oxidase / xanthine dehydrogenase to generate ROS by oxidizing NADH (69).

5.6. Roles of NAD$^+$ and NADH in aging

Cumulative evidence has suggested that NAD$^+$ and NADH could be critical factors in aging process by regulating sirtuins, PARP-1, tankyrases and oxidative stress. It has been suggested that Sir2 is a key enzyme mediating life span of yeast and C-*elegans* (21): A decrease or increase in gene copy of Sir2 shortens or extends the replicative life span of yeast, respectively (70); and increased gene copy of the Sir2 gene homolog in C-*elegans* also extends its life span (71). It has been further suggested that calorie restriction modulates Sir2 activity and extends the life span of yeast by decreasing NADH levels (72). Recently it was found that deficiency of SIRT6, a human homolog of Sir2, leads to aging-like phenotype and genomic instability in mice (73). However, latest studies have suggested increasingly complicated roles of Sir2 in aging (74): While it increases replicative life span, Sir2 may decrease chronological life span of yeast at least under certain conditions (75). Thus, many future studies are still needed to further elucidate the complex effects of sirtuins on chronological and replicative life span of various species.

Telomere and telomerases have been indicated as key factors in cellular aging (76). Since the NAD$^+$-dependent tankyrases are mediators of telomerase activity (77), NAD$^+$ may also affect aging processes through tankyrases. It was reported that there is strong positive correlation between the PARP activities of mononuclear blood cells and the longevity of thirteen mammalian species, which may result from greater PARP-1-mediated DNA repair capacity (78, 79). A role of PARP-1 in aging has been further raised by the recent report that PARP-1 interacts with and inhibits the catalytic activities of the protein of Werner syndrome --- a human disease of premature aging (80, 81). Because oxidative stress plays a critical role in aging process (82, 83), NAD$^+$ and NADH may also affect aging through their influence on oxidative stress.

5.7. Roles of NAD$^+$ and NADH in carcinogenesis and cancer treatment

It has been reported that selective inhibition of NAD$^+$ synthesis can induce apoptosis of tumor cells (84). Because PARP-1 plays critical roles in regulating DNA repair, genomic stability, cell cycle progression and gene expression (17), extensive studies have been conducted to determine the roles of PARP-1 in carcinogenesis (85). It has been found that PARP inhibitors can restore sensitivity of resistant tumors to methylating agents or topoisomerase I inhibitors, and may also decrease the toxic side effects of certain anticancer drugs (85). Many studies have suggested important roles of telomerases and telomere in carcinogenesis (86). Thus, NAD$^+$-dependent tankyrases --- the enzymes that regulate telomerase activities (64, 87) --- may affect carcinogenesis by affecting telomere metabolism.

Increasing evidence has indicated that sirtuins play significant roles in carcinogenesis and cancer treatment. A recent study provides intriguing evidence suggesting that cancer cells, but not noncancerous cells, may require SIRT1 for survival (88): Decreased levels of SIRT1 by RNA silencing selectively induced apoptosis and/or growth arrest in human epithelial cells by affecting FoxO4, caspase-3 and caspase-7, 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 producing increased p53 acetylation and suppressing antiapoptotic factor bcl-2 (89). This study further raises the possibility that SIRT1 may be used as a target for cancer treatment. Intriguingly, this study also suggests a link among NAD$^+$ metabolism, carcinogenesis and aging: Aging-related decreases in HIC1 expression may promote carcinogenesis by producing increased SIRT1 activity and consequent inhibition of p53; and diet-induced increases in NAD$^+$ levels might also promote carcinogenesis by activating SIRT1 and inhibiting p53. Future studies are required to further investigate the associations among NAD$^+$ metabolism, sirtuins, aging and carcinogenesis, which could provide crucial information for understanding some of the most essential questions in biology. Due to the critical roles of NAD$^+$ and NADH in cell death and various cellular functions including gene expression and signal transduction, it is conceivable that future studies would further elucidate important roles of NAD$^+$ and NADH in carcinogenesis and cancer treatment.

5.8. NAD$^+$ and NADH in immunological functions

Recent studies have suggested that the ecto-ARTs can produce mono-ADP-ribosylation of P2X$_7$R by consuming extracellular NAD$^+$, thus significantly promoting opening of P2X$_7$R. The P2X$_7$R opening can lead to death of certain types of immune cells, particularly CD4+CD25+ T cells (Treg cells) --- a type of T cells that inhibit the activation of other types of T cells (46). Based on this information, it has been proposed that NAD$^+$ may be used to modulate immunological functions (46). A recent study has also indicated that cADPR is a second messenger mediating the lipopolysaccharide-induced proliferation of human peripheral blood mononuclear cells (90). A number of recent studies have further indicated important roles of PARP-1 in inflammatory responses, due to its major effects on NF-κB (17). Since cell necrosis is a significant initiator of inflammatory responses and NAD$^+$ and NADH could determine cell death modes, NAD$^+$ and NADH may also significantly affect initiation of inflammatory responses.
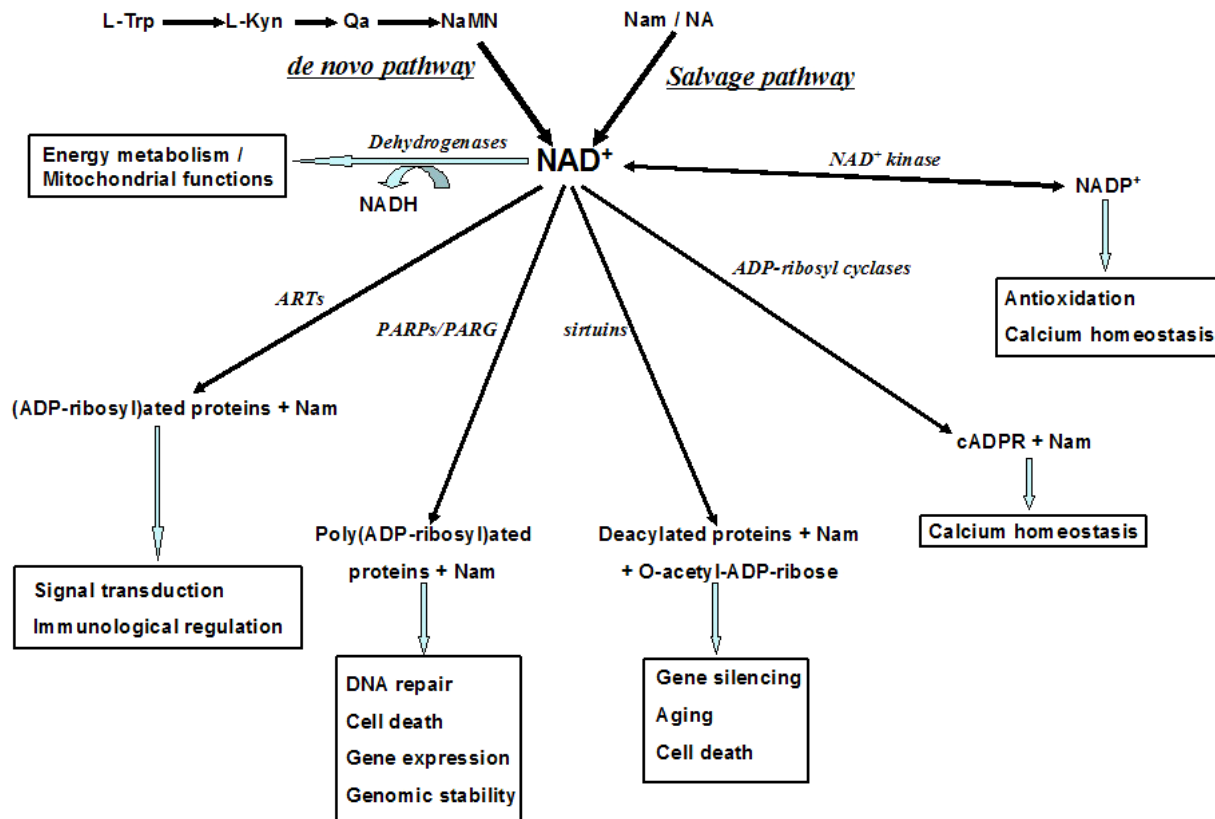
Figure 2. Diagrammatic presentation of the metabolism and biological activities of NAD$^+$. NAD$^+$ can be generated from the salvage pathway or the *de novo* pathway, which are mediated by nicotinamide mononucleotide adenylyltransferases (NMNATs). NAD$^+$ produces a number of biological effects through multiple NAD$^+$-dependent enzymes, including NAD$^+$/NADH-dependent dehydrogenases, poly(ADP-ribose) plymerases (PARPs), sirtuins, mono(ADP-ribosyl)transferases (ARTs), ADP-ribosyl cyclases, and NAD$^+$ kinase. The biological activities in open rectangles are the major NAD$^+$-mediated activities. Abbreviations used: Kyn: Kynurenine; Qa: Quinolinic acid; Nam: Nicotinamide; NA: Nicotinic acid; PARG: Poly(ADP-ribose) glycohydrolase; cADPR: Cyclic ADP-ribose; RyR: Ryanodine receptors.

5.9. Biological functions of extracellular NAD$^+$

Recent studies have indicated that extracellular NAD$^+$ may affect cellular functions by at least two mechanisms: First, extracellular NAD$^+$ can be used by the ecto-enzyme CD38, a human homolog of ADP-ribosyl cyclases, to generate cADPR, which is subsequently transported into cells by CD38 to act as a potent agonist of ryanodine receptor-gated calcium channels in astrocytes and other cell types (27, 28); and second, even 10 μM extracellular NAD$^+$ can produce T cell death, because ecto-ART2 can use NAD$^+$ to produce mono-ADP-ribosylation of P2X$_7$R thus potently promoting its opening (46, 91). Collectively, these findings suggest that extracellular NAD$^+$ may be a novel extracellular messenger, particularly considering the significant biological effects of P2X$_7$R and ryanodine receptor-gated calcium channels.

5.10. Summary of the biological activities of NAD$^+$ and NADH

Based on the discussions above, Figures 2 and 3 are drawn to provide diagrammatic presentations of the major biological activities of NAD$^+$ and NADH. It is noteworthy that much of the fundamental information supporting the diagrams is generated by recent studies, indicating that our knowledge about NAD$^+$ and NADH is undergoing an exciting, rapid growing phase. It is expected that these diagrams would be further evolving in the coming years.

6. NAD$^+$ AND NADH IN CELL DEATH

6.1. NAD$^+$ and NADH in PARP-1 toxicity
6.1.1. PARP-1 is a critical mediator of oxidative cell death both *in vitro* and *in vivo*

Ischemia-reperfusion leads to extensive oxidative damage of proteins, phospholipids and DNA in the brains (92-94). Oxidative stress has been indicated as a key mediator of ischemic brain damage (92, 95). Excessive PARP-1 activation appears to mediate cell death induced by oxidative stress (96, 97), NMDA-induced excitotoxicity (96) and oxygen-glucose deprivation (97). In cerebral ischemia, PARP-1 activity is increased due to not only oxidative stress-induced DNA damage, but also oxidative stress-induced increases in PARP-1 expression (98). Several lines of evidence

NMNH

NMNATs

Dehydrogenases

NADH

Energy metabolism /
Mitochondrial functions

NAD⁺

NAD(P)H oxidase

$NAD^+ + ROS$

XO / XH

$NAD^+ + ROS$

Opening of IP₃-gated
Ca²⁺ channels

↓Opening of RyR

VDAC inhibition

Iron release from ferritin
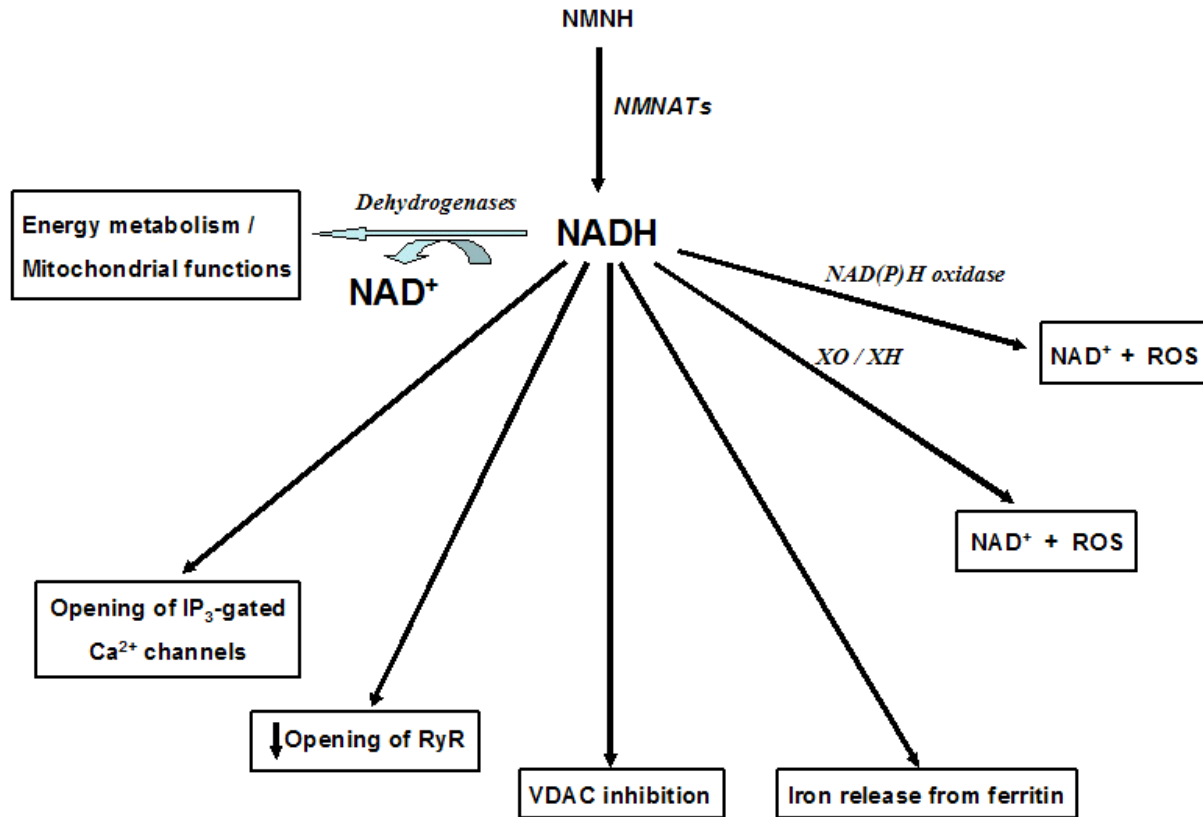
Figure 3. Diagrammatic presentation of the metabolism and biological activities of NADH. NADH is converted from NAD⁺ via the dehydrogenase-mediated reactions, or generated through a *de novo* pathway via the action of nicotinamide mononucleotide adenylyltransferases (NMNATs). The biological activities in open rectangles are the major NADH-mediated activities. Abbreviations used: NMNH: Reduced form of nicotinamide mononucleotide; RyR: Ryanodine receptors; VDAC: Voltage-dependent anion channels; ROS: Reactive oxygen species; XO: Xanthine oxidase; XD: Xanthine dehydrogenase.

have indicated that PARP-1 activation plays a key role in ischemic brain injury: First, increased PARP activities have been found in animal models of cerebral ischemia (99, 100), and in human brains after cardiac arrest (101); second, both pharmacological and genetic inhibition of PARP-1 decreases infarct size by up to 80% in the brains subjected to transient or permanent ischemia (97, 100); and third, PARP inhibition can produce long-term beneficial effects on experimental stroke recovery (102). These findings, combined with the findings indicating critical roles of PARP-1 in oxidative cell death *in vitro* and in ischemic damage of other organs (103), demonstrate that PARP-1 mediates ischemic brain injury. Figure 4 is a diagrammatic presentation of the roles of PARP-1 in ischemic brain damage.

A number of studies have indicated that oxidative stress plays a significant role in the pathogenesis of Parkinson's disease (PD) (83, 104, 105). Oxidative damage has also been indicated as one of the pathogenic factors in Alzheimer's disease (AD) (106-110). Thus, it is conceivable that PARP-1 may mediate neuronal injury in PD and AD. PARP-1 activation appears to play a key role in the neuronal death induced by MPTP, a model toxin for PD, in both cell culture

studies (111, 112) and in animal model studies (113-115). Increased nuclear PARP activity has also been found in the brains and peripheral cells of AD patients (114, 116). Recent studies have suggested that PARP-1 activation also mediates the β-amyloid-induced neuronal death, which is an *in vitro* model for AD (57, 117). Cumulative evidence has further indicated that PARP-1 activation is an important pathological factor in traumatic brain injury (118), diabetes (119), hypoglycemic brain injury (120), and shock and inflammation (17, 103). PARP-1 has become a promising therapeutic target for multiple diseases (17, 103, 121).

6.1.2. Mechanisms of PARP-1-mediated cell death

While it has been long hypothesized that PARP-1 induces cell death by depleting NAD⁺ and ATP (122), not until recently there has been no direct demonstration of this hypothesis. Two recent studies have, by directly delivering NAD⁺ into astrocytes, provided evidence demonstrating that NAD⁺ depletion is a key step mediating PARP-1-induced cell death (24, 29). This finding, together with the report that liposomal NAD⁺ delivery can partially prevent peroxynitrite-induced mitochondrial depolarization (13), indicates a
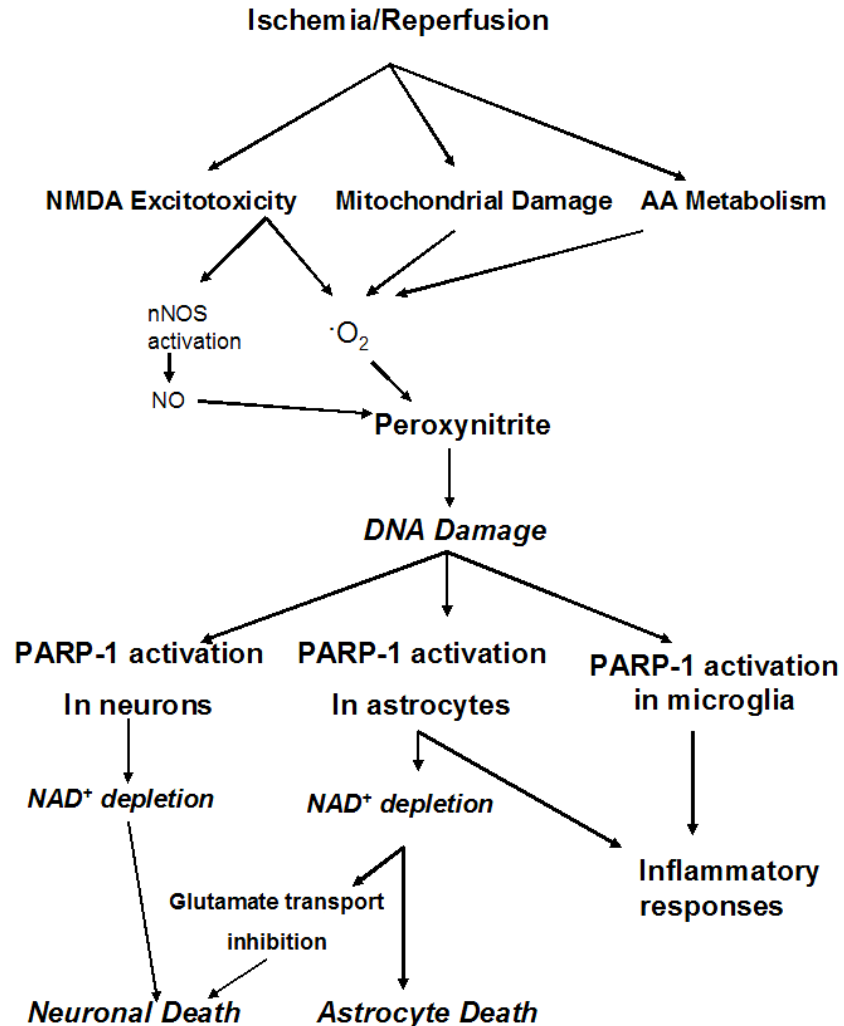
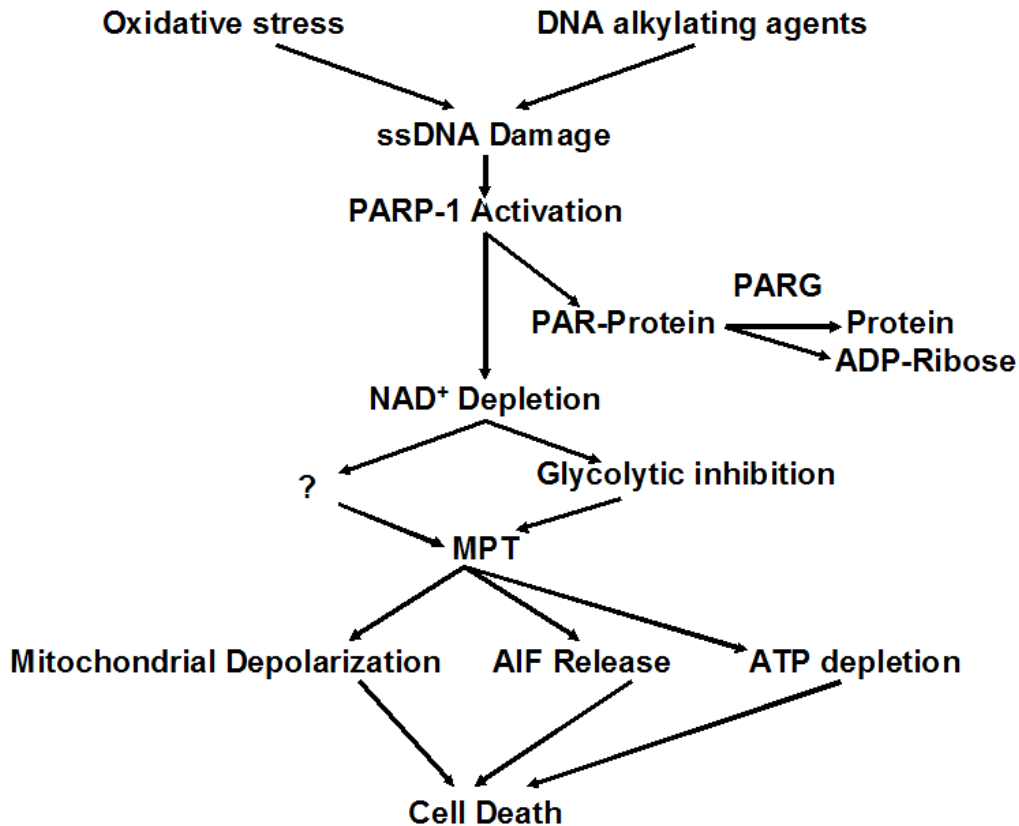**Figure 4.** Diagrammatic presentation of the roles of PARP-1 in ischemic brain injury.

key role of NAD$^+$ depletion in PARP-1-mediated cytotoxicity. Several studies have also shown that MPT (24) and apoptosis inducing factor (AIF) translocation (123) are important steps leading from NAD$^+$ depletion to cell death. There has also been information suggesting the mechanisms linking NAD$^+$ depletion and mitochondrial damage: NAD$^+$ depletion could mediate PARP-1-induced glycolytic inhibition (29), which would decrease pyruvate supply to TCA cycle thus reducing mitochondrial transmembrane H$^+$ gradient; and supply of TCA cycle substrates prevented PARP-1-induced neuronal and astrocyte death (124). Based on these studies, we propose a new diagram regarding the mechanisms of PARP-1-induced cytotoxicity (Figure 5).

Recent studies have also suggested other novel mechanisms underlying PARP-1 cytotoxicity. It was reported that ADP-ribose generated by PARP-1/PARG can produce TRPM2 opening, leading to increased intracellular calcium concentrations and cell death (55-57). It was also suggested that SIRT1 is an essential link between NAD$^+$ depletion and cell death (125). A latest

study has further indicated significant interactions between PARP-1 and SIRT1: SIRT1 deficiency leads to significant increases in PARP-1 activity, resulting in AIF-mediated cell death (126). While there have been multiple seemingly diverse mechanisms regarding PARP-1 toxicity, future studies may elucidate a common pathway linking these mechanisms. However, like the existence of diverse apoptotic cascades, there may also be differential PARP-1-mediated cell death cascades that are selectively activated depending on intensity of insults and cell types.

Several recent studies have indicated that PARP-1 inhibition produced completely different effects on the ischemic brain damage in male and female animals: While it profoundly decreased brain injury in male mice, PARP-1 inhibition markedly exacerbated ischemic brain damage in female mice (127-129). These results could have significant clinical implications: Gender may be considered an important factor for design of therapeutic strategies for ischemic stroke and possibly other diseases. The mechanisms underlying these

Figure 5. Diagrammatic presentation of the mechanisms of PARP-1 cytotoxicity. Abbreviations used: ssDNA damage: Single strand DNA damage; PARP-1: Poly(ADP-ribose) polymerase-1; PARG: Poly(ADP-ribose) glycohydrolase; AIF: Apoptosis inducing factor; MPT: Mitochondrial permeability transition.

intriguing findings still remain unclear. However, due to the critical roles of NAD$^+$ metabolism in PARP-1 toxicity, NAD$^+$ might be an important factor in the effects of gender on the brain injury.

6.1.3. Roles of PARG in cell survival

　　PARP-1-generated poly(ADP-ribose) (PAR) is rapidly degraded by the pivotal PAR-catabolizing enzyme PARG, resulting a half life of PAR less than 1 min (130). PARG digests PAR into ADP-ribose, which is then rapidly converted to AMP by a Mg^{2+}-dependent activity (131). PARG is an endo-exoglycosidase existing in low abundance in cells, which has little homology to known proteins. Due to the indispensable role of PARG in modulating poly(ADP-ribosyl)ation (130), it is not surprising that several studies have suggested that PARG plays significant roles in regulating gene expression, cell cycle and cell differentiation (132-134).

　　It has been proposed that PARG inhibition may prevent PARP-1-mediated cell death by the following mechanisms (135, 136): 1) PARG inhibition could slow the rapid PAR turnover thus preventing NAD$^+$ depletion. 2) PARP-1 can auto-poly(ADP-ribosyl)ate itself, leading to PARP-1 auto-inhibition (15). Therefore, PARG inhibition could prevent removal of PAR from PARP-1,

thus indirectly inhibiting PARP-1 activation. 3) Ca^{2+}-Mg^{2+}-dependent endonucleases (CME) mediate DNA fragmentation in certain apoptotic cascade (137). It has been found that CME is a substrate of PARP-1, and poly(ADP-ribosy)lation of CME leads to CME inhibition (137, 138). Thus, PARG inhibition could prevent removal of PAR from CME, leading to persistent CME inhibition. Recent studies have also provided novel potential mechanisms underlying the protective effects of PARG inhibition: PARP-1/PARG activities can generate ADP-ribose from hydrolysis of PAR, leading to activation of TRPM2 receptors and consequent cell death (55-57).

　　A number of *in vitro* and *in vivo* studies have supported the hypothesis that PARG may be a new target for decreasing oxidative cell death and ischemic tissue injuries (139): First, the mice that have genetic deletion of the 110 kDa PARG isoform have significantly decreased ischemic damage of intestine (140) and kidney (141) compared with wild type mice. Second, treatment with novel PARG inhibitors can decrease ischemic injury of brain (142) and intestine (140) and reduce development of septic shock-like syndrome (143). 3) PARG inhibitors can decrease death of various types of cells induced by oxidative stress and other PARP

activators (135, 136, 144-146). 4) Decreases of PARG levels by antisense oligonucleotide treatment (147) or RNA silencing (148) led to reduced PARP-1-mediated cell death. However, complete genetic deletion of PARG was reported to produce embryonic lethality of mice (149). Collectively, the studies regarding the roles of PARG in cell survival suggest that only partial inhibition of PARG could be beneficial. Increasing evidence has indicated that PARG has highly complicated properties (139, 150). Thus, extensive future studies are needed for elucidating the roles of PARG in cell survival.

6.1.4. Roles of other PARPs in cell death

A recent study has shown that overexpression of tankyrase 2 can cause rapid cell death (151). It has also been indicated that PARP-2 is a new executioner of cell death in focal cerebral ischemia, while it can decrease hippocampal injury after global ischemia (152). These studies suggest that in addition to PARP-1, other PARPs could also mediate cell death, and that the results obtained by using such non-specific PARP inhibitors as 3-aminobenzamide should be interpreted cautiously.

6.2. Important roles of the interactions among PARP-1, caspase-3 and calpains in determinations of cell death modes

Caspase-3 is one of the pivotal components in apoptotic cascades (153-155). Important roles of calpains in both apoptosis and necrosis have also been strongly indicated (156). In contrast, PARP-1-induced cell death appears to be mainly 'non-apoptotic', which has been named 'programmed necrosis' (157, 158), although several studies have suggested that PARP-1 activation can also mediate apoptosis under certain conditions (159, 160).

Increasing evidence has indicated close interactions among PARP-1, calpains and caspase-3. There are several lines of evidence indicating that PARP-1 and caspase-3 is each other's lethal enemy: Excessive PARP-1 activation can prevent caspase-3 activation by depleting ATP, and could block the activation of caspase 3-dependent DNA fragmentation factors DFF40 (161). Vice versa, PARP-1 is a known substrate for caspase-3-dependent cleavage that abolishes PARP-1 activity (156). It has also been found that the caspase 3-produced N-terminus cleavage product of PARP-1 can promote UV-mediated apoptosis by preventing ATP depletion (162). The mutual killing relationship between caspase-3 and PARP-1 may be explained as follows: Increasing evidence has indicated caspase-3 and PARP-1 as the key mediators of apoptosis and 'programmed necrosis', respectively. Because these two enzymes could be the master mediators of these death modes, the mutual inhibition of caspase-3 and PARP-1 may be needed to ensure uninterrupted execution of a specific cell death cascade. Several studies have further deepen our understanding about the interplays between caspase-3 and PARP-1: The study by Garnier et al. showed that caspase-3-dependent cleavage of PARP-1 could mediate ischemic preconditioning (163); and PARG was also found to be a substrate of caspase-3 (164).

The significant interactions among calpains, PARP-1 and caspase-3 are indicated by the following findings: First, both PARP-1 and procaspase-3 can be cleaved by calpains that are, like caspase-3, cysteine-dependent proteases (165). The cleavage was found to produce inhibition of PARP-1 and caspase-3 (165); second, caspase 3 can degrade calpastatin --- the endogenous calpain inhibitor, leading to activation of calpains (156, 166); and third, PARP-1 activation may lead to calpain activation by TRPM2-dependent increases in intracellular Ca^{2+} concentrations (55-57).

Based on the above discussion, it is tempted to propose that under a majority conditions, PARP-1, caspase-3 and calpains may be three master mediators of three cell death modes --- 'programmed necrosis', apoptosis, and mixed-type cell death. The complex interactions among these 'master mediators' may be the pivotal events in deciding cell death modes. Future studies are warranted to determine the mechanisms that mediate these complex interactions under various pathological conditions.

6.3. Potential roles of NAD$^+$ and NADH in apoptosis

Cumulative evidence has suggested that NAD$^+$ and NADH may play important roles in apoptosis: NADH / NADPH depletion has been found to be a very early event in apoptosis (167). It has also been reported that selective inhibitors of NAD$^+$ synthesis can induce apoptosis (84). NAD$^+$ and NADH may affect apoptosis through several potential mechanisms: First, NADH / NAD$^+$ ratio is a major index of cellular reducing power, which can modulate MPT --- a mediator of apoptosis under many conditions (36). Second, NAD$^+$ and NADH also play a key role in cellular energy metabolism that is a critical factor determining cell death modes. Third, NAD$^+$ levels mediate the activity of caspase-dependent endonuclease DFF40--- a key executioner of DNA fragmentation in apoptotic cascade (68). Fourth, NAD$^+$-dependent sirtuins may mediate apoptosis (168). In contrast to the extensiveness of the studies on PARP-1 --- a mediator of programmed necrosis (157, 158), there have been much less studies regarding the roles of NAD$^+$ and NADH in apoptosis. Future studies on this topic are critical for our comprehensive understanding about the roles of NAD$^+$ and NADH in cell demise.

6.4. Distinct properties of NADH in cell death

Although NAD$^+$ and NADH are extremely closely related, NADH appears to have multiple distinct properties compared with NAD$^+$: First, while NAD$^+$ is not permeable to mitochondrial membranes, increased cytosolic NADH can lead to increased NADH in mitochondria through the action of NADH shuttles (1), which may increase mitochondrial membrane potential thus preventing MPT. Second, increased NADH can enhance NADH / NAD$^+$ ratio – an index of reducing potential, which may block MPT (36). Third, cytochrome oxidase can directly use extramitochondrial NADH to increase mitochondrial membrane potential (38). Fourth, NADH can directly interact with VDAC, a major component of MPT pore (37).

With a ratio of cytosolic NAD$^+$ / NADH as high as 700 to 1, it is likely that NADH, like intracellular Ca^{2+}, is very tightly regulated in cells. In other words, cells could be sensitive to the changes of cytosolic NADH. There is evidence suggesting that both marked decreases and increases in cytosolic NADH may produce detrimental effects: NADH / NADPH depletion was found to be a very early event in apoptosis (167). While a decreased NADH / NAD$^+$ ratio can lead to decreased reducing potential in cells, a high cytosolic NADH level may also exacerbate oxidative damage by inducing iron release from ferritin and by providing substrates for NAD(P)H oxidase and xanthine oxidase / xanthine dehydrogenase (69) to generate ROS. Similarly, while increased cytosolic NADH can lead to increased NADH in mitochondrial via the NADH shuttles thus promoting oxidative phosphorylation, the increased cytosolic NADH may also block oxidative phosphorylation by promoting conversion of pyruvate to lactate, and by inhibiting VDAC permeability. Collectively, increasing evidence appears to suggest that the biological effects of altered cytosolic NADH levels are particularly complicated. These highly complex and intriguing effects of altered cytosolic NADH levels on cell injury should be further investigated.

Our recent study has indicated that NADH treatment decreases PARP-1-mediated astrocyte death with a significantly different dose response compared with that of NAD$^+$ treatment (30). The minimal protective doses for NADH and NAD$^+$ were 10 μM and 1 milimolar, respectively. However, at concentrations greater than 0.5 milimolar, the protective effects of NADH started to decline, while the NAD$^+$-produced protective effects dose-dependently increased at NAD$^+$ concentrations between 1 – 10 milimolar. While the exact mechanisms underlying these observations remain unclear, it is possible that excessively increased cytosolic NADH levels produced by high concentrations of extracellular NADH may lead to cytotoxicity via such mechanisms as inducing reductive stress, inhibiting VDAC and converting pyruvate to lactate.

7. THERAPEUTIC POTENTIAL OF NAD$^+$

7.1. *In vitro* studies have suggested therapeutic potential of NAD$^+$ and NADH

The recent studies by our research group have provided first evidence indicating that extracellularly applied NAD$^+$ and NADH can significantly decrease PARP-1 cytotoxicity (18, 24, 29, 30). It has also been shown that extracellular NAD$^+$ can prevent transaction-induced axonal injury, while the role of SIRT1 in this protection remains controversial (16, 169). These results raise the possibility that NAD$^+$ and NADH may be used *in vivo* to decrease PARP-1-mediated tissue injury. This possibility has been further enhanced by the observations that NAD$^+$ levels are significantly decreased by a PARP-mediated mechanism in the brains subjected to ischemia-reperfusion (100, 170), and the excitotoxin glutamate decreases NADH / NAD$^+$ ratios in a brain slice model (171).

7.2. Limitations of PARP inhibitors as potential therapeutic agents

While many studies have shown that PARP inhibitors can decrease cell injury under various pathological conditions (17), some studies have suggested that PARP inhibitors may also produce toxic effects. One of the major problems for PARP inhibitors in treating PARP-1-related diseases is: PARP-1 is a key enzyme for repairing single-strand DNA damage (15). While PARP-1 inhibition can decrease cell injury by preventing NAD$^+$ depletion, this inhibition could also compromise DNA repair capacity of cells. Because it is known that DNA damage can induce cell death by activating multiple cell death-inducing pathways such as that mediated by p53, PARP inhibitors may produce significant toxic side effects. As reported by Nakayama et al., PARP inhibitors can lead to increased ischemic brain injury in a model of mild brain ischemia (172). PARP inhibitors also promote γ irradiation-induced neuronal death (173). Our recent study has shown that the PARP inhibitor DPQ can not decrease peroxynitrite-induced DNA damage when applied either before or after peroxynitrite treatment (174). Because PARP-1 is also important for neurotrophic effects of nerve growth factor (175), learning and memory (176, 177), and regulation of gene expression, cell cycle progression and genomic stability (15), it is conceivable that PARP inhibitors may produce multiple unwanted side effects. Thus, it is of great clinical significance to find new approaches that can not only decrease PARP-1 toxicity at more delayed time points by blocking PARP-1-mediated downstream events, but also avoid the toxic effects produced by direct PARP-1 inhibition. Our latest studies have suggested that NAD$^+$ administration may be one of these novel approaches.

7.3. *In vivo* studies have suggested the therapeutic potential of NAD$^+$ for PARP-1-mediated diseases

One of the major concerns for drug delivery into the brains is that the drug delivery is often significantly limited by blood-brain barriers (178). A number of studies, including our own studies, have found that the drug delivery by the intranasal approach can produce neuroprotective effects in several brain disease models (179). This approach could have the following merits over traditional approaches: First, it may deliver drugs into the brains by bypassing blood-brain barriers, which is one of the major obstacles for treating brain diseases; second, it could reduce the probability that the protective effects of certain drugs on the CNS may result from the drug effects on peripheral systems; and third, it may decrease the amount of drugs needed to affect CNS, which could significantly decrease the cost of treatments. Notably, our study has shown that intranasal delivery of gallotannin (GT), a PARG inhibitor (135, 136), was dramatically more effective than i.v. injection of GT in decreasing ischemic brain damage (180): GT can decrease infarct formation by at least 50% even when administered 5 hrs after ischemic onset, while i.v. injection of GT did not produce significant protective effects even when administered immediately after reperfusion.

Based on this information, we applied intranasal delivery approach in delivering NAD$^+$ into the brains to test our hypothesis that NAD$^+$ may be used to decrease ischemic brain damage (181). We found that intranasal administration with NAD$^+$ is capable of increasing NAD$^+$ levels in rat brains. In a rat model of 2-hour transient focal ischemia, intranasal administration with 10 – 20 mg / kg NAD$^+$ at 2 hr after ischemic onset can decrease infarct formation and neurological deficits by greater than 80%. These results suggest that NAD$^+$ may be used as a novel therapeutic agent for treating cerebral ischemia and other PARP-1-mediated diseases.

8. PERSPECTIVES

Increasing evidence has suggested that NAD$^+$ and NADH play critical roles not only in energy metabolism and mitochondrial functions, but also in cell death, aging and most of the major cellular functions. These findings have fundamental changed our understanding about NAD$^+$ and NADH, suggesting the critical importance of these molecules in various aspects of life.

Numerous studies have indicated that ATP and Ca^{2+} play particularly important roles in virtually every major biological activities as well as in cell death and aging: Intracellular Ca^{2+} mediates numerous biological processes such as muscle contraction, neurotransmitter release, learning and memory as well as cell death (83, 106, 182-186); similarly, ATP not only acts as the basic energy molecule for most biological processes, but also mediates signal transduction by acting as the substrate for numerous protein kinases and acting as a crucial extracellular signaling molecule (1, 47, 187). Based on the increasing information indicating the critical biological activities of NAD$^+$ and NADH, I attempt to hypothesize that NAD$^+$ and NADH, together with ATP and Ca^{2+}, are four most fundamental components in life. These three specific molecules and one specific ion appear to be of paramount importance in most biological activities, cell death and aging. Future studies that further test this hypothesis may be warranted.

While remarkable progresses have been made, many crucial questions about the biological properties of NAD$^+$ and NADH remain unanswered. In my viewpoint, the following research topics may be of particular significance:

First, future studies are needed to determine the regulations of subcellular NAD$^+$ and NADH distribution under both biological and pathological conditions in various cell types, in light of the recent evidence suggesting the existence of NMNATs and PARP activities in subcellular organells. It is of particular interest to determine potential post-translational regulation of NADH shuttles and the enzymes involving in NAD$^+$ and NADH synthesis.

Second, it is warranted to further determine the interactions among NAD$^+$ / NADH-dependent proteins, including PARPs, ARTs, sirtuins, ADP-ribosyl cyclases / ADPR hydrolases and dehydrogenases. There have been several pieces of information suggesting significant interactions among these proteins, for example, recent studies have suggested close interactions between PARP-1 and SIRT1: SIRT1 was reported to mediate PARP-1-induced cell death (125); and SIRT1 deficiency led to increased PARP-1 activity (126). The latter finding suggests that SIRT1 could be an inhibitory factor of PARP-1. If this notion is solidly established, PARP-1 and SIRT1 would, like PARP-1 and caspase-3, appear to be mutually inhibitory, since it is known that PARP-1 can lead to inhibition of SIRT1 by depletion NAD$^+$. Because both PARP-1 and SIRT1 can affect multiple cellular functions and these two enzymes have common substrates such as histones and p53, future investigation into the interactions between these two critical enzymes is needed.

Third, it is of interest to determine the regulation of the NAD$^+$ / NADH-dependent enzymes on the levels of both post-translational modifications and gene expression. For example, recent studies have suggested that both cADPR and ADP-ribose can significantly affect calcium homeostasis by interacting with ryanodine receptors and TRPM2 receptors, respectively. Because nearly all of the known ADP-ribosyl cyclases are also cADPR hydrolases, potential post-translational modifications of these bifunctional enzymes may determine the levels of cADPR and ADP-ribose, thus determining the relative contributions of ryanodine receptors and TRPM2 receptors to calcium homeostasis.

Fourth, extensive studies would be needed to further to determine the biological and pathological implications of the notion that extracellular NAD$^+$, possibly also NADH, may be novel extracellular signaling molecules. The following questions remain to be answered: (a) How are extracellular NAD$^+$ and NADH levels regulated? (b) Are there receptors for NAD$^+$ and NADH on plasma membranes of cells? (c) What are the relationships between NAD$^+$ / NADH-mediated extracellular signaling and the purinergic receptor-mediated signaling? The recent finding that the ecto-ARTs mediate P2X$_7$R opening suggests that these interactions do exist.

Fifth, to search for the new strategies for slowing aging and treating various diseases including cerebral ischemia and cancer by targeting at NAD$^+$ / NADH metabolism and NAD$^+$ / NADH-dependent enzymes. Cellular and tissue NAD$^+$ levels may be directly modulated by direct NAD$^+$ administration, as we have conducted in the brain ischemia study. Alternatively, NAD$^+$ and NADH metabolism may be modulated by targeting at the enzymes involving in NAD$^+$ and NADH synthesis. Many studies have indicated significant therapeutic potential of several NAD$^+$ / NADH-dependent enzymes, such as PARP-1 and sirtuins. Recent findings have further implicated novel therapeutic potential of NAD$^+$/NADH-dependent

enzymes, for example, in light of the latest findings that ADP-ribose and O-acetyl-ADP-ribose are novel modulators of calcium homeostasis, the enzymes mediating the metabolism of these two factors may be targeted for modulating calcium homeostasis, that plays crucial roles in cell death, aging and various diseases (83, 106, 182-186).

During the last 10 years our understanding about NAD$^+$ and NADH has been dramatically improved, which may be exemplified by the numerous critical findings about PARPs and sirtuins. It is expected that our understanding about NAD$^+$ and NADH would be further rapidly improved, considering that many crucial questions in this field still remain unanswered. It is conceivable that future studies would further establish the roles of NAD$^+$ and NADH as fundamental mediators of cell death, aging and various biological functions. Our solid grasp of the biological properties of these molecules would be not only critical for answering some fundamental questions about life, but also essential for novel strategies for slowing aging and treating many diseases.

9. ACKNOWLEDGMENTS

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