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NAD^+ and NADH in brain functions, brain diseases and brain aging

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1. ABSTRACT

Numerous studies have suggested that NAD^+ and NADH mediate multiple major biological processes, including calcium homeostasis, energy metabolism, mitochondrial functions, cell death and aging. In particular, NAD^+ and NADH have emerged as novel, fundamental regulators of calcium homeostasis. It appears that most of the components in the metabolic pathways of NAD^+ and NADH, including poly(ADP-ribose), ADP-ribose, cyclic ADP-ribose, O-acetyl-ADP-ribose, nicotinamide and kynurenine, can produce significant biological effects. This exquisiteness of NAD^+ and NADH metabolism could epitomize the exquisiteness of life, through which we may grasp the intrinsic harmony life has evolved to produce. The exquisiteness also suggests a central regulatory role of NAD^+ and NADH in life. It is tempted to propose that NAD^+ and NADH, together with ATP and Ca^{2+}, constitute a Central Regulatory Network of life. Increasing evidence has also suggested that NAD^+ and NADH play important roles in multiple biological processes in brains, such as neurotransmission and learning and memory. NAD^+ and NADH may also mediate brain aging and the tissue damage in various brain illnesses. Our latest studies have suggested that NADH can be transported across the plasma membranes of astrocytes, and that NAD^+ administration can markedly decrease ischemic brain injury. Based on this information, it is proposed that NAD^+ and NADH are fundamental mediators of brain functions, brain senescence and multiple brain diseases. Because numerous properties of NAD^+ and NADH remain unclear, future studies regarding NAD^+ and NADH may expose some fundamental mechanisms underlying brain functions, brain pathologies and brain aging.

2. INTRODUCTION

A number of recent studies have substantially improved our understanding regarding NAD^+ and NADH. These two molecules can no longer be considered classic coenzymes of numerous dehydrogenases. Instead, NAD^+ and NADH appear to modulate nearly all of the major biological processes, including calcium homeostasis, energy metabolism, mitochondrial functions, gene expression, cell death and aging (1-3). As proposed in a previous review, NAD^+ and NADH, together with ATP and Ca^{2+}, may be four most fundamental regulatory factors in life (1).

While there is distinct insufficiency of the studies regarding the roles of NAD^+ and NADH in brains, many studies have provided information suggesting emerging novel paradigms for the roles of NAD^+ and NADH in brain functions, brain diseases and brain aging. Since there is no recent literature that generalizes the cumulating information in this field, this review has been written to provide an overview regarding this interesting topic. Through this generalization novel notions regarding NAD^+ and NADH in brains may be generated, and new research directions may be exposed.

3. NAD^+ AND NADH METABOLISM IN BRAINS

3.1. NAD^+ and NADH synthesis in brains

Vitamin B3, including nicotinic acid (niacin) and nicotinamide (niacinamide), is essential for the *de novo* synthesis of NAD^+. The *de novo* pathway and the salvage pathway are two major known pathways of NAD^+ biosynthesis (4, 5). The *de novo* pathway is necessary for NAD^+ generation when niacin is not available (4, 5), through which mammals can synthesize nicotinamide-containing nucleotides via the kynurenine pathway: After its generation from L-Tryptophan, L-kynurenine is used for the production of quinolinic acid. Nicotinate mononucleotide is subsequently generated from quinolinic acid, which is used for NAD^+ synthesis. In contrast, in the salvage pathway NAD^+ is generated directly from nicotinamide-containing molecules (4-6).

It is noteworthy that the kynurenine pathway leads to generation of several neuroactive intermediates, including quinolinic acid, kynurenic acid and 3-hydroxykynurenine: Quinolinic acid is an agonist of the excitotoxic N-methyl-D-aspartate (NMDA) receptor; kynurenic acid is an antagonist of both NMDA receptors and α7 acetylcholine receptor; and 3-hydroxykynurenine is a free radical generator (7-9). Interestingly, the enzymes in the kynurenine pathway, which determine the levels of these neuroactive compounds, are preferentially localized in astrocytes and microglia in brains (8). Due to the significant biological effects of those compounds in brains, it is conceivable that alterations of the *de novo* NAD^+ synthesis pathway may significantly affect brain functions. Indeed, cumulative evidence has suggested that under physiological conditions these neuroactive intermediates may modulate several neurotransmitter systems (7-9). Of particular interest, quinolinic acid and kynurenic acid have been indicated in the pathogenesis of epilepsy, Huntington's chorea, as well as several inflammatory neurological disorders (7-9). Based on this information, many studies have been conducted to determine the therapeutic potential of the drugs targeting at the kynurenine pathway for multiple neurological diseases (7-9).

Increasing evidence has also indicated significant biological activities of nicotinamide and nicotinic acid --- the other two important components in NAD^+ metabolism. Nicotinamide has been shown to enhance energy metabolism, activate Akt and inhibit poly(ADP-ribose) polymerases (PARPs) and sirtuins (10-13). Many studies have suggested the therapeutic potential of nicotinamide for certain diseases such as cerebral ischemia (10-13). Nicotinic acid can also significantly affect brain functions by such pathways as inducing glutamate release (14).

There have been no sufficient studies regarding the NAD^+ and NADH synthesis in brains. Spector determined the unidirectional influx of nicotinamide across the cerebral capillaries in rat brains, suggesting that nicotinamide can be transported rapidly and bidirectionally through the blood-brain barriers (BBB) by a high capacity transport system (15). This information is of significance for understanding the effects of nicotinamide administration on brain functions. It was also suggested that in rat cortical astrocytes, nicotinate is transported through a H^+-coupled, saturable transport system (16). Our latest study has suggested that intranasal NAD^+ administration can significantly increase NAD^+ levels in brains and profoundly reduce ischemic brain damage (1). However, the potential transport mechanisms of NAD^+ and NADH across BBB are largely unknown.

Current studies have indicated presence of three isoforms of human NMNATs --- NMNAT1 – 3 (5, 6, 17). While NMNAT1 is a nuclear enzyme, NMNAT2 and NMNAT3 may be localized in Golgi complex and mitochondria, respectively (5, 6, 17). This new information suggests that there could be relatively independent NAD^+ synthesis machineries in these subcellular organelles (6). Future studies are needed to determine the properties of NMNATs in neurons and glial cells under both physiological and pathological conditions. Recently a novel human NMNAT cDNA was cloned from human brains (18), which shares only 35% amino acid sequence with the human NMNAT-1. The expression of this new NMNAT is highly restricted to brain, heart and muscle tissues. A recent study also suggested the presence of a new pathway for NADH synthesis: NADH could be generated directly from the reduced form of nicotinamide mononucleotide (NMNH) and ATP through the catalysis of NMNATs (6). It remains to be determined if the same mechanism also occurs in brains.

3.2. Catabolism of NAD^+ and NADH in brains

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:

First, PARPs --- A family of enzymes that consume NAD^+ to produce nicotinamide and poly(ADP-ribose) (PAR) on target proteins (19). While for long time PARP-1 was the sole known PARP, recent studies have indicated the existence of at lease ten members in the PARP family (20, 21). PARP-1 is the most intensively studied PARP, which appears to play important roles in regulation of DNA repair, gene expression, cell cycle and genomic stability (1, 19, 21). Rapidly growing evidence has also indicated critical roles of PARP-1 in a variety of biological and pathological processes, including inflammation, cell death, learning and memory and functions of neurotrophic factors (19, 20). Excessive PARP-1 activation has been indicated as an important factor in ischemic injuries of various organs, MPTP-induced parkinsonism, traumatic brain damage, hypoglycemic brain damage, diabetes and shock (1, 19).

Second, mono(ADP-ribosyl)transferases (ARTs) --- A family of enzymes that use NAD^+ as a substrate to produce mono(ADP-ribosyl)ation of proteins (22, 23). While most mono(ADP-ribosyl)ation appears to be mediated by bacteria toxins, recent studies have suggested that ART1-5 are expressed in various cell types (24, 25). Of particular interest, it was indicated that the ectoenzyme ART2 on the plasma membranes of Treg cells --- a subset of T cells which mediate immunological activities --- can produce mono(ADP-ribosyl)ation of $P2X_7$ receptors, leading to Treg cell apoptosis (26, 27). There is a lack of information regarding ARTs in neurons and glial cells. However, our studies have shown that treatment of astrocytes with 10 mM NAD^+ for 24 hours did not decrease cell survival (28), and intranasal NAD^+ administration profoundly decreased ischemic brain injury (29). These results appear to argue against the possibility that extracellular NAD^+ promotes death of astrocytes and neurons by promoting ART-mediated $P2X_7R$ opening.

Third, the NAD^+-dependent histone deacetylases, also called Sir2 family proteins or sirtuins, produce deacetylation of histones and non-histone proteins by consuming NAD^+. This process can profoundly affect several key biological processes, including aging, carcinogenesis and cell death (30, 31).

Fourth, 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 (2). The major known mammalian ADP-ribosyl cyclase is CD38 (2). As discussed below, the cyclic ADP-ribose generated by CD38 could play critical roles in many biological processes in brains and other tissues and organs.

It has been indicated that PARP-1 plays a key role in consuming NAD^+ under conditions when DNA damage occurs (32). However, under physiological conditions there are similar levels of NAD^+ in the brains of PARP-1 knockout mice and the brains of wild-type mice (33). This observation suggests that, at least in brains, PARP-1 is not a major NAD^+-consuming enzyme under physiological conditions. In contrast, recent studies have raised an intriguing possibility that CD38 plays a key role in NAD^+ metabolism under physiological conditions: There were significant increases in NAD^+ in brain, lung, and kidney in the $CD38^{-/-}$ mouse (34, 35). Aksoy *et al.* determined the NADase activities and NAD^+ levels in various tissues from both wild-type and CD38 deficient mice (35). They found that the NADase activity is nearly absent in the plasma membranes, mitochondria, sarcoplasmic reticulum, and nuclei in most tissues from CD38 deficient mice. The tissue levels of NAD^+ in CD38 deficient mice are 10- to 20-fold higher than that in wild-type animals (35). Based on this information, I attempt to propose that CD38 is a key regulator of cellular NAD^+ levels under physiological conditions, while PARP-1 is the key factor determining intracellular NAD^+ levels when significant DNA damage occurs.

3.3. Subcellular distribution of NAD^+ and NADH

Mitochondrial membranes are impermeable to NAD^+ and NADH, while nuclear membranes could be freely permeable to NAD^+ and NADH. It has been reported that mitochondria of such cell types as myocytes contain a large portion of intracellular NAD^+ (36-38). However, the relative distribution of NAD^+ and NADH in the cytosol and mitochondria of neurons and astrocytes remains unclear, which requires future investigation. It is also of great interest to determine the potential alterations of the subcellular distribution of NAD^+ and NADH in neurons and glial cells under aging and various pathological conditions. Di Lisa *et al.* suggested that mitochondrial permeability pore (MPT) opening can lead to mitochondrial NAD^+ release and subsequent hydrolysis of NAD^+ by NAD^+ glycohydrolase (36). However, it remains unclear if similar MPT-dependent NAD^+ release also occurs in neurons and astrocytes. Future studies are also warranted to determine if MPT may lead to mitochondrial NADH release.

4. NAD^+ AND NADH TRANSPORT IN BRAINS

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

It was generally thought that NAD^+ and NADH can not be transported across plasma membranes of cells. However, recent studies suggest that Connexin 43 hemichannels could mediate NAD^+ gradient-dependent NAD^+ flux across fibroblast plasma membranes (39). Both the study by Varderio *et al.* and the study by our research group have also suggested that NAD^+ can be transported across the plasma membranes of murine astrocytes (28, 40, 41). However, our studies suggested that $P2X_7$ receptors ($P2X_7R$), instead of connexin43 hemichannels, mediate the NAD^+ transport in astrocytes. We found that bzATP, a $P2X_7R$ agonist, can induce NAD^+ release from murine astrocytes (42). The NAD^+ release was blocked by both the P2 receptor antagonist PPADS and the RNA silencing of $P2X_7R$, suggesting that activation of $P2X_7R$ can lead to NAD^+ release (42). Because $P2X_7R$ has been indicated as

a key factor under such pathological conditions as spinal cord injury (43), our results raise the possibility that the $P2X_7R$-mediated NAD^+ release may contribute to the tissue injuries under these pathological conditions.

A study by Verderio *et al.* also suggested that the shear stress produced by vigorous washes can lead to NAD^+ release (40). Smyth *et al.* detected constitutive and nerve-evoked overflow of NAD^+ (44). It is of great interest to further determine the physiological and pathological stimuli that can induce NAD^+ release, considering the findings suggesting signaling functions of extracellular NAD^+ (26, 27). Future studies are also warranted to determine if $P2X_7R$ is the common pathway mediating NAD^+ release induced by various stimuli.

Recently we have also provided first evidence suggesting that NADH can be transported across the plasma membranes of astrocytes by a $P2X_7R$-mediated mechanism (45): A critical role of $P2X_7R$ in the NADH transport is indicated by the findings that a reduction of $P2X_7R$ by RNA silencing led to decreased NADH transport, and transfection of $P2X_7R$-deficient HEK293 cells with mouse $P2X_7R$ cDNA increased NADH transport. Our 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 needed 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.

4.2. NADH transport across mitochondrial membranes

While mitochondrial inner membranes are not permeable to NADH and NAD^+ (46), the reducing equivalents of cytosolic NADH can be shuttled into mitochondria by the malate-aspartate shuttle (MAS) or the glycerol-3-phosphate shuttle (GPS) (46, 47). MAS consists of multiple proteins, including cytosolic malate dehydrogenase, aspartate transaminase, mitochondrial aspartate-glutamate carrier, and mitochondrial malate dehydrogenase, which mediates the following biological process: In cytosol oxloacetate is generated from aspartate by aspartate transaminase, which is converted to malate by cytosolic malate dehydrogenase with conversion of NADH to NAD^+. The malate is subsequently transported into mitochondria, which is used by the mitochondrial TCA cycle enzyme malate dehydrgenase for generating NADH from NAD^+. The NADH is subsequently oxidized through the Complex I of the electron transport chain, leading to generation of three ATP.

GPS is mainly composed of cytosolic glycerol-3-phosphate dehydrogenase (cGPD) and mitochondrial glycerol-3-phosphate dehydrogenase (mGPD) that is localized at the outer surface of the inner mitochondrial membranes. Through this shuttle glycerol-3-phosphate is generated with conversion of NADH to NAD^+ in cytosol. Subsequently the glycerol-3-phosphate is converted to dihydroxyacetone by the mGPD with generation of $FADH_2$ from FAD. The $FADH_2$ is subsequently used for generation of 2 ATP through the Complex II of the electron transport chain.

It appears that there are several major differences between these two shuttles: MAS leads to increased NADH in mitochondria, which then feeds into the Complex I leading to generation of three ATP, while GPS converts cytosolic NADH to mitochondrial $FADH_2$, which feeds into the Complex II leading to generation of 2 ATP. Another interesting difference between these two shuttles is that MAS is much more complicated than GPS. It remains unclear why two types of shuttles are needed in many cell types. It is tempted to propose that each of these two shuttles could have their distinct biological advantages: MAS could be subjected to greater regulation at multiple steps, and can lead to higher levels of ATP generation; while GPS could lead to relatively rapid generation of ATP because of its relative simplicity.

It is noteworthy that cytosolic NADH can be oxidized to NAD^+ through the NADH shuttles, the lactate dehydrogenase-catalyzed pyruvate-lactate conversion and other dehydrogenase-caralyzed reactions. Because these pathways could have major effects on cellular energy metabolism and other cellular functions, the regulation of the NADH shuttles may profoundly affect cellular functions due to its impact on the fate of cytosolic NADH. It is also likely that alterations of the NADH shuttles may produce major pathological consequences. Interestingly, Lu *et al.* found a nearly 60-fold increase in mGPD expression after ischemic insults, which was the most dramatic change of gene expression in their study (48). This result raises the possibility that the GPS upregulation might be one of the rapid cellular responses to certain stress conditions, which may be programmed to efficiently shuttle cytosolic NADH into mitochondria for ATP generation.

There is distinct insufficiency of the information regarding the NADH shuttles in brains. Several studies have suggested that MAS is the major NADH shuttle in neurons, while GPS is the major NADH shuttle in oligodendrites (47). However, the nature of the NADH shuttles in astrocytes is elusive: The studies using a MAS inhibitor suggested that GPS may exist in astrocytes, while another study argued against the significance of GPS in brains (47, 49). The studies on these shuttles have been significantly limited by the limited number of MAS inhibitors and the absence of selective GPS inhibitors (47, 49). Thus, molecular approaches and more specific inhibitors targeting at the NADH shuttles are critically needed.

Recently several studies have used MAS- or GPS-deficient mice to determine the biological functions of the shuttles. The latest study by Pardo *et al.* provided critical information regarding the regulation of NADH shuttles in neurons (50). Because they found that ARALAR --- the neuronal Ca^{2+}-binding mitochondrial aspartate-glutamate carrier, has Ca^{2+} binding domains facing the extramitochondrial space and plays a role in MAS, they determined the effects of Ca^{2+} signals on the NADH

shuttling activity. It has been well established that large $[Ca^{2+}]_i$ can significantly increase mitochondrial NADH generation by activating three key dehydrogenases in the mitochondria, including pyruvate dehydrogenase, NAD^+-isocitrate dehydrogenase and oxoglutarate dehydrogenase (51). Pardo *et al.* indicated a novel mechanism by which small Ca^{2+} signals that fall below the activation range of the Ca^{2+} uniporters can affect mitochondrial NADH levels: The small Ca^{2+} signals can enhance NADH shuttling from cytosol to mitochondria by activating ARALAR. Since it is increasingly clear that the NADH shuttles could produce profound impact on cellular activities, future studies are warranted to further determine the post-translational regulation of the NADH shuttles in neurons and glial cells under both physiological and pathological conditions. It may be of particular interest to determine the potential effects of oxidative stress and protein phosphorylation on the NADH shuttles.

5. ROLES OF NAD^+ AND NADH IN BRAIN FUNCTIONS

5.1. Roles of NAD^+ and NADH in the calcium homeostasis of brains

5.1.1. General information about the calcium homeostasis in brains

Ca^{2+} is one of the most critical factors in nearly all of the major biological processes in brains, including neurotransmitter release, long-term potentiation and long-term depression, brain development, and neuronal plasticity (52-56). Calcium dyshomeostasis has also been indicated in brain aging and in various brain diseases including ischemic brain injury and Alzheimer's disease (56-64). Thus, elucidation of the regulatory mechanisms of the calcium homeostasis is of critical importance for understanding brain functions, the pathologies of many brain diseases as well as brain aging.

There are multiple cellular machineries for regulating the calcium homeostasis of neurons and glial cells. This investigator attempts to categorize these machineries into two major systems: The 'Intracellular Ca^{2+} ($[Ca^{2+}]_i$)-Increasing System (ICIS)' and the '$[Ca^{2+}]_i$-Decreasing System (ICDS)'. ICIS is mainly composed of the ICIS on plasma membranes as well as intracellular ICIS. The ICIS on plasma membranes consist of various proteins on plasma membranes which allow Ca^{2+} influx into cells, including voltage-dependent Ca^{2+} channels, glutamate receptors, non-selectively Ca^{2+} channels, transient reduction potential (TRP) channels such as TRPM2 and TRPM7, as well as $P2X_7$ receptors. The intracellular ICIS mainly consists of the Ca^{2+}-releasing channels of intracellular Ca^{2+} stores, including the IP_3-gated Ca^{2+} channels and ryanodine receptors on ER membranes, the NAADP-mediated Ca^{2+} channels on lysosome membranes, the Na^+/Ca^{2+} exchangers of mitochondria as well as mitochondrial permeability transition (MPT) pores (65-68).

The ICDS mainly consists of various Ca^{2+}-pumping proteins and Ca^{2+}-buffering proteins. The Ca^{2+}-pumping proteins include the Ca^{2+} ATPase and Na^+/Ca^{2+} exchangers on plasma membranes, the Ca^{2+} ATPase on ER membranes and the Ca^{2+} uniporters on mitochondrial membranes (66-69); while the Ca^{2+}-buffering proteins include such proteins as calbindin and parvalbumin (70).

5.1.2. Major pathways by which NAD^+ and NADH regulate calcium homeostasis

Based on a fast growing body of information, this investigator proposes that NAD^+ and NADH are novel, key mediators of calcium homeostasis. NAD^+ may activate the ICIS by the following mechanisms:

First, ADP-ribosyl cyclases can generate cADPR from NAD^+, which is a most potent endogenous agonist of ryanodine receptors (71). The ecto-enzyme ADP-ribosyl cyclases or unidentified ADP-ribosyl cyclases locating within the cytosol generate cADPR from NAD^+ in many cell types, which is a potent Ca^{2+} mobilizing messenger via activating type 2 and 3 ryanodine receptors (71, 72).

Second, cumulating evidence has demonstrated that transient receptor potential (TRP) family proteins play crucial roles in a variety of cellular functions (73, 74), e.g., TRPM7 receptors were reported to mediate oxygen-glucose deprivation-produced neurotoxicity (75, 76). NAD^+ may lead to increased Ca^{2+} influx into cells by activating TRPM2 receptors after its conversion to ADP-ribose, cADP-ribose or O-acetyl-ADP-ribose: ADP-ribose, that could be generated from NAD^+ by NAD^+ glycohydrolases or PARPs / poly(ADP-ribose) glycohydrolase (PARG), is an activator of TRPM2 receptors (77, 78); Sir2 family proteins can generate O-acetyl-ADP-ribose that could directly bind to the cytoplasmic domain of TRPM2 channels and activate the channels (79); and recent studies have suggested that cADPR can also affect calcium homeostasis by gating TRPM2 receptors (80). The ADP-ribose / TRPM2 receptor pathways could mediate oxidative cell death by the following mechanisms (81-83): Oxidative damage induces PARP-1 activation and PAR formation. The subsequent generation of ADP-ribose from PAR can lead to opening of the Ca^{2+}-permeable TRPM2 receptors leading to cell death.

Third, NAD^+ can also modulate calcium metabolism by promoting mono(ADP-ribosyl)ation of $P2X_7R$: The ecto-ARTs-mediated mono(ADP-ribosyl)ation of $P2X_7R$ has been shown to promote opening of $P2X_7R$ (26), which can lead to Ca^{2+} influx (84).

In addition, NAD^+ may indirectly affect calcium homeostasis after its conversion to $NADP^+$ that can be converted to NAADP --- an agonist of the calcium channels on lysosome membranes (2, 72). Due to the critical roles of energy metabolism and mitochondrial activity in calcium homeostasis, it is conceivable that NAD^+ and NADH may also affect calcium homeostasis by mediating energy metabolism and mitochondrial functions.

It has been reported that NADH can also affect ryanodine-sensitive Ca^{2+} channels: Hypoxia-induced increases in NADH can directly increase Ca^{2+} release from IP_3-gated Ca^{2+} channels on ER membranes of cerebellar Purkinje cells and nerve growth factor-differentiated PC12 cells (85); and a recent study has further suggested that the

GAPDH associated with IP_3-gated calcium channels can locally generate NADH to promote the opening of IP_3-gated Ca^{2+} channels (86).

5.1.3. Cyclic ADP-ribose is a major NAD^+-dependent factor mediating calcium homeostasis

Pawlikowska *et al.* assessed the ADP-ribosyl cyclase activity in cortical neuronal and astrocyte cultures: No detectable ADP-ribosyl cyclase activity was found in neuron-enriched / astrocyte-poor cultures. In contrast, astrocyte cultures had an ADP-ribosyl cyclase activity of 0.84 nmol cADPR / mg protein / hr (87). The demonstration of extracellular ADP-ribosyl cyclase and cADPR hydrolase activities in astrocytes implicates a role of extracellular cADPR in signal transduction and in cell-cell communications in brains. CD38 activities have also been demonstrated *in vivo* in adult rat cerebellum, which may generate cADPR by using the trace amounts (11.5 $^+$/- 3.8 nM) of NAD^+ found in the basal interstitial fluid of the cerebellum. These results have implicated that the cADPR generated by the ectoenzyme CD38 may modulate the calcium homeostasis in cerebellum (88). Ceni *et al.* also showed that endogenous concentrations of cADPR are much higher in embryonic and neonate mouse brain compared with the adult tissues (89).

cADPR is known to modulate intracellular calcium levels and to be involved in multiple calcium-dependent processes, including synaptic transmission, plasticity and neuronal excitability (89). It has been suggested that metabotropic glutamate receptor activation could induce CD38 activation by a mechanism mediated by NO, cyclic GMP and protein kinase G, leading to cADPR-dependent Ca^{2+} release from ryanodine receptors (90). Hashii *et al.* has further indicated that cADPR can not only activate ryanodine receptors as a direct agonist but also interact with L-type voltage-activated calcium channels as an indirect agonist (91). It may be particularly interesting to further investigate the regulatory mechanisms of ADP-ribosyl cyclase activities under both physiological and pathological conditions. In addition to the studies indicating that the NO / cyclic GMP pathway can activate the enzyme, it has also been suggested that muscarinic acetylcholine receptors may regulate ADP-ribosyl cyclase activities by interacting with trimeric G proteins (92).

5.2. Roles of NAD^+ and NADH in the energy metabolism and mitochondrial functions of brains

NAD^+ and NADH belong to the most important factors in energy metabolism, which mediate nearly all major aspects of energy metabolism (1, 3, 46). NAD^+ and NADH produce these effects by acting as the coenzymes for the dehydrogenases in metabolic pathway, and by acting as electron donors of the mitochondrial electron transport chain. The two major intracellular locations where NAD^+ and NADH affect energy metabolism are cytosol and mitochondria.

5.2.1. The effects of NAD^+ and NADH on energy metabolism in cytosol

In cytosol NAD^+ and NADH mediate glycolysis by acting as the co-factors for the rate-limiting glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). NAD^+ and NADH also mediate other important energy metabolism-related reactions occurring in cytosol, such as the lactate dehydrogenase-catalyzed lactate-pyruvate conversions. Cytosolic NADH can further directly affect mitochondrial oxidative phosphorylation, since the reducing equivalents of NADH can be shuttled into mitochondria through the NADH shuttles.

It is noteworthy that GAPDH can mediate not only glycolysis but also multiple other biological processes: First, GAPDH can affect cellular redox state by influencing the glucose flux through pentose phosphate pathway, from which NADPH is generated (46). Second, it was found that GAPDH binds to the IP_3-gated Ca^{2+} channels in neurons to mediate the IP_3-gated Ca^{2+} channel activities by locally generating NADH (86). Third, GAPDH has been established as a mediator of apoptosis under many conditions: GAPDH binds to Siah which is then translocated into nucleus to mediate apoptosis (93-96). Fourth, GAPDH was also found to mediate GABA(A) receptor activity by phosphorylating the receptors (97). These effects of GAPDH implicate the exquisite cellular mechanisms that coordinate glycolytic activity with other biological processes. Considering the rapidly increasing evidence indicating multiple functions of GAPDH as well as the finding showing distinct abundance of GAPDH genes in human genome, it is likely that additional novel functions of GAPDH may be discovered. Future studies are warranted to further determine how NAD^+ and NADH can modulate the interactions between glycolysis and other biological processes by affecting GAPDH under both normal and disease state.

The lactate-pyruvate conversions may be of particular importance for brain energy metabolism, since it has been suggested that astrocytes may provide lactate as an energy fuel for neurons, which has been called astrocyte-neuron lactate shuttle (ANLS). Some researchers have challenged this hypothesis and defended the classical notion that glucose is the major energy substrate of neurons (98). Aubert & Costalat suggested the critical significance of determining the changes of the NADH/NAD^+ ratios in neurons and astrocytes for further testing the ANLS hypothesis (98). It is noteworthy that lactate generation is a causative factor for tissue acidosis under such pathological conditions as cerebral ischemia. Because increasing evidence has indicated critical roles of acidosis in such diseases as ischemic brain damage (99, 100), it is conceivable that the NAD^+/NADH-dependent conversions between lactate and pyruvate could produce major effects on the energy metabolism and integrity of brains under physiological and pathological conditions.

5.2.2. The effects of NAD^+ and NADH on mitochondrial energy metabolism

NAD^+ and NADH play pivotal roles in both of the two major machineries of mitochondrial energy metabolism: The tricarboxylic acid (TCA) cycle and the electron transport chain. NAD^+ and NADH are the coenzymes for the three rate-limiting enzymes in mitochondrial TCA cycle (51). NADH is also one of the

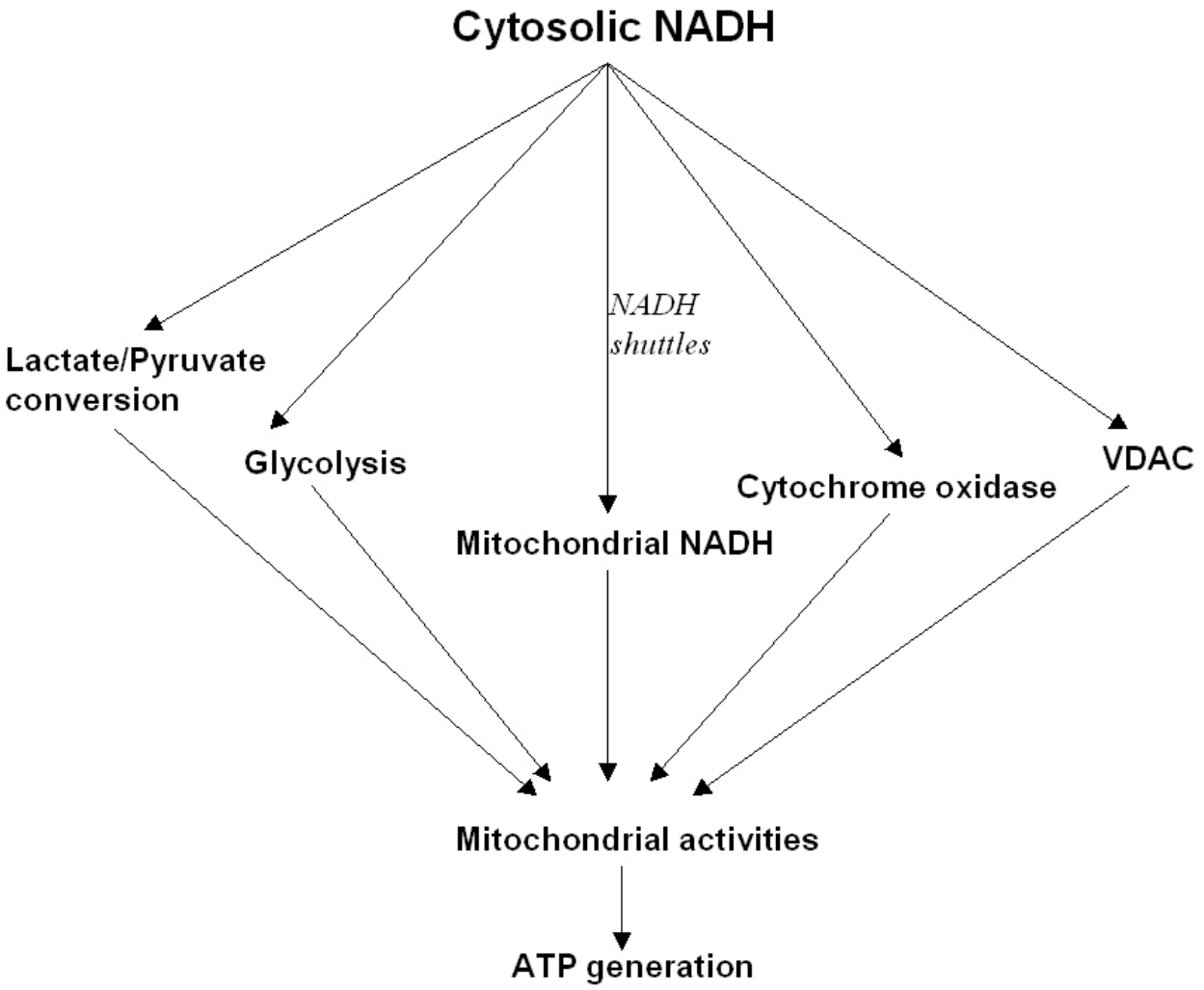

Figure 1. Diagrammatic presentation of the effects of cytosolic NADH on mitochondrial activities and ATP generation.

major electron donors for electron transport chain (46). Recent studies have suggested novel mechanisms by which NAD^+ and NADH may modulate energy metabolism. For examples, NAD^+ may affect energy metabolism by regulating Sir2 family proteins, which can modulate glycolysis / glycogenesis (101, 102). A latest study reported that Sir2 can mediate the activity of acetyl-CoA synthease (103).

In addition to their major roles in mitochondrial TCA cycle and electron transport chain, NAD^+ and NADH can affect mitochondrial activities via other mechanisms: First, NADH can directly interact with and inhibit voltage-dependent anion channels (VDAC), that is a component of MPT pore and controls the transport of small molecules across mitochondrial membranes (104); second, NAD^+ / NADH ratio is an important regulator of mitochondrial permeability transition (MPT) (105); and third, cytosolic NADH could be oxidized directly by cytochrome oxidase to increase mitochondrial membrane potential (106). 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 (65); and by regulating Sir2 family proteins that may influence mitochondrial metabolism via affecting p53 activity (107, 108). A diagrammatic presentation of the effects of cytosolic NADH on mitochondrial activity and ATP generation is shown in Figure 1.

NAD(P)H imaging has been widely used in brain research for assessing energy metabolism and mitochondrial activities. As an example of this interesting field, some recent studies have investigated the mechanisms underlying the excitatory stimulation-produced biphasic NAD(P)H fluorescence transients in hippocampal slices. It has been suggested that the oxidation phase is a consequence of mitochondrial metabolism, while the reduction phase may result from glutamate uptake-triggered astrocytic glycolysis (109). However, the latest study of Brennan *et al.* suggested that NAD(P)H transients report mitochondrial dynamics, instead of glycolytic metabolism (110).

5.3. Roles of NAD+ and NADH in neuronal activity and neuron-astrocyte interactions

Piper *et al.* observed high basal levels of PARP activity and DNA strand breaks in certain neuronal populations of rat brains, which appear to result from glutamate neurotransmission involving NMDA receptors and neuronal nitric oxide synthase activity (111). They further found that inhibition of NMDA receptors decreased basal PARP activity and DNA damage and increased NAD^+ in primary cultured neurons (111), suggesting that the basal NMDA receptor-mediated signaling is sufficient to affect basal NAD^+ metabolism of the neurons. Laschet *et al.* also reported that GAPDH is a GABA(A) receptor kinase which could link glycolysis to neuronal inhibition (97): The GABA(A) receptors are modulated by glycolysis-dependent phosphorylation, which mediates fast inhibition in the brain. It is of interest to know if intracellular NAD^+ and NADH may influence the activities of GABA(A) receptors by affecting GAPDH.

Several recent studies have suggested significant roles of CD38 in neuron-astrocyte interactions: It was found that addition of NAD^+ to astrocyte-neuron co-cultures results in a delayed intracellular calcium transient in neurons, which is significantly decreased by glutamate receptor antagonists. These data suggest that astrocyte-to-neuron calcium signalling can be triggered by the CD38/cADPR system in astrocytes: The cADPR can increase cytosolic Ca^{2+} levels by activating raynodine receptors, which may cause glutamate release from astrocytes (40).

The levels of neuroactive compounds in the kynurenine pathway, such as quinolinic acid and kynurenic acid, are determined by the enzymes in the pathway, which are preferentially localized in astrocytes and microglia in brains (8). This information raises the possibility that the generation of the neuroactive compounds in glial cells may play significant roles in neuron-glial cell interactions. The astrocyte-neuron lactate shuttle hypothesis suggests that astrocytes can provide lactate as an energy fuel for neurons. This theory implicates that the properties of NADH and NAD^+ in astrocytes could mediate neuronal energy metabolism due to their effects on the lactate generation in astrocytes.

5.4. NAD+ and NADH in learning and memory

An ADP-ribosyl transferase activity was found in the hippocampal CA1 tissues, which was dramatically stimulated by NO and attenuated by two different inhibitors of ARTs (112). ART inhibitors were found to block long-term potentiation --- an important process in learning and memory. These results suggest that the NAD^+-dependent ARTs may play a significant role in long-term potentiation (112). Cohen-Armon *et al.* reported that the PARP-1 activity in neurons can mediate several forms of long-term memory in Aplysia (113). They proposed that rapid and transient decondensation of chromatin structure by poly(ADP-ribosyl)ation promotes the gene expression necessary for long-term memory without DNA damage. The study of Satchell further suggested a significant role of PARP activity in learning and memory (114): While moderate concentrations of PARP inhibitor INH2BP improved spatial memory acquisition after traumatic brain injury, high doses of the PARP inhibitor severely impair the cognitive functions of animals. Their study further suggested that the detrimental effects of the profound PARP inhibition may result from the inhibition of nuclear poly(ADP-ribosyl)ation of 14-3-3γ --- a protein involving in learning and memory.

Ca^{2+} released from presynaptic and postsynaptic intracellular stores plays important roles in activity-dependent synaptic plasticity, including long-term depression of synaptic strength. The study of Reyes-Harde *et al.* suggests that cADPR could mediate long-term depression (115): They found that blockade of cGMP-dependent protein kinase, cADPR receptors, or ryanodine-sensitive Ca^{2+} stores prevented the induction of long-term depression at Schaffer collateral-CA1 synapses. Due to the critical roles of Ca^{2+} homeostasis, energy metabolism and gene expression in learning and memory, NAD^+ and NADH may further affect cognitive functions by affecting these important biological properties.

5.5. Roles of NAD+ and NADH in the gene expression of brains

NAD^+ may affect gene expression through multiple mechanisms: First, sirtuins can affect gene expression by several pathways: Yeast Sir2 can silence gene transcription (116); sirtuins can also mediate the activities of the transcriptional factors such as p53 and forkhead transcription factors (117); and mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription (118). Second, PARP-1 can affect several transcriptional factors such as p53, AP-1 and NF-κB (32, 119). Third, NAD^+ is required for the activities of other PARPs such as tankyrases, which could also affect gene expression (120). Fourth, NADH regulates the activity of the corepressor carboxyl-terminal binding protein --- a transcriptional factor for development, cell cycle regulation and transformation (121); and NADH also regulates Clock:BMAL1 and NPAS2:BMAL1 --- the heterodimeric transcription factors modulating the gene expression in circadian clock (122). A diagrammatic presentation of the effects of NAD^+ and NADH on gene expression is shown in Figure 2.

5.6. Summary of the functions of the metabolites in the NAD+/NADH metabolic pathways

Cumulative evidence has suggested that nearly all of the metabolites in the metabolic pathways of NAD^+ and NADH can produce significant biological effects. A diagrammatic presentation of the effects of these metabolites is shown in Figure 3.

6. NAD+ AND NADH IN BRAIN DISEASES

6.1. NAD+ and NADH in ischemic brain injury

6.1.1. General information about ischemic brain injury

Stroke is one of the leading causes of death and long-term disability in the world. Ischemic stroke accounts for a great majority of stroke. While the only FDA-approved drug for treating stroke can produce significant

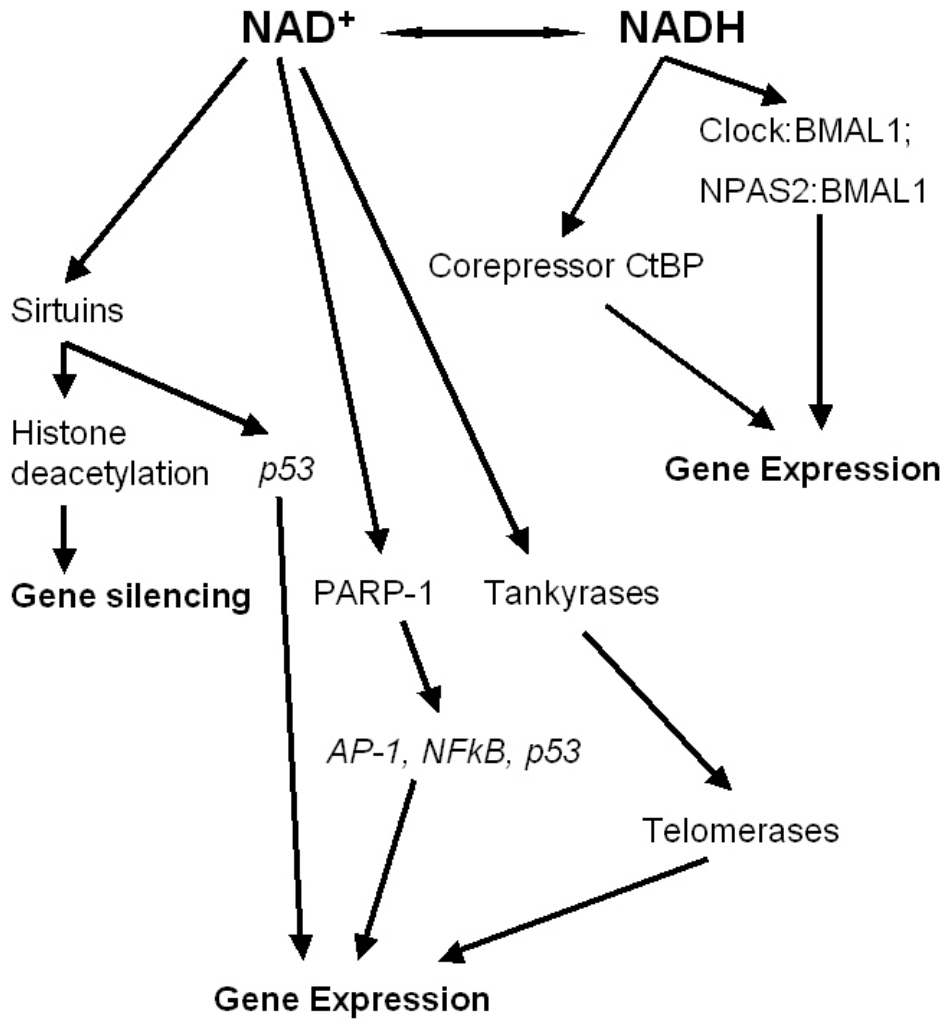

Figure 2. Diagrammatic presentation of the effects of NAD^+ and NADH on gene expression.

therapeutic effects for a small population of stroke patients when administered within a few hours after stroke, tissue plasminogen activator (tPA) can cause hemorrhage and other neurotoxic effects (123-125). Thus, further studies into the mechanisms of ischemic brain injury are of critical significance for establishing new treatments for stroke. Previous studies have provided significant amount of information regarding the mechanisms underlying ischemic brain damage. These studies have suggested that multiple interrelated factors, including oxidative stress, calcium disregulation, excitotoxicity, impaired energy metabolism, PARP-1 activation, apoptotic mechanisms, inflammation, activation of acid-sensing channels, protein aggregation, and cerebrovascular alterations, play important roles in ischemic brain injury (99, 126-140).

The promising results from the recent clinical trials using the free radical scavenger NXY-059 (141) further support the notion that oxidative damage plays a key role in ischemic brain damage (126, 127). Oxidative stress also plays a critical role in the brain injury induced by experimental intracerebral hemorrhage (142, 143). Due to the key roles of reactive oxygen species in ischemic brain injury, it is of interest to further understand the properties of oxygen in ischemic brain damage. Recently the oxygen levels in brains following ischemia-reperfusion were clearly characterized (144). Interestingly, hyperbaric oxygen is highly neuroprotective against brain damage induced by ischemia or subarachnoid hemorrhage (145-147).

Several research directions may be of particular interest for identifying the new strategies that can decrease ischemic brain damage with wide window of opportunity, i.e., the drugs can still be effective when applied multiple hours after ischemia: First, to identify the pathological mechanisms during the late phases after stroke; second, to determine the major differences between human and experimental animals in their biological responses to ischemic insults; and third, to search for new drug delivery approaches that can more effectively carry drugs to the brain regions of interest. With the increasing evidence suggesting critical roles of NAD^+ and NADH in calcium

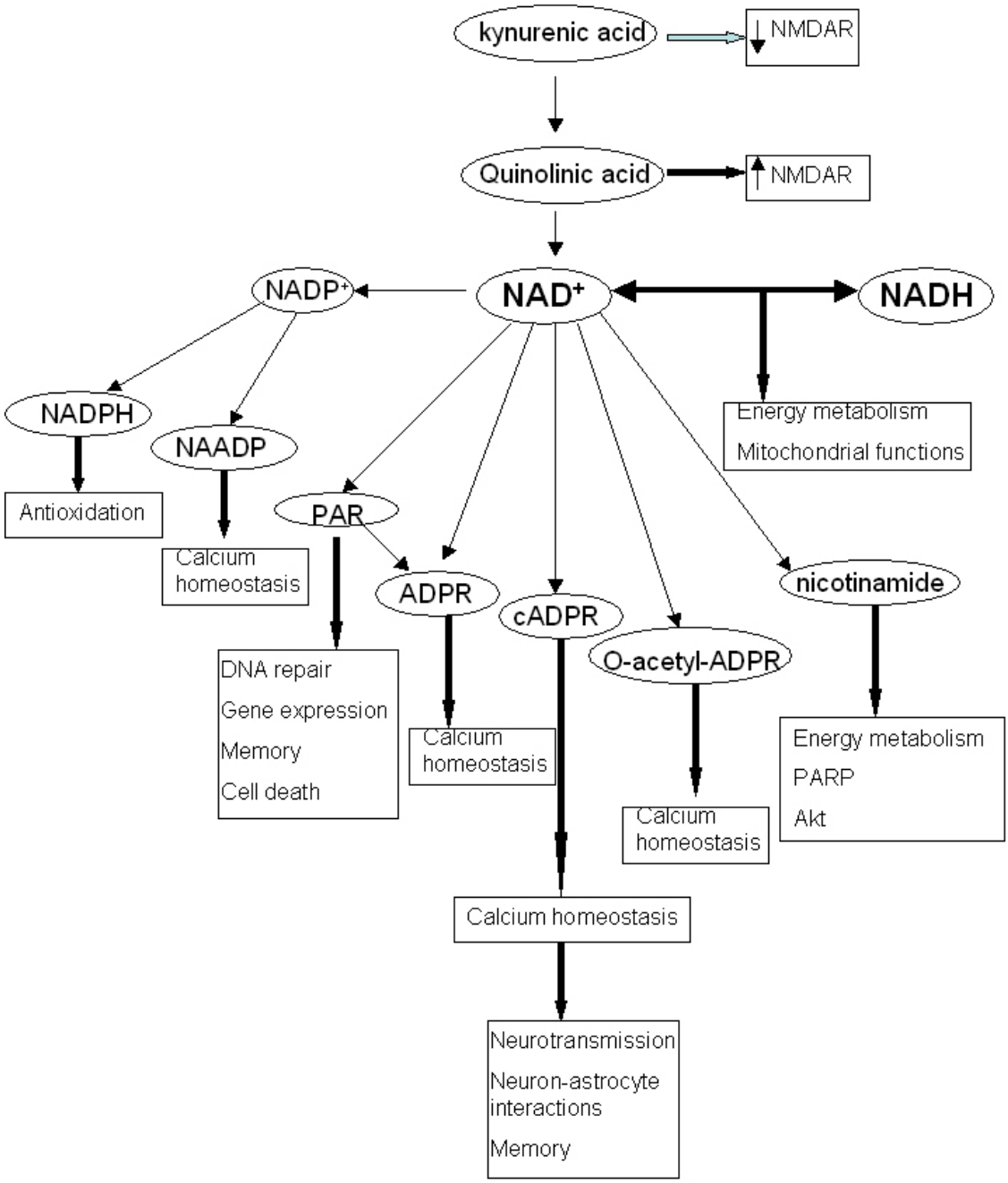

Figure 3. Diagrammatic presentation of the biological activities of the metabolites in NAD+ metabolic pathways. The molecules in open ovals are the metabolites in the NAD+ metabolic pathways; and the biological processes in open rectangles are the processes that are mediated by the metabolites. Abbreviations used: ADPR: ADP-ribose; cADPR: cyclic ADP-ribose; O-acetyl-ADPR: O-acetyl-ADP-ribose; PAR: poly(ADP-ribose); NMDAR: NMDA receptors.

homeostasis, mitochondrial functions and other key biological processes, it is of value to further understand the roles of NAD+ and NADH in ischemic brain damage.

6.1.2. Changes of NAD+ and NADH levels in cerebral ischemia

NAD+ decreases have been found in ischemic brains in which PARP-1 plays a key pathological role in the brain injury (33, 148). These results, combined with the *in vitro* studies suggesting that NAD+ depletion mediates PARP-1 cytotoxicity (29), suggest that the NAD+ decreases may also contribute to the PARP-1 toxicity *in vivo*. Several animal studies have further suggested that NAD+ loss may be a significant factor determining the relationships between PARP and ischemic brain damage. In a mild ischemia model in which there was no NAD+ decrease,

PARP activation could be beneficial by promoting DNA repair (149). Recent studies have also indicated that PARP-1 plays either detrimental or beneficial effects in male or female animals subject to ischemic insults (150, 151). A role of NAD^+ in this intriguing gender effect has been implicated by the study using a perinatal brain injury model (151). Our latest study has shown that intranasal NAD^+ administration can profoundly decrease infarct formation and neurological deficits, when administered at 2 hrs after ischemic onset (29). These results further support the notion that the NAD^+ decreases significantly contribute to ischemic brain injury.

The classic hypothesis for PARP-1 cytotoxicity proposes that PARP-1 activation produces NAD^+ depletion, thus leading to ATP depletion and cell death (152,153). While increasing evidence supports the NAD^+ depletion hypothesis, the role of ATP in PARP-1 toxicity remains unclear. Many, but not all, *in vitro* studies have found correlation between PARP activation and ATP depletion (154). There has been evidence arguing against the close associations among NAD^+ depletion, ATP depletion and ischemic brain injury. Paschen *et al.* reported that in animals subjected to reperfusion of various durations, the extent of ATP depletion was more pronounced than that of NAD^+ (155). Since their study did not demonstrate an important role of PARP activation in the brain damage in their experimental model, it is difficult to discuss the implications of their results for understanding the relationships among PARP-1, NAD^+ decreases, ATP decreases, and brain injury. However, their results do suggest that ATP can decrease at least partially by NAD^+-independent mechanisms, e.g., ATP can be converted to adenosine or xanthine under ischemic conditions. Future studies are needed to further elucidate the relationships among NAD^+, ATP, PARPs and ischemic brain injury.

6.1.3. Roles of PARP-1 in ischemic brain injury

Oxidative stress and nitrosative stress play critical roles in many diseases (58, 59, 127, 156-164). Numerous studies have indicated oxidative stress as a key mediator of ischemic brain damage (126, 127). A number of *in vitro* studies have also indicated excessive PARP-1 activation as a key factor mediating cell death induced by oxidative and nitrosative stress (165, 166), NMDA-induced excitotoxicity (165), oxygen-glucose deprivation (166) and zinc (167). It has been proposed that the cell death induced by PARP-1 activation is 'programmed necrosis' (168), although some studies have suggested that PARP-1 activation could mediate apoptosis (169).

Increased PARP activities have been found in animal models of cerebral ischemia (33, 170), and in human brains after cardiac arrest (171). Many *in vivo* studies using various PARP inhibitors have indicated that PARP-1 mediates ischemic brain injury of male animals. The studies using PARP-1 knockout mice have further demonstrated a key role of PARP-1 in ischemic brain damage of male mice (33, 166). It was also found that PARP inhibition can produce long-term protective effects on experimental stroke (172).

While it was hypothesized more than 20 years ago that PARP-1 induces cell death by depleting NAD^+ and ATP (153), until recently there has been no direct demonstration of this hypothesis. Recent studies have, by delivering NAD^+ into cells, provided evidence demonstrating that NAD^+ depletion mediates PARP-1 cytotoxicity (28, 41, 173). It has also been indicated that two mitochondrial alterations, including MPT and apoptosis inducing factor (AIF) translocation, are important components leading from NAD^+ depletion to cell death (41). A few mechanisms have been suggested for linking PARP-1 activation to mitochondrial alterations, including NAD^+ depletion-produced glycolytic inhibition (28, 174) or SIRT1 inhibition (175), and activation of JNK pathways (176). A latest study has further indicated significant interactions between PARP-1 and SIRT1: SIRT1 deficiency leads to increased PARP-1 activity, resulting in AIF-mediated cell death (177). Recent studies have also suggested other novel mechanisms underlying PARP-1 cytotoxicity: The ADP-ribose generated by PARP-1/PARG can activate TRPM2 receptors, leading to increased intracellular calcium concentrations and cell death (81-83); extracellular signal-regulated kinases 1/2 can regulate PARP-1 activity by directly phosphorylating the enzyme (178); and our latest study also suggested that low concentrations of NADH can block PARP-1-induced astrocyte death (179). It is of significance to determine if these seemingly diverse mechanisms may be linked by a central pathway. There may also be differential PARP-1-triggered cell death machineries in different cell types or under different intensities of insults.

Recent studies have indicated that PARP-1 inhibition produced completely different effects on the ischemic brain damage in male and female animals: Although it significantly decreases brain injury in male animals, PARP-1 inhibition markedly exacerbates ischemic brain damage in female animals (150, 151, 180). These results suggest the existence of profoundly different cell death programs in male and female animals. These findings also highlight the need for further determining the effects of estrogen and androgen as well as other sex-related factors in determining cell death programs. Future studies are warranted to determine if similar observations may be found in human, and if similar properties may be observed in other brain diseases.

In summary, cumulative evidence has indicated that PARP-1 inhibition can be greatly beneficial under multiple pathological conditions, with significant window of opportunity. However, this potential therapeutic approach could have several limitations: First, PARP-1 activation is clearly detrimental under relatively severe ischemic conditions, while it is less important under mild ischemic conditions under which there is no NAD^+ decrease; second, PARP-1 activation is highly toxic in male animals, while it is protective in female animals; and third, the toxicity of PARP-1 is more pronounced in transient focal ischemia, while it is less critical in permanent brain ischemia. Based on this information, it is proposed that the extent of NAD^+ decreases may be a key factor determining the roles of PARP-1 activation under different conditions.

While most of the studies regarding the roles of PARP in cell death have focused on PARP-1, recent studies have suggested the significance of other PARPs in cell injury. For examples, it has been indicated that PARP-2 mediates the cell death in focal brain ischemia, while it is beneficial in a model of global ischemia (181). A recent study has shown that overexpression of tankyrase 2 can produce rapid cell death (182). It is of interest to determine the relative contributions of PARP-1 and other PARPs in cell death under certain pathological conditions, and to elucidate the roles of NAD^+ and NADH in the effects of other PARPs on cell survival.

6.1.4. Roles of PARG in ischemic brain injury

Poly(ADP-ribose) glycohydrolase (PARG) is a key enzyme in PAR catabolism (183), which is an endo-exoglycosidase present in low abundance in cells. PARG digests PAR into ADP-ribose that is converted to AMP by a Mg^{2+}-dependent activity (184). Multiple *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 damage (185): *In vivo* studies have shown that genetic PARG inhibition significantly decreased ischemic damage of intestine (186) and kidney (187); and PARG inhibitors can also decrease ischemic injury of brain (188) and intestine (186). *In vitro* studies have further shown that inhibition of PARG by PARG inhibitors (189-193), PARG antisense oligonucleotides (194) or RNA silencing (195) can decrease the death of various types of cells induced by oxidative stress and other PARP activators. A latest study reported that both genetic and pharmacological inhibition of PARG significantly decreased spinal cord injury and inflammation (196).

Our latest study shows that intranasal delivery with the PARG inhibitor gallotannin (GT) decreased infarct formation by 60 – 70 % in a rat model of transient (2-hr) focal brain ischemia, when the drug was administered either at 2 or 5 hours after ischemic onset (197). We further found that intranasal GT administration abolished nuclear translocation of AIF. The GT administration also markedly increased PAR formation in the ischemic brains at 6 - 26 hrs after ischemia, suggesting that GT acts as a PARG inhibitor *in vivo*. Collectively, our study suggests that PARG inhibition can markedly decrease ischemic brain injury, probably in part by blocking AIF translocation. The observations that GT increased PAR formation and decreased ischemic brain injury also suggest that increased PAR formation is not a reliable marker of PARP activation.

PARG inhibition may prevent PARP-1-mediated cell death by the following mechanisms (189, 190): First, PARP-1 can auto-poly(ADP-ribosyl)ate itself, leading to PARP-1 auto-inhibition (32). Therefore, PARG inhibition could prevent removal of PAR from PARP-1, thus indirectly inhibiting PARP-1 activation. Second, PARG inhibition could slow the rapid PAR turnover thus preventing NAD^+ depletion. Third, PARP-1/PARG activities can generate ADP-ribose from hydrolysis of PAR, leading to activation of TRPM2 receptors and cell death (81-83). Fourth, Ca^{2+}-Mg^{2+}-dependent endonucleases (CME) mediate DNA fragmentation in certain apoptotic cascades (198). CME is a substrate of PARP-1, and poly(ADP-ribosy)lation of CME leads to CME inhibition (198, 199). Therefore, PARG inhibition could prevent removal of PAR from CME, leading to persistent CME inhibition.

It was reported that complete genetic deletion of PARG leads to embryonic lethality of mice (200). This observation may be accounted for by the fact that PAR metabolism mediates many biological processes, thus complete blockage of PAR metabolism would produce detrimental effects. Collectively, current studies appear to suggest that partial inhibition of PARG could be protective against genotoxic damage, while complete PARG inhibition could be lethal. With the increasing evidence suggesting that PARG has highly complicated properties (185, 201), numerous studies are still needed before we can have a solid understanding about the properties of PARG under both physiological and pathological conditions.

6.1.5. Roles of NAD^+ and NADH in ischemic preconditioning

The development of ischemic tolerance in the brain, whereby a brief period of sublethal 'preconditioning' ischemia reduces injury from subsequent severe ischemia, may involve the activation of multiple intracellular signaling events. Understanding of the mechanisms underlying ischemic preconditioning could be of great significance for finding new strategies for decreasing ischemic brain damage. Multiple mechanisms underlying ischemic preconditioning have been reported, such as NMDA receptor activation, Hsp70 upregulation, increased resistance to oxidative DNA damage, PKC-delta activation, PARP-1 cleavage, NO signaling, sirtuin activation, and delta-opioid receptor activation (202-208).

Garnier *et al.* reported that PARP-1 cleavage by caspase-3 may mediate the chemical ischemia-induced preconditioning effects (205). The rationale for this mechanism is: Mild ischemic insults may modestly activate caspase-3, which is known to cleave PARP-1 thus deactivating PARP-1. This effect of mild ischemia may prevent PARP-1 activation induced by subsequent severe insults. A recent study has also suggested that sirtuins mediate ischemic preconditioning: The sirtuin activator resveratoral mimics the preconditioning effects; while the sirtuin inhibitor sirtinol blocks the preconditioning effects (208). It remains unclear how sirtuins mediate ischemic preconditioning. A recent study by Chen and his colleagues have shown that ischemic preconditioning profoundly decreased oxidative DNA damage induced by brain ischemia-reperfusion (209). Consequently, detrimental DNA damage-induced changes, including NAD^+ depletion, were attenuated during reperfusion in preconditioned brains (209).

6.2. Roles of NAD^+ and NADH in traumatic brain injury

Multiple studies have indicated that PARP-1 activation plays a significant role in traumatic brain injury

(TBI) (19, 114, 210). This finding is not surprising, considering the strong evidence indicating oxidative stress as a key pathological factor in TBI (211, 212). Wallis *et al.* reported that treatment with PARP inhibitors was strongly protective. Interestingly, major protection was also produced by the inhibitors of mono(ADP-ribosyl)ation (213). These findings suggest that both poly(ADP-ribosyl)ation and mono(ADP-ribosyl)ation could mediate acute traumatic neuronal injury. The study by Clark and his colleagues has also shown nuclear AIF translocation in a TBI model, which may contribute to the traumatic brain injury. Since AIF is a NADH oxidase (214), it is of interest to determine if this property of NADH contributes to the potential biological effects of AIF translocation on traumatic brain damage.

However, many properties of NAD^+ and NADH under TBI conditions remain unclear. Since TBI shares multiple pathological mechanisms with ischemic brain damage, it is postulated that many alterations of NAD^+/NADH-dependent properties which have been observed in cerebral ischemia may also be found in TBI. Based on our current knowledge about NAD^+ and NADH in cellular functions and cell death, it may be of particular interest to determine the roles of sirtuins, the NADH shuttles and the NAD^+/NADH-dependent changes of calcium homeostasis in TBI.

6.3. Roles of NAD^+ and NADH in Parkinson's disease

6.3.1. General information about PD

Parkinson's disease (PD) is a common and debilitating idiopathic neurodegenerative disease characterized by tremor, rigidity, bradykinesia and balance difficulties. These motor abnormalities are attributed to depletion of brain dopamine, which results from the profound loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (215). Based on our current understanding of PD, multiple strategies have been proposed to treat PD by targeting at various steps in the cell injury cascade of PD, e.g., by blocking oxidative stress and mitochondrial dysfunction (216) and inhibiting apoptosis (217).

Multiple lines of evidence has suggested that oxidative stress could play a significant role in the pathogenesis of PD (58, 216, 218): First, dopamine, the major neurotransmitter in the SNpc, is a potent oxidative stress generator (219); second, decreased GSH levels occur in the presymptomatic stage of PD, which is associated with incidental Lewy body disease and may be the earliest biochemical marker of nigral cell death (219); third, oxidative stress may mediate α-synuclein aggregation and formation of Lewy bodies – a major hallmark of PD (219, 220); fourth, the free radical generator iron is accumulated in the SNpc with increasing age (219); and fifth, oxidative damage appears to mediate the neuronal death in the two major models for PD, i.e., the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model and 6-hydroxydopamine (6-OHDA) model (221, 222).

Several years ago this investigator proposed a new hypothesis for neurodegenerative diseases, aging and cell death --- The deleterious network hypothesis (57-61): Oxidative stress, calcium misregulation and energy impairments are closely related to each other, constituting a deleterious network. Various genetic and environmental factors can trigger the network by initiating one or more of the three components, leading to neurodegenerative disorders, aging and cell death. Increasing number of studies seems to support this hypothesis. Cumulative evidence has also suggested that oxidative stress may be a key factor that impairs NAD^+ and NADH metabolism in aging or under pathological conditions by such mechanisms as activating PARP-1.

6.3.2. Roles of PARP-1 in PD

A number of studies have indicated that oxidative stress plays a significant role in the pathogenesis of PD (60, 216, 218). Because it has been indicated as one of the key mediators of oxidative stress-induced cell death (1, 19, 165, 166), PARP-1 may mediate neuronal injury in PD. PARP-1 activation appears to play a key role in the neuronal death induced by MPTP in both cell culture studies (223, 224) and in animal model studies (225-227). Yang *et al.* also reported that nicotinamide treatment can decrease MPTP-induced neurotoxicity *in vivo* by inhibiting oxidative stress, increasing NAD^+ and ATP production and inhibiting PARP (228).

Several recent studies have suggested a novel hypothesis for PD, which could further deepen our understanding regarding the roles of PARP-1 in PD: 1-Methylnicotinamide, which is generated from nicotinamide by nicotinamide N-methyltransferase, may contribute to the pathogenesis of PD (229-231). The evidence supporting their hypothesis includes: 1) 1-Methylnicotinamide injection into rat SNpc significantly decreased dopamine content in the striatum (229); 2) 1-methylnicotinamide destroyed several subunits of cerebral complex I (230); 3) nicotinamide N-methyltransferase is present in the human brain, which is increased in PD brains; and 4) nicotinamide N-methyltransferase can convert nontoxic pyridines such as 4-phenylpyridine into MPP^+ like compounds. Based on this information, the following mechanism for PD pathology has been proposed (230): Excessive PARP-1-mediated consumption of NAD^+ generates excessive amount of nicotinamide, which is methylated to 1-methylnicotinamide in cytoplasm. The 1-methylnicotinamide can increase superoxide generation from Complex I leading to impairments of Complex I, which would further cause oxidative DNA damage and PARP-1 activation. Genetic or environmental factors may accelerate this vicious cycle resulting in the neuronal death in PD. It is of great interest to further test the validity of this hypothesis.

6.3.3. Other information regarding NAD^+ and NADH in PD

A latest study by Hara *et al.* suggested a significant pathological role of GAPDH in PD (93): The widely used PD medicine deprenyl and a related agent, TCH346, in subnanomolar concentrations, prevent S-nitrosylation of GAPDH, the binding of GAPDH to Siah, and nuclear translocation of GAPDH. In mice treated with

MPTP, low doses of deprenyl block the binding of GAPDH to Siah1 in the dopamine-enriched corpus striatum. It is of interest to further determine the relationships among PARP-1 activation, GAPDH translocation, NAD^+/NADH levels and neuronal injury in PD.

NADH has been used to boost endogenous dopamine production, since NADH indirectly supplies reducing equivalents to the rate-limiting, tyrosine hydroxylase-catalysed step of dopamine synthesis (232). NADH has been used as medication in clinical trials to treat PD patients. As reported by Birkmayer *et al.*, beneficial clinical effects were observed in about 80% of the patients (233). Another clinical study also showed beneficial effects of NADH application in treating PD patients (234). In their study NADH significantly increased bioavailability of plasma levodopa, which is used to attenuate the striatal dopamine deficits in PD. This effect may partially underlie the beneficial effects of NADH. However, there is also evidence arguing against applications of NADH for treating PD: A placebo-controlled trial did not show any clear benefit of NADH. Thus, future studies that apply more effective NADH administration strategies and larger scale studies are needed to further determine the therapeutic potential of NADH for PD.

6.4. Roles of NAD^+ and NADH in Alzheimer's disease

Oxidative damage has been indicated as one of the pathogenic factors in AD (58, 235-238). Thus, it is conceivable that PARP-1 may mediate the neuronal injury in AD. Increased nuclear PARP activity has also been found in the brains and peripheral cells of AD patients (226, 239). Recent studies have suggested that PARP-1 activation also mediates the β-amyloid-induced neuronal death, which is an *in vitro* model for AD (83, 240).

Several recent studies have suggested the therapeutic potential of sirtuins for AD (241-243). Chen *et al.* reported that both overexpression of SIRT1 deacetylase and application of the SIRT1 agonist resveratrol markedly reduced β-amyloid-mediated NF-κB signaling and produced major neuroprotective effects (241). Qin *et al.* also reported that the decreases in β-amyloid content in the brain during caloric restriction can be reproduced in murine neurons *in vitro* by manipulating cellular SIRT1 through mechanisms involving the regulation of the serine/threonine Rho kinase ROCK1. ROCK1 is known for its capacity to inhibit the non-amyloidogenic α-secretase processing of the amyloid precursor protein (242). These results suggest that SIRT1 activation may underlie the beneficial effects of caloric restriction on amyloid neuropathology.

Demarin *et al.* reported that after treatment with NADH, the subjects had significantly improved cognitive functions compared with the subjects treated with placebo ($p < 0.05$) (244). The kynurenine pathway is a major route of L-tryptophan catabolism leading to production of multiple biologically active molecules. Among them, the neurotoxin quinolinic acid is considered to be involved in the pathogenesis of several inflammatory neurological diseases. There is evidence suggesting that quinolinic acid is also involved in the pathogenesis of AD (245).

6.5. Roles of NAD^+ and NADH in multiple sclerosis

Multiple sclerosis (MS) is a chronic demyelinating disease, which appears to result from the aberrant immune responses of genetically susceptible individuals to one or more myelin antigens upon induction by certain undefined factors (246). Oxidative stress could play a significant role in the demyelination and axonal damage in both MS and experimental autoimmune encephalomyelitis (EAE) --- an animal model of MS (247). Since it could mediate both inflammation and oxidative damage (19, 154), PARP-1 might be involved in the pathogenesis of MS.

Diestel *et al.* suggested that 7-ketocholesterol, a lipid breakdown product found in the brain and cerebrospinal fluid (CSF) of MS patients and in EAE, contributed to microglial activation-induced neuronal damage by a PARP-1-dependent pathway: It was found that the 7-ketocholesterol levels in the CSF of MS patients were high enough for producing neuronal damage by activating microglia in living brain tissues; and 7-ketocholesterol can rapidly enter the cell nucleus and activate PARP-1, leading to inflammatory responses. In a study using a monkey EAE model of MS, abnormally higher levels of PARP-1 activation was observed in the astrocytes surrounding demyelinated EAE plaques (248). Collectively, cumulating evidence supports the hypothesis that PARP-1 may contribute to MS pathology.

There is insufficient information regarding the roles of NAD^+ and NADH in MS. Because NAD^+ and NADH can significantly affect both immunological functions and cell survival by multiple mechanisms (1), it may be valuable to investigate the roles of NAD^+ and NADH in the pathogenesis of MS. The recent studies suggesting an important role of NAD^+ in axon degeneration (249) further implicate the potential value of this line of research.

6.6. Roles of NAD^+ and NADH in Huntington's disease (HD)

Vis *et al.* observed strong glial expression of PARP-immunoreactivity in HD brains (250): While PARP immunoreactivity was predominantly seen in astrocytes, large motor neurons displayed decreased staining compared with controls. This result contrasts sharply to the PAR staining of the brain tissues from AD and MPTP-treated animals, where PAR was observed mainly in neurons (251). It is of interest to determine the mechanism underlying these major differences among the patterns of PAR staining in these neurodegenerative diseases.

It was reported that Sir2 activation through increased *sir-2.1* dosage or treatment with the sirtuin activator resveratrol decreased early neuronal dysfunction induced by mutant polyglutamines in transgenic *elegans* (252). Additionally, resveratrol rescued mutant polyglutamine-specific cell death in neuronal cells derived from HdhQ111 knock-in mice. Therefore, Sir2 activation may protect cells against the toxicity of mutant polyglutamines. A latest study also suggested that the

cytotoxicity of mutant Huntingtin (mHtt) requires its nuclear translocation, which appears to be mediated via a ternary complex of GAPDH-Siah-mHtt (93).

6.7. Roles of NAD+ and NADH in axon degeneration

Axon degeneration can be induced by various insults (253), which often occurs in neurodegenerative diseases. The studies on Wallerian degeneration slow (Wlds) mice have suggested important mechanisms underlying axon degeneration (253). The mutation of Wlds mice leads to overexpression of a chimeric protein (Wlds) consisting of NMNAT1 and the ubiquitin assembly protein Ufd2a. It was found that injury-induced axon degeneration is delayed by the mutation. Recent studies have suggested that the increased NMNAT1 expression resulting from the mutation mediates the protective effects of the Wallerian mutation (249, 254). However, the mechanisms underlying the protective effects of increased NMNAT1 expression are unclear. One study suggested that the increased NMNAT1 expression produces its effects by affecting SIRT1 --- a member of sirtuins (254). However, Wang *et al.* suggested that NMNAT1 may affect axon degeneration by preventing NAD^+ loss in degenerating axons: They found that the degeneration of axonal segments that have been separated from their soma can still be prevented by the exogenous administration of NAD^+ or nicotinamide.

Other mechanisms for the Wallerian mutation-produced delay in axon degeneration have also been suggested, including increased intra-axonal calcium, mitochondrial dysfunction and impaired axonal transport (253). Because NAD^+ can significantly affect calcium homeostasis and mitochondrial function (1), the multiple potential mechanisms for the Wallerian mutation-produced delay in axon degeneration may be essentially linked to each other.

6.8. Roles of NAD+ and NADH in brain tumors

There have been studies implicating sirtuins in brain tumors: It was suggested that SIRT2 may act as a tumor suppressor gene in human gliomas possibly through the regulation of microtubule network and may serve as a novel molecular marker for gliomas (255). It was also reported that human SIRT2, that is most predominantly expressed in the brain, is severely reduced in a large number of human brain tumor cell lines (256). Thus, the authors proposed that the absence of SIRT2, a potential tumor suppressor, may play a key role in cellular transformation and development of cellular malignancy.

Extensive studies have been conducted regarding the roles of PARPs in carcinogenesis and cancer treatment. Tentori *et al.* reported that in short-term cultures of glioma cells derived from surgical specimens, PARP inhibitor enhanced chemosensitivity to temozolomide (257). They also found that systemic administration of a novel PARP inhibitor increases the antitumor activity of temozolomide against glioma (258).

7. NAD+ AND NADH IN BRAIN AGING

Cumulative evidence has suggested that NAD^+ and NADH could play critical roles in aging process by regulating sirtuins, PARP-1, tankyrases, oxidative stress, calcium homeostasis and mitochondrial functions (1). There have also been some studies suggesting significant roles of NAD^+ and NADH in brain senescence. However, considering that NAD^+ and NADH may profoundly affect several aging-mediating factors, including oxidative stress, mitochondrial dysfunction and calcium misregulation (60, 259-263), the insufficiency of the studies regarding the roles of NAD^+ and NADH in brain senescence is obvious. It is likely that future studies investigating the roles of NAD^+ and NADH in brain aging may suggest novel mechanisms of brain senescence as well as new strategies for slowing brain aging. Because aging is a major risk factor for multiple neurological diseases such as Alzheimer's disease, understanding of brain aging would also be essential for elucidating the pathogenesis of certain neurological diseases. The following sections provide an overview of the major information about NAD^+ and NADH in brain aging, based on which future studies may be designed.

7.1. Roles of PARPs in brain aging

It was reported that there is strong positive correlation between PARP activities of mononuclear blood cells and longevity of thirteen mammalian species, which may result from greater PARP-1-mediated DNA repair capacity (264, 265). A role of PARP-1 in aging has been further raised by the recent report that PARP-1 interacts with and inhibits the protein of Werner syndrome --- a human disease of premature aging (266, 267). Recent studies regarding PARP-1 in brain aging appear to support the hypothesis that decreased PARP-1 activity in response to genotoxic insults may contribute to brain aging. It was reported that PARP-1 is not activated by excessive oxidative/genotoxic stress in aged hippocampus, in contrast to a significant increase in PARP-1 activity in adults (268). There are also age-related alterations of PAR synthesis in rat cerebellum, including reduced PARP-1 activation in response to enzymatic DNA cleavage and cell type-specific loss of poly(ADP-ribosyl)ation capacity in granule cell layer and Purkinje cells *in vivo* (269). It was further suggested that *in vivo* factors other than the levels of PARP-1 protein and NAD^+ may be responsible for the age-associated decreases in PAR synthesis.

Telomere and telomerases have been indicated as key factors in cellular aging (270). Since the NAD^+-dependent tankyrases are mediators of telomerase activity (271), NAD^+ may also affect aging processes through tankyrases. It is of interest to determine the effects of tankyrases and telomerase on certain biological process in brains such as neurogenesis, which may affect the aging of brains.

7.2. NADH in aging brains

Repeated administration of NADH was found to improve the performance of old rats in the Morris water maze studies, suggesting the capacity of NADH to enhance the cognitive functions of old rats (272). Zarchin *et al.* also reported that exposures of adult rats and old rats to anoxia resulted in an approximately 36% and 10% elevations of NADH, respectively, suggesting a significant age-

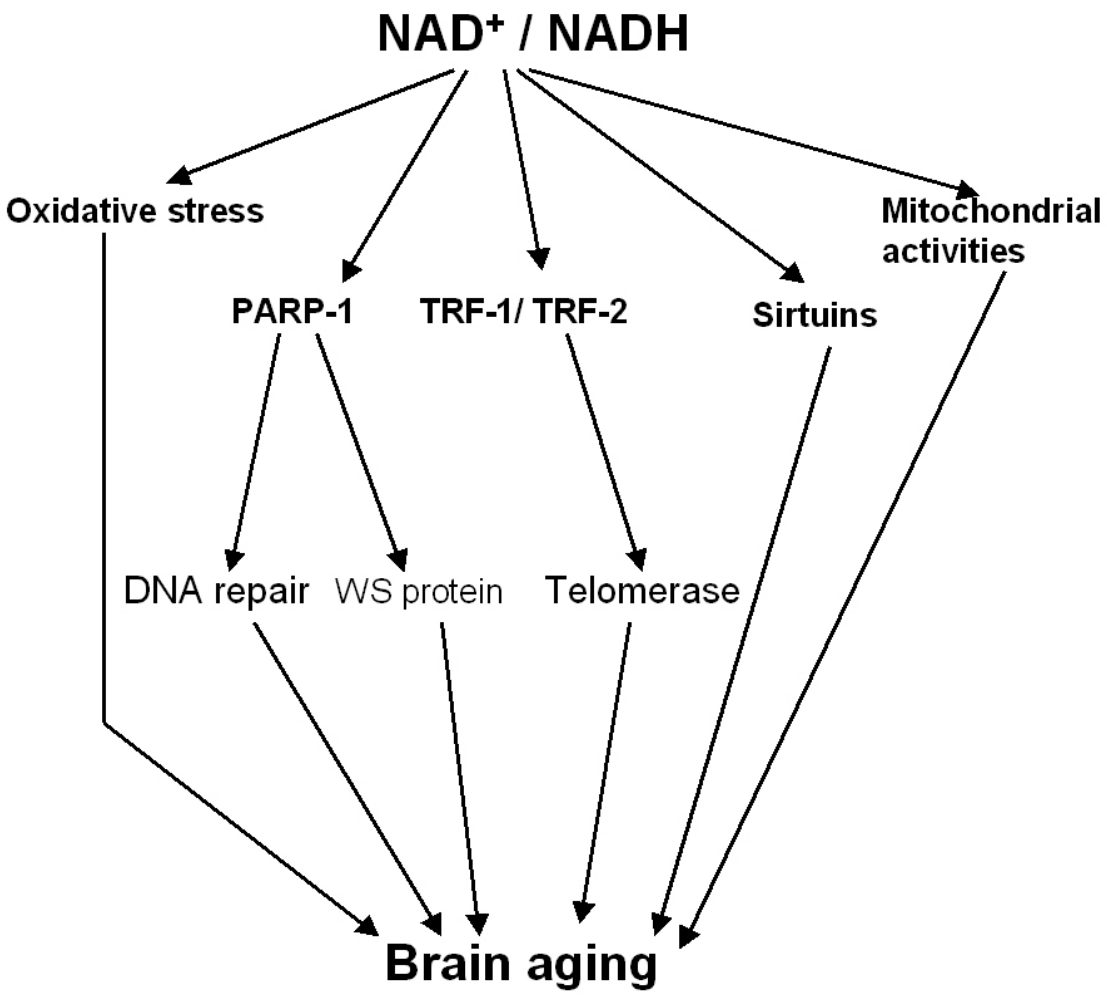

Figure 4. Diagrammatic presentation of the roles of NAD^+ and NADH in aging processes.

associated difference in the NADH-dependent responses to anoxia (273). Genova *et al.* investigated NADH oxidation in non-synaptic and synaptic mitochondria from brain cortex of 4- and 24-month-old rats. The NADH oxidase activity was significantly lower in non-synaptic mitochondria from aged rats (274).

7.3. Roles of sirtuins in aging

It has been suggested that Sir2 is a key enzyme mediating life span of yeast and C-*elegans* (275): A decrease or increase in gene copy of Sir2 shortens or extends the replicative life span of yeast, respectively (276); and increased gene copy of the Sir2 gene homolog in C-*elegans* also extends its life span (277). It was further suggested that caloric restriction modulates Sir2 activity and extends the life span of yeast by decreasing NADH levels (278). Recently it was found that deficiency of SIRT6, a human homolog of Sir2, produces aging-like phenotype in mice (279). However, there has been no significant information regarding the roles of sirtuins in brain senescence.

7.4. Summary

A diagrammatic presentation regarding the potential pathways by which NAD^+ and NADH may affect brain aging is shown in Figure 4. The distinct insufficiency of the information in this promising field suggests the need of research on this topic.

8. PERSPECTIVES AND FUTURE DIRECTIONS

A rapidly growing body of information has suggested that NAD^+ and NADH are major regulators of not only energy metabolism and mitochondrial functions, but also calcium homeostasis, gene expression, cell death and aging. This review has provided an overview of the metabolism and transport and NAD^+ and NADH in brains. The article also reviewed the literature regarding the roles of NAD^+ and NADH in brain functions, brain diseases and brain senescence. Based on this overview, it is proposed that NAD^+ and NADH are central regulators of brain functions, brain aging and multiple brain diseases.

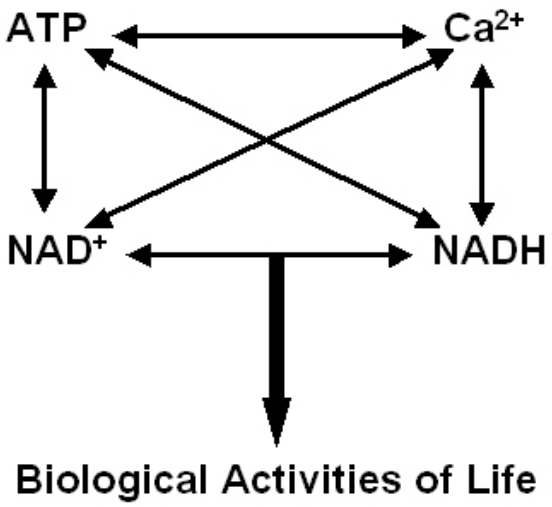

Figure 5. Diagrammatic presentation of the Central Regulatory Network of life.

As stated in a previous review (1), ATP and Ca^{2+} appear to be particularly important regulators of most biological activities: ATP is not only the fundamental energy molecule, but also a mediator of protein kinases and extracellular signaling (46, 84, 280); and intracellular Ca^{2+} mediates numerous biological processes such as muscle contraction, neurotransmitter release and cell death (56-63, 130, 281, 282). Thus, it was proposed that NAD^+ and NADH, together with ATP and Ca^{2+}, may be four most fundamental regulatory components in life (1). Obviously, NAD^+ and NADH are closely related with ATP and Ca^{2+}: NAD^+ and NADH are major regulators of ATP metabolism and calcium homeostasis; and ATP and Ca^{2+} also significantly affect the metabolism and transport of NAD^+ and NADH. Thus, it is tempted to propose that ATP, NAD^+, NADH and Ca^{2+} constitute a Central Regulatory Network of life: The close interactions among these four components may play central roles in regulating most biological processes; and alterations of this central network may also mediate aging, cell death and many disease processes (Figure 5). Future investigation into the relationships among these components may expose some fundamental properties of life.

Cumulative evidence has indicated that nearly all of the metabolites generated from the NAD^+ metabolic pathways have certain biological functions (Figure 3). This exquisiteness of the NAD^+ and NADH metabolism can only be attributed to the long evolutional process of life. This exquisiteness implicates the exquisiteness of life, through which we may sense the intricate harmony life can produce. The exquisiteness also implicates the central regulatory roles of NAD^+ and NADH in life.

Obviously, there is insufficient information about many fundamental properties of NAD^+ and NADH in brains. Much fruitful future investigation may be expected in this increasingly interesting field. The following research field may be of particular interest:

First, it is needed to investigate the roles of sirtuins in brain functions under both physiological and pathological conditions. Increasing evidence has indicated critical roles of sirtuins in aging, gene expression, cell death and energy metabolism. However, there is distinct lack of information about the biological properties of sirtuins in brains. It is likely that future studies on this topic may establish significant roles of sirtuins in brain functions, brain senescence and brain pathologies.

Second, cumulative evidence has suggested significant roles of NAD^+ and NADH in regulating calcium homeostasis. Due to the critical roles of calcium in brain functions, it is warranted to further investigate the roles of the NAD^+/NADH-dependent changes of calcium homoeostasis in brain functions, brain aging and brain diseases.

Third, while many studies have investigated PARP-1 in brain injuries, the roles of other PARPs such as tankyrases in brain functions are largely unknown. It may be valuable to investigate the roles of these proteins in brains under both physiological and pathological conditions.

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