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NAD^+ and NADH in ischemic brain injury

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1. ABSTRACT

NAD^+ and NADH have been emerging as the common mediators of energy metabolism, mitochondrial functions, calcium homeostasis, aging and cell death. NAD^+ and NADH can affect cell death by various mechanisms, such as influencing energy metabolism, mitochondrial permeability transition pores, and apoptosis-inducing factor. Because energy failure, calcium disregulation and cell death are the key components in the tissue damaging cascade initiated by cerebral ischemia, it is likely that NAD^+ and NADH play significant roles in ischemic brain damage. Many studies, including the findings that poly(ADP-ribose) polymerase-1 mediates ischemic brain injury and that NAD^+ administration can decrease ischemic brain damage, have suggested significant roles of NAD^+ and NADH in the debilitating illness. However, there is still distinct insufficiency of the information regarding the roles of NAD^+ and NADH in ischemic brain injury. Because increasing evidence has indicated critical functions of NAD^+ and NADH in various biological processes, future studies on the roles of NAD^+ and NADH in cerebral ischemia may expose essential mechanisms underlying ischemic brain injury and suggest novel therapeutic strategies for the illness.

2. INTRODUCTION

Accumulating evidence has suggested that NAD^+ and NADH possess biological functions that are well beyond their classical functions (1-4). NAD^+ and NADH appear to play critical roles in not only energy metabolism and mitochondrial functions, but also in calcium homeostasis, aging, and cell death (1-4). Many progresses about the novel biological functions of NAD^+ and NADH have been made during the last decade. For examples, the NAD^+-dependent histone deacetylase Sir2 appears to play a critical role in aging (5); extensive biological functions of poly(ADP-ribose) polymerases (PARPs) in DNA repair, gene expression, inflammation, and cell death have been found; and cyclic ADP-ribose --- a molecule generated from NAD^+ --- appears to be a key calcium-mobilizing messenger (6, 7).

Stroke is one of the leading causes of death and long-term disability. While it can decrease brain injury in a small population of stroke patients, tissue plasminogen activator (tPA) --- the only FDA-approved drug for treating stroke --- has significant limitations such as short therapeutic window and multiple toxic side effects (8-10). Therefore, it is of significance to further investigate the

mechanisms underlying ischemic brain injury, in order to establish new therapeutic strategies 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, excitotoxicity, altered calcium homeostasis, energy failure, apoptotic mechanisms, PARP-1 activation, and inflammation, play important roles in ischemic brain injury (11-26).

Decreases in NAD^+ have been found in ischemic brains (27). NAD^+ and NADH may play important roles in ischemic brain injury, since these molecules can significantly affect cell death, calcium homeostasis and energy metabolism. The goal of this review is to provide up-to-date generalization about the roles of NAD^+ and NADH in cell death, and to generalize the information about NAD^+ and NADH in ischemic brain injury. Through the generalization, future directions for the research regarding NAD^+ and NADH in brain ischemia may be suggested.

3. GENERAL INFORMATION ABOUT NAD$^+$ AND NADH

3.1. NAD$^+$ Synthesis in cells

Because NADH is generated from NAD^+ via numerous NAD^+/NADH-dependent dehydrogenases, NAD^+ biosynthesis plays a pivotal role in the metabolism of both NAD^+ and NADH. Two major NAD^+ biosynthesis pathways are the *de novo* pathway and the salvage pathway (28, 29). In the *de novo* pathway L-tryptophan is used as the precursor for NAD^+ generation: L-Tryptophan is converted to L-kynurenine, followed by generation of quinolinic acid from L-kynurenine. Quinolinic acid is subsequently converted to nicotinate mononucleotide that can be used by nicotinamide mononucleotide adenylyltransferases (NMNATs) for NAD^+ synthesis. In the salvage pathway, NAD^+ is generated by NMNATs using nicotinamide or nicotinic acid as precursors (28-30).

3.2. NAD$^+$ catabolism in cells

There are at least four major families of NAD^+-consuming enzymes that can profoundly affect various biological activities, which include poly(ADP-ribose) polymerases (PARPs), mono(ADP-ribosyl) cyclases, sirtuins and ADP-ribosyl cyclases: First, PARPs are a family of enzymes that consume NAD^+ to produce poly(ADP-ribibose) (PAR) on target proteins (31). Most studies about PARPs have focused PARP-1, which appears to play essential roles in regulation of various biological processes, including DNA repair, gene expression, genomic stability, and cell death (1, 31, 32). It has also been found that excessive PARP-1 activation mediates ischemic brain injury, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism, traumatic brain damage, hypoglycemic brain damage, diabetes and shock (1, 31). Second, mono(ADP-ribosyl)transferases (ARTs) are a family of NAD^+-consuming enzymes that produce mono(ADP-ribosyl)ation of proteins (33, 34). Expression of ART1-5 has been found in various cell types (35, 36). Of particular interest, the ecto-enzyme ART2 on the plasma membranes of Treg cells --- a subset of T cells that mediates immunological activities --- can produce mono(ADP-ribosyl)ation of $P2X_7$ receptors thus leading to Treg cell apoptosis (37, 38). Third, the NAD^+-dependent histone deacetylases (or called sirtuins or Sir2 family proteins) produce deacetylation of histones and non-histone proteins. Many studies have indicated critical roles of sirtuins in aging, carcinogenesis and cell death (39, 40). Fourth, ADP-ribosyl cyclases / cyclic ADP-ribose hydrolases can both consume NAD^+ to generate cyclic ADP-ribose and hydrolyze cyclic ADP-ribose into free ADP-ribose (41). A number of studies have suggested significant biological functions of the mammalian ADP-ribosyl cyclase CD38 (2, 6, 7).

There is increasing evidence suggesting that PARP-1 mediates intracellular NAD^+ levels when extensive DNA damage occurs (2, 42), while CD38 may mediate intracellular NAD^+ levels under physiological conditions (43). Future studies are needed to further determine the relative contributions of the multiple NAD^+-consuming enzymes to the changes of intracellular NAD^+ and NADH levels under various biological and pathological processes.

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

4.1. PARPs in cell death

Oxidative stress plays critical roles in many diseases (12, 44-54). Numerous studies have indicated oxidative stress as a key mediator of ischemic brain damage (11, 12). Many *in vitro* studies have also shown that excessive PARP-1 activation mediates cell death induced by oxidative stress (55, 56), NMDA-induced excitotoxicity (55) and oxygen-glucose deprivation (56). The cell death induced by excessive PARP-1 activation appears to be a mixture of classical necrosis and apoptosis: While there is ATP depletion in the cells, some apoptosis-associated changes, such as chromatin condensation and apoptosis-inducing factor (AIF) translocation, also occur in the cells. Cumulative evidence appears to support the proposition that the cell death induced by PARP-1 activation is 'programmed necrosis' (57).

It was proposed about 20 years ago that PARP-1 activation induces cell death by depleting NAD^+. We have provided direct evidence indicating that NAD^+ depletion is a key step mediating PARP-1-induced cell death (58, 59). Several studies have also indicated that mitochondrial permeability transition (MPT) (59) and AIF translocation (60) are major steps linking NAD^+ depletion to cell death. Our results further suggest that NAD^+ depletion could induce mitochondrial alterations by producing glycolytic inhibition (58), which can reduce pyruvate supply to tricarboxylic acid cycle (61). The suggestion has been supported by the findings that pyruvate treatment after PARP-1 activation significantly decreases PARP-1-induced cell death in both cell cultures and brain slices (61-63).

Recent studies have suggested novel mechanisms underlying PARP-1 cytotoxicity. It has been suggested that the ADP-ribose generated by PARP-1/poly(ADP-ribose)

glycohydrolase (PARG) can cause TRPM2 opening, leading to increased intracellular calcium concentrations and cell death (64-66). Recent studies have further indicated interactions between PARP-1 and SIRT1: It was suggested that SIRT1 is a key link between NAD^+ depletion and cell death (67); and SIRT1 deficiency was also found to produce significant increases in PARP-1 activity, leading to AIF-mediated cell death (68). However, it has also been suggested that PAR, instead ADP-ribose monomers, is a PARP-1-produced signal leading to PARP-1-induced AIF translocation and cell death (69, 70).

Cumulative evidence has indicated that multiple protein kinases, including c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs), could contribute to PARP-1-mediated cell death (71-74): It was reported that JNK is required for PARP-1-induced cell death (73, 75). A recent study further found that JNK1 could activate PARP-1 by directly phosphorylating the enzyme (74). It has also been suggested that direct phosphorylation of PARP-1 by ERK1/2 can induce PARP-1 activation (71). A latest study reported that direct interactions between phosphorylated ERK2 and PARP-1 can induce DNA damage-independent PARP-1 activation (72).

Several reasons may account for the seeming diversity of the mechanisms underlying PARP-1 toxicity: 1) There may be differential PARP-1-mediated cell death cascades that are selectively activated depending on cell types and intensities of insults; second, inclusion of such nutritional factors as pyruvate can profoundly affect PARP-1 toxicity (61, 62), suggesting that the differential nutritional compositions of the experimental media used in various studies may contribute to the variability of the experimental findings (76); and third, multiple factors may form a detrimental network that leads to PARP-1-initited cell death, thus inhibition of any one of the major components of the network may block PARP-1 toxicity. While the reported mechanisms for PARP-1 toxicity are seemingly diverse, increasingly unified pathways linking these mechanisms may be exposed by future studies.

Cumulative evidence has suggested that other PARPs can also mediate cell death: A recent study reported that PARP-2 mediates the survival of $CD4^+CD8^+$ double-positive T-cells during thymopoiesis (77). It has been found that overexpression of tankyrase 2 can also produce rapid cell death (182). Tankyrase 1 overexpression was also shown to antagonize both Mcl-1L (myeloid cell leukemia-1 long)-mediated cell survival and Mcl-1S (myeloid cell leukemia-1 short)-induced cell death (78). Future studies are needed to further elucidate the roles of various PARPs in cell survival and the relationships among NAD^+, PARPs and cell survival.

4.2. Roles of PARG in cell death

PARG is the key enzyme for PAR catabolism (79), which generates ADP-ribose from PAR (80). Three isoforms of PARG have been found in the nucleus, mitochondrial and cytosol, respectively (81). Multiple studies have indicated critical roles of PARG in gene expression, cell cycle, cell differentiation (82-84) and changes of chromatin structure (85). Cumulating evidence has also supported the hypothesis that PARG inhibition may prevent PARP-1-mediated cell death by several mechanisms (86, 87): First, it has been suggested that PARP-1/PARG activities can generate ADP-ribose by hydrolyzing PAR, leading to activation of TRPM2 receptors and consequent cell death (64-66). Thus, PARG inhibition could decrease cell death by blocking ADP-ribose generation from PAR. Second, PARP-1 can auto-poly(ADP-ribosyl)ate itself, leading to PARP-1 auto-inhibition (42). Therefore, PARG inhibition could prevent removal of PAR from PARP-1 thus indirectly inhibiting PARP-1 activation. Third, PARG inhibition may block the rapid PAR turnover leading to prevention of NAD^+ depletion. Fourth, Ca^{2+}-Mg^{2+}-dependent endonucleases (CME) can mediate DNA fragmentation in certain apoptotic cascades (88). Because CME are poly(ADP-ribosy)lated thus being inhibited under physiological conditions (88, 89), PARG inhibition may prevent removal of PAR from CME leading to sustained CME inhibition.

Nearly all of the studies using various structurally different PARG inhibitors, including GPI 18214 (90), GPI 16552 [N-bis-(3-phenyl-propyl)9-oxo-fluorene-2,7-diamide] (91), gallotannin and nobotanin B (86, 87, 92-94), have supported the hypothesis that PARG may mediate oxidative cell death (1, 95): The PARG inhibitor gallotannin and nobotanin B can decrease cell death induced by various PARP activators *in vitro* (86, 87, 92-94); the PARG inhibitor GPI 18214 is beneficial for septic shock-like syndrome (90) and inflammatory bowel disease (96); and the PARG inhibitor GPI 16552 can also decrease ischemic brain injury (91) and spinal cord injury (97). The cell culture studies in which PARG activity was decreased by RNA silencing or antisense approaches also showed that PARG inhibition is cytoprotective: Decreases of PARG levels by RNA silencing (98) or antisense oligonucleotide treatment (99) led to reduced PARP-1-mediated cell death. In a latest study in which cells have increased PARG activity, the increased PARG activity leads to accelerated NAD^+ depletion and increased cell death (81). This study further suggests a protective effect of PARG inhibition.

However, the studies using PARG knockout mice have generated variable results: The mice that have genetic deletion of the 110 kDa PARG isoform have decreased spinal cord injury (97) and ischemic damage of kidney (100) and intestine (101), compared with wild type mice. In contrast, there are studies suggesting that genetic deletion of PARG produces detrimental effects (69, 102). Since PARG can significantly affect gene expression and other biological properties (82-84) and the *PARG* gene is closely associated with inner mitochondrial membrane translocase 23 (*TIM23*) gene (103), it may be warranted to consider the potential genetic alterations in the PARG knockout mice. One study has reported that in PARG knockout mice there are marked changes in the gene expression of cyclooxygenase 2 and heat shock protein 70 (102) --- two proteins that are important for cellular functions and cell survival.

Our latest study has provided new information regarding the relationships between PARG and CME (104): We found that the DNA alkylating agent *N*-methyl-N'-nitro-*N*-nitrosoguanidine (MNNG) can dose-dependently increase DNA fragmentation and chromatin condensation. Post-treatment with the CME inhibitor aurintricarboxylic acid abolished the MNNG-induced DNA fragmentation and chromatin condensation, suggesting that CME plays a key role in the MNNG-induced DNA fragmentation and chromatin condensation. We also found that decreases in the PARG in the cell nucleus by PARG antisense oligonucleotides can also prevent the MNNG-induced chromatin condensation. Collectively, our observations suggest that CME and PARG are two critical components in the MNNG-induced nuclear alterations in astrocytes. Together with the well established finding that CME is inhibited by poly(ADP-ribosyl)ation, our findings are consistent with our hypothesis that PARG inhibition may decrease genotoxic agent-induced cell injury at least in part by inhibiting CME. These studies also suggest that PARG and CME may be important targets for reducing genotoxic agent-induced chromatin condensation and DNA fragmentation.

Based on the cumulating information about PARG in cell death, it appears that partial PARG inhibition can be cytoprotective, while complete PARG inhibition is deleterious. Future studies are warranted to apply conditional PARG knockout mice or more selective PARG inhibitors to further determine the role of PARG in cell death.

4.3. Roles of NAD$^+$ and NADH in apoptosis

Increasing evidence has suggested that NAD$^+$ could also play significant roles in apoptosis. It has been found that selective inhibitors of NAD$^+$ synthesis can induce apoptosis (105); and NAD(P)H depletion is a very early event in apoptosis (106). There are several potential mechanisms underlying the roles of NAD$^+$ and NADH in apoptosis: NAD$^+$ and NADH may affect apoptosis by mediating energy metabolism ---- a critical factor determining cell death modes, or by influencing MPT --- a mediator of apoptosis under many conditions (107). NAD$^+$ may also influence apoptosis through NAD$^+$-dependent sirtuins (108). Moreover, it has been reported that NAD$^+$ mediates the functions of caspase-dependent endonuclease DFF40 --- an executioner of DNA fragmentation in certain apoptotic cascades (109).

AIF is a NADH oxidase, which can act as both a pro-death factor and a pro-survival factor (110-112). It has been indicated that translocation of AIF from mitochondria to the nucleus mediates caspase-independent apoptosis (112) and PARP-1 cytotoxicity (60). However, AIF is also closely associated with mitochondrial Complex I activity (113). Genetic deletion of AIF has been shown to produce skeleton muscle atrophy, dilated cardiomyopathy and neurodegeneration (112). Our latest study has shown that intranasal gallotannin administration can abolish AIF translocation in ischemic brains (114). We have also found that treatment of a CME inhibitor can profoundly decrease MNNG-induced chromatin condensation, despite nuclear translocation of AIF (unpublished observation). This finding suggests that nuclear AIF translocation is not sufficient to induce chromatin condensation under certain conditions. While it has been established that AIF is a NADH oxidase and NAD$^+$ depletion mediates PARP-1-induced AIF translocation (59), many future studies are still needed to further determine the roles of NAD$^+$ and NADH in AIF functions.

4.4. NADPH in cell death

NAD$^+$ could significantly affect NADPH levels: NAD$^+$ is required for generation of NADP$^+$ --- the essential substrate for NADPH generation. A rapidly growing body of evidence has indicated that NADPH oxidase mediates cell death under both *in vitro* and *in vivo* conditions (115). For examples, NADPH oxidase mediates ROS generation induced by oxygen-glucose deprivation in neurons (116); NADPH oxidase activation in astrocytes was found to mediate β-amyloid-induced neuronal death (117); and inhibition of NADPH oxidase is protective against ischemic brain injury (118, 119). Considering these intriguing findings, it is increasingly possible that NAD$^+$ and NADH may also affect cell survival by influencing NADPH levels and NADPH oxidase activity.

5. NAD$^+$ AND NADH IN ISCHEMIC BRAIN INJURY

5.1. Roles of PARPs in ischemic brain injury

There are increased PARP activities in both animal models of cerebral ischemia (27, 120) and in human brains after cardiac arrest (121). A number of *in vivo* studies applying various PARP inhibitors have indicated that PARP-1 mediates ischemic brain injury (31). These studies, together with the studies showing great resistance of PARP-1 knockout mice against ischemic insults (27, 56), have demonstrated a critical role of PARP-1 in ischemic brain damage.

Consistent with the *in vitro* findings showing PARP-1-induced NAD$^+$ depletion, there are also significant NAD$^+$ decreases in the ischemic brains in which PARP-1 plays a key pathological role (27, 122). These results, combined with the *in vitro* studies suggesting that NAD$^+$ depletion mediates PARP-1 cytotoxicity (58), suggest that the NAD$^+$ decreases may also contribute to the PARP-1-mediated brain injury *in vivo*. Our recent study has shown that intranasal NAD$^+$ administration can profoundly reduce infarct formation and neurological deficits (123). These results have further suggested that NAD$^+$ decreases play a significant role in ischemic brain injury.

The extent of NAD$^+$ loss could determine if PARP-1 activation plays a beneficial or detrimental role in brain ischemia: It was indicated that PARP activation could be beneficial by promoting DNA repair in a mild ischemia model, in which there was no NAD$^+$ decrease (124). This study suggests that PARP inhibitors should not be used for treating brain ischemia when ischemic insults can not produce significant NAD$^+$ loss in the brains.

Several recent studies have suggested that PARP-1 activation could be a cytoprotective mechanism against

ischemic insults for female rats, although it has been established that PARP-1 activation is a key pathological mechanism in the ischemic brain injury of male rats (125-127). These results suggest that there are significantly different cell death programs in male and female animals. It remains unclear why PARP-1 activation plays dramatically different roles in the ischemic brain injury of male and female animals. Since estrogen is neuroprotective in both neuronal culture studies and in the studies using animal models of brain ischemia (128, 129), the estrogen of female animals may account for the protective effects of PARP-1 activation against ischemic insults in female animals: Estrogen might prevent PARP-1-induced NAD$^+$ depletion by decreasing oxidative stress in female animals under ischemic conditions, so that PARP activation mainly leads to enhanced DNA repair thus producing neuroprotective effects. There are also other potential mechanisms underlying the gender effects on the roles of PARP-1 activation in ischemic brain injury: It has been suggested that estrogen may prevent PARP-1 binding to DNA strand breaks and subsequent PARP-1 activation by anchoring PARP-1 to estrogen receptor α (130).

In summary, it appears that PARP-1 activation plays complex roles in ischemic brain injury: The severity of ischemic insults and the gender of the animals could determine if PARP-1 activation is detrimental or beneficial. It is possible that the extent of NAD$^+$ decreases is a key factor determining the roles of PARP-1 activation under different conditions. Future studies on the roles of PARP-1 are warranted, which may not only expose new mechanisms of ischemic brain damage, but also suggest important information for designing therapeutic strategies for brain ischemia.

While PARP-1 has been the focus of most previous studies regarding the roles of PARP in cerebral ischemia, recent studies have suggested significant roles of other PARPs in ischemic brain damage. Of particular interest, it was reported that PARP-2 knockout mice is also resistant to ischemic insults in a model of focal brain ischemia (181). In contrast, PARP-2 knockout mice are more vulnerable to ischemic insults in a model of global ischemia (181). These studies highlight the differences of the cell death programs triggered in focal ischemia models and global ischemia models, which suggest the necessity of designing differential treatment strategies for different modes of brain ischemia.

5.2. Roles of PARG in ischemic brain injury

There are two studies that determined the effects of structurally different PARG inhibitors on ischemic brain damage, both of which have indicated that PARG inhibition could decrease ischemic brain damage (91, 114). It was reported that a novel PARG inhibitor can decrease ischemic brain injury even when administered after reperfusion (91). Our latest study has also shown that intranasal delivery of the PARG inhibitor gallotannin can decrease ischemic brain damage by inhibiting PARG (114). Intranasal delivery of gallotannin within 5 hours after ischemic onset markedly decreased the infarct formation and neurological deficits of rats. The gallotannin administration also increased PAR in the ischemic brains, suggesting that gallotannin acts as a PARG inhibitor *in vivo*. Moreover, the gallotannin treatment abolished nuclear translocation of AIF in the ischemic brains, suggesting that prevention of AIF translocation may contribute to the protective effects of gallotannin. In contrast, intravenous injection of gallotannin at 2 hours after ischemic onset did not reduce ischemic brain damage. Collectively, our findings suggest that PARG inhibition can significantly decrease ischemic brain injury, possibly by blocking AIF translocation.

5.3. Roles of NAD$^+$ and NADH in ischemic preconditioning

A recent study has shown that ischemic preconditioning profoundly decreased oxidative DNA damage induced by brain ischemia-reperfusion, which led to decreased DNA damage-induced changes including NAD$^+$ depletion (131). It was also reported that PARP-1 cleavage by caspase-3 may mediate the chemical ischemia-induced preconditioning effects, which may be accounted for by the following mechanism (132): Mild ischemia may only modestly activate caspase-3, which can cleave PARP-1 thus deactivating PARP-1. This effect may prevent the PARP-1 activation induced by subsequent severe insults. A recent study has also suggested that sirtuins could mediate ischemic preconditioning: It was found that the sirtuin inhibitor sirtinol blocks the preconditioning effects (133), while the sirtuin activator resveratoral mimics the preconditioning effects.

5.4. Therapeutic potential of NAD$^+$ for ischemic brain injury

The *in vitro* studies by our research group have provided the first *in vitro* evidence indicating that treatment with NAD$^+$ can abolish PARP-1 cytotoxicity (58, 59, 76, 134). These results prompted us to propose the hypothesis that NAD$^+$ administration can decrease ischemic brain injury. We have used a rat model of transient focal ischemia to test our hypothesis (123): We found that intranasal delivery with 10 mg / kg NAD$^+$ at 2 hours after ischemic onset decreased infarct formation by nearly 90%. In contrast, intranasal delivery with nicotinamide at the dose of 10 mg / kg did not reduce ischemic brain damage. These results provide the first *in vivo* evidence that NAD$^+$ administration may be a novel strategy for decreasing brain injury in cerebral ischemia.

NAD$^+$ may have distinctive merits as a cytoprotective agent: First, *in vitro* studies have shown that NAD$^+$ is protective even when applied at 3-4 hrs after PARP-1 activation, suggesting that NAD$^+$ administration may have long window of opportunity in decreasing tissue injury; second, NAD$^+$ can produce nearly complete protection against PARP-1 cytotoxicity (58, 59); and third, NAD$^+$ may decrease cell death by multiple pathways such as enhancing both energy metabolism and the activities of sirtuins.

Our latest study has provided evidence suggesting that intranasal NAD$^+$ administration may also decrease

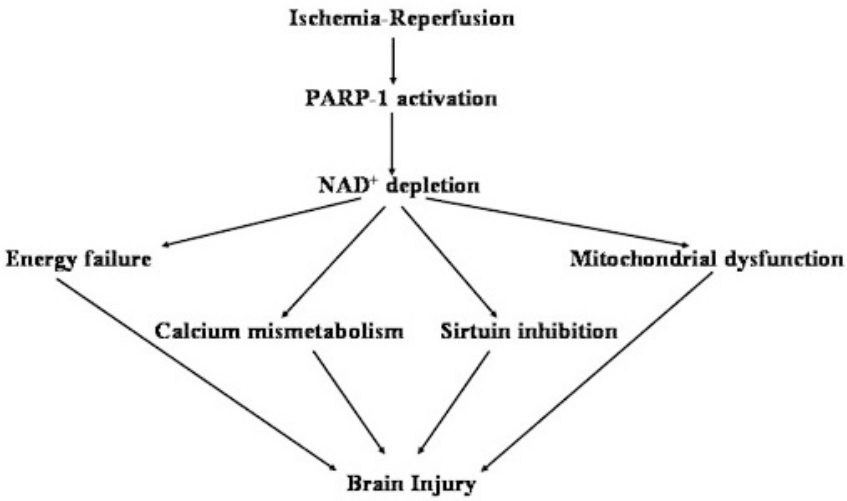

Figure 1. Diagrammatic presentation of the potential mechanisms by which NAD^+ depletion may contribute to ischemic brain injury.

traumatic brain injury (135). Because PARP-1 activation plays significant roles in multiple neurological diseases, it is likely that NAD^+ administration may be used for treating a number of CNS diseases.

6. SUMMARY AND PERSPECTIVES

Our understanding regarding the novel biological functions of NAD^+ and NADH have been greatly improved in recent years. In particular, a number of studies have suggested new roles of NAD^+ and NADH in cell death. There are also studies that suggest significant roles of these molecules in ischemic brain injury. Alterations of NAD^+ metabolism may contribute to ischemic brain damage through multiple mechanisms (Figure 1), which warrants future investigation. The following lines of studies may be of particular interest:

First, while it has been shown that NAD^+ administration can decrease ischemic brain damage, the mechanisms underlying the protection remain unclear. Future studies investigating the mechanisms may provide valuable information for elucidating the roles of NAD^+ metabolism in ischemic brain injury.

Second, increasing evidence has suggested that sirtuins play significant roles in cell death, while there has been little information regarding the roles of sirtuins in ischemic brain injury. Studies on the roles of various isoforms of sirtuins in cerebral ischemia may suggest important mechanisms underlying ischemic brain damage.

Third, extensive studies have been conducted on PARP-1 in ischemic brain damage. However, there have been only few studies that determine the roles of other isoforms of PARPs in brain ischemia. Since some *in vitro* studies have suggested that other PARPs are also involved in cell death, it is warranted to investigate the roles of other PARPs in ischemic brain damage.

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Abbreviations: AIF: apoptosis-inducing factor, CME: Ca^{2+}-Mg^{2+}-dependent endonucleases, ERK: extracellular signal-regulated kinase, GT: gallotannin, JNK: c-Jun N-terminal kinases (JNKs), MNNG: *N*-methyl-N'-nitro-*N*-nitrosoguanidine, MPT: mitochondrial permeability transition, NMDA: *N*-methyl-*D*-aspartate**,** PAR: poly(ADP-ribose), PARG: Poly(ADP-ribose) glycohydrolase, PARP-1: poly(ADP-ribose) polymerase-1, ART: mono(ADP-ribosyl)transferases, TIM23: inner mitochondrial membrane translocase 23, tPA: tissue plasminogen activator.

Key Words: Ischemia; brain injury; poly(ADP-ribose) polymerase; poly(ADP-ribose); cell death; mitochondria; oxidative damage

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