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Oxidative Stress and NAD⁺ in Ischemic Brain Injury: Current Advances and Future Perspectives

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Abstract

Numerous studies have indicated oxidative stress as a key pathological factor in ischemic brain injury. One of the key links between oxidative stress and cell death is excessive activation of poly(ADP-ribose) polymerase-1 (PARP-1), which plays an important role in the ischemic brain damage in male animals. Multiple studies have also suggested that NAD⁺ depletion mediates PARP-1 cytotoxicity, and NAD⁺ administration can decrease ischemic brain injury.

A number of recent studies have provided novel information regarding the mechanisms underlying the roles of oxidative stress and NAD⁺-dependent enzymes in ischemic brain injury. Of particular interest, there have been exciting progresses regarding the mechanisms underlying the roles of NADPH oxidase and PARP-1 in cerebral ischemia. For examples, it has been suggested that androgen signaling and binding of PARP-1 onto estrogen receptors could account for the intriguing findings that PARP-1 plays remarkably differential roles in the ischemic brain damage of male and female animals; and some studies have suggested casein kinase 2, copper-zinc superoxide dismutase, and estrogen signaling can modulate the expression and activity of NADPH oxidase.

This review summarizes these important current advances, and proposes future perspectives for the studies on the roles of oxidative stress and NAD⁺ in cerebral ischemia. It is increasingly likely that future studies on NAD⁺- and NADP⁺-dependent enzymes, such as NADPH oxidase, PARP-1, and sirtuins, would expose novel mechanisms underlying the roles of oxidative stress in cerebral ischemia, and suggest new therapeutic strategies for treating the debilitating disease.

Keywords

Brain ischemia; oxidative stress; NAD⁺; poly(ADP-ribose)polymerase-1; sirtuins; cell death

1. GENERAL INFORMATION ABOUT THE ROLES OF OXIDATIVE STRESS AND NAD⁺ IN BRAIN ISCHEMIA

Stroke is one of the leading causes of disability and death around the world. Ischemic stroke is the major form of stroke. Extensive studies on the disease have discovered many important mechanisms underlying ischemic brain injury, and suggested some potential

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therapeutic strategies. However, tissue plasminogen activator (tPA) has been the only federal drug administration (FDA)-approved drug for treating ischemic stroke, and the drug has only limited success due to the toxic side-effects and the narrow window of opportunity of the drug [1]. A number of clinical trials on stroke therapies have failed [2], which suggest that our understanding on the pathological mechanisms of brain ischemia is still significantly deficient. Many future studies are critically needed to expose new mechanisms of ischemic brain damage.

A large number of studies have indicated that ischemic brain injury is caused by several key pathological factors that interact with each other, which include oxidative stress, impaired calcium homeostasis, mitochondrial dysfunction, and inflammation [3–6]. Compelling evidence has indicated that oxidative stress plays significant roles in not only ischemic brain damage [3, 7], but also other neurological diseases including Parkinson's disease [8–10] and Alzheimer's disease [11–15]. While numerous studies regarding the role of oxidative stress in ischemic brain damage have been reported [3, 7], future studies on this topic remain to be crucial to elucidate the new mechanisms of ischemic brain damage.

In brain ischemia-reperfusion, oxidative stress generated from such resources as activated NADPH oxidase (NOX) or impaired mitochondria leads to oxidative DNA damage. Single strand DNA damage can lead to excessive activation of PARP-1, which has been shown to play a key role in oxidative stress-induced cell death in cell culture studies [16, 17], and to play an important role in ischemic brain injury in animal model studies [18, 19]. It has also been indicated that NAD⁺ depletion plays a critical role in PARP-1-mediated cytotoxicity [20–22]. Previous studies have also found that ischemia/reperfusion (I/R) can induce significant decreases in the NAD⁺ levels in the brain [19], and NAD⁺ administration can significantly decrease ischemic brain injury in an animal model of cerebral ischemia [23].

Collectively, a number of studies have suggested the importance of NOX, PARP-1 and NAD⁺ in oxidative cell death in the brains subjected to I/R. During recent years there have been multiple exciting progresses regarding the roles of oxidative stress in brain ischemia. The particularly interesting findings include those regarding NOX, PARP-1, and NAD⁺-dependent enzymes such as sirtuins. The aim of this article is to provide an overview of these key recent discoveries.

2. SOURCES OF OXIDATIVE STRESS IN BRAIN ISCHEMIA-REPERFUSION

Oxidative stress refers to the cytotoxic consequences of a mismatch between the production of reactive oxygen species (ROS) or free radicals (FRs) and the ability of cells to defend against them. ROS include the oxygen-centered radicals possessing unpaired electrons such as superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$), or covalent molecules such as hydrogen peroxide (H_2O_2). Under physiological conditions, $O_2^{\cdot-}$ is generated during the electron-transport process in mitochondria [24]. In addition, $O_2^{\cdot-}$ is formed in the endoplasmic reticulum by cytochromes P450 [25] and in the cytoplasm by enzymes such as xanthine oxidase [26]. The plasma membrane of many cell types can also produce $O_2^{\cdot-}$ by activation of enzymes such as phospholipase A2 and NAD(P)H oxidase [27]. H_2O_2 is formed from $O_2^{\cdot-}$ spontaneously or by action of superoxide dismutase (SOD) [28]. H_2O_2 is in turn reduced to water by glutathione peroxidase or converted to O_2 and H_2O by catalase. Although the reactivity of H_2O_2 toward organic compounds is relatively low, it can form more reactive $OH^{\cdot}$ by interacting with transitional metals [29].

Nitric oxide (NO) is an important reactive nitrogen species which has also been shown to play an important role in ischemic brain injury [30]. NO is synthesized from L-arginine by nitric oxide synthase (NOS) [31]. NOS is known to exist in three isoforms: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3) [32].

It is established that a burst of ROS generation occurs during the reperfusion phase: During the phase of brain ischemia, the lack of oxygen and glucose leads to reversal of glutamate transporters, resulting in glutamate release and glutamate-produced excitotoxicity [33]. Hypoxic conditions in the brain can also cause brain acidosis due to generation of lactate from pyruvate, which can activate acid-sensing Ca^{2+} channels [34] and exacerbate oxidative damage [35]. The openings of both ionotropic glutamate receptors and acid-sensing Ca^{2+} channels can lead to Ca^{2+} influx into the neurons [34, 36], leading to impairments of mitochondria and activation of various Ca^{2+} -dependent proteases and phospholipases. During the reperfusion phase, various cytosolic ROS-generating enzymes as well as the enzymes of mitochondrial electron transport chain can obtain sufficient oxygen to generate ROS. In brain ischemia-reperfusion, the cytosolic ROS-generating enzymes include NOX [37], neuronal NOS [38], and the enzymes involving in arachidonic acid metabolism such as phospholipase A2 [39].

3. MECHANISMS UNDERLYING THE ROLES OF NADPH OXIDASE IN ISCHEMIC BRAIN INJURY

NOX exists in seven isoforms in a number of tissues and cell types [40, 41]. An important recent advance regarding the sources of ROS during brain ischemia/reperfusion (I/R) is the discoveries indicating that NOX in neurons/astrocytes can significantly contribute to the generation of ROS [42–45]. Multiple studies have also shown that inhibition of NOX by both genetic and pharmacological approaches can significantly decrease oxidative damage and reduce ischemic brain injury [37, 46, 47].

Latest studies have provided multiple pieces of novel information regarding the roles of NOX in brain ischemia. A recent study has indicated an interesting crosstalk between SOD-1 activity and NOX expression [37]: The expression of gp91phox --- a NOX subunit --- was decreased or increased in SOD1-overexpressing mice or SOD-1 deficient mice, respectively. This finding suggests that ROS itself is a positive modulator of NOX activity. It also suggests a detrimental positive feedback mechanism of ROS generation in brain ischemia: Increased superoxide levels may increase NOX activity thus further enhancing ROS generation [37]. Kim et al. also reported that casein kinase 2 (CK2) is a key negative modulator of NOX in the brain [48]: CK2 α could regulate NOX activity by binding onto Rac1 and inhibit Rac1 --- a positive modulator of NOX; and this binding is decreased in transient focal ischemia leading to exacerbated brain injury. This study has suggested that CK2 is a novel neuroprotective protein in brain ischemia.

A latest study has also indicated that estrogen decreases oxidative damage in cerebral ischemia at least partially by attenuating the activation of NOX [49]: The binding of estrogen onto extranuclear estrogen receptor α can lead to Akt activation, phosphorylation/inactivation of Rac1, and subsequent inhibition of NOX. This study suggests that estrogen

can enhance cellular antioxidant capacity both by acting directly as an antioxidant and by inhibiting NOX activity.

Because increasing evidence has indicated a key role of NOX in ischemic brain injury, it is of great clinical significance to modulate the enzyme in the disease. Multiple strategies may be used to inhibit the enzyme, which includes direct applications of NOX inhibitors and manipulation of such modulators of NOX as Rac1 and CK2 [40, 41, 50]. Moreover, prevention of ROS generation from NOX can also be achieved by inhibition of pentose phosphate pathway, from which NADPH is generated. It is of great interest to design potent and specific activators and inhibitors for both NOX and those enzymes that can modulate NOX activity.

4. MECHANISMS UNDERLYING THE ROLES OF PARP-1 IN ISCHEMIC BRAIN INJURY

4.1. Roles of PARP-1 in brain ischemia

A number of studies have indicated that PARP-1 activation plays an important role in ischemic brain injury: First, multiple *in vitro* studies have shown that excessive PARP-1 activation mediates the cell death induced by oxidative stress, oxygen-glucose deprivation (OGD) and *N*-methyl-*D*-aspartic acid (NMDA)-produced excitotoxicity [18, 51]; second, increased PARP activities have been found in animal models of cerebral ischemia [19, 52] and in human brains after cardiac arrest [53]; and third, numerous studies applying various PARP inhibitors or PARP-1 knockout mice have shown that inhibition of PARP-1 can profoundly decrease ischemic brain damage of male animals [18, 19].

There are at least two major mechanisms underlying the toxic effects of PARP-1 activation in ischemic brain damage: 1) PARP-1 activation in neurons induces neuronal injury by such mechanisms as NAD⁺ depletion; and 2) PARP-1 activation in microglia leads to increases in inflammatory responses, resulting in neuronal death. It has been shown that PARP-1 inhibition can lead to inhibition of NF- κ B activation and promote neurogenesis [54]. However, since inflammation can produce both detrimental and beneficial effects in brain ischemia [55], future studies are warranted to determine if PARP-1 may both attenuate and exacerbate ischemic brain injury through its effects on different components in the brain ischemia-induced inflammation.

It is known that single strand DNA (ssDNA) damage can induce rapid dimerization and subsequent activation of PARP-1 [56]. It is also established that brain I/R can induce significant DNA damage by generating ROS and reactive nitrosative species (RNS), particularly peroxynitrite [57]. Therefore, I/R appears to induce PARP-1 activation by generating oxidative DNA damage. However, it has also been suggested that calcium signaling in the nucleus can induce PARP-1 activation via DNA damage-independent pathway [58]. Because there are extensive impairments of calcium homeostasis in ischemic brains [4], it remains possible that the altered nuclear calcium signaling in ischemic neurons, astrocytes and microglia might also partially contribute to the PARP-1 activation in ischemic brains. It has also been shown that brain ischemia can induce increased gene expression of PARP-1 [59, 60], which appears to be at least partially mediated by I/R-induced superoxide generation [60]. These studies have suggested that brain ischemia can modulate PARP-1 activity both by producing ssDNA damage [56] and by increasing the expression of the enzyme.

However, a number of recent studies have suggested that PARP-1 inhibition is beneficial only in male mice, while PARP inhibition produces either negligible or detrimental effects in female mice [61–63]. Latest studies have suggested some mechanisms underlying the

sexual dimorphism of PARP-1 inhibition on ischemic brain damage: First, it has been suggested that testicular androgen is required for the protective effects of PARP-1 inhibition, and androgen is also required for ischemia-induced increases in PARP expression [64]. Collectively, these results suggest that androgen is a key factor affecting both the signaling pathway triggered by PARP-1 activation as well as the ischemia-initiated pathway leading to PARP expression. Thus, the different levels of androgen in male and female mice may underlie the differential effects of PARP-1 activation on ischemic brain damage. Second, the study by Szabo and his colleagues has suggested that PARP-1 can form a complex with estrogen receptor α [65], which leads to binding of the complex to DNA. The authors suggest that this interaction between PARP-1 and estrogen receptor α may prevent excessive activation of PARP-1 by ssDNA.

Another important advance regarding the role of PARP-1 in ischemic brain damage is the findings that further suggest the beneficial effects of PARP-1 under certain mild ischemic conditions. Yang et al. recently reported that there is significant intranuclear matrix metalloproteinase (MMP) activity in the neurons in ischemic brains, which leads to cleavage of PARP-1 and X-ray cross-complementary factor 1 (XRCC1). Inhibition of the intranuclear MMP activity has been shown to prevent PARP-1 cleavage and oxidative DNA damage, suggesting that PARP-1 activity is critical for repairing the DNA damage induced by oxidative stress. This observation is consistent with both the concept that PARP-1 is a key DNA repair enzyme [56] and the finding that PARP may promote recovery following sublethal transient global ischemia [66]. Therefore, for potential applications of PARP inhibitors in clinical settings, precaution is needed to avoid inhibiting PARP-1 excessively, so as to maintain the DNA repair functions of the enzyme.

4.2. Mechanisms of PARP-1-Mediated Neurotoxicity

Direct evidence indicating NAD⁺ depletion as a key factor in PARP-1-induced cell death has been demonstrated by recent studies [20, 21]. These studies have further suggested that NAD⁺ depletion could induce mitochondrial depolarization and mitochondrial permeability transition (MPT) at least partially by producing glycolytic inhibition and subsequent reductions of pyruvate supply to tricarboxylic acid (TCA) cycle [20, 21]. A latest study by Alano et al. has provided further evidence indicating that NAD⁺ depletion mediates PARP-1-induced neuronal death [67]: Depletion of intracellular NAD⁺ by delivery of NAD⁺ glycohydrolase into neurons is sufficient to induce apoptosis-inducing factor (AIF) release and neuronal death. NAD⁺ depletion-induced glycolytic inhibition appears to, at least partially, mediate the toxicity of NAD⁺ depletion.

Multiple studies have also indicated c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) in PARP-1-induced cytotoxicity [68–71]: JNK1 can mediate PARP-1-induced mitochondrial impairments and cell death [70, 72]; and ERK1/2 has also been shown to induce PARP-1 activation by phosphorylating directly the enzyme [68].

There is increasing amount of information regarding the roles of mono(ADP-ribose) and poly(ADP-ribose) (PAR) in PARP-1 toxicity: Several studies have suggested that PARP-1/poly(ADP-ribose) glycohydrolase-generated ADP-ribose can cause opening of TRPM2 receptors, resulting in elevated intracellular Ca²⁺ concentrations and cell death [73–75]. In contrast, it has also been suggested that PAR, instead of ADP-ribose monomers, mediates PARP-1-induced translocation of AIF and cell death [76, 77]. However, under certain experimental conditions, it appears that AIF translocation can still be blocked by NAD⁺ supply, despite the significant existence of PAR in the cells [67].

5. ROLES OF NAD⁺ AND NAD⁺-DEPENDENT SIRTUINS IN ISCHEMIC BRAIN DAMAGE

5.1. Roles of NAD⁺ in Ischemic Brain Damage

The first evidence that NAD⁺ can profoundly decrease PARP-1-mediated astrocyte death was provided in 2003 [20]. Since 2004 a number of studies have further demonstrated that NAD⁺ can reduce the death of astrocytes, neurons, and myocytes induced by various insults, including oxidative stress [21], DNA alkylating agents [20], oxygen-glucose deprivation [22], zinc [78], and axonal damage [79]. These results strongly indicated that NAD⁺ is a novel cytoprotective agent.

Several studies have also suggested mechanisms underlying the protective effects of NAD⁺: NAD⁺ can decrease PARP-1-mediated cell death by preventing glycolytic inhibition, MPT and AIF translocation [20, 21]; it has also been suggested that NAD⁺ can decrease OGD-induced neuronal death by enhancing DNA repair activity [22]; and Pillai et al. found that PARP-1-mediated myocyte cell death was prevented by repletion of cellular NAD⁺ levels either by extracellularly added NAD or overexpressing NAD⁺ biosynthetic enzymes, which appears to be mediated by NAD-induced increases in Sir2a activity [80]. Due to the increasingly important roles of NAD⁺ in various biological functions, including calcium homeostasis, energy metabolism and gene expression [81, 82], it is warranted to further investigate the roles of NAD⁺ in oxidative and ischemic cell death.

In a rat model of transient focal brain ischemia, intranasal administration of NAD⁺ was shown to profoundly decrease the ischemic brain damage [83]: NAD⁺ administration at 2 hrs after ischemic onset can reduce infarct formation by up to approximately 85%. This observation has provided the first evidence that NAD⁺ administration may become a novel strategy for treating ischemic stroke, and NAD⁺ metabolism could be a new target for treating brain ischemia. However, the mechanisms underlying the protective effects of NAD⁺ *in vivo* remain unclear. Our recent study has suggested that NAD⁺ administration could also decrease the neonatal hypoxia-ischemia-induced brain damage [84]. Protective effects of NAD⁺ have also been found in a model of myocardial ischemia: Cardiac-specific overexpression of nicotinamide phosphoribosyltransferase can increase the NAD⁺ content in the heart and reduce I/R-induced myocardial infarction [85].

There is evidence implicating that NAD⁺ administration may decrease brain injury in not only cerebral ischemia, but also multiple other diseases [86]: Because excessive PARP-1 activation could also mediate the cell injury in several major neurological diseases such as Parkinson's disease and traumatic brain injury [16], it is speculated that NAD⁺ may also decrease the cell injury in these diseases. This speculation has been supported by a preliminary study showing that intranasal NAD⁺ administration reduces traumatic brain injury [23]. Interestingly, a latest study has demonstrated that as low as 1–10 μ M NAD⁺ can decrease the survival of a variety of cancer cells including neuroblastoma cells and glioma cells, by generating oxidative stress and increasing intracellular Ca²⁺ concentrations [87]. These studies have suggested that NAD⁺ may become a potential therapeutic agent not only for acute and chronic degenerative diseases, but also for cancer.

5.2. Roles of Sirtuins in Ischemic Brain Injury

Sir2 has been shown to mediate the aging process of yeast [88]. Sirtuins are the mammalian homolog of Sir2, which are NAD⁺-dependent histone deacetylases. Sirtuin family proteins include SIRT1–SIRT7, in which SIRT1 has been most extensively studied [89]. A number of studies have suggested that SIRT1 can decrease cell death by reducing the levels of p53 [90] or by inhibiting NF- κ B [91, 92]. It has also been indicated that SIRT1 deficiency can

lead to increases in PARP-1 activity, resulting in AIF-mediated cell death [93], suggesting that SIRT1 may also produce inhibitory effects on PARP-1. Because both p53 and PARP-1 play significant roles in ischemic brain injury [3, 7, 94], it is possible that SIRT1 also produces protective effects in brain ischemia by its effects on p53 and PARP-1. In addition, because SIRT1 can reduce neuronal injury by inhibiting microglia activation [91, 92], SIRT1 may also attenuate ischemic brain damage through its effects on inflammation.

Neuronal culture studies have produced conflicting results regarding the roles of SIRT1 in neuronal injury. A recent study has indicated that Icariin can decrease oxygen-glucose deprivation (OGD)-induced neuronal death by increasing SIRT1 levels [95]. However, it has been suggested that SIRT1 plays a detrimental role in zinc-induced neuronal death, due to its consumption of NAD⁺ [78]. *In vivo* studies have also suggested that resveratrol --- a potent SIRT1 activator, can produce preconditioning effects of ischemic brain injury by activating SIRT1 and subsequent inhibition of mitochondrial uncoupler protein 2, leading to enhancement of mitochondrial ATP synthesis efficiency [96]. However, a latest study applying transgenic mice overexpressing SIRT1 solely in neurons did not show protective effect of the SIRT1 overexpression on ischemic brain damage [97].

Collectively, the studies regarding the roles of sirtuins in brain ischemia are far from sufficient. The insufficiency is further highlighted by the fact that a majority of the studies on the roles of SIRT1 in neuronal injury have applied resveratrol as the key pharmacological modulator of the enzyme. However, resveratrol may affect cell injury by multiple pathways, such as decreasing oxidative stress and inhibiting inflammation [98, 99]. Because latest studies have suggested that sirtuins are fundamental modulators of multiple cellular functions [100–102], future studies are needed to elucidate the roles of sirtuins in ischemic brain injury. Design of specific and potent activators and inhibitors of sirtuins is of particular importance for these future studies.

6. PERSPECTIVES

A large number of studies have compellingly indicated crucial roles of oxidative stress in ischemic brain damage. However, many future studies are still needed to elucidate the mechanisms underlying the generation of ROS under ischemia/reperfusion conditions, and the mechanisms underlying the ROS-initiated tissue injury cascade. In contrast, only recently an important role of NAD⁺ in brain ischemia has been suggested. Many mysteries still remain unsolved regarding the roles of NAD⁺ in cerebral ischemia. The following research directions regarding oxidative stress and NAD⁺ in ischemic brain damage may be of particular interest and significance:

First, it is warranted to determine the interaction between oxidative stress and altered NAD⁺ metabolism in brain ischemia, and to elucidate the role of this interaction in ischemic brain damage.

Second, future studies are needed to further elucidate the interactions between oxidative stress and other key pathological factors in brain ischemia, including inflammation, calcium dysregulation and mitochondrial alterations.

Third, it is necessary to further determine the relative contributions of microglial NADPH oxidase, neuronal NADPH oxidase and astrocytic NADPH oxidase to the oxidative damage in brain ischemia.

Fourth, ROS can produce both tissue damage and physiological redox signaling. It is of significance to elucidate the pathways underlying the detrimental effects of ROS and the pathways of the potentially beneficial redox signaling of ROS.

Fifth, it is warranted to conduct comprehensive studies on the roles of sirtuins in brain ischemia.

Sixth, future studies are needed to further determine if NAD⁺ can also decrease the brain injury in other brain ischemia models, and to elucidate the mechanisms underlying the protective effects of NAD⁺ on transient focal ischemia.

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