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Review

Nicotinamide provides neuroprotection by modulating cellular energy, inflammatory response, and apoptosis in rats subjected to ischemia-induced brain damage

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ABSTRACT

Cerebral ischemia is a result of reduced blood flow to tissues. The consequence is a rapid depletion of energy stores and the subsequent triggering of a complex cascade of cellular events including membrane depolarization, calcium influx, excessive production of free radicals, and the activation of necrotic as well as apoptotic pathways. The above reactions further perpetuate initial ischemic neuronal damage. Cytokines such as tumor necrosis factor-α, interleukin-6, and inducible nitric oxide synthase are involved in post-ischemic inflammatory responses that further exacerbate ischemic brain damage. Neuroprotective effects of nicotinamide (NAm), an amide form of vitamin B3, have been well-documented in other earlier studies. NAm can significantly reduce brain infarctions and improve neurologic outcome following ischemic stroke in various strains of both male and female rats. The protective effect of NAm is largely dose-specific at 500 mg/kg. However, a higher dose (750 mg/kg) is required in hypertensive rats. NAm also prevents energy depletion by facilitating neuronal adenosine triphosphate (ATP) production during cerebral ischemia. It also blocks cellular inflammatory response and has the ability to inhibit early apoptotic phosphatidylserine exposure and late nuclear DNA degradation induced by an experimental stroke as demonstrated in some studies. Accordingly, NAm exhibits multifaceted neuroprotective effects and can prevent neuronal death by inhibiting post-ischemic ATP depletion, inflammation, and necrotic as well as apoptotic events.

KEYWORDS: cerebral ischemia, nicotinamide, neuroprotection

1. INTRODUCTION

Stroke is one of the main causes of death and disability in adults [1-3]. Brain injury is expected to occur when cerebral blood flow drops to a level of less than 25% of normal values [4]. The consequence of decrease in blood flow is a failure in metabolic energy support, and the event initiates severe disturbances in cellular mechanisms essential for neuronal functioning [5, 6]. Ischemia leads to membrane depolarization, pH value reduction, and alteration of trans-membrane ionic gradients, resulting from massive energy consumption. Depolarization of cell membranes is potentially reversible at early stages, but if structural lesions continue to develop, it can lead to permanent damage. Depending on the size and location of the cerebral infarction, various neurologic deficits can ensue, including focal motor weakness, sensory loss, vision damage, speech comprehension issues, expression impairment as well as cognitive and memory disturbances. Ischemia also elicits the release of excessive excitatory amino acids and an increase in intracellular calcium. The influx of

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calcium into the cells through voltage and glutamate receptors subsequently activates the expression of lipases, proteases, and endonucleases that eventually cause cerebral damage [7].

Ischemia-reperfusion injury induces alterations in the expression of metalloproteinases, which further breaks down the blood-brain barrier and induces activation of inflammatory cascades as well as the disruption of basement membranes and the extracellular matrix [8]. Post-ischemic inflammatory response therefore contributes to brain injury. Ischemia-reperfusion brain injury inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) are subsequently massively produced by activated resident microglia and other immune cells [5, 6, 9]. The consequences of this activity further disrupt basement membranes and the extracellular matrix of cerebral endothelium [10]. Additionally, ischemia-reperfusion injury is believed to result in the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that increase blood-brain barrier permeability by activating metalloproteinases. Consequently, recruitment neutrophils, monocytes/macrophages and lymphocytes infiltrate into the ischemic brain tissues, and these events increase the susceptibility of brain damage via inflammation and apoptosis.

The current treatment modality for acute ischemic stroke is still confined to thrombolysis and revascularization of the obstructed blood vessel. Thrombolytic therapy, however, is effective and safe only at very early stages (up to 6 hours) [9, 11]. For example, intravenous administration of tissue plasminogen activator (tPA) within 3 hours of symptom onset has been proven to be effective with regard to restoring cerebral blood flow in the case of acute ischemic stroke [12-15]. However, delayed administration of tPA increases the risk of hemorrhagic transformation, and this often comes with fatal results. Therefore, a safe and effective neuroprotective agent is ultimately needed not only to benefit all stroke patients but also to protect the ischemic brain tissue against reperfusion injury after thrombolytic therapies.

Nicotinamide (NAm), the amide form of vitamin B3 (niacin), is a soluble B group vitamin [16]. NAm is a precursor of the coenzyme β-nicotinamide adenine dinucleotide (NAD^+) and a poly-ADP-ribose polymerase-1 (PARP-1) inhibitor, which is used to generate adenosine triphosphate (ATP) in the mitochondrial electron-transport chain and to prevent energy depletion [17, 18]. NAm treatment is considered as a novel form of neuroprotection and is critical for preserving neuronal survival in several forms of neurodegenerative disorders [19]. It is known that exposure to free radicals [20-22] and oxidative stress [23] decreases neuronal survival and increases membrane phosphatidylserine (PS) exposure. Treatment with NAm however prevents membrane PS expression and enhances neuronal survival [24]. Meanwhile, NAm provides neuroprotection against malonate (a mitochondrial complex II inhibitor)-induced damage to the striatum [25] and nitric oxide-induced injury in the rat hippocampus [26]. NAm also prevents cellular degeneration following transient [27, 28] and permanent focal cerebral ischemia. Therefore, NAm has the ability to protect brain neurons against ischemia-induced energy imbalances or oxidative stress.

2. Dose-specificity of NAm in ischemic stroke

The usage of NAm is well-established and is associated with little or no adverse effects in the treatment of a variety of disorders, even with large daily doses [29]. Thus, it is relatively convenient to use this drug for various clinical trials incorporating stroke patients. However, it is imperative to determine the optimal treatment dose of NAm for damaged nervous tissues, since NAm-induced neuroprotection is dose-specific both *in vivo* and *in vitro*. In animal models of cerebral ischemia including transient or permanent middle cerebral artery (MCA) occlusion, either intraperitoneal (i.p.) or intravenous (i.v.) injection with NAm of 500 mg/kg dose significantly reduces brain infarction [30-33] and ameliorates neurological outcome. However, a higher concentration of NAm at 750 mg/kg is needed to achieve neuroprotection in hypertensive stroke rats (SHR) [34]. It has been reported consistently that increased dose requirements are required for other neuroprotective agents in the case of SHR [35, 36]. In cellular models, the protection of neurons against oxygen-glucose deprivation requires an exact concentration of 12.5 mM [20, 21].

Treatment with NAm at a concentration range of 7.5 to 15 mM can significantly protect neurons against nitric oxide (NO)-induced cytotoxicity [21]. Either pre-treatment or post-treatment with NAm can increase neuronal survival, decrease degradation of genomic DNA, and can prevent membrane PS exposure after NO-induced cytotoxicity. Thus, NAm effectively prevent NO-induced apoptosis [21]. Moreover, the neuroprotection ability of NAm has been demonstrated to be effective in various rat strains such as Wistar, Sprague-Dawley, and Fisher 344, and has similar potency for both male and female rats following cerebral ischemia [30-32, 34, 37-40].

3. Therapeutic window of NAm in ischemic stroke

NAm not only reduces the number of brain infarctions but also improves the neurobehavioral outcomes caused by ischemia of the middle cerebral artery (MCA) occlusion. In our previous reports, NAm, given intraperitoneally, protects against ischemic brain damage with a therapeutic window of up to 2 hours following permanent MCA occlusion in rats [30, 41]. The cerebral infarction of rats is attenuated on day 3 and day 7 [32]. Additionally, NAm reduces cerebral infarction with a therapeutic window of up to 4 hours and improves neurobehavioral outcomes with a therapeutic window of up to 6 hours after a transient MCA occlusion [37]. Moreover, NAm not only maintains genomic DNA integrity and membrane PS asymmetry, but it also prevents membrane reversal with a therapeutic window of up to 6 hours in an *in vitro* model of the NO-induced neuronal apoptosis [21]. The combination of these anti-apoptosis effects can potentially ameliorate cerebral infarction and neurobehavioral deficits, thus mitigating brain injury with ischemic stroke. Furthermore, administration of NAm using an i.v. route can extend the therapeutic window beyond 2 hours. Therefore, delayed treatment with NAm via an i.v. route greatly improves neuroprotection as compared to that using an i.p. injection [31, 33]. Thus, NAm treatment depends on different models of MCA occlusion in terms of the appropriate therapeutic window. These results indicate that the therapeutic window for reducing cerebral infarction and neurobehavioral deficit in transient MCA occlusion is around 6 hours post-insult, while that for reducing cerebral infarction in a permanent MCA occlusion is 2 hours.

4. Cellular energy management using NAm in ischemic stroke

NAm has been shown to protect neuronal damage against neurochemical toxin-induced lesions in rats [25, 42, 43]. ATP is essential for maintaining brain function. It has been shown that the pathophysiology of ischemic lesions begins with an imbalance between energy supply and energy demand that results in rapid energy depletion and neuronal damage [30]. NAm, a precursor of NAD^+, can be directly utilized by cells to synthesize NAD^+ [19, 44, 45]. ATP is produced through a respiratory electron transport chain by utilizing NAD^+ in the mitochondria [46-48]. Furthermore, NAm can significantly increase NAD^+ levels, thus preventing the depletion of neuronal ATP and boosting the amount of ATP in the ischemic brain [25, 42, 49, 50]. The above-mentioned results suggest that NAm offers neuroprotection through the maintenance of NAD^+ levels in tissues that are at risk of infarction after cerebral ischemia.

During cell injury involving DNA-strand breaks, PARP is required for DNA repair. PARP-1, a highly abundant nuclear protein, binds to DNA-strand breaks and cleaves NAD^+ into nicotinamide and ADP-ribose [51]. Overactivation of PARP-1, however, rapidly consumes tissue stores of NAD^+ and ATP, thereby resulting in cell dysfunction and cell death. In an ischemia-induced neuronal injury, NO release is an important stimulus for DNA damage and PARP-1 activation [52]. It is known that PARP-1 activation contributes to neuronal damage after cerebral ischemia. In rats with cerebral ischemia, PARP-1 inhibitors and PARP-1 gene deletion exhibit cerebral protection by reducing brain infarction [53-57]. NAm is a PARP-1 inhibitor, and it also serves to prevent DNA damage. Thus, NAm provides a substrate for PARP-1, maintains the PARP-1 integrity, and prevents DNA damage after cerebral ischemia [45, 58]. Accordingly, NAm improves the energy supply to the ischemic brain by boosting the amount of ATP and by counteracting PARP-1 activation [38]. NAm also preserves a cellular

energy reserve for ATP-dependent DNA repair and maintains PARP-1 integrity. Finally, NAm protects neurons against ischemia by rectifying the ischemia-induced energy imbalance.

5. Inflammatory response to NAm in ischemic stroke

Inflammatory response and oxidative stress are well-known to exacerbate the brain damage caused by an ischemia-reperfusion brain injury [59-65]. Both experimentally and clinically, the inflammatory response partially exaggerates initial brain injury post-ischemia [60, 62, 64-67]. Stroke patients with systemic or cerebral inflammation exhibit grave clinical outcomes [68-70]. Following stroke, resident microglial cells activate and produce numerous proinflammatory mediators [10]. These cellular mediators damage cerebral endothelial permeability, and as a result, peripheral leukocytes are recruited into the ischemic brain [71]. The proinflammatory cytokines include iNOS, TNF-α, IL-1β, and IL-6 [60, 61]. These inflammatory cytokines are produced by a variety of activated cells containing endothelial cells, microglia, neurons, monocytes/macrophages, and fibroblasts [66]. In addition, cytokines elicit the synthesis of adhesion molecules such as P-selectin, E-selectin, and intercellular adhesion molecule-1 that facilitate the adhesion of activated leukocytes to endothelial cells. Consequently, activated leukocytes infiltrate and migrate into the ischemic brain parenchyma [72-74]. In several animal models, cerebral ischemia has been found to induce recruitment and activation of inflammatory cells, including neutrophils, T cells, and monocytes/ macrophages into the ischemic brain tissues [60, 61, 64, 65]. These infiltrating inflammatory cells and activated microglia further contribute to secondary ischemic brain injury [60, 66, 75, 76].

Clinical studies have suggested that the progression of brain damage can be reduced by attenuating inflammation during ischemia-reperfusion [77, 78]. The inflammatory responses are inhibited by blocking iNOS and cyclo-oxygenase-2 expression [79]. NAm modulates inflammatory responses by directly inhibiting the expression of IL-1β, IL-6, and TNF-α [80, 81]. This suggests that NAm can exhibit potent anti-inflammatory activity after ischemia-reperfusion.

Additionally, both PARP-1 and nuclear factor kappa B (NFκB) play a crucial role in the inflammatory response. NFκB, a family of inducible transcription factors, regulates the genes involved in inflammatory responses, apoptosis, cell proliferation and differentiation [82]. PARP-1 is required for NFκB-dependent gene expression and acts as a coactivator of NFκB. NFκB is normally sequestered in the cytoplasm by NFκB inhibitors (IκB) [83, 84]. Ischemia activates an upstream kinase (IκB kinase), which results in the phosphorylation of IκB leading to the release of NFκB into the nuclei. Subsequently, it induces the expression of NFκB-dependent proinflammatory cytokines such as iNOS, IL-6, IL-1β, and TNF-α [80, 83, 84]. Therefore, NFκB-driven signals play a pivotal role in mediating inflammatory responses after cerebral ischemia.

Our studies indicated that NAm effectively inhibits the production of the TNF-α, IL-6, and NO in lipopolysaccharide (LPS)-treated Raw 264.7 and BV2 cells [85]. Furthermore, NAm-treated animals show significant decrease in the number of neutrophils and activated microglia/ macrophages in the ischemic brain after an ischemic insult. Additionally, the NAm-treated animals show significant reduction of brain infarction, increase in neuronal survival and improvement in neurobehavioral outcome. Consequently, NAm prevents neuronal damage after brain ischemia owing to its anti-inflammatory properties. Our previous study also showed that NAm significantly reduces specific NFκB binding activity and nuclear translocation *in vitro* in LPS-stimulated BV2 and Raw 264.7 cells and *in vivo* in the ischemic neurons [85]. Moreover, we observed that NAm significantly decreases the phosphorylation of IκB and attenuates NFκB translocation from cytoplasm to nucleus, and thus downregulates the iNOS synthase expression in the ischemic brain. These results indicate that NAm exhibits robust anti-inflammatory actions against the neuroinflammation elicited by cerebral ischemia.

6. The anti-apoptotic effects of NAm in ischemic stroke

The ROS, NO, and proinflammatory cytokines cause neuronal apoptoic death following ischemia

injury [86, 87]. Apoptosis, also termed as 'programmed cell death' is a major neuronal injury after ischemia. Apoptosis occurs through two pathways that involve the loss of membrane asymmetry and DNA fragmentation [88, 89]. In normal cells, PS residues are found in the inner membrane of the cytoplasmic membrane. During apoptosis, loss of membrane phospholipid asymmetry leads to the exposure of membrane PS residues on the membrane surface that assists microglia to target the injured cells for phagocytosis [19, 90-92]. The cleavage of genomic DNA into fragments is a delayed event that occurs late in apoptosis [93, 94]. Oxidative stress is one of the eliciting factors that lead to neuronal apoptosis due to the release of excessive ROS after cerebral ischemia [95-97]. In experimental stroke models, the generation of NO causes neuronal apoptosis in a variety of cell types such as cortical neurons, hippocampal neurons and mesencephalic neurons [93, 98-100]. Treatment with NAm enhances neuronal survival during NO toxicity and prevents NO-induced programmed cell death [21]. Additionally, NAm prevents the exposure of membrane PS residues, blocks inflammatory cell activation [20, 21, 101, 102], and inhibits DNA destruction [20-22]. Thus, NAm provides robust neuroprotection against oxidative stress following stroke.

Caspases are a family of cysteine proteases that are responsible for cellular morphological alterations that occur during apoptosis. NO elicits the activation of caspase 1 and 3, and they mediate membrane PS exposure and DNA fragmentation [103, 104]. Several studies have demonstrated that NAm reduces NO-induced neuronal apoptosis and increases neuronal viability in a concentration-specific manner [19-21]. Treatment with Nam at a concentration range of 7.5 to 15 mM significantly protects neurons against NO-induced apoptosis [21]. Furthermore, NAm protects cell apoptosis against NO toxicity with a therapeutic window of up to 6 hours *in vitro*. Moreover, NAm prevents neuronal apoptosis by decreasing genomic DNA fragmentation after an NO-induced injury. Administration of NAm consistently maintains DNA integrity and prevents membrane PS exposure in the case of NO-induced neuronal damage. These results suggest that NAm maintains genomic DNA integrity and membrane PS asymmetry through the modulation of cysteine protease activity and prevention of the cleavage of PARP-1 [20, 21].

7. CONCLUSION

A substantial number of studies have indicated that NAm has multifaceted properties that act against brain damage after ischemic brain injury. In animal models of stroke, NAm treatment significantly reduces cerebral infarction, increases neuronal survival and improves neuronal behavior. NAm offers neuroprotective effects by enhancing the maintenance of DNA integrity and the preservation of membrane asymmetry after PS exposure, and also prevents oxidative stress-induced programmed cell death. These protective effects are linked to its regulation ability to reduce the activation of caspase 1 and 3, and the DNA-repair enzyme PARP-1. NAm, as a precursor to NAD^+ has the potential to reduce ischemia-induced energy depletion via increasing ATP reserves in neurons. Additionally, NAm prevents secondary neuronal damage owing to its anti-inflammatory actions. In LPS-stimulated RAW 264.7 and BV2 cells, NAm effectively inhibits NFκB translocation and binding activity as well as the production of TNF-α, IL-6, and NO. Moreover, NAm attenuates the phosphorylation of IκB, NFκB translocation and binding activity and then reduces the iNOS expression in the ischemic brain. Also, animals treated with NAm exhibit significant reductions in immune cell infiltration into the ischemic brain. These anti-energy depletory, anti-inflammatory and anti-apoptotic effects offered by NAm can potentially prevent ischemia-induced brain injury (Figure 1).

NAm not only reduces brain infarction and improves neurological outcome, but also provides a wide therapeutic window following a prolonged recovery period in rats. Furthermore, the neuro-protection remains effective in different ischemic animal models including transient or permanent MCA occlusion and in different conditions of diabetic or hypertensive subjects. NAm also exhibits neuroprotective effects in various rat strains as well as in male and female rats following cerebral ischemia. More importantly, NAm is already used clinically in large doses and has very few side effects. Thus, NAm can be considered as a

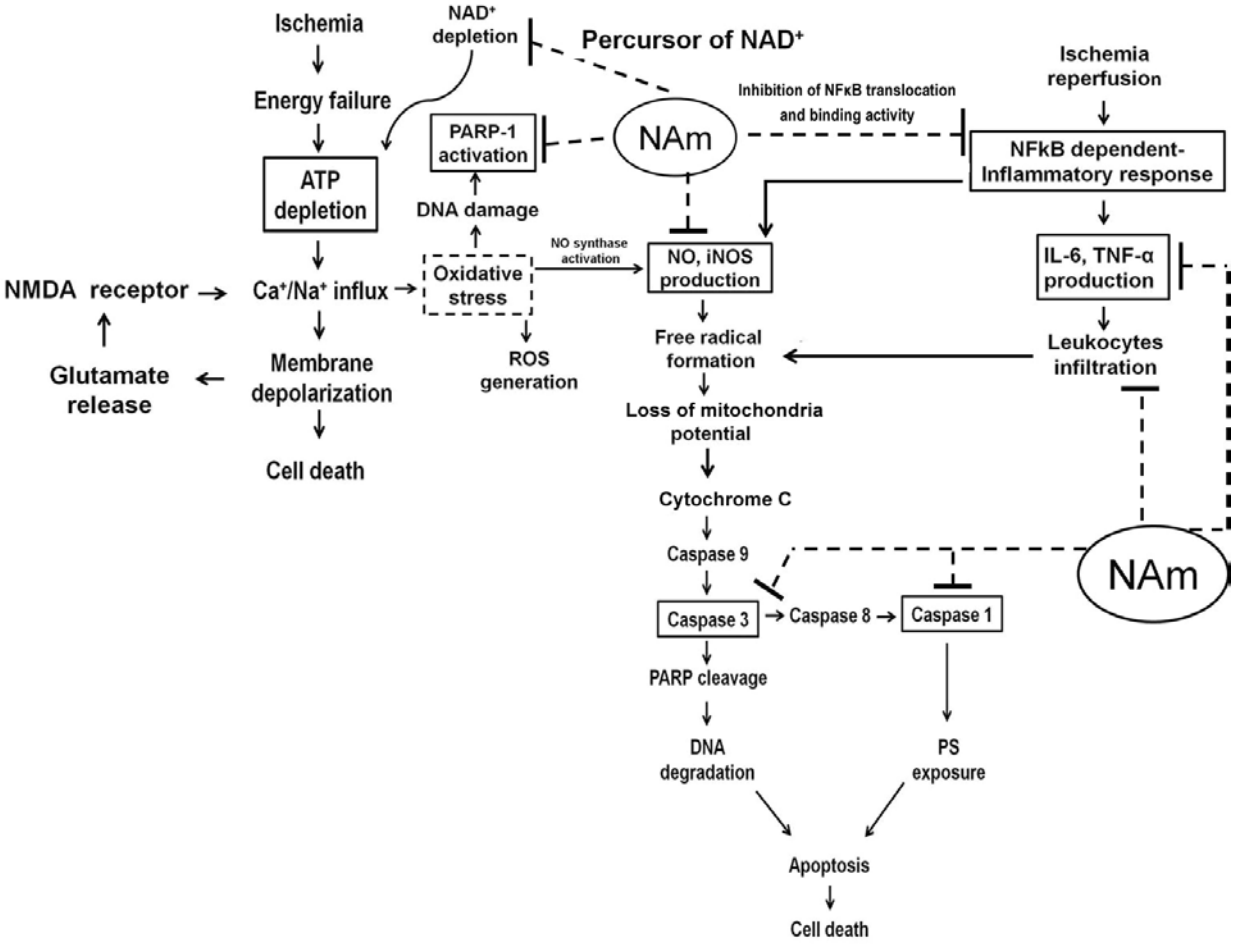

Figure 1. Nicotinamide (NAm) provides neuroprotection through multiple pathways. (1) NAm regulates poly-ADP-ribose polymerase-1 (PARP-1), nuclear factor kappa B (NFκB), and cysteine protease activity. (2) NAm attenuates nitric oxide (NO), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) production and (3) decreases leukocyte infiltration to prevent cell death post-ischemia.

candidate that can act as a neuroprotective agent and may be suited for use in clinical trials conducted on patients with ischemic stroke.

CONFLICT OF INTEREST STATEMENT

The authors declare there is no conflict of interest.

ABBREVIATIONS

NAm, nicotinamide; TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; RNS, reactive nitrogen species; tPA, tissue plasminogen activator; NAD^+, nicotinamide adenine dinucleotide; PARP-1, poly-ADP-ribose polymerase-1; ATP, adenosine triphosphate; PS, phosphatidylserine; i.p., intraperitoneal; i.v., intravenous; SHR, hypertensive stroke rats; NO, nitric oxide; MCA, middle cerebral artery; NFκB, nuclear factor kappa B; LPS, lipopolysaccharide.

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