Cellular NAD Replenishment Confers Marked Neuroprotection Against Ischemic Cell Death
Role of Enhanced DNA Repair

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Background and Purpose—NAD\(^+\) is an essential cofactor for cellular energy production and participates in various signaling pathways that have an impact on cell survival. After cerebral ischemia, oxidative DNA lesions accumulate in neurons because of increased attacks by ROS and diminished DNA repair activity, leading to PARP-1 activation, NAD\(^+\) depletion, and cell death. The objective of this study was to determine the neuroprotective effects of NAD\(^+\) repletion against ischemic injury and the underlying mechanism.

Methods—In vitro ischemic injury was induced in rat primary neuronal cultures by oxygen-glucose deprivation (OGD) for 1 to 2 hours. NAD\(^+\) was replenished by adding NAD\(^+\) directly to the culture medium before or after OGD. Cell viability, oxidative DNA damage, and DNA base-excision repair (BER) activity were measured quantitatively up to 72 hours after OGD with or without NAD\(^+\) repletion. Knockdown of BER enzymes was achieved in cultures using AAV-mediated transfection of shRNA.

Results—Direct NAD\(^+\) repletion in neurons either before or after OGD markedly reduced cell death and OGD-induced accumulation of DNA damage (AP sites, single and double strand breaks) in a concentration- and time-dependent manner. NAD\(^+\) repletion restored nDNA repair activity by inhibiting serine-specific phosphorylation of the essential BER enzymes AP endonuclease and DNA polymerase-\(\beta\). Knocking down AP endonuclease expression significantly reduced the prosurvival effect of NAD\(^+\) repletion.

Conclusion—Cellular NAD\(^+\) replenishment is a novel and potent approach to reduce ischemic injury in neuronal cultures. Restoration of DNA repair activity via the BER pathway is a key signaling event mediating the neuroprotective effect of NAD\(^+\) replenishment. (Stroke. 2008;39:2587-2595.)

Key Words: ischemia ■ DNA damage ■ base-excision repair

NAD is an essential cell survival factor that participates in various critical cellular processes, including energy metabolism, ADP-ribose cyclase synthesis, and class III histone deacetylase activity.\(^1\) NAD also acts as the substrate of poly(ADP-ribose) polymerase 1 (PARP-1), which, once activated, catalyzes transfer of ADP-ribose moieties from NAD to target proteins. A large body of literature shows that cerebral ischemia/reperfusion results in PARP-1 overactivation and consequent decline of NAD levels in the brain.\(^2,3\) As NAD is essential for the mitochondrial electron transport reaction, NAD depletion is thought to suppress mitochondrial function and ATP generation, leading to the release of apoptosis-inducing factor (AIF) and eventually cell death.\(^4\) In nonproliferating cells such as neurons, however, NAD is highly compartmentalized, and the mitochondrial pool of NAD is not readily depleted by PARP-1 activation.\(^1,5\) Thus, neuronal NAD depletion is most likely to menace cell survival by repressing other NAD-dependent signaling pathways.\(^5,6\)

Oxidative DNA damage is a severe consequence of oxidative stress that, if not repaired, results in cell death via activation of several pathways.\(^7\) Endogenous oxidative damage to nDNA that produces base damage, apurinic/apyrimidinic abasic site (AP sites), and strand breaks occurs rapidly after cerebral ischemia/reperfusion and is an important trigger of ischemic cell death.\(^8,9\) In the brain, DNA base excision repair (BER) functions as the major repair mechanism for oxidative DNA damage, and neuronal BER activity is highly regulated after ischemia and reperfusion. Although BER activity is markedly upregulated in the brain after sublethal insults,\(^10,11\) including the neuroprotective paradigm of ischemic preconditioning,\(^12\) it rapidly declines after lethal ischemic
injury, leading to the accumulation of cytotoxic oxidative DNA lesions.10,12 Our recent study suggests that the rapid downregulation of BER activity after lethal ischemic injury is likely attributable to the aberrant serine/threonine-specific phosphorylation of the BER rate-limiting enzymes AP endonuclease (APE) and DNA polymerase-β (βpol).13 Because the post-translational disabling of BER enzymes is reversible at least during the early stage of ischemic injury,13 preventing or inhibiting this undesirable process represents a legitimate strategy to restore DNA repair function in ischemic brain.

The current study was aimed at investigating the neuroprotective effects of direct exogenous supplementation with NAD⁺ (NAD replenishment) in an in vitro model of ischemic neuronal injury induced by oxygen-glucose deprivation (OGD). Despite the long-held assumption that NAD⁺ is cell membrane impermeable, recent reports indicate that exogenous NAD⁺ can gain limited access into certain types of cells, including cultured hippocampal neurons and cerebral astrocytes.5,14,15 The data presented here demonstrate that cellular NAD⁺ replenishment confers remarkable neuroprotection against ischemic injury and that this neuroprotective effect is mediated at least in part via restoration of DNA repair activity in neurons.

Materials and Methods

Primary Culture and Oxygen-Glucose Deprivation

Primary cultures of hippocampal or cortical neurons were prepared from embryonic day 17 Sprague-Dawley rat embryos as previously described.16 Experiments were conducted at 12 days in vitro, when cultures consisted of ~97% of neurons. To model ischemia-like conditions in vitro, cultures were exposed to transient oxygen and glucose deprivation (OGD). To replenish cellular NAD levels, NAD⁺ was added directly to culture medium either before or after OGD and incubated for up to 3 hours. Alamar blue fluorescence (AccuMed International) was used to measure the viability of the cultured neurons at 24 to 72 hours after OGD.16 OGD-induced cell death was quantified by measuring lactate dehydrogenase (LDH) release from damaged cells into the culture medium.16 using a commercial kit (Sigma-Aldrich).

Overall BER Activity

The in vitro DNA incorporation repair assay was performed to measure the overall BER activity in nuclear protein extracts.10,12,13 The repair substrate used in the assay consisted of purified pcDNA plasmids containing the oxidative adduct 8-oxoG. The repair products were analyzed by autoradiography and densitometry analysis.

Western Blot

Western blot analysis was performed using standard procedures.10 The working dilutions for the following monoclonal antibodies were used per the manufacturer’s suggestions: OGG1 (1:1000), βpol (1:500), and ligase-I (1:1000) (NeoMarkers), and APE1 (1:1000) (Novus Biologicals).

Statistical Analysis

Data are presented as mean±SEM. Comparisons were made using analysis of variance (ANOVA) and post hoc Scheffe’s tests. A level of P<0.05 was considered statistically significant.
Results

NAD Replenishment Confers Remarkable Neuroprotection Against OGD

To investigate whether NAD⁺ replenishment is neuroprotective against OGD, NAD⁺ at various concentrations was added directly to cultures 30 minutes prior to 1, 1.5, or 2 hours of OGD, and the NAD⁺-containing medium was replaced with normal medium after 3 hours of incubation. Using 2 independent measurements (Figure 1A and 1B), NAD⁺ treatment remarkably attenuated OGD-induced cell death at 24 hours in a concentration-dependent manner. At 5 mmol/L, NAD⁺ completely prevented cell death induced by 1 hour of OGD; at 15 mmol/L, NAD⁺ completely prevented LDH release, and restored cell viability to 85% of normal levels after 2 hours of OGD. The protective effect of NAD⁺ was confirmed using morphological assessments, including phase contrast, DAPI nuclear staining, and propidium iodide/DAPI double staining (Figure 1E through 1G). At all concentrations tested (0.3 to 15 mmol/L), NAD⁺ itself did not cause cell death in control non-OGD neurons (not shown).

To determine the time window of efficacy, NAD⁺ (15 mmol/L) was added to cultures before or after 2 hours of OGD for 3 hours. Whereas NAD⁺ added before or immediately after OGD offered complete or near-complete neuroprotection (Figure 1C and 1D), NAD⁺ treatment initiated 0.5
or 1 hour after OGD showed only partial neuroprotection. In contrast, no neuroprotection was detectable when NAD⁺ treatment was delayed for 2 hour (Figure 1C and 1D).

NAD⁺ Attenuates OGD-Induced DNA Damage

The nuclear accumulation of oxidative DNA lesions is the trigger for PARP-1 activation and subsequent NAD depletion after ischemia, presumably impairing DNA repair, as NAD is an essential cofactor for the BER pathway. Therefore, the next objective was to determine the effect of NAD⁺ on OGD-induced oxidative DNA damage, with emphases on the early stages of post-OGD injury. Two types of DNA lesions, AP sites and single strand breaks (SSB) were measured quantitatively in cultures challenged with 2-hour OGD. Immediately (0 hours) after OGD, measurement of both AP sites and SSB showed a 4- to 5-fold increase over non-OGD controls in vehicle-treated cultures (Figure 2A and 2B). The levels of DNA lesions showed burst increases (~10-fold for AP sites, ~20-fold for SSB) 0.5 hour after OGD, reaching peaks at 6 hours. NAD⁺ given before OGD nearly completely...
prevented the induction of AP sites and SSB (Figure 2A and 2B). NAD$^+$ given immediately after OGD had no effect on the initial induction (0 hours after OGD) of DNA damage, but significantly reduced the levels of DNA lesions 0.5 hour after OGD and thereafter.

The effect of NAD$^+$ on OGD-induced SSB is illustrated at the cellular level (Figure 2C). Consistent with the role of SSB in activating PARP-1, NAD$^+$-treated neurons showed diminished formation of poly(ADP-ribose)polymers (PARS) as well as SSB after OGD. Notably, in neurons treated with the PARP-1 inhibitor 3-aminobenzomide (3-AB), OGD-induced formation of PARS was prevented but the induction of SSB was not reduced. This result may explain the partial loss of 3-AB neuroprotection after prolonged post-OGD reperfusion (supplemental Figure II, available online at http://stroke.ahajournals.org).

The effect of NAD$^+$ on OGD-induced DNA damage was further examined using the Comet assay, which detects DNA strand damage at more advanced stages. Using this assay, increases in DNA tail length began to be detected at 0.5 hour after 2 hours OGD (Figure 2D and 2E); thereafter, the increases in tail length were more robust. NAD$^+$ given immediately after OGD nearly completely prevented the increases of tail length, whereas delayed NAD$^+$ treatment (0.5 hour after the completion of OGD) partially reduced the tail length (Figure 2E).

**NAD Restores DNA Repair Activity After OGD**

The accumulation of DNA lesions in ischemic brain results from both increased oxidative DNA damage and impaired DNA repair. We next determined whether NAD$^+$ treatment affected DNA repair activity in neurons after OGD. Nuclear protein extracts were assessed for AP endonuclease activity and $\beta$pol activity, the 2 rate-limiting steps in the BER pathway, and total BER activity. As shown (Figure 3), all 3 repair activities showed marked reduction (75%) in neurons 0.5 to 6 hours after 2 hours OGD. NAD$^+$ given before OGD completely prevented the diminishment of DNA repair activ-
ities, whereas NAD⁺ given immediately after OGD restored the repair activities up to 80% of normal levels (Figure 3).

NAD Restores DNA Repair by Attenuating Phosphorylation of BER Enzymes

To elucidate the mechanism by which NAD⁺ restored BER activities after OGD, we first examined the expression levels of the essential BER enzymes OGG1, APE1, βpol, and ligase-1. None of the enzymes showed reduced expression either 0.5 or 2 hours after OGD, whereas APE1 and βpol showed a modest 25% to 30% reduction 6 hours after OGD (Figure 4A and 4B). These results indicate that the diminished BER activity 0.5 to 6 hours after OGD was not attributable to reduced expression of BER enzymes, but may be attributable to posttranslational disabling of BER enzymes.

Our recent study suggests that BER enzymes are disabled by serine-specific phosphorylation after focal ischemia. To determine whether this mechanism was occurring in OGD, we treated nuclear protein extracts from OGD neurons with alkaline phosphatase. Alkaline phosphatase-mediated serine dephosphorylation completely restored both APE1 (Figure 4C) and βpol activity (Figure 4F). Coimmunoprecipitation was performed using either anti-APE1 or anti-βpol antibody to detect serine phosphorylation on these enzymes after OGD. As expected, OGD induced robust serine phosphorylation in both APE1 (Figure 4D) and βpol (Figure 4G), whereas NAD⁺ given immediately after OGD abolished serine phosphorylation after OGD (Figure 4E and 4H).

Functional Integrity of BER Is Critical for the Neuroprotective Effect of NAD⁺

To determine whether enhanced DNA repair by NAD⁺ is essential for its neuroprotective effect against OGD, we examined the effect of knockdown of APE1, the rate-limiting enzyme in the BER pathway. AAV (carrying the shRNA sequence targeting APE1) infection for 3 days resulted in approximately 90% reduction in APE1 expression (Figure 5A), APE activity (Figure 5B), and total BER activity (Figure 5C). The neuroprotective effect of NAD⁺ against OGD was
markedly reduced, but not completely eliminated, in APE1-deficient neurons, showing significantly increased formation of AP sites (Figure 5D) and reduced cell survival (Figure 5E and 5F) compared to nontransfected neurons or neurons transfected with the scramble shRNA sequence.

Discussion

The current study demonstrated that NAD replenishment in primary neurons conferred robust neuroprotection against OGD-induced cell death. Moreover, the results suggest that restoration of DNA repair activity and reduced accumulation of cytotoxic DNA lesions are an important mechanism underlying the neuroprotective effect of NAD\textsuperscript{+}. A diagram illustrating this hypothesized pathway is shown (Figure 6).

The data show that exogenous supplementation of NAD\textsuperscript{+} to cultured neurons attenuated OGD-induced cell death in both concentration- and time-dependent manners. At the optimal concentration range (3 to 15 mmol/L), NAD\textsuperscript{+} nearly completely inhibited cell death after OGD for at least 72 hours in cultures. The efficacy of NAD\textsuperscript{+} appears to be greater than MK801 and 3-AB (supplemental Figure II), the 2 well characterized neuroprotective agents capable of inhibiting PARP-1–mediated excitatory cell death in the OGD model. The optimal concentration range of NAD\textsuperscript{+} for neuroprotection is comparable to that used in cultured cerebral astrocytes,\textsuperscript{5,15} but substantially higher than that required for cytoprotection against myocyte cell death.\textsuperscript{6} Although the precise mechanism by which exogenous NAD\textsuperscript{+} gains internalization into mammalian cells is not understood, it has been reported that plasma membrane connexin-43 channels can actively transport extracellular NAD\textsuperscript{+}.\textsuperscript{20} In support of this notion, we found that lowering the temperature of neuronal cultures to 4°C could block NAD\textsuperscript{+} uptake (supplemental Figure I). Therefore, the expression level and activity of NAD\textsuperscript{+}-transporting channels may be a determining factor for the concentration-dependency of NAD\textsuperscript{+} in a given cell type. It is likely that neurons and astrocytes possess very low levels of NAD\textsuperscript{+}-transporting activity, thus a millimolar range of exogenous NAD\textsuperscript{+} is required for its neuroprotective effect. It is a concern whether a therapeutic concentration of NAD\textsuperscript{+} can be reached in the brain following systemic administration. In a recent study,\textsuperscript{21} nasal delivery of NAD\textsuperscript{+} to rats reduced ischemic cerebral infarction, suggesting that NAD\textsuperscript{+} replenishment may be a feasible therapeutic strategy.
We investigated the effect of NAD\textsuperscript{+} replenishment on oxidative DNA damage, a critical intracellular event that contributes to OGD-induced cell death. The levels of cell-killing oxidative DNA lesions were remarkably reduced in NAD\textsuperscript{+}-treated OGD neurons. Although reduced ROS production could be an underlying mechanism for this effect, it is likely that NAD\textsuperscript{+} also prevents the accumulation of DNA lesions by enhancing the BER DNA repair pathway. In agreement with results from the focal ischemia/reperfusion model,\textsuperscript{13} BER activities were greatly compromised without subsequent recovery in neurons 0.5 hour after OGD, thus facilitating the accumulation of DNA damage. The loss of BER activities in OGD neurons was attributable to serine- and threonine-specific phosphorylation of the BER rate-limiting enzymes APE1 and β-pol, because the enzymatic activities from OGD neurons could be completely restored by alkaline phosphatase. Thus, it is likely that NAD\textsuperscript{+} reinstated the BER activities in OGD neurons by inactivating the protein kinase(s) or activating the phosphatase(s) that control the phosphorylation states of BER enzymes. Although we did not investigate the precise phosphorylation sites on BER enzymes in this study, a previous report has demonstrated that β-pol can be phosphorylated at Ser44 and Ser55 by protein kinase C, which resulted in the loss of its enzyme activity.\textsuperscript{22} Further study will be needed to elucidate whether protein kinase C or other protein kinases are involved in the modification of BER enzymes.

APE-1 knockdown only partially eliminated the prosurvival effect of NAD\textsuperscript{+} in OGD neurons, suggesting that, although the functional integrity of BER is essential for NAD neuroprotection, NAD\textsuperscript{+} replenishment must activate additional prosurvival mechanisms. NAD\textsuperscript{+} replenishment may restore substrate delivery to the mitochondria under severe NAD-consuming conditions, thus resuming energy production.\textsuperscript{23} Moreover, among the NAD\textsuperscript{+}-dependent cellular processes particularly worth noting are the class III histone deacetylases, also called SIRT proteins.\textsuperscript{24} The nuclear-localized SIRT1 plays a critical role in cell survival through regulating chromatin remodeling and suppressing the apoptotic proteins p53 and Ku70.\textsuperscript{25} A protective role for SIRT1 has been demonstrated in the brain and heart against PARP-1–dependent cell death,\textsuperscript{6,14} and in a brain slice model of OGD injury.\textsuperscript{26} The deacetylase activity of SIRT proteins is greatly affected by changes in cellular NAD\textsuperscript{+} levels. Whereas reduced cellular levels of NAD attenuate the SIRT deacetylase activity,\textsuperscript{25} increased NAD\textsuperscript{+} biosynthesis activates it.\textsuperscript{14} Thus, it is possible that NAD\textsuperscript{+} replenishment in OGD neurons may promote neuronal survival via activating SIRT deacetylase activity, in addition to its role in enhancing DNA repair as demonstrated in the present study.

In summary, the results presented here elucidate a novel intracellular signaling pathway by which NAD\textsuperscript{+} replenishment confers remarkable neuroprotection against OGD-induced cell death in primary cultured neurons. Further characterization of the neuroprotective effects of NAD\textsuperscript{+} replenishment, either through exogenous NAD\textsuperscript{+} delivery or endogenous NAD\textsuperscript{+} biosynthesis, and the underlying mechanisms for NAD\textsuperscript{+} neuroprotection, may enhance the candidacy of NAD as a novel therapeutic strategy for stroke.

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**Disclosures**

None.

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