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Nicotinamide pre-treatment ameliorates NAD(H) hyperoxidation and improves neuronal function after severe hypoxia

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Abstract

Prolonged hypoxia leads to irreversible loss of neuronal function and metabolic impairment of nicotinamide adenine dinucleotide recycling (between NAD⁺ and NADH) immediately after reoxygenation, resulting in NADH hyperoxidation. We test whether addition of nicotinamide (to enhance NAD⁺ levels) or PARP-1 inhibition (to prevent consumption of NAD⁺) can be effective in improving either loss of neuronal function or hyperoxidation following severe hypoxic injury in hippocampal slices. After severe, prolonged hypoxia (maintained for 3 min after spreading depression) there was hyperoxidation of NADH following reoxygenation, an increased soluble NAD⁺/NADH ratio, loss of neuronal field excitatory post-synaptic potential (fEPSP) and decreased ATP content. Nicotinamide incubation (5 mM) 2 hr prior to hypoxia significantly increased total NAD(H) content, improved neuronal recovery, enhanced ATP content, and prevented NADH hyperoxidation. The nicotinamide-induced increase in total soluble NAD(H) was more significant in the cytosolic compartment than within mitochondria. Prolonged incubation with PJ-34 (>1hr) led to enhanced baseline NADH fluorescence prior to hypoxia, as well as improved neuronal recovery, NADH hyperoxidation and ATP content on recovery from severe hypoxia and reoxygenation. In this acute model of severe neuronal dysfunction prolonged incubation with either nicotinamide or PJ-34 prior to hypoxia improved recovery of neuronal function, enhanced NADH reduction and ATP content, but neither treatment restored function when administered during or after prolonged hypoxia and reoxygenation.

Keywords

Nicotinamide; Hypoxia; Brain; Spreading Depression; Hippocampus; PARP-1

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Introduction

A major factor contributing to neuronal damage following severe hypoxia-ischemia is depletion of ATP associated with impaired mitochondrial function (Fiskum et al., 2008; Paschen et al., 2000). ATP generation and cellular metabolic status depend on metabolic recycling of the nicotinamide adenine dinucleotide couple [NAD^+/NADH] (Chance et al., 1962). One index of metabolic dysfunction after restoration of substrate following hypoxia-ischemia is significant decrease in NADH level, which has been termed hyperoxidation (Perez-Pinzon et al., 1998a; Perez-Pinzon et al., 1998b; Rosenthal et al., 1995). Although the basis for hyperoxidation remains unclear it occurs directly after reoxygenation following severe hypoxia and occurs together with irreversible loss of neuronal responses (Foster et al., 2008).

Possible causes of hyperoxidation include either lack of enzymatic regeneration of NADH following hypoxia-ischemia or changes in NAD^+ cofactor availability. Since mitochondria are impermeable to NAD(H) [total content of both NAD^+ and NADH], cytosolic and mitochondrial NAD(H) form two separate but linked pools (Alano et al., 2007; McKenna et al., 2006). Most enzymatic recycling of NAD^+ to NADH occurs within the mitochondrial tricarboxylic acid [TCA] cycle, so damage to TCA cycle enzymes, for example, could reduce regeneration of NADH, perhaps via reactive oxygen species [ROS] upon reoxygenation (Assaly et al., 2012). Alternatively, decreased mitochondrial NAD^+ could arise from leakage of NAD^+ through the mitochondrial permeability transition pore [mPTP] (Assaly et al., 2012; Fiskum et al., 2008). Reduced cytosolic NAD^+ availability could occur from overutilization in a number of NAD^+ - requiring reactions so that the available pool is drained, or lack of sufficient synthesis of NAD^+ (from its precursor, nicotinamide) (Alano et al., 2010; Bai et al., 2011; Liu et al., 2009; Ying, 2008).

One major enzymatic reaction which requires NAD^+ is somatic DNA repair by poly(ADP-ribose) polymerase -1 (PARP-1); intensive activation of PARP-1 could lead to depletion of the cytosolic pool of NAD^+ (Alano et al., 2007) and secondary energy failure resulting from impairment of NAD^+ -dependent steps of glycolysis (Suh et al., 2005). Provision of direct mitochondrial substrates after ischemia (such as pyruvate or ketone bodies) can potentially prevent mitochondrial failure and neuronal death by bypassing glucose utilization in aerobic glycolysis (Alano et al., 2010; Alano et al., 2004) (Ying et al., 2002). Sirtuins, another group of NAD^+ dependent deacetylating proteins, are regulated by the availability of NAD^+ , the NAD/NADH ratio, nicotinamide, leading to transcriptional adaptation by deacetylation of histone or transcriptional factors. Increased intracellular levels of NAD^+ modulate the activation of SIRT activity and protects neurons against excitotoxicity (Liu et al., 2008).

Exogenous nicotinamide (NAM) has also been reported to improve energy metabolism and neuronal function before ischemia and after brain injury, presumably by enhancing the synthesis of NAD^+ (Ghosh et al., 2012; Klaidman et al., 2003; Yang et al., 2004; Yang et al., 2002c). However, NAD^+ synthesis from nicotinamide is energy-requiring, indicating that pre-treatment would be optimal if possible (ie, when energy reserves are sufficient) rather than treatment directly after ischemia, when energy levels are low and only slowly being restored. Nicotinamide has also been suggested as a dietary treatment for this reason

(Stein and Imai, 2012). Multiple reports have suggested that NAD⁺ precursor therapy after ischemia and head injury can significantly limit cellular NAD⁺ impairment and cell death during recovery (Hoane et al., 2008; Klaidman et al., 2003; Liu et al., 2009; Peterson et al., 2012; Vonder Haar et al., 2011; Yang et al., 2002a). However, treatment with nicotinamide after ischemia may help in improving metabolic activity in the penumbra, but has proven less effective at improving the outcome in the ischemic core (Liu et al., 2009), and demonstrates a limited time window of efficacy to within a few hours of ischemia (Ayoub et al., 1999). To overcome this limited window of treatment after a metabolic insult nicotinamide pre-treatment has been performed in animal models, with administration preceding an experimental intervention, such as ischemia or head injury (Ayoub et al., 1999; Bai and Canto, 2012; Kim et al., 2011; Klaidman et al., 2003; Liu et al., 2009). This pre-treatment regimen has shown significant improvement in brain function compared to the baseline recovery from the metabolic insult, but in humans this form would require a chronic dietary treatment before an unpredictable disease onset. Because NAD⁺ synthesis is an energy requiring metabolic process, we wanted to determine if in our model supplementation of nicotinamide prior to the metabolic insult (hypoxia) would be more or less efficacious than treatment after hypoxia.

Our initial hypothesis explaining hyperoxidation and metabolic dysfunction after severe hypoxia is that consumption of NAD⁺ occurs due to increased activity of NAD⁺-requiring enzymes, such as PARP-1. This hypothesis best explains our previous data showing reduced NADH levels during a second, severe hypoxia after an initial hyperoxidation (Foster et al., 2008). We assess this hypothesis by measuring direct soluble NAD⁺/NADH ratio, NADH tissue fluorescence, and neuronal function, comparing whether nicotinamide incubation (to increase metabolically available NAD⁺) or PARP-1 antagonism (to reduce loss of NAD⁺) can reduce hyperoxidation and enhance neuronal recovery.

Material and Methods

Slice preparation

Animal use was approved by Duke University and Durham VA Medical Center Animal Care and Use Committees. Acute transverse hippocampal slices (400 μ m) were prepared from adult male Fischer 344 rats (2–3 months old, Harlan, Indianapolis, IN, USA) as described earlier (Shetty et al., 2012). Hippocampal slices were incubated in oxygenated (95 % O₂ - 5% CO₂) ACSF (in mM: NaCl 124, KCl 3.0, NaH₂PO₄ 1.25, NaHCO₃ 24, CaCl₂ 2.0, MgSO₄ 2.0, and dextrose 10, pH 7.4) at 36°C for 1 hr. Subsets of slices were treated with 5 mM nicotinamide for minimum 2 hour at 36°C before using for electrophysiological and biochemical manipulation, or PJ-34, a PARP-1 inhibitor, was added in some experiments (1 μ M).

Electrophysiological recording and synaptic stimulation

Schaffer collateral/commissural pathway was stimulated with a bipolar electrode situated in the stratum radiatum of the CA1 hippocampal region. An extracellular glass electrode filled with 0.2 M NaCl was coupled to an Axon Multiclamp Direct Current (DC) amplifier, and placed in CA1 stratum radiatum to record both evoked field excitatory post-synaptic

potential (fEPSP) and DC extracellular potential. After plotting an I/O curve to establish the stimulus required for saturation values for fEPSP, the stimulus current was adjusted to evoke an fEPSP of ~50% of maximum amplitude.

Hypoxia

Hippocampal slices were deprived of oxygen following 10 min of baseline recordings by replacing the 95% O₂- 5% CO₂ humidified gas mixture above the slices and in the buffer with 95% N₂ - 5% CO₂ (to maintain the bicarbonate pH buffering). As soon as hypoxic spreading depression (HSD) occurred (as evident by the negative change in DC extracellular potential), the hypoxia was continued for a set amount of time (between 1 and 5 min) followed by reoxygenation for up to 120 min (Foster et al., 2008). The percentage of neuronal recovery was assessed, i.e. the difference in fEPSP peak amplitude measured at 40 minute following reoxygenation compared to the baseline control prior to hypoxia.

NADH fluorescence imaging

NADH fluorescence in hippocampal slices was measured using 290 to 370 nm excitation filter and a 420 nm long pass emission filter (Omega Optical, Brattleboro, VT, USA, xenon light source, DG-4, Sutter), as described previously (Foster et al., 2008). Though baseline fluorescence imaging also detects NADPH as well as NADH, NADPH is less metabolically active and a minor contributor to the overall fluorescence (Chance et al., 1962). Slices in the interface chamber were imaged through a Nikon upright microscope (UM-2) with a compound lens (4x, N.A 0.13) using a linear, cooled 12 bit CCD camera with 1280 × 1024 digital spatial resolution (Sensicam QE). Images were acquired every 5 sec with ~0.5 s exposure for each image. The imaging analysis consisted of creating a region of interest (ROI) within stratum radiatum (SR), between the stimulating and recording electrodes, and then calculating the average pixel value within each region of interest for each image. The net difference across the image series was then calculated as $100 \times [(ROI: \text{image} - ROI: \text{baseline}) / ROI: \text{baseline}]$.

NAD⁺/NADH measurement

Total soluble NAD⁺ and NADH levels were measured from hippocampal slices using NAD⁺/NADH assay kit based on enzymatic cycling reaction. Hippocampal slices (4 each) were homogenized in ice cold NAD⁺/NADH extraction buffer (Abcam: NAD⁺/NADH extraction kit) and centrifuged at 16000 g for 5 min at 4 °C. The supernatant was filtered immediately through 10kDa cutoff microspin column to separate the NADH consuming enzymes at 4°C. Fifty µl of ultra filtrates were heated at 60°C for 30 min in a heating block to decompose NAD⁺ for NADH measurement. Both the heated (NADH) and unheated samples [total NAD(H)] were mixed separately with NAD⁺/NADH cycling assay mix for 5 min in a 96 well micro plate. The color was developed with NADH developer solution and the absorbance was measured at 450 nm (micro plate reader) after 2 hour. An aliquot of homogenized sample before ultrafiltration was used to measure the protein content using standard biorad procedure by reading the absorbency at 595 nm. The concentrations of NAD⁺ and NADH were expressed in nmol per mg protein based on standard NADH readings.

Mitochondrial isolation for NADH measurement

Hippocampal slices treated with and without nicotinamide were washed with ice cold wash buffer (Abcam mitochondrial isolation kit) and then homogenized with ice cold mitochondria isolation buffer (2ml/10–20 slices) using ice cold tissue grinder with 25–40 passes with the grinder at 4°C. The volume was made up to 2ml with mitochondrial isolation buffer in 2ml centrifuge tube. After centrifugation at 1000 g for 10 minute at 4°C the supernatant was collected, the volume made up to 2ml with extraction buffer and then centrifuged at 12,000g for 15 minutes. The pellet was collected for mitochondrial fractions and supernatant for cytosolic fractions. Supernatant was centrifuged again at 15,000 g × 30 minute at 4°C and the resulting supernatant was used as a cytosolic fraction. The mitochondrial pellet was washed 3 times with 2ml of mitochondrial isolation buffer and collected the pellet by centrifugation at 12,000g × 15 min. This mitochondrial pellet was used for the NADH extraction procedure.

ATP assay

Hippocampal slices, subjected to hypoxia and reoxygenation (in interface chamber) and variously treated with and without nicotinamide, were frozen in liquid N₂ and homogenized with ice cold perchloric acid (PCA) containing EDTA. Supernatant collected after centrifugation was used for ATP determination by fluorescence assay kit (From abcam) using the ATP standards ranging from 50 to 400 pmol. The PCA precipitated proteins were solubilized in 0.1N NaOH and used for the protein measurement using standard biorad procedure by reading the absorbency at 595 nm to express the ATP concentration from the sample as nmol/mg protein.

Data analysis

Results are shown as mean ± SEM; differences between the groups were analyzed by analysis of variance (ANOVA) with pairwise comparisons. Finding of a p value < 0.05 was considered statistically significant.

Results

Effects of nicotinamide incubation on neuronal function during hypoxia

Previously we have shown that hypoxia in hippocampal slices leads to HSD, and that restoring oxygen less than 30 sec after HSD occurrence typically leads to full fEPSP recovery (Foster et al., 2008). Similarly, a short hypoxia duration (ie, restoring oxygen 1 min after HSD) was also nearly fully reversible in our current experiments (detailed in Fig. 2A). Therefore, we analyzed progressively longer durations of hypoxia, next waiting 3 min after onset of HSD before restoring the oxygen to the tissue, to assess neuronal recovery.

Figure 1 depicts a typical, severe hypoxic episode, with reoxygenation at 3 min after occurrence of HSD (shown by the vertical line). Fig. 1 shows the NADH images (Fig. 1A) as well as the NADH time response (Fig. 1B), fEPSP (Fig. 1C) and DC extracellular voltage (V_o) (Fig. 1D); representative traces from a nicotinamide-supplemented slice are superimposed with data from a control slice. Note in the control slice a transient fEPSP recovery occurs prior to the HSD occurrence after initially waning, there is a large increase

in NADH during the hypoxia (reduction), and subsequent fEPSP failure persists after HSD occurrence (red trace, Fig. 1C; HSD as shown in the lower Vo trace). The NADH fluorescence trends lower than baseline after reoxygenation (termed hyperoxidation; Fig. 1B; dark blue areas in Fig. 1A). In contrast to this moderate hypoxia (ie, reoxygenation at 3 min after HSD), shorter, reversible episodes of hypoxia (ie, reoxygenation at 1 min after HSD) did not exhibit any hyperoxidation (Fig. 2). The DC extracellular changes noted with both the first and second hypoxic episodes confirm the presence of HSD for each episode. However the duration of hypoxia prior to onset of HSD was significantly reduced during a second hypoxic episode (3.5 ± 0.5 min for 2nd vs 7.0 ± 0.3 min for 1st hypoxia).

Next, we analyzed the effect of nicotinamide incubation prior to hypoxia (5 mM for minimum 1 hr, tissue NAD(H) content shown in Fig. 3) on this moderate duration of irreversible hypoxia, also shown as subtraction images in Fig. 1 (A) to the right (+NAM). The mean time to HSD onset was not statistically significant with nicotinamide incubation: duration of hypoxia prior to HSD was 7.01 ± 0.3 min for the control and 8.0 ± 0.5 min for nicotinamide groups (Fig. 1D). Because the slices were re-oxygenated at 3 min after HSD, the total hypoxia duration averaged approximately 10 – 11 min for control and nicotinamide treated slices. Results obtained by slices exposed to 10 minutes total duration of hypoxia in both conditions were similar to those obtained with 3 min of persistent hypoxia after HSD as expected (data not shown). In contrast to the control hypoxia the degree of hyperoxidation of NADH in the nicotinamide treated slices after 15 min of reoxygenation was significantly less than that noted in untreated slices: $-6.3 \pm 2.4\%$ vs $-17.5 \pm 2.0\%$ ($p < 0.05$ nicotinamide vs control; Fig. 1A and B, 2B). Nicotinamide pre-treatment also improved the recovery of the fEPSP amplitude significantly during reoxygenation after hypoxia (Fig. 1C). However, equivalent treatment with nicotinamide beginning after hypoxia and reoxygenation demonstrated no effect on neuronal recovery and hyperoxidation at 60 min, in comparison to the control (data not shown), indicating that the metabolic enhancement afforded by nicotinamide pre-incubation was primarily acting during hypoxia but also possibly shortly after reoxygenation.

The presence of immediate NADH hyperoxidation during reoxygenation after the first hypoxic episode was associated with a decreased maximum reduction of NAD⁺ during the 2nd hypoxic exposure in control slices: $45.0 \pm 0.3\%$ vs $27.6 \pm 2.2\%$ ($p < 0.01$; Fig. 1B and 2E). In contrast to the control situation, slices incubated with nicotinamide showed an equivalent NADH reduction peak during both the 1st and 2nd hypoxic exposure (Fig. 1B). Thus, the nicotinamide incubation led to decreased hyperoxidation upon reoxygenation, improved neuronal recovery, and a more pronounced second hypoxic NADH response, all suggesting improved neuronal function during reoxygenation. However, when reoxygenation was delayed to 5 min after HSD occurrence nicotinamide pre-treatment improved neither hyperoxidation of NADH nor fEPSP recovery, indicating that the insult was likely too severe to benefit from nicotinamide treatment (Fig. 2C, D, E).

NAD(H) and ATP content after hypoxia with/without nicotinamide

To assess whether NAD(H) was significantly decreased after the moderate hypoxia we next studied NAD(H) content biochemically. The total soluble NADH content (protein free)

steadily decreased over time in control slices after slicing (data not shown). Total NAD(H) content, NADH, and NAD⁺ all increased significantly following nicotinamide slice incubation for 1hr (5 mM), (Fig. 3A, B, C) and remained stable for 4 hour at 36°C. Nicotinamide incubation increased the total NAD(H) content from 1.6 ± 0.2 nmol per mg protein to 2.6 ± 0.3 nmol per mg protein (Fig. 3A; $p < 0.001$), but without a significant change in the baseline NAD⁺/NADH ratio (9.87 ± 1.6 vs 8.89 ± 0.8 of control, Fig. 3F: black bars). Both reduced NADH (0.27 ± 0.04 nicotinamide vs 0.16 ± 0.02 control; nmol per mg protein; $p < 0.05$) and oxidized NAD⁺ (2.5 ± 0.2 nicotinamide vs 1.3 ± 0.1 control; nmol per mg protein; $p < 0.001$) were significantly increased with nicotinamide. The enhanced NAD(H) level was more significant in the cytosolic compartment (252 ± 6.4 %, $p < 0.001$) than within mitochondria (112 ± 4.3 %) (Fig. 3D).

In contrast to the NADH imaging, the soluble NAD(H) content remains stable at 10 min hypoxia and up to 2 hour after reoxygenation, in both control and nicotinamide treated slices (Fig. 3E). However, hyperoxidation of NADH was notably averted in nicotinamide incubated slices: the ratio of NAD⁺/NADH was 15.3 ± 1.5 vs control 28.0 ± 2.5 ($p < 0.01$; Fig. 3F). Because there was no net loss of tissue total NAD(H) levels when compared to matching control slices, the severe shift toward oxidation during reoxygenation may indicate insufficient metabolic turnover of NAD⁺ to NADH.

To determine whether nicotinamide incubation exerted any effect on cellular ATP (Fig. 4) we also measured total ATP levels in hippocampal slices. Basal ATP concentration in hippocampal slices was 8.1 ± 0.5 nmol/mg protein and nicotinamide treatment did not alter basal ATP content. Total ATP content with nicotinamide incubation was not statistically significant compared to the control under basal conditions. However, ATP levels decreased significantly ($p < 0.05$) in control hypoxia slices after 40 min of reoxygenation but not in nicotinamide treated slices (Fig. 4).

PARP-1 inhibition prevents NADH hyperoxidation

Because of the significant neuronal injury after severe hypoxia it is likely that DNA repair enzymes are induced, possibly even early before reoxygenation, leading to somatic PARP-1 activation and NAD⁺ consumption required for DNA repair (Liu et al., 2009). Therefore, we studied NADH imaging, hyperoxidation, recovery and second hypoxic events in the presence of a PJ-34 (PARP-1 inhibitor), with maintained hypoxia 3 min after the occurrence of HSD (Fig. 5). We first equilibrated rat hippocampal slices briefly (10 min) with 1 μ M PJ-34 (Abdelkarim et al., 2001) prior to exposure to hypoxic conditions; at this concentration PJ-34 prevented NADH hyperoxidation (Fig. 5A, D). PJ-34 application also prevented the decrease of the NADH peak during the second hypoxic episode (Fig. 5A, E; 53 ± 1 % vs 28 ± 1 % for control; $p < 0.001$). However, Fig. 5B shows that brief PJ-34 administration did not promote neuronal recovery after hypoxia and reoxygenation (Fig. 5B, F). In addition, the mean time for HSD was occurred significantly earlier in the presence of PJ-34 (Fig. 5C): 4.52 ± 0.4 min vs. 7.01 ± 0.3 min control ($p < 0.001$).

However, slices equilibrated with PJ-34 by more than 1 hr prior to hypoxia also showed a significant improvement in NADH reduction (Fig. 6A) and recovery of neuronal fEPSPs (Fig. 6B). Additionally, no change in the time to HSD onset during hypoxia was observed

during prolonged incubation with PJ-34 (Fig. 6C). Hyperoxidation of NADH following PJ-34 pre-treatment was significantly improved following 3 min reoxygenation post HSD hypoxia (Fig. 6D) at 15 minute, 30 minute and 40 min of reoxygenation. In addition, the NADH reduction peak of a second hypoxia event after hypoxic reoxygenation was similar to the 1st hypoxic episode (Fig. 6E). However, the maximum NADH reduction peak of the 1st hypoxia following prolonged PJ-34 pre-incubation was significantly less than the control hypoxic slices. On the other hand, NADH fluorescence analysis showed that prolonged PJ-34 incubation (1–2hr) significantly increased ($8.86 \pm 1.5\%$ vs $-2 \pm 1.5\%$ of control; $-1.4 \pm 1.5\%$ with NAM; $p < 0.05$, $n = 4$) the baseline NADH fluorescence intensity in hippocampal slices compared to a steady baseline fluorescence with or without nicotinamide treatment (Fig 6F). Therefore, the increased baseline NADH fluorescence with prolonged PJ-34 incubated slices prior to hypoxia resulted in a smaller NADH peak compared to the baseline NADH during hypoxia.

As in the control hypoxic experiments (Fig. 3E) the total soluble NAD(H) content did not change with PJ-34 administered during hypoxia and reoxygenation (Fig. 7A). Similarly, there was no change of the total soluble NAD(H) following hypoxia and reoxygenation in slices pre-treated with PJ-34 at 1 to 2hr. However prolonged incubation with PJ-34 significantly prevented steady loss of total NAD(H) during incubation after the slicing (7B). Similar to the imaging experiments, direct biochemical tissue measurements revealed that the soluble NAD⁺/NADH ratio decreased significantly during hypoxia in both control and PJ-34 treated hippocampal slices (control hypoxia 1.42 ± 0.3 vs. control baseline 8.96 ± 0.8 ; PJ-34 hypoxia 2.1 ± 0.2 vs its control baseline 8.7 ± 0.6 ; Fig. 7C) and also with prolonged (1 to 2hr) PJ-34 pre-incubation (hypoxia 1.72 ± 0.19 vs its control baseline 8.23 ± 1.06). Similar to NADH imaging analysis the addition of PJ-34 significantly prevented the hyperoxidation and severe increase in the NAD⁺/NADH ratio (Fig. 7C; PJ-34 6.9 ± 1.5 , prolonged PJ-34 7.63 ± 0.7 vs. corresponding control 28 ± 3.3 ; $p < 0.001$). However, total ATP content is significantly maintained in prolonged PJ-34 incubation (1–2hr) during reoxygenation compared to shorter duration of pretreatment (Fig. 7D).

Discussion

Our findings demonstrate that nicotinamide incubation prior to hypoxia led to enhanced NAD(H) tissue levels (particularly within the cytosol), preserved ATP content, improved recovery of neuronal function after otherwise irreversible hypoxia, and significantly reduced hyperoxidation, as measured by both soluble NAD⁺/NADH ratios and NADH imaging. Additionally, pre- incubation with PJ-34 (>1hr) prior to hypoxia significantly improved neuronal recovery and ATP content during reoxygenation. In contrast, attenuation of PARP-1 activity to prevent NAD⁺ consumption by PJ-34 administered briefly before and during hypoxia and reoxygenation hastened the onset of HSD and also significantly decreased hyperoxidation, but failed to improve neuronal recovery and ATP content.

Neuronal fEPSP recovery in our data is highly sensitive to hypoxic duration following the onset of HSD, which if prolonged can be associated with NADH hyperoxidation, both during *in vivo* ischemia experiments (Dora et al., 1986; Kogure et al., 1980; Rosenthal et al., 1995) as well in *in vitro* hippocampal slice data (Foster et al., 2005; Foster et al., 2008;

Perez-Pinzon et al., 1998a; Perez-Pinzon et al., 1998b). Hyperoxidation and decreased NADH fluorescence peak during a second hypoxia has been suggested to be either loss of NAD(H) content or a severe impairment of metabolism affecting the NAD⁺/NADH ratio (Foster et al., 2008; Rosenthal et al., 1995). Surprisingly, our biochemical analysis shows that hyperoxidation is not associated with a net loss of soluble NAD(H) content. Though hyperoxidation arises shortly after reoxygenation (or upon reperfusion after ischemia, within 15 min), it is not clear if the relevant changes underlying hyperoxidation occur during the period of hypoxia/ischemia, or after the restoration of substrate, when a high level of reactive oxygen species (ROS) would be expected (Assaly et al., 2012; Foster et al., 2006). For example, during the persistent hypoxia, damage to TCA cycle enzymes and mitochondria could be accumulating (ie, such as persistent mitochondrial permeability transition, toxic Ca²⁺ levels), so that even upon reoxygenation there is less capacity for new regeneration of NADH from the existing pool of NAD⁺. After reoxygenation, the enhanced ROS formation may also cause protein damage leading to additional enzymatic dysfunction and impairment of the TCA cycle. Further, immediately upon reoxygenation there is consumption of nearly all accumulated NADH by complex 1 due to the rapid, immediate energy demands, promoting the hyperoxidized state (Kirsch and De Groot, 2001).

In this model of acute hippocampal slices, our data demonstrate that NAD(H) content is significantly increased during nicotinamide incubation prior to hypoxia. Alternative treatment schemes could include an immediately available form of nicotinamide, such as nicotinamide riboside (Canto et al., 2012) or NAD⁺ itself (Pittelli et al., 2011). In our slice model the entire slice is exposed to the hypoxia and persists with a low energy state after reoxygenation, more similar to the ischemic core than the penumbra. Thus, it is not surprising that immediate treatment after reoxygenation with nicotinamide did not restore function in our model. More severe hypoxia (i.e., 5 min duration hypoxia after HSD or nearly 13 min total) also did not show any neuroprotection in response to nicotinamide administration, indicating that metabolism was too severely dysfunctional after this prolonged hypoxia to be reversible. In comparison, *in vivo* nicotinamide treatment of ischemia has been shown to reduce infarct volume but not eliminate the stroke region altogether, indicating that this treatment cannot “rescue” severely dysfunctional brain regions (Liu et al., 2009; Yang et al., 2002c).

The nicotinamide pre-treatment regimen clearly led to enhanced NAD(H) content, particularly in the cytoplasmic fraction. Increased NAD⁺ level upon reoxygenation can facilitate the conversion of lactate to pyruvate and assist the turnover of NAD⁺ into NADH in the TCA cycle, augment NADP⁺ and glutathione function for better buffering of reactive oxygen species (ROS), and prevent occurrence of mitochondrial permeability transition (Houtkooper and Auwerx, 2012; Klaidman et al., 2003; Yang et al., 2002b). Since ROS generation and secondary damage likely occur upon reoxygenation the enhanced NAD(H) may help mitochondria recover more rapidly from the severe hypoxia in our model, as soon as the oxygen is restored (Yang et al., 2002c). Prevention of the ATP drop following the hypoxia by nicotinamide pre-treatment confirms improved metabolism, likely due to equilibration of reducing equivalents between mitochondria and cytosol (McKenna et al., 2006). The enhanced cytoplasmic NAD(H) noted from our fractionation results may also

lead to augmented energy production from anaerobic glycolysis even during hypoxia, since glucose is still available in our conditions.

There are several NAD⁺ consuming metabolic reactions within cells, including PARP-1 (a DNA repair enzyme within the nucleus) and SIRT1s, which are regulated by NAD⁺ levels. SIRT1 has a lower affinity for NAD⁺ than PARP-1, hence is less likely to consume NAD(H) than PARP-1 or lead to glycolytic energy failure (Alano et al., 2010; Bai et al., 2011; Kim et al., 2011) after severe hypoxia. In our model SIRT1 activation, particularly due to increased NAD⁺ levels with nicotinamide, may lead to neuroprotection (Bai and Canto, 2012). Activation of another NAD⁺-dependent enzyme, SIRT3, may also improve recovery from excitotoxicity, through increased mitochondrial function, elevated superoxide dismutase, and enhanced ROS buffering (Houtkooper and Auwerx, 2012; Houtkooper et al., 2012). Like NMDA or glutamate treatment in tissue cultures, our hypoxia model may also involve an element of excitotoxicity, with SIRT1 or SIRT3 activation occurring due to the enhanced NAD(H) from nicotinamide (Liu et al., 2008).

PARP-1 accounts for 85% of the nuclear PARP activity activated by somatic DNA damage, and NAD⁺ is a critical cofactor in the DNA repair process and PARP-1 activation can deplete the cytosolic NAD⁺ pool (Bai and Canto, 2012; Clark et al., 2007; Moroni et al., 2001). In addition to being a NAD⁺ precursor, nicotinamide has also been suggested to be a direct PARP-1 inhibitor (Klaidman et al., 2003). PARP-1 activation and the loss of cytosolic NAD⁺ leads to impairment of both glycolysis and lactate to pyruvate conversion (via lactate dehydrogenase), as well as a secondary mitochondrial impairment through release of apoptosis-inducing factor (AIF) (Alano et al., 2010; Alano et al., 2004; Kauppinen and Swanson, 2007). A previous report suggested that treatment with nicotinamide prevented excitotoxic cell death by preserving SIRT1 levels and reducing Par accumulation (marker for PARP-1 activation)(Liu et al., 2009).

In our current data prolonged PARP-1 antagonism prior to hypoxia showed improved neuronal recovery and reduced hyperoxidation, likely due to stabilization or increase in the level of NAD(H) prior to the hypoxic episode. In contrast, PARP-1 inhibition immediately before hypoxia and reoxygenation led to a more rapid onset of HSD, possibly via induction of an enhanced metabolic rate but with less available substrate during the hypoxia (Bai and Canto, 2012). Similarly, PARP-1 knockout mice showed excessive metabolic activity and increased oxygen consumption (Bai et al., 2011). The prolonged PJ-34 treatment may therefore sufficiently enhance metabolism that there is more resistance to the hypoxia and better metabolic recovery.

Thus, prolonged incubation with either PARP-1 inhibitors or nicotinamide can be viewed as a therapeutic option in hypoxia-ischemia. Recovery of neuronal function following hypoxic injury can be ameliorated by the enhanced metabolic function induced by nicotinamide, prolonged PARP1-inhibition or other NAD(H) precursors. The critical difference between these treatments is that potentially nicotinamide may be administered chronically as a dietary supplement, possibly enhancing tissue NAD(H) content sufficiently to provide neuroprotection against episodes of hypoxia-ischemia.

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Abbreviations

fEPSP	field excitatory post-synaptic potential
HSD	hypoxic spreading depression
NAM	nicotinamide
ACSF	artificial cerebrospinal fluid
CA1	cornu ammonis region 1
DG	dentate gyrus
SR	stratum radiatum
ROI	region of interest
NAM	nicotinamide
NAD⁺	nicotinamide adenine dinucleotide
NAD(H)	total NAD ⁺ and NADH content
PARP-1	poly(ADP-ribose) polymerase -1
AIF	apoptosis-inducing factor
H	hypoxia
reox	reoxygenation
SIRT-1	silent mating-type information regulation 1
TCA cycle	tricarboxylic acid cycle
ROS	reactive oxygen species

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Highlights

- Nicotinamide treatment increased total NAD(H) content in hippocampal slices.
- Nicotinamide decreased hyperoxidation of NADH and enhanced ATP content.
- Nicotinamide increased NADH response to subsequent hypoxia after reoxygenation.
- Existing pools of NAD(H) insufficient to maintain neuronal function with hypoxia.
- PARP-1 inhibition alone is insufficient to recover neuronal function after hypoxia.
- Prolonged pre-incubation with PARP-1 inhibitor (>1hr) improved neuronal function after hypoxia.

Figure 1. Nicotinamide incubation before hypoxia enhances neuronal recovery following prolonged hypoxia after hypoxic spreading depression

(A) Left panel shows images of NADH autofluorescence in hippocampal slices taken during the progression of irreversible hypoxia (3 min persistent hypoxia [H] after hypoxic spreading depression occurrence [HSD]) and reoxygenation (reox); the right series of NADH images is following 5 mM nicotinamide (NAM) incubation then the same duration of hypoxia. In both series of images a later second episode of hypoxia is also performed after 40 min reoxygenation. The top images show unsubtracted raw NADH fluorescence with the region of interest (ROI) indicated by a gray rectangle in the stratum radiatum (SR) of the CA1 region. The asterisk indicates the position of the recording electrode. The scale bar indicates 500 μ m. The subsequent NADH images are taken at the marked times during and after hypoxia, and represent subtractions from the control image; the scale bar represents change in fluorescence intensity compared to the control image, ranging from +50% (red) to -50% (violet). Note that the CA1 region is severely affected by the hypoxic episodes. (B) shows the NADH responses (from the CA1 ROI), (C) shows the CA1 fEPSP, and (D) the CA1 extracellular voltage, during the first and second hypoxic episodes, comparing with and without nicotinamide incubation. The vertical solid and dotted vertical lines indicate the initiation of HSD during the 1st hypoxic exposure with control and nicotinamide treated slices respectively. The critical differences induced by the nicotinamide incubation include reduced hyperoxidation after the first hypoxic episode, an increased NADH response with the second hypoxic episode, and significant improvement in neuronal fEPSP recovery after the first hypoxia.

Figure 2. NADH fluorescence and neuronal recovery after different hypoxic durations (A) The percentage of NADH decrease at various times of reoxygenation (15, 30, 40 minutes) following 1 min prolongation of hypoxia after hypoxic spreading depression (HSD), comparing hypoxia with and without nicotinamide [+NAM] incubation. (B) NADH fluorescence after 3 min prolonged hypoxia following HSD and (C) after 5 min of hypoxia after HSD. Note that the level of hyperoxidation is reduced at 15 min after reoxygenation at 1 and 3min post HSD in the +NAM condition. (D) Degree of fEPSP recovery at 40 min of reoxygenation following 1, 3, and 5 minute prolongation of hypoxia after HSD. (E) Maximum level of NADH fluorescence after the onset of HSD during hypoxia in control and +NAM slices, comparing the first and second hypoxic exposure, following 1, 3 and 5 min of hypoxia prolongation after HSD during the first hypoxic episode. The NADH response during the second hypoxic episode is significantly enhanced in the +NAM condition. All panels show error bars (SEM, n = 8) and statistical significance as determined by ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3. Nicotinamide incubation enhances NAD(H) content

Total NAD(H) levels (A), NADH (B), NAD⁺ (C) show a significant enhancement after incubation with 5 mM nicotinamide incubation (1hr). (D) Cytosolic extract showed the significant enhancement of total NAD(H) with nicotinamide (E) No significant change in the total NAD(H) from the slices subjected to 10 minutes hypoxia or up to 120 minute of reoxygenation following 10 minute hypoxia in either control or nicotinamide treated slices. (F) Ratio of NAD⁺/NADH during and after hypoxia at 40 min reoxygenation, with or without nicotinamide treatment; note the significant decrease in the ratio during reoxygenation with nicotinamide treatment. (* p<0.05, *** p<0.001, versus baseline, ## p<0.01, vs control hypoxia).

Figure 4. Nicotinamide incubation restores ATP levels

Total ATP content shows a significant decline after 40 min reoxygenation following 10 minute hypoxia, and restoration of levels following nicotinamide treatment. Error bar show SEM, n = 5 (* p < 0.05, versus baseline).

Figure 5. PARP-1 inhibition improves NADH hyperoxidation without enhancing neuronal fEPSP recovery

(A) NADH fluorescence during reoxygenation after hypoxia post HSD with PARP-1 inhibitor (PJ-34) shows decreased NADH hyperoxidation and enhanced second hypoxic NADH response compared to control, but (B) shows no fEPSP recovery in either situation. (C) The DC extracellular potential shows HSD occurs significantly earlier in PJ-34 condition. (D) NADH fluorescence change after various duration of reoxygenation showing improved hyperoxidation in all groups compared to control. Error bar, SEM, $n = 5$. *** $p < 0.001$, versus reoxygenation control. (E) comparison of maximum NADH reduction responses between 1st and 2nd hypoxic episodes with PJ-34 (* $p < 0.05$, *** $p < 0.001$, against respective 1st hypoxia, ### $p < 0.001$ against 2nd hypoxic control). (F) neuronal fEPSP recovery with PJ-34 at 40 min of reoxygenation following 3 min hypoxia after HSD. Error bar, SEM, $n = 5$. (*** $p < 0.001$, against baseline fEPSP).

Figure 6. Prolonged incubation with PJ-34 improved NADH hyperoxidation and fEPSP recovery (A) NADH fluorescence during reoxygenation after hypoxia post HSD with prolonged PJ-34 preincubation shows decreased NADH hyperoxidation and enhanced second hypoxic NADH response, (B) shows fEPSP recovery upon reoxygenation. (C) Shows occurrence of HSD events during hypoxia. (D) NADH fluorescence change after various duration of reoxygenation showing improved hyperoxidation in all groups compared to control. Error bar, SEM, $n = 5$. *** $p < 0.001$, ** $p < 0.01$ versus reoxygenation control. (E) comparison of maximum NADH reduction responses between 1st and 2nd hypoxic episodes with PJ-34 pre-incubated slices over the baseline fluorescence (** $p < 0.01$ against respective 1st hypoxia, ## $p < 0.01$ against 2nd hypoxic control). (F) shows the percentage of changes in NADH baseline fluorescence after 1 hour of recording in an interface chamber at 36°C with or without nicotinamide or PJ-34 (* $p < 0.05$, against control).

Fig. 7. PARP inhibitor (PJ-34) improves NAD⁺/NADH ratio

(A) Total NAD(H) level with PJ-34 before and after hypoxic reoxygenation in PJ-34 pre-treated slices (B) decrease in the baseline level of NAD(H) during incubation, PJ-34 stabilizing the loss during the incubation (**p<0.01 vs initial baseline; ## p<0.01 vs corresponding control). (C) Control NAD⁺/NADH ratio was 8.9 ± 0.8 , during hypoxia 1.5 ± 0.29 and after 40 min reoxygenation 28 ± 3.3 . In contrast, the NAD⁺/NADH ratio significantly improved in both PJ-34 pre-treated slices after 40 min reoxygenation. Error bar show SEM, n = 4 (***) p<0.001, against respective baseline control, ### p<0.001 against 40 min reox control). (D) Total ATP content shows a significant preservation after 40 min reoxygenation following 10 minute hypoxia, and restoration of levels following PJ-34 pretreatment(>1hr) compared PJ-34 treatment during hypoxia. Error bar show SEM, n = 5 (* p<0.05, versus baseline).