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BRAIN RESEARCH

Research Report

Visualization and quantification of NAD(H) in brain sections by a novel histo-enzymatic nitrotetrazolium blue staining technique

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ABSTRACT

A histo-enzymatic technique for visualizing and quantifying endogenous NAD(H) in brain tissue was developed, based on coupled enzymatic cycling reactions that reduce nitrotetrazolium blue chloride to produce formazan. Conditions were used where the endogenous level of nicotinamide adenine dinucleotides (NAD(H)) was the rate limiting factor for formazan production. Spontaneous degradation of NAD^+ that occurs during incubation of thawed tissue was minimized by the addition of nicotinamide mononucleotide, an inhibitor of NAD^+ glycohydrolases. Cryostat sections of brains obtained from rats immediately after decapitation and 30 min later were used to determine the effects of ischemia alone on brain NAD(H) levels and neuroanatomic distribution. The ischemic insult resulted in a greater than 50% decline in the rate of formazan generation in the CA1 pyramidal neuronal layer of the hippocampus and in the parietal cortex and striatum, but not in the CA3 and dentate gyrus (DG) subregions of the hippocampus. The ischemia-induced changes in NAD(H) levels were confirmed by utilizing spectrofluorimetric measurements of NAD(H) present in perchloric acid extracts of brain samples. This new histo-enzymatic technique is suitable for visualizing and quantifying relative NAD(H) levels in the brain. This assay could prove useful in identifying region-selective NAD(H) catabolism that may contribute to neurodegeneration.

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1. Introduction

NAD(H) plays critical roles in cell energy metabolism, calcium homeostasis, aging and death. It affects cell survival by various mechanisms including controlling cellular bioenergetics, mitochondrial permeability transition pore opening, and apoptosis-inducing factor (Di Lisa et al., 2001; Ying, 2006, 2007, 2008a, 2008b; Ying et al., 2007; Liu et al., 2009; Xia et al., 2009). Since energy failure, mitochondrial calcium dysregulation and cell death are the key components in the tissue-damaging cascade initiated by cerebral or myocardial ischemia, it is likely that NAD(H) plays a significant role in ischemic brain and heart injury. NAD(H) levels

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Abbreviations: 3-AB, 3-aminobenzamide; Abs, optical absorbance; cADPR, cyclic ADP-ribose; DG, dentate gyrus; Nam, nicotinamide; NAD^+, NADH, NAD(H), nicotinamide adenine dinucleotides; NBT, nitrotetrazolium blue chloride; NMN, nicotinamide mononucleotide; NMNAT, nicotinamide mononucleotide adenylyltransferase; PARP, poly ADP-ribose polymerase; PCA, perchloric acid

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in ischemic tissue can fall, due to permeation through leaky membranes, by inhibition of *de novo* NAD^+ synthesis or the NAD^+ salvage pathway, and by enzymatic degradation through activation of NAD^+ glycohydrolases (Klein et al., 1981b; Schaper and Schaper, 1983; Snell et al., 1984).

Incubation of heart or brain sections with tetrazolium salts is used as a technique to visualize the damaged regions of post-ischemic tissue and to evaluate the size of the infarcted area. This technique depends upon redox enzyme activities and cofactors present in cells that are capable of reducing the colorless, soluble tetrazolium salt to the intensely dark-colored formazan (Klein et al 1981a; Schaper and Schaper, 1983; Liszczak et al., 1984; Bederson et al., 1986; Ridenour et al., 1992). The tetrazolium salt is reduced to formazan by diaphorases in the presence of NADH that serves as the electron donor (Klein et al., 1981a). Conversely, the non-stained tissue is considered dead due to the lack of enzymatic activity resulting from either lack of substrate, hydrolysis of cofactors (NAD(H)), or direct inactivation of enzymes, e.g., by proteolysis. Some authors have also suggested that decreased NADH-producing dehydrogenase enzyme activities play a key role (Nachlas and Shnitka, 1963) whereas others (Klein et al. 1981a, 1981b) argue that the loss of cofactors and substrates following short-term ischemia is responsible for differences in the staining. Moreover, Schaper and Schaper (1983) observed that the decreased tissue content of NAD^+ after myocardial ischemia, rather than reduced dehydrogenase enzyme activities, was the basis for histo-enzymatic reactions employing tetrazolium salts. One of the important limitations of the current tetrazolium-based techniques is that they are often used on gross tissue slices (Bederson et al., 1986; Khalil et al., 2006) that do not allow visualization of micro-anatomic changes within the affected tissue.

The primary aim of the present study was to modify and improve the tetrazolium staining technique allowing identification of relative NAD(H) levels in different brain sub-regions and cell types. The secondary aim was to determine if this histo-enzymatic method can be used to quantify changes in brain NAD(H) after global cerebral ischemia. Our data indicate that the slower formazan accumulation rates in ischemic brain tissue are due to lower NAD(H) levels. To prevent NAD^+ hydrolysis in brain tissue slices during the staining procedure, we added the NAD^+ glycohydrolase inhibitor, nicotinamide mononucleotide (NMN), to the assay incubation medium. Our NAD(H) histo-enzymatic technique enables identification of microscopic (intracellular) changes in brain tissue NAD(H) content following global cerebral ischemia that was not previously possible and should therefore be applicable to many experimental paradigms where cell-selective NAD(H) catabolism may be important.

2. Results

2.1. *NAD(H)-dependent enzymatic generation of formazan in tissue sections*

We modified the tetrazolium staining technique to allow its use as a tool to visualize and estimate relative NAD(H) levels in tissue sections. The schematic diagram of the cyclic enzymatic assay that converts tetrazolium salt to formazan in tissue is shown in Fig. 1. Our assay medium contained oxidizable substrates (malate and glutamate) for tissue dehydrogenases that reduce NAD^+ to NADH. In the presence of NADH, diaphorases convert nitrotetrazolium blue chloride (NBT) to the darker-colored formazan. Prior to histo-enzymatic measurements, we utilized spectrofluorometric measurements of NAD(H) in homogenates of brain tissue slices after they had been incubated at different times to determine if measures were needed to inhibit possible spontaneous NAD(H) degradation that might occur during tissue incubation. As Fig. 2 shows, after 2 min of incubation, 85 % of the NAD(H) was hydrolyzed and after 5 min, the NAD(H) was almost completely degraded. This rapid and extensive NAD(H) degradation was prevented when the incubation medium contained

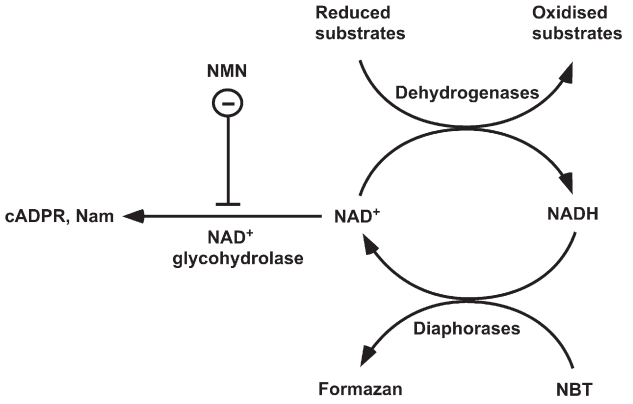

Fig. 1 – Diagram illustrating the enzymatic cycling reactions of the NAD(H)-dependent formazan generation in tissue sections. NBT is reduced to dark-colored water-insoluble formazan by tissue diaphorases in the presence of NADH, which is oxidized to NAD^+. In turn, NAD^+ is reduced back to NADH by various dehydrogenases in the presence of their oxidizable substrates. NAD^+ can be degraded in the tissue by NAD^+ glycohydrolases. This is inhibited by NMN that prevents NAD^+ hydrolysis to cyclic ADP-ribose (cADPR) and nicotinamide (Nam). Since our assay medium contains excess of substrate for dehydrogenases the limiting factor for the rate of formazan production is the tissue NAD(H) level.

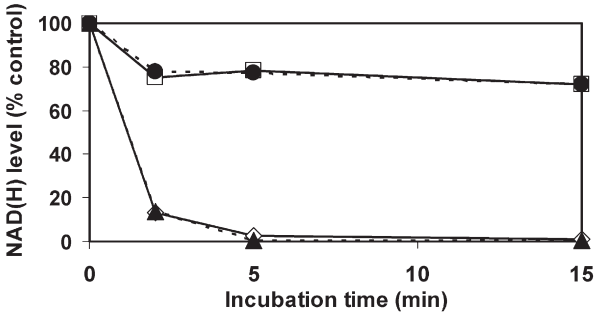

Fig. 2 – Rapid endogenous NAD(H) degradation in homogenates from control rat forebrains during incubation at 37 °C. Incubation medium consisted of 100 mM Tris-base buffer, 5 mM malate, 5 mM glutamate (◇), plus 5 mM NMN (□), plus 1 mM 3-AB (▲), or plus 1 mM AMP and 5 mM NMN (●).

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