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Research Report

Ischemic tolerance following low dose NMDA involves modulation of cellular stress proteins

*Monique C. Saleh, Barry J. Connell, Tarek M. Saleh**

Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, P.E.I., Canada C1A 4P3

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ABSTRACT

Mild NMDA receptor activation is correlated with neuroprotection in models of cerebral ischemia. Neuroprotection with NMDA manifests as a form of ischemic tolerance and involves the induction of cellular stress systems sensitive to disturbances in cellular calcium homeostasis. Unilateral micro-injection of 10, 160 and 320 μM NMDA into the prefrontal cortex of a rat 30 min prior to permanent occlusion of the middle cerebral artery (MCAO) significantly reduced the area of infarct observed after 4 h of ischemia. The highest dose of NMDA (320 μM) prevented the propagation of ischemic damage through a direct toxicity on neuronal tissue adjacent to the injection site as demonstrated in thionin-stained sections. As a result, the degree of ischemia-induced damage was similar to that measured in rats pretreated with the low dose of NMDA (10 μM). Expression of heat shock protein (HSP) 70 and glucose-regulated protein (GRP) 94 in cortical samples taken from the region of infarct following MCAO was significantly reduced in rats pretreated with 10 μM NMDA compared to saline-injected control rats and rats pretreated with higher doses of NMDA. Furthermore, 10 μM NMDA did not appear to influence expression of m-calpain or GRP78, however, higher doses of NMDA did significantly induce expression of both proteins as assessed by Western blotting. In summary, our data demonstrate an *in vivo* rodent model of ischemic tolerance in which 30 min of neuronal preconditioning with 10 μM NMDA confers protection against a 4 h period of MCAO-induced ischemia. This effect may involve modulation of cellular stress signals, in particular HSP70 and GRP94.

1. Introduction

It is widely accepted that glutamate excitotoxicity contributes significantly to the cell death induced by periods of ischemia, both acute and chronic. More recently, the phenomenon of NMDA-receptor mediated neuronal preconditioning has been proposed suggesting that subtoxic doses of glutamate (Lin et al., in press) or other NMDA-receptor agonists (Soriano et al., 2006), may in fact confer ischemic tolerance to neurons through a variety of mechanisms. Evidence suggests that increased synaptic NMDA receptor activity promotes neuroprotection by activating prosurvival signal molecules, including cAMP response element-binding protein-dependent (CREB) gene expression (Lee et al., 2005; Papadia et al., 2005), brain-derived neurotrophic factor (BDNF; Shieh et al., 1998; Tao et al., 1998) and phophatidylinositol-3-kinase-Akt (PI3K-Akt)

* *Corresponding author*. Fax: +1 902 566 0832.
E-mail address: tsaleh@upei.ca (T.M. Saleh).

Abbreviations: CPP(±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; ER, endoplasmic reticulum; GABA, gamma-aminobutyric acid; GRP, glucose-regulated protein; HSP, heat shock protein; ip, intraperitoneal; iv, intravenous; MCA, middle cerebral artery; MCAO, middle cerebral artery occlusion; NMDA, N-methyl-D-aspartic acid; TTC, 2,3,5-triphenol tetrazolium chloride

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(Papadia et al., 2005; Soriano et al., 2006). Other proposed mediators of ischemic tolerance include heat shock proteins, (Kirino et al., 1991), ATP-dependent potassium channels (Heurteaux et al., 1995), superoxide dismutase (Toyoda et al., 1997) and caspase-3 (McLaughlin et al., 2003).

Induction of cellular stress proteins in models of ischemic preconditioning has been shown to reduce endoplasmic reticulum (ER) stress and prevent neuronal cell death after ischemia (Hayashi et al., 2003). Induction of GRP78 was shown to correspond with the ischemic tolerance time window as well as an inhibition of protein kinase-like ER eIF2alpha kinase (PERK) phosphorylation (Hayashi et al., 2003). Conversely, toxic doses of NMDA (20 mM) which were shown to contribute to retinal cell damage were correlated with significant increases in GRP78 protein expression in retinal ganglion cells (Shimazawa et al., 2007). However, a lower dose of NMDA (0.8 nM), while having no adverse effects on retinal cell viability, was shown to significantly increase expression of HSP72, an inducible heat shock protein (Ahn et al., 2008). Inducers of ER stress such as tunicamycin are correlated with increased cell death as well as induction of cellular stress proteins such as GRP78 and CHOP (Shimazawa et al., 2007). Reducing ER stress through inhibition of mediators such as eukaryotic translation initiation factor 2 subunit alpha kinase (eIF2alpha) has been shown to reduce apoptosis in retinal endothelial cells (Li et al., 2008) and protect against kainic acid-induced excitotoxicity in cultured hippocampal neurons (Sokka et al., 2007). In summary, these findings suggest that early indicators of cellular stress in response to subthreshold stimuli may signify pro-survival modifications in neurons which ultimately confer a degree of ischemic tolerance when later challenged.

Studies of cross-tolerance indicate that NMDA-receptor activation and the ensuing influx of calcium are common threads in the mechanism of preconditioning (Tauskela et al., 2001). Calcium (Ca^{2+})-sensitive mediators of cellular stress such as HSP70 and m-calpain are induced in models of cerebral ischemia (Gaspary et al., 1995; Siman et al., 1996). Pre-conditioning of differentiated C6 glioma cells with 100 μM NMDA was correlated with increased expression of HSP70 24 h later and was protective against a toxic dose of glutamate (Singh and Kaur, 2006). In fact, the time course of HSP70 protein induction in cortical neurons has been shown to correlate well with the period of ischemic tolerance while other stress-related proteins, GRP75 and 78, were not reliably enhanced (Chen et al., 1996). Furthermore, the use of anti-HSP70 antibodies or anti-oxidants such as quercetin which inhibit HSP70 was shown to remove the tolerance previously induced by a period of sublethal ischemia (Nakata et al., 1993).

The following experiment was designed to investigate the potential role for NMDA preconditioning in an *in vivo* rodent model of focal ischemia. Specifically, the effect of pre-injecting NMDA at several concentrations directly into the prefrontal cortex, which is affected by occlusion of the middle cerebral artery (MCAO), on the final lesion area 4 h post-MCAO was examined. In addition, we hypothesized that NMDA preconditioning in this cortical region involves modulation of endoplasmic reticulum stress elements such as GRP78 kDa and 94 kDa as well as mobilization of cytosolic Ca^{2+}-sensitive proteins, namely HSP70 and m-calpain.

2. Results

2.1. NMDA effects on infarct area

Our laboratory has previously shown that the 3-point MCA occlusion model used in this study produces, after 4 h, a reproducible lesion localized to the prefrontal cortex including the insular cortex comprising approximately 32% of the affected hemisphere (Saleh et al., 2001). Micro-injection of 200 ηl of saline 30 min prior to a 4 h period of MCAO produced a lesion occupying approximately 34.2±1.9% of the ipsilateral side (Fig. 1). Infarct area was significantly reduced following pretreatment with 10 μM NMDA resulting in a lesion of only 15.0±1.3% of the hemisphere (Fig. 1). Mid-range doses did not appear to offer protection against ischemia with infarct areas being similar to that measured in saline-injected controls. The highest doses of NMDA studied (160 and 320 μM) also significantly reduced infarct area (respectively 22.6±4.2% and 11.2±1.8% of the ipsilateral side). When NMDA (10 or 320 μM) was co-injected with the competitive NMDA-receptor antagonist, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP; 10 μM), the extent of ischemic injury measured in the cortex was similar to that observed with saline injection, indicating that the previous neuroprotective effects observed at these doses were due to the binding of NMDA to its receptor.

To assess the degree of cellular damage incurred following injection of NMDA directly into the cortex, histological observation of micro-injection tracts and morphology of neuronal tissue immediately adjacent to the injection site was qualitatively assessed using light microscopy (Fig. 2). In sham-operated controls, micro-injection of saline or 10 μM NMDA did not adversely affect cell viability as assessed by staining with 2,3,5-triphenol tetrazolium chloride (TTC; Fig. 2).

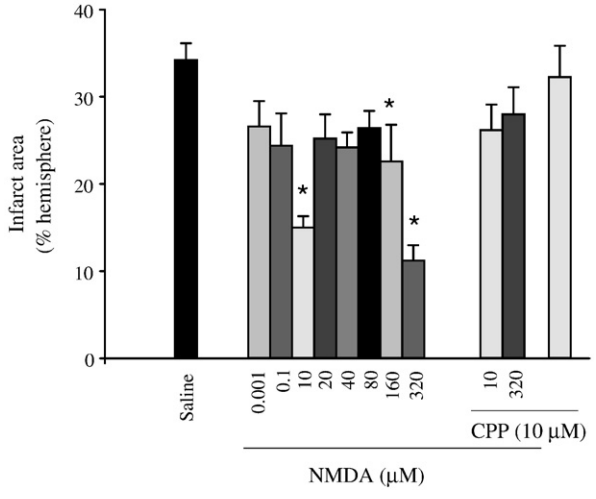

Fig. 1 – Bar graph illustrating the average area of infarct (mean±S.E.M.) as a percent of the ipsilateral hemisphere in all drug-treated groups (NMDA, N-methyl-D-aspartic acid; CPP, (±)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid). Asterisk indicates significantly different from saline-treated rats (ANOVA; $p<0.05$).