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# NECROSTATIN-1 ATTENUATES MITOCHONDRIAL DYSFUNCTION IN NEURONS AND ASTROCYTES FOLLOWING NEONATAL HYPOXIA–ISCHEMIA

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**Abstract—Receptor interacting protein (RIP)-1 kinase activity mediates a novel pathway that signals for regulated necrosis, a form of cell death prominent in traumatic and ischemic brain injury. Recently, we showed that an allosteric inhibitor of RIP-1 kinase activity, necrostatin-1 (Nec-1), provides neuroprotection in the forebrain following neonatal hypoxia–ischemia (HI). Because Nec-1 also prevents early oxidative injury, we hypothesized that mechanisms involved in this neuroprotection may involve preservation of mitochondrial function and prevention of secondary energy failure. Therefore, our objective was to determine if Nec-1 treatment following neonatal HI attenuates oxidative stress and mitochondrial injury. Postnatal day (p) 7 mice exposed to HI were injected intracerebroventricularly with 0.1 μL (80 μmol) of Nec-1 or vehicle. Nec-1 treatment prevented nitric oxide (NO•), inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine increase, and attenuated glutathione oxidation that was found in vehicle-treated mice at 3 h following HI. Similarly, Nec-1 following HI prevented: (i) up-regulation of hypoxia inducible factor-1 alpha (HIF-1α) and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) expression, (ii) decline in mitochondrial complex-I activity, (iii) decrease in ATP levels, and (iv) mitochondrial structural pathology in astrocytes and in neurons. Up-regulation of glial fibrillary acidic protein (GFAP) following HI was also prevented by Nec-1 treatment. No differences by gender were observed. We conclude that Nec-1 immediately after HI, is strongly mitoprotective and prevents secondary energy failure by blocking early NO• accumulation, glutathione oxidation and attenuating mitochondrial dysfunction.**


**Key words: astrocytes, hypoxia–ischemia, mitochondria, neurons, receptor interacting protein-1 kinase, regulated necrosis.**

## INTRODUCTION

Following neonatal hypoxic–ischemic (HI) brain injury, a broad variety of neuronal death phenotypes are observed in the rodent forebrain (Northington et al., 2007). Although hybrid forms along a continuum between apoptosis and necrosis were originally described in the immature model (Portera-Cailliau et al., 1997), they are also found in adult models and in cell culture (Degterev et al., 2005; Festjens et al., 2006). Regulated necrosis, also called programmed cell necrosis (Galluzzi et al., 2011), has mechanistic similarities to apoptotic–necrotic hybrids and is classically described upon activation of the death receptor (tumor necrosis factor (TNF)-receptor superfamily) in the setting of caspase inhibition and/or mitochondrial failure (Han et al., 2009). Regulated necrosis proceeds with subsequent receptor-interacting protein (RIP)-1 kinase activation and formation of RIP-1–RIP-3 complex (necrosome) (Holler et al., 2000; Cho et al., 2009; Kim et al., 2010).

A selective and potent allosteric inhibitor of RIP-1 kinase, necrostatin-1 (Nec-1), prevents the progression of regulated necrosis in adult murine models of traumatic and ischemic cerebral and ischemic myocardial injury (Degterev et al., 2005; Lim et al., 2007; Smith et al., 2007). Similarly, inhibition of RIP-1 kinase activity provides neuroprotection in the cortex, hippocampus and thalamus at subacute and chronic stages of degeneration following neonatal HI in the mouse model, an effect that appears to be more robust in male mice compared to female mice (Northington et al., 2011a). RIP-1 kinase inhibition immediately after neonatal HI, using Nec-1, also

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*Abbreviations:* ANOVA, analysis of variance; ATP, adenosine-5′-triphosphate; BNIP3, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EM, electron microscopy; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GSH, reduced glutathione; GSSG, oxidized glutathione; HI, hypoxia ischemia; HIF-1α, hypoxia inducible factor-1 alpha; iNOS, inducible nitric oxide synthase; NADH, nicotinamide adenine dinucleotide; Nec-1, necrostatin-1; NO•, nitric oxide; OD, optical density; p, postnatal day; PBS, phosphate buffered saline; PCR, Polymerase Chain Reaction, PMSF, phenylmethanesulfonyl fluoride; qRT PCR, quantitative reverse transcription PCR, RIP, receptor interacting protein; siRNA, silencing RNA; TNF, tumor necrosis factor.

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decreases protein oxidative modification and inflammatory markers expression within first 24 h following HI, suggesting that histologic techniques may lack sensitivity to detect smaller acute changes (Northington et al., 2011a). Other intermediate steps between prevention of necrosome formation and neuroprotection are mostly unknown.

Mitochondrial dysfunction and energy failure is a recognized hallmark of necrotic cell death (Eguchi et al., 1997; Nicotera and Lipton, 1999) and well described following neonatal HI (Blomgren and Hagberg, 2006; Northington et al., 2007). Nitric oxide (NO•) inhibits *in vitro* NADH dehydrogenase (complex I) function, ultimately depleting intracellular adenosine-5′-triphosphate (ATP) and promoting a switch from apoptosis to necrosis (Nicotera et al., 1998; Leist et al., 1999; Riobo et al., 2001). In cell cultures, Nec-1 has no direct antioxidant effects; however, it prevents NO• formation and mitochondrial complex I dysfunction while increasing reduced glutathione (GSH) levels and preventing glutamate-induced programmed necrosis (Xu et al., 2007; Davis et al., 2010). From these data we hypothesized that the neuroprotection afforded by Nec-1 treatment following HI *in vivo* was mediated by prevention of secondary energy failure via blockade of NO• accumulation with resultant mitochondrial preservation. Our main goal was to determine if Nec-1 would provide biochemical and ultrastructural protection of mitochondrial following neonatal HI. We evaluated levels of NO•, inducible nitric oxide synthase (iNOS) and 3-nytrotyrosine as well as gluthatione oxidation and markers of mitochondrial function (complex I activity and ATP levels) and ultrastructure in mice treated with Nec-1 or vehicle following neonatal HI. We also investigated the expression of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) which in conditions triggering ROS accumulation, such as neonatal HI, inserts into the mitochondrial membrane triggering a necrotic-like cell death, similar to regulated necrosis associated with energy failure (Vande Velde et al., 2000; Kubli et al., 2008).

## EXPERIMENTAL PROCEDURES

### Animals

All experiments were performed with approval by the Institutional Animal Care and Use Committee at Johns Hopkins University – School of Medicine and followed the Guide for the Care and Use of Laboratory Animals provided by the National Institutes of Health, US Department of Health and Human Services (NIH Publications No. 80-23, revised in 1996). All efforts were made to minimize the number of animals used and their suffering.

### Neonatal mouse hypoxic–ischemic brain injury model and tissue preparation

We used the Vannucci model adapted for neonatal mice to induce HI in C57B6 mice at postnatal day (p) 7 (ligation of the right common carotid artery under anesthesia with isofluorane and subsequent exposure to $FiO_2$ = 0.08 for 45 min) (Graham et al., 2004). Within 15 min after hypoxia, the mice were exposed to a second brief period of anesthesia with isofluorane followed by intracerebroventricular injection of 0.1 μl of 80 μmol of Nec-1 (5-(1H-indol-3-ylmethyl)-3-methyl-2-sulfanylideneimidazolidin-4-1, Calbiochem-EMD Chemicals Group, Gibbstown, NJ), an allosteric inhibitor of RIP-1 kinase, or vehicle (methyl-β-cyclodextrin, Sigma, St. Louis, MO, USA). Pups were returned to the dam until they were killed at 3 h, 24 h (p8) and 96 h (p11) ($n$ = 5–12/treatment/gender/time) for biochemical analysis and perfusion. Controls were age-matched and gender-matched naive littermates not exposed to HI or to treatment with vehicle or Nec-1.

Mice were killed with an exposure to 20% (v/v) mixture of isoflurane in propylene glycol via one-drop exposure method (Markovic and Murasko, 1993). Animals were then decapitated and forebrain tissue was rapidly dissected and frozen in isopentane on dry ice. Pieces of fresh tissue from forebrain were obtained for the experiments described below.

### Nitric oxide colorimetric assay

NO• was assessed indirectly by measuring the levels of oxidized forms (nitrites and nitrates) in samples using the Nitric Oxide Colorimetric Assay Kit (ab65328; Abcam, Cambridge, MA, USA). A standard curve was generated to measure levels between 1 and 100 μM of nitrite per well. Within 2 weeks of freezing, tissues were homogenized at 1:10 (w/v) using ice-cold homogenization buffer prepared in 20 mM Tris/HCl, containing 1 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 10 mM benzamidine and protease inhibitors (Complete® Protease Inhibitor Cocktail tablets, Roche Apply Science, Indianapolis, IN) ($n$ = 6–8 mice/group). A 5-μl aliquot of homogenized tissue was used to determine total protein concentration using Bradford assay. A 200-μL aliquot of homogenized tissue was centrifugated at 10,000$g$ at 4 °C for 2 min and the clarified supernatant recovered. The clarified sample was deproteinated to improve NO• stability by adding ice-cold 5% metaphosphoric acid in a 1:1 ratio (v:v) (Sigma–Aldrich), mixing and spinning at 10,000$g$ for 5 min. Clarified deproteinated samples (supernatants) and standards were exposed to nitrate reductase and cofactors for 1 h at room temperature to transform nitrate to nitrite. Following application of the enhancer supplied by manufacturer, Griess reaction reagents were applied to convert nitrite to a purple azo cromophore compound and developed over 10 min providing a lower limit of detection of 1 μM at 540 nm using a linear model in a microplate reader.

### iNOS, 3-nitrotyrosine, GFAP and BNIP3 protein expression

Ipsilateral forebrain samples were obtained and frozen at 3 h, 24 h (p8) and p11 post-HI from naive control, vehicle and Nec-1-treated mice ($n$ = 5–9 mice/treatment/gender/time). Protein homogenates were prepared as previously described (Northington et al., 1996) and concentrations were determined using Bradford assay. Twenty-μg aliquots of homogenized protein were diluted 2:1 (v:v) in loading buffer containing 20% (w/v) glycerol and loaded onto 15% SDS–PAGE. Protein was transferred to nitrocellulose membrane, stained with Ponceau S, blocked with 2.5% nonfat dry milk (or 0.1% bovine serum albumin (BSA) for 3-nitrotyrosine) and with 0.1% Tween-20 in 50 mM Tris-buffered saline (TBST, 50 mM Tris/HCl and 150 mM NaCl, pH 7.4). Nitrocellulose membranes were consecutively incubated overnight at 4 °C with primary antibodies at 1:200 (except for 3-nitrotyrosine at 1:40,000). After exposure to each primary antibody, membranes were washed with 2.5% nonfat dry milk (or TBST for 3-nitrotyrosine), exposed to secondary antibodies for 1 h and then developed with enhanced chemiluminescence using SuperSignal kit (Thermo Scientific, Rockford, IL). To quantify protein immunoreactivity, films were scanned using Adobe Photoshop (Adobe Systems Inc., San Jose, CA), and optical density (OD) was determined with NIH Image J Software (NIH, Bethesda, MD) adjusted for background. The reliability of sample loading and protein transfer was evaluated by staining nitrocellulose membranes with Ponceau S before immunoblotting.