Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI

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Necrostatin-1 inhibits receptor-interacting protein (RIP)-1 kinase and programmed necrosis and is neuroprotective in adult rodent models. Owing to the prominence of necrosis and continuum cell death in neonatal hypoxia–ischemia (HI), we tested whether necrostatin was neuroprotective in the developing brain. Postnatal day (P)7 mice were exposed to HI and injected intracerebroventricularly with 0.1 μ L of 80 μ mol necrostatin, Nec-1, 5-(1H-Indol-3-ylmethyl)-(2-thio-3-methyl) hydantoin, or vehicle. Necrostatin significantly decreased injury in the forebrain and thalamus at P11 and P28. There was specific neuroprotection in necrostatin-treated males. Necrostatin treatment decreased necrotic cell death and increased apoptotic cell death. Hypoxia–ischemia enforced RIP1–RIP3 complex formation and inhibited RIP3–FADD (Fas-associated protein with death domain) interaction, and these effects were blocked by necrostatin. Necrostatin also decreased HI-induced oxidative damage to proteins and attenuated markers of inflammation coincidental with decreased nuclear factor- κ B and caspase 1 activation, and FLIP ((Fas-associated death-domain-like IL-1 β -converting enzyme)-inhibitory protein) gene and protein expression. In this model of severe neonatal brain injury, we find that cellular necrosis can be managed therapeutically by a single dose of necrostatin, administered after HI, possibly by interrupting RIP1–RIP3-driven oxidative injury and inflammation. The effects of necrostatin treatment after HI reflect the importance of necrosis in the delayed phases of neonatal brain injury and represent a new direction for therapy of neonatal HI.

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Introduction

Cells appear to be able to switch or coactivate alternative biochemical and structural processes of cell death if the initially activated mechanisms are occluded genetically, pharmacologically, or physiologically. The coactivation of apoptotic and necrotic cell deaths is prominent in the developing brain after hypoxia–ischemia (HI) and excitotoxicity (Northington *et al*, 2007; Portera-Cailliau *et al*, 1997).

Cells undergoing this hybrid form of demise display morphologic features of both apoptosis and necrosis, which we have termed ‘continuum’ cell death (Portera-Cailliau *et al*, 1997) and is similar to programmed necrosis, which has been described *in vitro* (Festjens *et al*, 2006). This hybrid form of cell death occurs upon cytokine-mediated death receptor activation, in the setting of caspase inhibition and seems to be under the control of the adaptor proteins receptor-interacting protein (RIP)1 and RIP3 kinase and prominently involves reactive oxygen species (ROS) (Kim *et al*, 2007; Zhang *et al*, 2009).

Necrostatin, 5-(1H-indol-3-ylmethyl)-3-methyl-2-sulfanylideneimidazolidin-4-one, a small molecule inhibitor of programmed cell necrosis, has shown promise as a neuroprotectant in adult rodent models of myocardial ischemia as well as traumatic and ischemic brain injury (Degterev *et al*, 2005; Lim *et al*, 2007; You *et al*, 2008). Necrostatin protects by inhibiting RIP1 kinase activation (Degterev *et al*,

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2008) with possible downstream antiinflammatory effects (You *et al*, 2008) and perhaps other mechanisms. Although necrostatin has no intrinsic antioxidant activity, it blocks tumor necrosis factor (TNF)-induced activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Kim *et al*, 2007) and nitric oxide-mediated necrosis (Davis *et al*, 2010) by the inhibition of both Nox1 and mitochondrial-derived oxygen-free radicals. *In vitro*, necrostatin has little effect on classic apoptotic cell death, but rather, strongly inhibits programmed cellular necrosis (Degterev *et al*, 2005). In neonatal brain HI, the prominence of necrosis and the hybrid 'continuum' cell death (Northington *et al*, 2007), the known role of death receptor activation (Graham *et al*, 2004; Northington *et al*, 2001a), and the importance of oxidative injury and inflammation (Barks *et al*, 2008; Sheldon *et al*, 2004) led us to test the possibility that necrostatin could inhibit these mechanisms. We tested the efficacy of necrostatin to block neurodegeneration and cell death after neonatal HI and sought to identify the possible molecular mechanisms of action. This study shows that necrostatin could be an important translational neuroprotective drug for treating brain injury in newborns after HI by blocking a toxic cascade involving RIP1–RIP3 complex formation, oxidative injury, and inflammation.

Materials and methods

Animal Model

Animal studies were performed with the approval of the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine and carried out with standards of care and housing in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services 85–23, 1985.

Hypoxic-Ischemic Injury and Drug Administration

Postnatal day (P)7 mice were exposed to HI as described previously using the Vannucci model as adapted for neonatal mice (permanent ligation of the right common carotid and $\text{FiO}_2 = 0.08$ minutes $\times$ 45 minutes) (Ditelberg *et al*, 1996; Graham *et al*, 2004). Fifteen minutes after the hypoxic exposure, the pups were briefly reanesthetized with isoflurane and 0.1 μL of 80 μmol necrostatin, Nec-1, 5-(1H-Indol-3-ylmethyl)-(2-thio-3-methyl) hydantoin (Alexis Biochemical, Plymouth Meeting, PA, USA) or vehicle, methyl- β -cyclodextrin (Sigma, St Louis, MO, USA) was injected intracerebroventricularly. The pups were returned to the dam until their killing ($n = 7$ to 12 per group) at 3 hours, 24 hours (P8), P11, or P28 for histologic ($n = 7$ to 12 per group) or biochemical analysis ($n = 6$ per group). Additional groups included naive controls ($n = 6$) and pups injected with either 0.1 or 0.2 μL of 80 μmol necrostatin or vehicle ($n = 6$ each) for histologic analysis at P28. The necrostatin or vehicle-only groups were used to

determine whether the drug alone causes deleterious effects on normal brain development.

Tissue Preparation and Histology

Histology: Animals were killed with an overdose of pentobarbital, 65 mg/kg intraperitoneally, and exsanguinated with cold 0.1 mol/L phosphate-buffered saline (pH 7.4) by intracardiac perfusion. Mice were perfusion fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 minutes at 4 mL/min by intracardiac perfusion. The brains were postfixed in 4% paraformaldehyde for 12 to 16 hours, then cryoprotected in 20% glycerol, and frozen in isopentane (-30°C). Coronal sections of 60- μm thickness were cut on a sliding microtome for cresyl violet (CV) staining for injury analysis.

Injury Analysis: iVision software (iVision, Atlanta, GA, USA) was used to quantitate the percentage injury in each of the regions examined. Percentage injury was determined by measuring the total area of the contralateral cortex, hippocampus, or thalamus and comparing it with the uninjured area in the corresponding ipsilateral region. Injury is measured at four coronal levels for each region and combined for a single percentage injury score for each region of interest in each brain. As an error could be introduced into the measurements with uneven coronal sectioning of the brain, six naive sham brains were evaluated with the above-mentioned procedure and found to have side-to-side differences in area of 0.5% to 2.0%, suggesting that sectioning does not introduce a significant error into the measurements. As an additional assessment of injury at P8, optical densitometry (OD) of CV staining at multiple levels in the hippocampus was measured using the iVision software, corrected for background, and compared with OD of the contralateral hippocampus. The ipsilateral/contralateral OD ratio was compared between necrostatin- and vehicle-treated mice. Injection-only animals were analyzed to determine the consistency of CV staining and ipsilateral/contralateral OD ratios in these animals without visible injury were >0.95 .

To determine whether there was a gender effect of necrostatin on neonatal HI injury, cerebellar and brain stem sections were stained for SRY (SRY C-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then reanalyzed to determine whether sex was a determinate of necrostatin neuroprotection.

To determine whether there was an effect of necrostatin treatment on different cell death phenotypes after neonatal HI, CV-stained sections from necrostatin- and vehicle-treated mice at P11 were used to photograph the cerebral cortex (sensorimotor cortex) in random nonoverlapping fields, under oil immersion. The morphology of individual dying cells in five high-resolution digital images were classified as apoptotic, necrotic, and continuum, using specific criteria as described previously (Martin *et al*, 1998) and then counted by an observer blinded to the treatment group.

OxyBlot Analysis, Immunoprecipitation, and Protein Immunoblotting: Ipsilateral forebrain samples were obtained immediately after pentobarbital overdose and then

frozen ($n=6$ per group at 3 and 24 hours (P8) after HI). Cytosolic extracts obtained using NE-PER extraction reagents (Pierce Biotechnology, Rockford, IL, USA) were analyzed using the OxyBlot detection kit, (Chemicon International, Temecula, CA, USA) for detection of carbonyl modification of proteins as a measure of oxidative injury as described previously (Mueller-Burke *et al*, 2008). Films were scanned using Adobe Photoshop (San Jose, CA, USA) and quantitated with NIH Image J Software (NIH, Bethesda, MD, USA). The reliability of sample loading and protein transfer was evaluated by staining nitrocellulose membranes with Ponceau S before immunoblotting and by quantification of Coomassie-stained gels with optical densitometry.

Forebrain cytosolic extracts (250 μ g of protein) from vehicle- and necrostatin-treated (3 and 24 hours) and control mice were combined with anti-RIP3 and incubated overnight, gently mixing at 4°C. Immobilized Protein A (Thermo Scientific, Rockford, IL, USA), 100 μ L, was added to the antigen–antibody complex and gently mixed at room temperature for 2 hours. To remove any unbound protein, the samples were washed 4 times with 0.5 mL of immunoprecipitation buffer (Thermo Scientific) and centrifuged for 3 minutes at $2,500 \times g$. The supernatant was discarded after each wash. The pellet was washed with 0.5 mL distilled H₂O, centrifuged for 3 minutes at $2,500 \times g$, and the supernatant discarded. The beads were resuspended in 50 μ L of $2 \times$ treatment buffer and boiled for 5 minutes, then centrifuged at $14,000 \times g$ for 5 seconds. The supernatant (20 μ L per lane) was loaded onto a gel for SDS-PAGE. Blots were incubated overnight at 4°C with anti-RIP1, exposed to a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (0.2 μ g/mL) and developed with enhanced chemiluminescence.

To determine the specificity of anti-RIP3 immunoprecipitation, 2.5 μ g of antibody was preincubated with 5 μ g of recombinant RIP3 (Abnova, Taipei, Taiwan), and then immunodepleted by pull down with protein A-agarose beads. The cleared supernatant was then used to immunoprecipitate another aliquot of the sample from a 24-hour vehicle mouse determined to have abundant RIP3–RIP1 interaction in the immunoprecipitation studies.

Protein immunoblotting experiments for FLIP ((Fas-associated death-domain-like interleukin (IL)-1 β -converting enzyme)-inhibitory protein), TNF α , RIP1, and RIP3 were performed as described previously (Graham *et al*, 2004).

Antibodies: Our use of anti-FLIP (G-11) and anti-Fas-associated protein with death domain (FADD) (H181) antibodies (Santa Cruz Biotechnology) has been described previously (Graham *et al*, 2004). Anti-RIP1, (0.2 μ g/mL) (H-207, Santa Cruz Biotechnology) is a rabbit polyclonal antibody that does not cross-react with RIP2 or RIP3. Anti-RIP3 (2 μ g/mL) (Abcam, Cambridge, MA, USA) is a rabbit polyclonal antibody used for both immunoprecipitation and immunoblotting experiments. Anti-TNF α (0.1 μ g/mL) (Millipore, Billerica, MA, USA) is a rabbit polyclonal antibody raised against recombinant mouse TNF α .

Determination of Gene Expression by Real-Time Quantitative Reverse Transcriptase-PCR: Total RNA was

extracted from the forebrain using the PureLink Micro-to-midi total RNA purification system (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's specifications. Approximately 1 μ g of total RNA was used for the generation of complementary DNA (cDNA) using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Reverse-transcription protocol included 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C. Complementary DNA was then used to amplify *FLIP*, *TNF α* , *IL-1 β* , *IL-6*, and *IL-12p35* genes by real-time quantitative reverse transcriptase-PCR using 300 nmol/L concentration of specific primers designed for mouse sequences (Table 1). The amplification protocol included 40 cycles of 1 minute at 95.0°C, 1 minute at 60°C, and 2 minutes at 72°C. Fold difference in gene expression was corrected to *G6PDH* (reference gene) using the method of Pfaffl (2001). Melting curves confirm the amplification of a single gene product of the expected molecular weight.

Caspase Activity Assay: Cytosolic extracts obtained from the forebrain were assayed for caspase 1 or caspase 3 activity using the respective Colorimetric Activity Assay Kits, (APT161 and APT165; Millipore). Cytosolic extracts (400 μ g) were reconstituted in cell lysis buffer and diluted in the same volume of dithiothreitol (10 mmol/L) for caspase 1 and in an assay buffer for caspase 3 assay, as recommended by the manufacturer. YVAD- ρ NA (caspase 1, 200 μ mol/L) or Ac-DEVD-pNA (caspase 3, 30 μ g/dL) substrate was added to the mix and incubated for 60 minutes at 37°C. The chromophore ρ -NA (p-nitroaniline) produced after cleavage from the labeled substrate was measured at 405 nm using a Model 680 Microplate Reader (Bio-Rad). For caspase 3, a standard curve was generated using recombinant human caspase 3 protein (CC119, Millipore, Temecula, CA, USA). A unit of caspase 3 is defined as the amount of enzyme that cleaves 1.0 nmol of the labeled substrate per hour at 37°C.

Nuclear Factor- κ B p65 Transcription Factor Assay: Nuclear extracts, obtained by NE-PER extraction reagents (Pierce Biotechnology), were used to determine concentrations of the active nuclear factor (NF) κ B attached to the consensus sequence (5'-GGGACTTTCC-3') in the biotinylated capture probe bound to the streptavidin plate and detected by a primary rabbit anti-NF κ B p65 antibody (1:1,000) followed by a horseradish peroxidase-conjugated goat anti-rabbit secondary detection antibody (1:500). Positive (TNF α -treated HeLa whole-cell extract), specific competitor (NF κ B competitor oligonucleotide), and negative controls were used to establish accuracy of the results. Absorbance at 450 nm was determined using a microplate reader. Nuclear extracts (1 μ L) were visualized at $\times 400$ to confirm the presence of intact spherical nuclei.

Statistical Analysis: Mann–Whitney *U*-test was used to compare percentage injury, ipsilateral/contralateral OD ratios, cell death phenotype, and results from gene expression studies in necrostatin- and vehicle-treated mice, whereas Kruskal–Wallis ANOVA (analysis of variance) was used to determine progression of injury over time. Results were reported as medians with interquartile

Table 1 Primers used for real time qRT-PCR

Gene	Direction	Primer set	UniSTS
FLIP	Sense	5'-GGAGTCATTACATTAAAGATTATTTCT-3'	207162
	Antisense	5'-GATTAAGGACACAGATAGATTGTTT-3'	
IL-1 β	Sense	5'-TTCAAGGGGACATTAGGCAG-3'	125786
	Antisense	5'-TGTGCTGGTGCTTCATTCAT-3'	
IL-6	Sense	5'-TCCTTTTTCCTTATCTCTTTGCC-3'	130584
	Antisense	5'-GCCTCTAACTCAGAGATCTTCC-3'	
IL12p35	Sense	5'-TCACATCTCATCTCCCCAAA-3'	159124
	Antisense	5'-TCTGCTAACACATTGAGGGG-3'	
TNF α	Sense	5'-GGGACAGTGACCTGGACTGT-3'	144644
	Antisense	5'-CTCCCTTTGCAGAACTCAGG-3'	
G6PDH	Sense	5'-GAAGCCTGGCGTATCTTCAC-3'	Ref.
	Antisense	5'-GTGAGGGTTACCCACTTGT-3'	

Ref, reference gene.

range (25th to 75th percentile) and, in most cases, represented as box-and-whisker plots in which 75th and 25th percentiles (interquartile range) define the upper and lower limits of the box, respectively, and the median is represented as the line inside the box. Optical densitometry measurements of carbonyl-modified proteins were analyzed using paired analysis, whereas NF κ B p65, caspase activity, as well as TNF α and FLIP protein expression were analyzed with unpaired *t*-tests. Significance was assigned to *P*-values <0.05, and Bonferroni's correction was applied when multiple comparisons were performed. Statistical analyses were performed using Stata version 10.0 (Stata, College Station, TX, USA) and SPSS 14.0 (SPSS, Chicago, IL, USA).

Results

Necrostatin Provides Delayed and Persistent Neuroprotection by Blocking Injury Progression after P8 in the HI-Injured Ipsilateral Forebrain, Hippocampus, and Thalamus

Unilateral carotid artery ligation followed by 45 minutes of hypoxia produces a moderate-to-severe brain injury in immature mice (Ditelberg *et al*, 1996; Graham *et al*, 2004) as shown in this study in vehicle-treated mice (Figure 1A). This injury is attenuated by necrostatin when administered immediately after HI (Figure 1A). Injury within the ipsilateral hippocampus is evident by 24 hours (P8), in both necrostatin- and vehicle-treated controls and is not different between groups (Figure 1B). After 24 hours, injury in vehicle-treated mice progresses to a large area of porencephalic damage in the ipsilateral cortex and hippocampus, and the ipsilateral thalamus atrophies (Figures 1B to 1D). Progression of injury after P8, in necrostatin-treated mice is markedly attenuated with a much smaller area of porencephaly and significant preservation of the ipsilateral cortex, hippocampus, and thalamus when compared with vehicle-treated mice at P28 (Figure 1A).

There is no difference in percentage injury in necrostatin versus vehicle treatment when measured

at 24 hours after HI (P8) in the hippocampus (Figure 1B), cerebral cortex (Figure 1C), or thalamus (Figure 1D). When P8 hippocampal injury was further analyzed with OD for loss of CV staining as the marker of injury instead of percentage injury based on area measurements, no difference was found between necrostatin- and vehicle-treated mice (median ipsilateral/contralateral OD; median: necrostatin = 0.79, vehicle = 0.86, *P* = 0.34), thus confirming the measures of injured area. After P8, injury progresses in all regions in vehicle-treated mice but not in necrostatin-treated mice, and by P11, there is detectable neuroprotection in necrostatin-treated mice compared with those receiving vehicle, in the cortex (*P* = 0.01), hippocampus (*P* < 0.001), and thalamus (*P* = 0.003). Owing to the severe degree of injury in the cerebral cortex and hippocampus in vehicle-treated mice, no further progression of injury is seen between P11 and P28. Although less severely injured, there is no progression of injury in the cerebral cortex or hippocampus in necrostatin-treated mice either; thus, the neuroprotection evident at P11 in necrostatin-treated mice persists at P28. In contrast, thalamic injury continues to increase from P11 to P28 in vehicle-treated mice (Figure 1D) (*P* < 0.001). No additional injury in the thalamus is seen in necrostatin-treated mice after P8, thus increasing the difference between vehicle- and necrostatin-treated groups at P28 in the thalamus (Figure 1D).

When percentage injury in the cortex was stratified by gender, no effect was evident at P8, but significant neuroprotection was found in necrostatin-treated males at P11 (Figure 1E) (*P* = 0.02) and at P28 (Figure 1F) (*P* = 0.01). The median injury score in necrostatin-treated females and males was equivalent at P11, but necrostatin-treated females were not different from vehicle-treated females which showed wide variation in injury. In fact, females account for almost all of the variability in the combined data at both P11 and P28 (comparing Figure 1C with Figures 1E and 1F). At P28, in addition to having less injury than vehicle-treated males, necrostatin-treated males also had less cortical injury than necrostatin-treated females (*P* = 0.01) (Figure 1F).

Figure 1. Necrostatin provides delayed and persistent neuroprotection after neonatal HI with possible gender effects. (A) Representative coronal photomicrographs at the level of anterior hippocampus at postnatal day P8 and P28 comparing the degree of infarct after hypoxia–ischemia (HI) with subsequent injection of vehicle or necrostatin at P7. No difference is evident at P8; however, significant neuroprotection is seen at P28 after post-HI treatment with necrostatin. Percentage infarct at P8, P11, and P28 in the (B) hippocampus, (C) cerebral cortex, (D) and thalamus after HI and treatment with vehicle (white) or necrostatin (gray). Percentage cortical infarct by gender is also shown at (E) P11 and (F) P28 comparing females (white) and males (gray). Data are represented as box-and-whisker plots with 75th and 25th percentiles (interquartile range) defining the upper and lower limits of the box, respectively, and the median is represented by the line inside the box. * $P \leq 0.02$ (Mann–Whitney *U*-test corrected by Bonferroni). ○, outliers; ●, extremes. $n = 7$ to 12 per group, $n = 3$ to 8 per group when stratified by gender).

Necrostatin Blocks Necrotic Cell Death and Shifts the Cell Death Phenotype After Neonatal Hypoxia–Ischemia

In the sensorimotor cortex of vehicle-treated HI mice at P11, cells were found undergoing classic necrosis, apoptosis, and continuum forms of cell death (Figure 2A). Cellular necrosis was the dominant phenotype in the HI cerebral cortex at P11 (Figure 2A). Necrostatin significantly decreased the number of cells undergoing necrosis in the cortex at P11 after

neonatal HI (median; necrostatin = 112.5 (dark gray) versus vehicle = 300 (white), $P < 0.001$ versus vehicle). In contrast, there was an increase in apoptotic cells in necrostatin-treated mice (Figure 2A) (median; necrostatin = 37.5 versus vehicle = 0, $P = 0.01$ versus vehicle). Surprisingly, the number of cells classified as continuum did not differ between vehicle- and necrostatin-treated mice (Figure 2A). Caspase 3 activity in cytosolic extracts of the ipsilateral forebrain increased significantly in both vehicle- (white) or necrostatin (dark grey)-treated

Figure 2 Necrostatin shifts cell death phenotype and blocks RIP1–RIP3 interaction after neonatal HI. **(A)** Cell death phenotype analysis at postnatal day P11 after neonatal hypoxia–ischemia (HI) and treatment with either necrostatin or vehicle. Counts are no. of profiles per mm³ with necrotic, apoptotic, or continuum (hybrid) appearance viewed under oil immersion. Vehicle (white) versus necrostatin (dark gray): no. of necrotic profiles—* $P < 0.001$; no. of apoptotic profiles—* $P = 0.01$. Data are represented as box-and-whisker plots with 75th and 25th percentiles (interquartile range) defining the upper and lower limits of the box, respectively, and the median is represented by the line inside the box (Mann–Whitney U -test corrected by Bonferroni). ●, extremes. **(B)** Caspase 3 activity in cytosolic extracts at 24 hours after HI and treatment with vehicle (white) or necrostatin (dark gray) versus naive (light gray) mice represented as bars (mean $\pm$ s.e.m.). * $P = 0.01$ naive versus vehicle, $P = 0.001$ naive versus necrostatin treated, $n = 6$ per group. **(C)** Necrostatin inhibits interaction of receptor-interacting protein (RIP)3 and RIP1, after neonatal HI. Immunoprecipitation with anti-RIP3 reveals a small increase in interaction with RIP1 at 3 hours in vehicle-treated mice and a larger increase in RIP3–RIP1 interaction at 24 hours. Minimal RIP3–RIP1 interaction is detected in necrostatin-treated mice. In naive mice, significant amounts of RIP3 interact with FADD; HI blocks this interaction in both vehicle- and necrostatin-treated mice. At 24 hours after HI, there is detectable RIP3–FADD interaction in necrostatin-treated mice only. **(D)** Anti-RIP3 is specific for detection of RIP3. Immunodepletion with recombinant RIP3 blocks the ability to detect RIP3–RIP1 immunocomplexed proteins (lane 3 versus lane 2). Aliquots from the same 24 hours vehicle-treated sample were used for lanes 2 and 3. **(E)** Immunoblot for RIP1 and RIP3 proteins in the neonatal mouse brain. Both RIP1 and RIP3 are abundantly expressed in necrostatin- and vehicle-treated mice at 3 and 24 hours after HI/treatment. β -Actin is shown as a loading control.

mice at 24 hours after HI compared with naive (light grey), but necrostatin and vehicle mice did not differ significantly ($P = 0.01$ naive versus vehicle, $P = 0.001$ naive versus necrostatin treated) (Figure 2B).

Necrostatin Inhibits Interaction of Receptor-Interacting Protein-3 and Receptor-Interacting Protein-1 After Neonatal Hypoxia–Ischemia

Coimmunoprecipitation showed that the neonatal mouse brain after HI has enforced interaction between RIP1 and RIP3 (Figure 2C). There was a small increase in RIP3–RIP1 interaction at 3 hours and a large increase at 24 hours in vehicle-treated

mice (Figure 2C). Necrostatin largely occluded the RIP1–RIP3 interaction detected after HI (Figure 2C). Only a minimal amount of RIP3–RIP1 interaction is detected in necrostatin-treated mice at either 3 or 24 hours (Figure 2C). As a validation of this assay, anti-RIP3 antibody was preincubated with recombinant RIP3 protein, and then used for the immunoprecipitation assay; there was minimal pull down of RIP1 (Figure 2D). In naive mice, significant amounts of RIP3 interact with FADD, and HI blocks this interaction initially in both vehicle- and necrostatin-treated mice (Figure 2C). By 24 hours, there is again detectable RIP3–FADD interaction, but only in necrostatin-treated mice (Figure 2C). Differences in RIP3–RIP1 interaction are not due to significant

differences in levels of the two proteins after HI. Receptor-interacting protein-1, the pharmacologic target of necrostatin-1 (Degterev *et al*, 2008) and RIP3 are highly expressed in the developing brain. Abundant expression of 74 kDa full-length RIP1 and 57 kDa RIP3 is found in the neonatal forebrain at both 3 and 24 hours after HI in vehicle- and necrostatin-treated mice, respectively (Figure 2E).

Protein Oxidation is Attenuated by Necrostatin After Neonatal Hypoxia–Ischemia

Proteins of multiple molecular weights are extensively oxidatively damaged after neonatal HI (Figure 3B). In vehicle-treated mice (dark gray bars; Figure 3A) at 3 and 24 hours after HI, there was a 63 and 51% increase in carbonyl-modified proteins,

Figure 3 Protein oxidation is suppressed by necrostatin treatment. **(A)** Carbonyl-modified protein in the forebrain from naive animals (light gray) and at 3 and 24 hours after hypoxia–ischemia (HI) and treatment with vehicle (white) or necrostatin (dark gray). Data represented as bars (mean $\pm$ s.e.m.). $*P < 0.05$. **(B)** Representative OxyBlots with corresponding loading controls are shown for vehicle (V) and necrostatin (N) at 3 and 24 hours after HI and treatment. Position of molecular weight standards is shown for reference.

respectively (versus naive controls (light gray bars)); however, these differences did not reach significance. Treatment with necrostatin (white bars; Figure 3A) blocked this increase at both 3 and 24 hours after HI and necrostatin mice had 51% ($P = 0.03$) and 55% ($P = 0.008$) less oxidatively modified proteins than did vehicles at the same time point.

Cytokine Gene and Protein Expression, Nuclear Factor- κ B Activity, and Caspase 1 Activation are Suppressed in Necrostatin-Treated Neonatal Mice After Hypoxia–Ischemia

Cytokines increase in response to HI in the developing brain (Silverstein *et al*, 1997). When compared with vehicle-treated animals, necrostatin produced a 43, 73, 72, and 64% reduction in *IL-1 β* , *IL-6*, *IL-12p35*, and *TNF α* gene expression in the forebrain, respectively ($P < 0.05$; Figure 4A). Melting curves for each of the cytokines and the housekeeping gene showed that a single gene product was obtained in each case (Figure 4B). Immunoblots of crude homogenates used for the gene expression studies confirm that necrostatin suppresses *TNF α* protein expression after neonatal HI by $\sim 50\%$ (Figures 4C and 4D, vehicle: white bars, necrostatin: dark gray bars, $P = 0.02$ versus vehicle, $n = 3$ per group).

An approximately three-fold increase in nuclear NF κ B p65/50 levels occurs at 3 hours after HI in vehicle-treated mice (white bars, $P = 0.004$) versus naive controls (light gray bars), and necrostatin (dark gray bars) blocks this increase, bringing levels of NF κ B activity back to basal levels ($P = 0.02$, versus 3 hours vehicle, Figure 4D). Levels of NF κ B activity at 24 hours are not different between treatment groups or compared with controls. Caspase 1 activity increases by 23% at 3 hours ($P = 0.05$) and by 32% at 24 hours after HI ($P = 0.03$) in vehicle-treated mice (white bars, versus naive controls), and necrostatin treatment attenuates this response by 24% at 3 hours ($P = 0.04$) and 25% at 24 hours after HI ($P = 0.04$, dark gray bars versus vehicle, Figure 4E).

FLIP Gene and Protein Expression is Suppressed in Necrostatin-Treated Neonatal Mice After Hypoxia–Ischemia

FLIP protein turnover is high and posttranscriptional modification is minimal; therefore, FLIP protein levels are highly dependent on levels of gene expression (Kataoka *et al*, 2002), which is under the transcriptional control of NF κ B (Ichikawa *et al*, 2005). There is a 70% reduction in *FLIP* gene expression (range 90% to 60%, $P = 0.01$, Figure 5A) and a $> 50\%$ reduction in FLIP protein levels ($P = 0.04$, Figure 5B) in necrostatin-treated mice (dark gray bars) versus vehicle-treated mice (white bars). Both the 55 and 30 kDa FLIP isoforms are identified by the antibody and are similarly affected.

Figure 4 Cytokine gene and protein expression, NF κ B and caspase 1 activity after HI and necrostatin treatment. **(A)** Fold change in interleukin (IL)-1 β , IL-6, IL-12p35, and tumor necrosis factor (TNF) α gene expression in the forebrain 24 hours after HI and necrostatin treatment versus vehicle (reference line sitting at 1). Data represented as a box-and-whisker plot in which the 75th and 25th percentiles (interquartile range) define the upper and lower limits of the box, respectively, and the median is represented as the line inside the box. **(B)** Melting curves confirm single PCR product for all genes of interest. **(C)** Immunoblot for TNF α in protein fractions at 24 and 48 hours after hypoxia-ischemia (HI) (N: necrostatin treated, V: vehicle treated). β -Actin is shown as loading control. **(D)** TNF α protein expression is represented as bars (vehicle: white, necrostatin: dark gray, mean $\pm$ s.e.m., * P = 0.02 versus vehicle, n = 3 per group). **(E)** Nuclear factor (NF) κ B p65 levels in nuclear extracts and **(F)** caspase 1 activity in cytosolic extracts at 3 and 24 hours after HI and treatment with vehicle (white) or necrostatin (dark grey) versus naive (light gray) animals represented as bars (mean $\pm$ s.e.m.). * P < 0.05, n = 5 to 6 per group.

Discussion

Necrostatin, when administered after neonatal HI, provides robust and long-lasting neuroprotection primarily by blocking necrotic cell death in the mouse brain. Necrostatin blocks the interaction of RIP1 and RIP3 and facilitates the restoration of FADD and RIP3 binding. These necrostatin-mediated alterations in brain cell death and molecular signaling are associated with decreases in protein oxidation, decreases in NF κ B activation, caspase 1 activity, FLIP gene and protein expression, and decreases in gene and protein expression of proinflammatory cytokines.

This is the first study to show the neuroprotective efficacy of necrostatin in any model of neonatal brain

injury. Previous studies have shown that necrostatin is neuroprotective in adult mouse stroke and traumatic brain injury models as shown by histologic and behavioral outcomes (Degterev *et al*, 2005; You *et al*, 2008). Protection in necrostatin-treated neonatal mice is demonstrable in the ipsilateral forebrain and thalamus at 4 days (P11) and persists at 21 days (P28) after HI. Our techniques may lack the sensitivity for detecting small histologic/structural differences in necrostatin-treated mice at P8 due to the variability inherent in this model. However, necrostatin-treated mice did show prominent neuroprotection against biochemical damage at 3 and 24 hours after HI as revealed by OxyBlot and coimmunoprecipitation analyses. The protection against the delayed progressive injury in the thala-

Figure 5 FLIP gene and protein expression after neonatal HI and necrostatin treatment. Change in (A) *FLIP* gene and (B) protein expression in the forebrain 24 hours after hypoxia–ischemia (HI) and treatment with vehicle (white) or necrostatin (gray). Gene expression is represented as box-and-whisker plots in which 75th and 25th percentiles (interquartile range) define the upper and lower limits of the box, respectively, and the median is represented as the line inside the box. Melting curves for FLIP confirm a single PCR product. Protein expression is represented as bars (mean $\pm$ s.e.m.). Representative SDS-PAGE for FLIP protein expression is shown with β -actin as loading control in panel B. * $P < 0.05$ in all cases. $n = 3$ to 6 per group.

mus may result from necrostatin-mediated preservation of the forebrain and interruption of system-directed neurodegeneration (Northington *et al*, 2001b; Stone *et al*, 2008).

Gender differences have been found in neonatal rodent models of HI brain injury (Zhu *et al*, 2006). We analyzed cohorts of male mice and found that necrostatin provided significant neuroprotection in males when necrostatin-treated mice were compared with vehicle-treated mice. This study was not designed to determine the effect of gender, and the numbers in each group were small when separated for gender (the female group sizes were too small to analyze); nevertheless, a strong neuroprotective effect of necrostatin resolved in males. Necrostatin neuroprotection *in vivo* is associated with a block in translocation of apoptosis-inducing factor (AIF) to the nucleus (Asare *et al*, 2009). After HI, AIF nuclear translocation is enhanced in neonatal males in comparison with females (Zhu *et al*, 2006). Our results showing that necrostatin blocks RIP1–RIP3 interaction, oxidative damage, and inflammation could reflect mechanisms of action upstream of AIF translocation in males.

Necrostatin is a small molecule chosen from an extensive chemical library for its ability to inhibit cell death caused by TNF α stimulation in the setting of caspase inhibition (Degtarev *et al*, 2005). It is an allosteric inhibitor of RIP1 (Degtarev *et al*, 2005), which is a key signaling intermediate in the form of cell death described as programmed necrosis (Holler *et al*, 2000). *In vitro*, inhibition of RIP1 kinase blocks cell death caused by death receptor signaling in

the presence of caspase inhibition (Degtarev *et al*, 2008). We have described previously that a morphologic hallmark of delayed-ongoing neurodegeneration in the neonatal rodent brain after HI is apoptotic-necrotic continuum or hybrid cell death (Northington *et al*, 2007), which has similarities to programmed necrosis. Classic cellular necrosis also contributes significantly to the neurodegeneration caused by HI (Northington *et al*, 2001b). ‘Continuum’ cell neurodegeneration may result from failure of completion of caspase-dependent apoptotic cell death after neonatal HI (Northington *et al*, 2007). There is an abundance of biochemical evidence for activation of caspase-dependent pathways after neonatal HI (Blomgren *et al*, 2001; Nakajima *et al*, 2000; Northington *et al*, 2001a; Zhu *et al*, 2006), but there is a paucity of evidence that apoptosis is fully executed during the acute phase of neonatal brain injury induced by HI (Blomgren *et al*, 2007; Ishimaru *et al*, 1999; Martin *et al*, 2000; Northington *et al*, 2001b), thus resulting in a preponderance of continuum cell death driven by failure of completion of caspase-dependent apoptotic cell death after neonatal HI (Northington *et al*, 2007). When cell death phenotype analysis was conducted, we once again found a prominent role for necrotic cell death in neurodegeneration after HI (Northington *et al*, 2001b), and found that necrostatin significantly blocked this necrotic cell death with a three-fold reduction in the number of necrotic profiles. As a result, there was a major overall increase from <33% to ~60% in the contribution of apoptotic and continuum forms of cell death in necrostatin-treated

animals. This finding was surprising, given that we engaged the study with the hypothesis that necrostatin would act on programmed necrosis or continuum cell death in the neonatal HI brain. Nevertheless, this result validates the concept of the apoptosis-necrosis cell death continuum and further supports the idea that morphologically defined classic necrosis and continuum cell death are distinct entities, and that mechanistically classic necrosis and programmed necrosis might be more similar than previously realized. In this model, consistent with previous reports, there is no effect of necrostatin on caspase activity (Degterev *et al*, 2005), and in necrostatin-treated mice, there is an increase in the number of cells with an apoptotic profile. The decrease in FLIP gene and protein expression in necrostatin-treated mice may also support increased apoptosis (Kreuz *et al*, 2001) because FLIP normally acts as a dominant negative for caspase 8 (Micheau *et al*, 2001). The shift to a greater incidence of apoptosis in necrostatin-treated mice after HI is consistent with these findings. Overall, these findings support the interpretation that necrostatin acts directly on and reduces the predominant cellular necrosis after neonatal HI and favors an overall cell death profile that is 'cleaner' and less likely to result in proinflammatory cell disruption. A similar mechanism of neuroprotection in the injured neonatal brain may be operative with mild hypothermia (Mueller-Burke *et al*, 2008).

The receptor-interacting protein kinases, RIP1 and especially RIP3, have major roles as a molecular switch between programmed necrosis and apoptosis (Zhang *et al*, 2009), and possibly, on the basis of data presented in this study, between classic necrosis and apoptosis. Similar to RIP1, RIP3 contains an N-terminal kinase domain with ~40% homology to RIP1 and an RIP homotypic interaction motif, but does not contain a death domain (Cho *et al*, 2009), and both proteins are abundantly expressed in the neonatal brain. Both RIP3 and the kinase activity of RIP1 are essential for stable formation of the RIP1–RIP3 pronecrotic complex (complex II), and induction of complex II kinase activity is specifically required for programmed necrosis. Necrostatin can abolish RIP1–RIP3 interaction by the inhibition of RIP3 phosphorylation and can potentially inhibit complex II kinase activity and thus programmed necrosis (Cho *et al*, 2009) or classic necrosis as seen in this study. Receptor-interacting protein-3 also regulates ROS production (Cho *et al*, 2009) by stimulation of metabolic pathways that provide substrates for the respiratory chain and mitochondrial ROS production (Zhang *et al*, 2009). Our finding that necrostatin attenuates cellular necrosis and oxidative damage to proteins after neonatal HI is consistent with an anti-ROS/antinecrosis mechanism of action *in vivo*.

Both RIP1 and RIP3 interact with FADD (Cho *et al*, 2009). When the RIP kinases are complexed with FADD and exposed to TNF α , apoptosis seems to be the predominant form of cell death. Receptor-

interacting protein-1 is recruited to FADD in a TNF-dependent manner, whereas RIP3 is more constitutively associated with FADD (Cho *et al*, 2009). Our results are consistent with these *in vitro* studies. We find that RIP3 and FADD coimmunoprecipitate in the normal developing brain; RIP1 is recruited to complex with RIP3 after HI, and HI disrupts the association of FADD and RIP3. Importantly, necrostatin markedly reduces RIP1–RIP3 complex formation after HI, and by 24 hours after HI, partially restores the association of RIP3 and FADD. These findings are consistent a central role for pronecrotic RIP1–RIP3 complex formation in response to neonatal HI and with inhibition of RIP1–RIP3 activity as a major mechanism of necrostatin-mediated neuroprotection. The results of RIP3–FADD coimmunoprecipitation studies may indicate a generalized permissivity of the developing brain to apoptotic cell death and a shift toward a molecular environment more permissive for apoptosis and inhibitory to classic necrosis in necrostatin-treated mice after neonatal HI.

Our study shows that a single dose of necrostatin significantly decreases the oxidative damage to proteins during the first 24 hours after neonatal HI. We do not know how necrostatin acts to block oxidative protein modification, nor do we know the source of the ROS responsible for the oxidative damage to proteins. However, this oxidative injury to proteins can be attenuated dramatically by immediate post-HI treatment with necrostatin, as is also the finding with hypothermia (Mueller-Burke *et al*, 2008). Receptor-interacting protein-1 has been implicated in signaling cascades important to both mitochondrial and non-mitochondrial ROS production (Lin *et al*, 2004; Shen *et al*, 2004). There is no known direct antioxidant effect of necrostatin, but it does modulate redox mechanisms in experimental systems (Xu *et al*, 2007). Necrostatin increases glutathione levels and decreases ROS production (Xu *et al*, 2007), blocks nitric oxide-mediated cell necrosis and mitochondrial ROS production (Davis *et al*, 2010) and may also delay the opening of mitochondrial permeability transition pore (Lim *et al*, 2007). These effects may be reflected in the decrease in protein oxidation in the first 24 hours and the subsequent delayed appearance of neuroprotection after HI and necrostatin administration in our model.

We also determined that necrostatin treatment suppresses important brain proinflammatory cytokines and proximal regulators of cytokine expression after neonatal HI. In normal physiology, a primary function of RIP1 kinase is to transduce the NF κ B signal, and although apparently normal at birth, RIP-deficient mice fail to thrive and die during the first 3 days after birth with extensive lymphoid apoptosis associated with failure to activate NF κ B (Kelliher *et al*, 1998). A single intracerebroventricular dose of necrostatin does not have this effect because our mice survived for 3 weeks after injection of necrostatin, and in drug-only controls, there was no abnormality

of the brain seen on CV sections (Supplementary figure available online). *In vitro* studies establishing necrostatin as an inhibitor of RIP1 kinase failed to show an effect on NF κ B activation (Degterev *et al*, 2008), but the molecular actions of necrostatin *in vivo* are unknown. As FLIP is under the transcriptional control of NF κ B, the effects on FLIP gene and protein expression in necrostatin-treated mice serve as a reporter of decreased NF κ B activity in these mice, whether decreased NF κ B is a direct or indirect result of necrostatin treatment. Early inhibition of NF κ B is neuroprotective in this model (Nijboer *et al*, 2008). We find that HI induced a three-fold increase in NF κ B activity in vehicle-treated mice, which is not seen in necrostatin-treated mice. This suppression in NF κ B activity could contribute to the decrease in cytokine gene expression found in necrostatin-treated mice or result from the decreased cytokine expression. The 32% increase in caspase 1 activity after HI in vehicle-treated mice suggests a significant activation of the inflammasome (Bryant and Fitzgerald, 2009), which also signals for cytokine production. The effect of necrostatin to suppress this HI-induced increase in caspase 1 activity may act in conjunction with early inhibition of NF κ B activity to further suppress cytokine gene and protein expression (Bryant and Fitzgerald, 2009). We do not show that these protein, enzyme, and gene expression effects are a direct result of necrostatin treatment, and that the decrease in inflammatory markers may be related indirectly to necrostatin inhibition of necrosis. These results, overall, are consistent with the decreased neutrophil influx and microglial activation found in necrostatin-treated adult mice after traumatic brain injury (You *et al*, 2008).

Conclusions

Necrostatin is a potent neuroprotective drug for treating neonatal HI brain injury in mice by blocking necrotic cell death, previously believed to be unregulated and untreatable. The mechanisms of operation of necrostatin are related to the inhibition of RIP1–RIP3 interaction, decreased oxidative injury, and suppression of upstream proinflammatory signaling and multiple cytokines. Our results emphasize the importance of necrosis in neonatal HI and suggest a plausible biochemical mechanism that may underlie expression of the apoptosis–necrosis continuum.

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Conflict of interest

The authors declare no conflict of interest.

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