ORIGINAL RESEARCH

Proteasome Biology Is Compromised in White Matter After Asphyxic Cardiac Arrest in Neonatal Piglets

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***Background*—**Neurological deficits in hypoxic-ischemic encephalopathy, even with therapeutic hypothermia, are partially attributed to white matter injury. We theorized that proteasome insufficiency contributes to white matter injury.

***Methods and Results*—**Neonatal piglets received hypoxia-ischemia (HI) or sham procedure with normothermia, hypothermia, or hypothermia+rewarming. Some received a proteasome activator drug (oleuropein) or white matter–targeted, virus-mediated proteasome knockdown. We measured myelin oligodendrocyte glycoprotein, proteasome subunit 20S (P20S), proteasome activity, and carbonylated and ubiquitinated protein levels in white matter and cerebral cortex. HI reduced myelin oligodendrocyte glycoprotein levels regardless of temperature, and myelin oligodendrocyte glycoprotein loss was associated with increased ubiquitinated and carbonylated protein levels. Ubiquitinated and carbonyl-damaged proteins increased in white matter 29 hours after HI during hypothermia to exceed levels at 6 to 20 hours. In cortex, ubiquitinated proteins decreased. Ubiquitinated and carbonylated protein accumulation coincided with lower P20S levels in white matter; P20S levels also decreased in cerebral cortex. However, proteasome activity in white matter lagged behind that in cortex 29 hours after HI during hypothermia. Systemic oleuropein enhanced white matter P20S and protected the myelin, whereas proteasome knockdown exacerbated myelin oligodendrocyte glycoprotein loss and ubiquitinated protein accumulation after HI. At the cellular level, temperature and HI interactively affected macroglial P20S enrichment in subcortical white matter. Rewarming alone increased macroglial P20S immunoreactivity, but this increase was blocked by HI.

***Conclusions*—**Oxidized and ubiquitinated proteins accumulate with HI-induced white matter injury. Proteasome insufficiency may drive this injury. Hypothermia did not prevent myelin damage, protect the proteasome, or preserve oxidized and ubiquitinated protein clearance after HI. **(*J Am Heart Assoc*. 2018;7:e009415. DOI: 10.1161/JAHA.118.009415)**

Key Words: hypothermia • hypoxia • neonatal ischemia • oxidative stress • white matter disease

Intrapartum complications with birth asphyxia cause nearly 1 million neonatal deaths annually worldwide.[1,2] Perinatal hypoxic-ischemic encephalopathy (HIE) causes lifelong neurological and psychological disabilities in up to one third of survivors, with enormous personal, familial, and socioeconomic impact.[3–6] Therapeutic hypothermia reduces the risk of death or disability and is the standard of care for HIE.[7] However, hypothermia is not fully protective. Many hypothermia-treated survivors have persistent moderate-to-severe disabilities.[8–10]

The persistent disabilities may be attributed, in part, to white matter injury. We[11–13] and others[14–17] have shown that human neonates who received hypothermia for HIE exhibit white matter injuries on brain magnetic resonance imaging. In neonatal piglet, white matter apoptosis occurs after hypoxia-ischemia (HI) despite hypothermia.[18] Many mechanisms can cause white matter injury after HI,[19] as in other neurological disorders,[20,21] including oxidative stress and failed proteostasis. If permitted to accumulate, oxidatively damaged and misfolded proteins may trigger cell death.[22–24]

Oxidized, ubiquitinated, misfolded, and aggregated proteins are normally cleared by proteasomes to maintain cellular homeostasis.[25] This proteasome-driven cytoprotective mechanism may be disrupted during and after HI. Proteasomes are multisubunit and ATP-dependent multicatalytic proteinase complexes reliant on energetic and redox states in

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Clinical Perspective

What Is New?

- Proteasome insufficiency may contribute to white matter injury in neonatal hypoxic-ischemic encephalopathy, even with the use of therapeutic hypothermia.

What Are the Clinical Implications?

- Proteasome activation has potential as an adjuvant treatment to hypothermia to improve white matter protection.

compliance with the unfolded protein response. HI induces endoplasmic reticulum stress, which should activate the unfolded protein response and proteasomal clearance of damaged and modified proteins to prevent neural cell death.[23–25] However, we found that HI may attenuate the unfolded protein response[26] and potentially reduce proteasome activation from endoplasmic reticulum–associated degradation. Oligodendrocytes have high rates of myelin protein synthesis and turnover that render them highly vulnerable to failures in protein quality control.[27,28] Thus, we postulate that proteasome insufficiency contributes to white matter injury.

We studied the proteasome and white matter injury in a neonatal piglet model of HI, hypothermia, and rewarming. Because humans have a large cerebral cortex and correspondingly large cerebral white matter volume and complex ascending, descending, and commissural white matter tracts,[29] a pig model is ideal for studying white matter injury as well as comparing white matter to gray matter responses in preclinical and translational brain injury research. We tested the hypotheses that hypothermia provides incomplete white matter protection from HI, and that HI-induced white matter injury is associated with proteasome insufficiency and inadequate clearance of oxidized and ubiquitinated proteins. We also examined whether oleuropein (OLE), a naturally occurring proteasome activator,[30] protects the white matter and whether this protection depends upon the proteasome.

Methods

The data, analytical methods, and study materials are available to other researchers for the purposes of reproducing the results or replicating the procedure as described in this article or upon contacting the principal investigator (J.K.L., jklee@jhmi.edu). We studied brains from piglets of our past cohorts to conserve animals.[18,26,31] In addition, we conducted 98 new piglet experiments for the current study. Our protocols were approved by the Johns Hopkins University Animal Care and Use Committee. All procedures complied with the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. We ensured animal comfort at all points in the protocols, and animal care complied with the National Institutes of Health Guidelines.

Piglet Preparation

Neonatal male piglets (2–3 days old, 1.0–2.5 kg) were assigned by a randomization schedule to HI or sham procedure. "Naïve," male, and age-matched piglets that did not receive anesthesia, surgery, or HI were prepared as an additional control. Figure 1 summarizes the protocols, the number of animals in each group, and their triaging for outcome measurements. The HI protocol was carried out as we have previously published.[18,26,31] Anesthesia was induced with isoflurane 5% and 50%/50% nitrous oxide/oxygen by nose cone, and then the piglets were intubated for mechanical ventilation to maintain normocarbia. After intubation, the nitrous oxide was increased to 70% with 30% oxygen concentration. Isoflurane was decreased to 2% for placement of femoral arterial and venous catheters. Piglets then received intravenous normal saline with dextrose 5% (4 mL/kg/h) and fentanyl (20 μg IV bolus followed by 20 μg/kg/h). Arterial blood pressure was monitored continuously. We discontinued the isoflurane after the femoral catheters were placed, which takes ≈10 to 15 minutes. All piglets received vecuronium (0.2 mg/kg/h, IV) to prevent shivering during hypothermia and to provide the same anesthetic to all groups. We previously determined that this anesthetic does not affect cerebral cortical[31] or white matter[18] cell apoptosis or the unfolded protein response.[26] We maintained mean arterial blood pressure (MAP) above 45 mm Hg using phenylephrine or dopamine infusions, when necessary, to ensure that blood pressure remained above the lower limit of autoregulation.[32]

Cardiac Arrest HI Injury Protocol

Inspired oxygen was decreased to 10% for 45 minutes to produce whole-body hypoxia with a goal oxyhemoglobin saturation of 30% to 35%. We then provided the piglets with 5 minutes of room air, which is required for cardiac resuscitation. Then, the endotracheal tube was occluded to produce asphyxia. Piglets destined for histological evaluation or 20 hours of recovery for western blots received 7 minutes of asphyxia. Piglets that recovered for 6 or 29 hours for western blots or that were treated with OLE (Sigma-Aldrich, St. Louis, MO) or vehicle received 8 minutes of asphyxia (see below for OLE methods.) We resuscitated the piglets with manual chest compressions, 50% oxygen, and epinephrine (100 μg/kg, IV). Piglets that did not resuscitate after 3 minutes of compressions were excluded. After

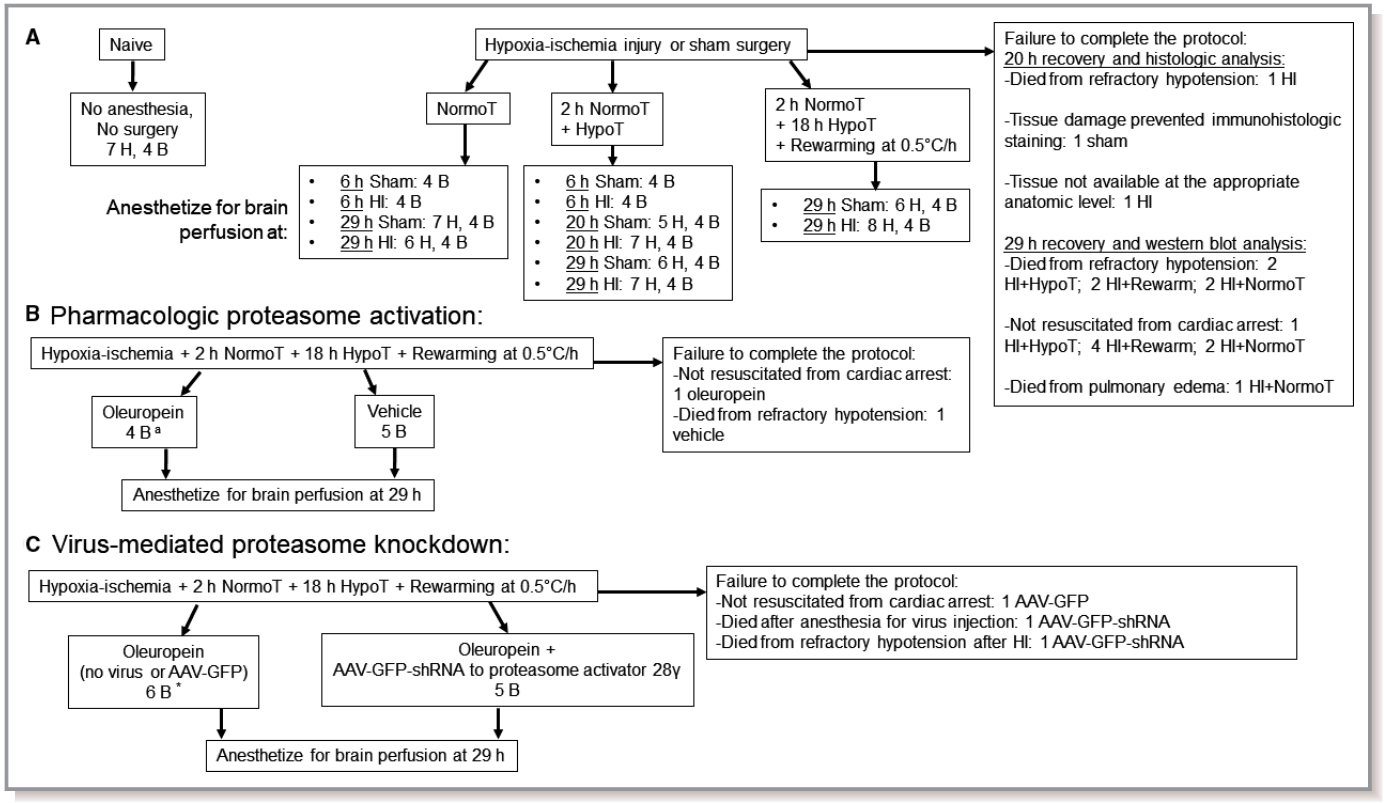

Figure 1. Study design. We previously reported the protocol completion rates for piglets that had histological evaluation after 29 hours of recovery from hypoxia-ischemia (HI).[31] We only show the number of additional piglets that did not complete the protocol or that could not be analyzed in the additional experiments for the current study. A, Randomization of piglets to treatment group and temperature. B, Piglets were randomized to receive proteasome activation with oleuropein or vehicle. C, Randomization of piglets to oleuropein or adeno-associated virus (AAV)-mediated proteasome genetic knockdown. *Four piglets received oleuropein without virus injection, and these piglets' brain tissue was also tested in the proteasome knockdown experiments. B indicates Sample size for biochemistry; GFP, Green fluorescent protein; H, Sample size for histology; h, Hour; hypoT, Hypothermia; normoT, Normothermia; shRNA, Short hairpin RNA.

resuscitation, inspired oxygen was lowered to 30% for the rest of the experiment. Piglets randomized to the sham procedure received the same duration of anesthesia, placement of femoral catheters, and FiO_2 30% throughout the experiment, but did not undergo HI. Arterial blood gases and electrolyte levels were checked every 1 to 4 hours. We adjusted ventilation and administered sodium bicarbonate and calcium to correct acidosis and hypocalcemia, as necessary.

Temperature Management

Before the start of the experiment, piglets were also randomized to 6, 20, or 29 hours of normothermia (rectal temperature, 38.0 to 39.5°C; normothermic for swine), sustained hypothermia (34.0°C), or hypothermia with rewarming at 0.5°C/h (the clinical rewarming rate for HIE[11]; Figure 1A). Normothermia was maintained with heating lamps and warming blankets. Hypothermia induction began 2 hours after resuscitation from HI or sham procedure to mimic clinical delays in cooling. We used 34.0°C as the hypothermia target because this approximates the 4°C decrease that is used in clinical therapeutic hypothermia (37°C human normothermia with cooling to ≈33°C). Only piglets that recovered for 29 hours received rewarming.

Ice packs and cooling blankets were used to induce and maintain hypothermia. Rectal and brain temperatures correlated within 0.2°C in our model.[18] Rewarming began 20 hours after resuscitation (after 18 hours of hypothermia) by increasing the blanket's circulating water temperature and by applying warm packs to reach normothermia. Piglets were deeply anesthetized and euthanized with beuthanasia (50 mg/kg of pentobarbital plus 6.4 mg/kg of phenytoin) for transcardial perfusion of cold PBS followed by 4% paraformaldehyde for brain fixation and histological measurements or cold PBS alone for fresh brain harvesting and biochemical measurements.

Brain Proteasome Enforcement

Some pigs were randomized to receive the proteasome activator, OLE,[30] or vehicle after HI, 2 hours of normothermia, 18 hours of hypothermia, and rewarming at 0.5°C/h

(Figure 1B). OLE is a putative proteasome activator that crosses the blood–brain barrier. It is a natural polyphenolic compound extracted from olive plant products. OLE 2.7 mg/kg IV in 1% DMSO was given 15 minutes after resuscitation from HI, followed by 0.7 mg/kg IV 1 hour after HI and then 0.7 mg/kg IV every 2 hours until the end of the experiment. Piglets assigned to receive vehicle were given 1% DMSO intravenously in equivalent dose volume and timing.

Virus-Mediated Proteasome Genetic Knockdown in White Matter

We conducted this experiment to verify that a molecular mechanism of OLE action is proteasome enforcement. Piglets were anesthetized as described above for stereotaxic injections of either adeno-associated virus (AAV) encoding green fluorescent protein (GFP; Vector Biolabs, Malvern, PA) or AAV encoding short hairpin RNA (shRNA) to proteasome activator subunit 28γ (PA28γ) and GFP (Vector Biolabs) according to a randomization schedule. The former pigs were controls for the latter experimental pigs (Figure 1C). Using a microinjector needle and glass syringe, we bilaterally injected AAV (titer 10^{13} genome copies/mL) 10 μL per side (equivalent to 10^{11} genome copies per side) into the subcortical white matter, including the corona radiata, using the injection landmarks: 1.24 mm rostral to the lambda suture, 7.5 mm lateral from the midline, and 7.5 mm deep. We used a stereotaxic atlas of the pig brain as a guide.[33] Then, the pigs emerged from anesthesia. Two days later, the piglets were reanesthetized for the HI injury protocol described above followed by 2 hours of normothermia, 18 hours of hypothermia, and rewarming at 0.5°C/h. Piglets also received the proteasome activator, OLE, as described above. Four HI piglets received OLE without AAV and 1 anesthetic. The piglets were deeply anesthetized and euthanized as described above for transcardial perfusion with cold PBS, and the subcortical white matter was dissected from fresh brain slabs on wet ice and then flash-frozen in isopentane cooled dry ice for western blots. Virus injection sites were verified grossly at brain removal from the skull (at the cortical surface) and at brain cutting by visualizing the needle injection tracks.

Immunohistochemistry

Fixed brains were embedded in paraffin and cut into 10-μm coronal sections. Proteasome subunit 20S (P20S) immunohistochemistry (IHC) staining was conducted as previously described.[18] P20S is the catalytic core protease subunit that degrades oxidized and ubiquitinated proteins.[25] Briefly, sections were blocked with 3% normal goat serum and incubated with the primary antibody, rabbit anti-P20S (1:100; GeneTex, Irvine, CA), the secondary antibody, goat antirabbit immunoglobulin G (1:20; Sigma-Aldrich), and rabbit peroxidase antiperoxidase soluble complex antibody (1:100; Sigma-Aldrich). We used 3,3′-diaminobenzidine substrate to disclose sites of antibody binding to P20S as brown followed by cresyl violet counterstaining to show the nucleus as blue.

One investigator (J.K.L.), who was blinded to the treatment group, classified and counted macroglia (astrocytes and oligodendrocytes) in the subcortical white matter of the motor and somatosensory gyri and in the internal capsule as P20S negative, trace positive, enriched, or highly enriched. These white matter regions are readily and consistently identifiable as individual structures in the pig brain. The cells were identified as astrocytes and oligodendrocytes based on their small size, <10 μm in diameter, distinct nuclear morphologies revealed by the cresyl violet staining, and small cytoplasmic volume (Figures 2A, B). P20S-negative cells were identified by absence of P20S brown staining. Trace-positive cells had distinct brown P20S granules. P20S-enriched glia had light brown, diffuse P20S staining without discernible granules. Glia that were highly enriched for P20S had very dark, diffuse P20S staining (Figure 2A). A co-investigator (L.J.M.) screened the slides for counter-reliability. Glia were counted in 10 nonoverlapping microscope fields of the motor subcortical white matter and in 10 nonoverlapping fields of the somatosensory subcortical white matter at ×1000 magnification with oil immersion (Figure 2C). Glia in the first bundle of the internal capsule were counted in 5 nonoverlapping fields at the same magnification. Mean glia counts were used for the analysis. Glial number reflects changes in white matter macroglia (astrocytes and oligodendrocytes) only.

We excluded white matter neurons from the cell counts; neurons were identified by their large size (typically 10–20 μm in diameter), characteristic large open nucleus with nucleolus and chromatin strands, and large oval cell body with the long axis oriented in parallel with the white matter alignment.[18,31] We also excluded microglia, which were identified by their irregular and elongated nuclear morphology and soma with branch points and processes. Transformed, activated, large microglia with a macrophage-like morphology were not encountered in the white matter of these piglets, as previously reported.[26] Furthermore, we did not count cells that were within 1 cell diameter of a vessel in an effort to exclude blood-borne cells.

Western Blots

After transcardial perfusion of the pigs with cold PBS, brains were removed from the skull. The somatosensory subcortical white matter and primary somatosensory cortex were dissected from fresh brain slabs on a metal plate chilled by wet ice. Samples were frozen in isopentane-chilled dry ice. We homogenized the tissue and mixed the homogenate with ice-

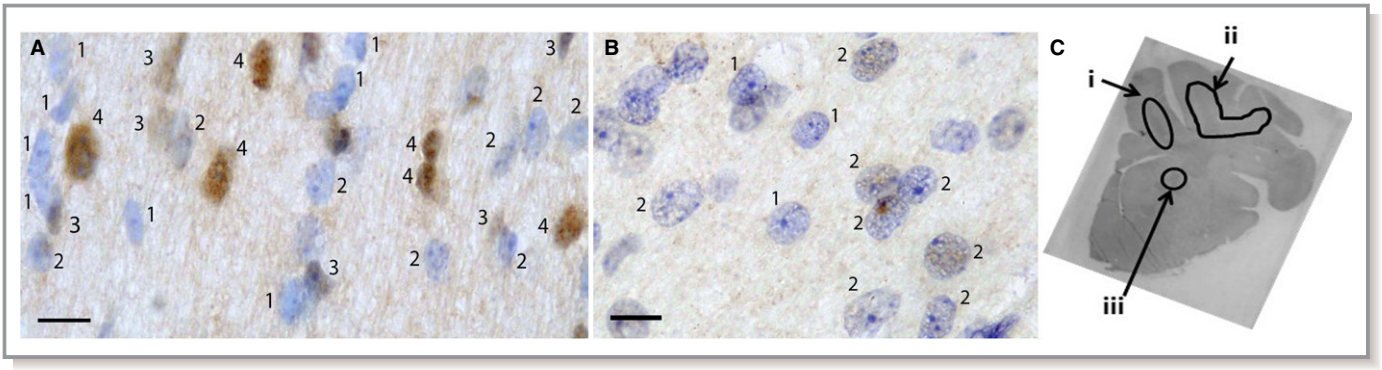

Figure 2. Glial cell classification using immunohistochemical detection of proteasome 20S (P20S) with 3,3′-diaminobenzidine (brown) and cresyl violet counterstain (blue). 1: P20S-negative (no brown granules). 2: trace positive for P20S (distinct brown granules). 3: P20S-enriched (diffuse, light brown stain with loss of individual granules). 4: P20S highly enriched (very dark, diffuse brown stain). A, A representative piglet that received hypoxia-ischemia (HI) and hypothermia for 20 hours. B, A piglet that received HI, hypothermia, and rewarming with 29 hours of recovery. Photos were taken of the subcortical white matter in the motor gyrus under a ×1000 lens with oil immersion. Scale bar=10 μm. C, Macrophotograph of the striatal anatomical level where the P20S glia enrichment was classified in the (i) subcortical white matter of the motor gyrus, (ii) subcortical white matter of the primary somatosensory gyrus, and (iii) first bundle of the internal capsule. This grayscale image of a hematoxylin & eosin–stained section was photographed at ×10.

cold RIPA buffer (Cell Signaling Technology, Danvers, MA), phosphatase inhibitor (Roche Applied Science, Branford, CT), and protease inhibitor cocktail (Invitrogen, Grand Island, NY) in a 0.1 g to 1 mL ratio. After the homogenate was centrifuged, protein concentration in the supernatant was measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Samples were boiled for 5 minutes, mixed with loading buffer, separated by SDS-PAGE on 4% to 12% Tris–glycine gels, and transferred to nitrocellulose membranes. We loaded a mixture of white matter from 4 naïve, unanesthetized piglets into 1 lane of each gel as a control. One piglet from each treatment group was run on each gel. Protein loading was quantified by membrane Ponceau S stain (Sigma Life Science, St. Louis, MO). Membranes were then washed, blocked in 5% nonfat milk, and incubated overnight with the primary antibodies, monoclonal rabbit antimyelin oligodendrocyte glycoprotein (MOG; 1:5000; Abcam, Cambridge, MA), polyclonal rabbit anti-P20S (1:2000; GeneTex), or monoclonal mouse antiubiquitin antibody (1:2000; Sigma-Aldrich), which detects mono- and polyubiquitinated proteins. After the primary antibodies, membranes were washed and incubated with the appropriate secondary antibodies (1:5000, antirabbit or -mouse immunoglobulin G; Jackson Immunoresearch, West Grove, PA). Western blots for piglets that received OLE were also incubated with the primary antibody, polyclonal rabbit anti-PA28γ (1:500; Cell Signaling Technology), followed by secondary antibody (1:1000), and with the primary antibody, polyclonal rabbit anti-GFP (1:10 000; Chemicon International, Temecula, CA) followed by secondary antibody (1:5000). These antibodies were used to verify proteasome enforcement/knockdown, and the GFP reporter verified virus-mediated gene transduction. We imaged the membranes with enhanced chemiluminescence (Bio-Rad, Hercules, CA) and iBrightCL1000 Imaging System (Invitrogen). We used ImageJ (NIH, Bethesda, MD) to analyze the immunoreactive band intensities and normalized (divided) the densities to the total protein in Ponceau S–stained membranes.

Carbonylated proteins were detected with the OxyBlot Protein Oxidation Detection Kit (Millipore, Burlington, MA). A white matter mixture from 4 naïve, unanesthetized piglets was loaded on each gel as a control. Two gels were run per Oxyblot experiment. Protein loading was measured by Coomassie stain on 1 gel, and the second was used to detect carbonylated proteins. Two negative controls were run on each gel by adding derivatization control solution to 2 samples. We used ImageJ (NIH) to analyze the immunoreactive band intensities normalized to (divided by) the total protein in Coomassie-stained gels.

Proteasome Activity

We measured proteasome activity using the Proteasome Activity Fluorometric Assay Kit I (UBPBio, Aurora, CO) per the manufacturer's instructions. First, we verified that the assay detects piglet proteasome activity using brain homogenate with and without the proteasome inhibitor, marizomib, 5 mmol/L (Sigma-Aldrich). Then, we tested subcortical white matter and sensorimotor cortex in naïve piglets and those that received HI+hypothermia for 20 hours and HI+hypothermia for 29 hours. We selected these groups because neonates treated for moderate or severe HIE receive more than 29 hours of hypothermia in clinical practice. Bovine P20S was used as a positive control, and wells loaded with only cell lysis buffer were negative controls. Reactions were run in 0.2 μg of cell extract protein. Data are presented as enzyme activity.

Putamen Neuropathology Methods

We counted viable neurons in the putamen as an estimate of HI injury severity in piglets. Putamen neuron counts reflect individual variability in the HI response because putamen neuronal injury was evident 1 day after HI in our model.[33,34] One investigator (C.E.O.), who was blinded to the treatment group, counted the viable neurons in putamen at ×400 magnification in 10 to 12 nonoverlapping fields of hematoxylin and eosin–stained sections. Viable neurons were identified as described above with the absence of ischemic necrotic or apoptotic morphology.[18,31] Two investigators (L.J.M., J.K.L.) screened the slides for counter-reliability.

Statistical Analysis

Analyses were conducted with SigmaPlot (v.14.0; Systat Software, San Jose, CA), and graphs were generated with GraphPad Prism (v.5; (GraphPad Software Inc, La Jolla, CA). Data are presented as dot plots or as means with SDs. Significance was assumed when the 2-sided *P* value was less than 0.05. Each anatomical region was analyzed separately. Western blot densities were normalized to (divided by) that of the naïve piglet white matter mixture on each gel. For the proteasome genetic knockdown experiment, western blot densities were normalized to (divided by) the median of piglets that received OLE without proteasome knockdown.

We first tested the data with the Shapiro–Wilk normality and Levene equal variance tests. IHC data that passed both normality and equal variance tests were analyzed by *t* test or one-way ANOVA with post hoc Holm–Sidak pairwise comparisons. Western blot data that passed normality and equal variance tests were analyzed by repeated-measures ANOVA with data blocked by gel and post hoc Holm Sidak pairwise comparisons. If tests of normality and equal variance were not both passed, IHC data were analyzed by Mann–Whitney U rank-sum tests or Kruskal–Wallis 1-way ANOVA on ranks and post hoc Dunn's pairwise comparisons, and western blot data were analyzed by Friedman repeated-measures ANOVA on ranks with data blocked by gel and post hoc Dunn comparisons. OLE western blots were analyzed by *t* test or Mann–Whitney U rank-sum test.

We used 2-way ANOVA to test the independent effects and interactions between HI and temperature on P20S IHC at 29 hours of recovery. Data were transformed with a log(x+1) transformation to generate normally distributed data for the 2-way ANOVA, when necessary.

To evaluate the relationship between MOG and protein ubiquitination or carbonylation at 29 hours, we analyzed paired (within pig) comparisons of MOG and ubiquitinated protein levels and separately MOG and carbonylated protein levels by segmented linear regression using GraphPad. The software determined the MOG breakpoint for the best fit model. We tested the null hypothesis that the change in ubiquitinated protein level (or carbonylated protein level) per change in MOG for data above and below the breakpoint was 0.

Variability in brain injury severity among piglets may influence variability in white matter damage as reflected by MOG and protein modification as well as proteasome responses. Because putamen neurons are highly vulnerable to hypoxia-asphyxia in this model,[35] we performed an exploratory subanalysis in which we used putamen viable neuron counts as an indicator of HI injury severity while taking into account temperature. We analyzed the mean of viable putamen neuron counts from 10 to 12 microscope fields as a percentage of the mean of normothermic shams. Among HI groups, we first determined the relationship between temperature and viable neuron count by 1-way ANOVA with post hoc Holm–Sidak tests. Second, we ran Pearson correlations between the number of P20S-enriched glia in white matter and viable putamen neurons. Finally, we used ANCOVA to explore the combined effect of temperature, viable putamen neuron count, and their interactions on the number of P20S-enriched glia in white matter. Because temperature and neuron counts are not independent (as determined by ANOVA in the first step), we cannot assess the independent effect of viable neuron counts on glial P20S. Rather, we designed this exploratory analysis to look at the interactive effect between temperature and viable neuron counts, as a reflection of HI brain injury severity, on white matter P20S expression in the model as a whole.[36]

We analyzed proteasome enzymatic activity first by repeated-measures 2-way ANOVA with group (naïve, HI+20 hours of hypothermia, or HI+29 hours of hypothermia) as factor 1 and assay reaction time as factor 2 in subcortical white matter and separately in cortex. Second, we analyzed proteasome activity by repeated-measures 2-way ANOVA with brain region (white matter or cortex) as factor 1 and assay reaction time as factor 2 with data stratified by 20 or 29 hours of recovery. Post hoc comparisons between groups at each assay reaction time point were conducted using the Holm–Sidak test.

We used 1-way ANOVA with post hoc Holm–Sidak tests or Kruskal–Wallis 1-way ANOVA on ranks with post hoc Dunn tests to compare temperature before hypothermia induction, MAP, pH, arterial partial pressure of carbon dioxide ($PaCO_2$), oxyhemoglobin saturation, hemoglobin, and sodium levels between treatment groups at each time point.

Sample Size

When IHC had been completed in 4 pigs per group, we conducted power estimates to compare the number of P20S highly enriched glia between pigs that recovered 20 hours and those that recovered 29 hours after HI+hypothermia. The difference in means was 23 with a within-group SD of 5.

A sample size of 4 would permit rejection of the null hypothesis with power >0.95 and an alpha level of 0.05. We increased the sample size for IHC to accommodate potentially greater variability and underestimation of the effect size. We reanalyzed histological tissue from piglets that recovered for 29 hours after HI or sham procedure from our previous studies on cortical[31] and white matter[18] apoptosis. We previously used n=4 to demonstrate differences by western blot in cell death measures after piglet HI, hypothermia, and rewarming.[31] Little information exists about the efficacy of AAV gene transduction or pharmacological proteasome activation in piglets. One report successfully used AAV transduction in 3 piglets for a semiquantitative analytical outcome.[37] For the current study, we tested 4 to 6 piglets/group for proteasome activation or knockdown.

Results

HI resuscitation rate was 93% in piglets destined for 29 hours of recovery and histological evaluation, as described previously for some of the pigs used here.[31] Figure 1 shows piglet randomization to treatment group and the number of piglets that did not complete the protocol for the 98 additional piglet experiments in this study. HI resuscitation rate was 82% and the protocol completion rate was 63% after HI among pigs analyzed by western blot. We used brain tissue from the western blot piglets for the proteasome activity assay. The success rate for the HI and OLE protocol, including piglets that did or did not receive AAV, was 71%.

Pig HI and Physiology

We previously reported temperature, pH, $PaCO_2$, oxyhemoglobin saturation, MAP, hemoglobin, and sodium levels in piglets that received HI or sham procedure, 29 hours of recovery, and histological evaluation.[18,31] Physiological data for this study of 60 additional piglets without proteasome modulation are shown in Table. Sham normothermic piglets were more acidotic than the other groups at baseline ($P<0.05$) and at 3 hours ($P<0.05$) and 20 hours ($P<0.05$) after resuscitation. Sodium levels after resuscitation also differed ($P<0.05$). $PaCO_2$, oxyhemoglobin saturation, and hemoglobin levels were similar before and after hypoxia-asphyxia. HI groups had similar levels of severe acidosis, hypercarbia, and hypoxia during hypoxia and asphyxia. Temperature before hypothermia induction was similar among groups, and rewarming did not overshoot normothermia. HI normothermic piglets had lower MAP than did sham normothermic and hypothermic pigs at 1 and 3 hours after resuscitation ($P<0.05$ for both). HI rewarmed pigs had lower MAP than sham hypothermia pigs at 28 hours ($P<0.05$). Phenylephrine was administered to 2 HI+normothermia, 2 HI+hypothermia, and 2 HI+rewarming piglets and to 1 sham+hypothermia piglet. Dopamine was given to 2 HI+normothermia piglets, 2 HI+hypothermia piglets, and 1 sham+rewarming piglet.

Hypothermia Did Not Protect Myelin After HI

We used MOG as a readout for myelin integrity. MOG in pig brain was detected on western blots as an immunoreactive band at 28 kD (Figure 3H), which is consistent with MOG's molecular weight in mammals.[38] MOG levels in somatosensory subcortical white matter differed at 29 hours ($P<0.05$; Figure 3A). In post hoc pairwise comparisons, each HI group had lower MOG immunoreactivity than that of each sham group ($P<0.05$ for all comparisons). For example, HI piglets that received sustained hypothermia ($P<0.05$) or hypothermia+rewarming ($P<0.05$) had lower MOG than shams that received the same temperature treatment. MOG level did not differ by temperature among HI groups ($P>0.05$ for all comparisons).

High Levels of Protein Ubiquitination and Oxidative Damage are Associated With Myelin Injury 29 Hours After HI

We assessed general protein damage by levels of ubiquitin-modified proteins. Immunoreactive mono- and polyubiquitinated proteins were detected by western blotting at 15 to 250 kD throughout the length of the gels. The most intense bands were in the range of 50 to 250 kD (Figure 3I). Ubiquitinated protein levels were not statistically different among shams during normothermia, hypothermia, and hypothermia+rewarming (Figure 3B), but protein ubiquitination differed after HI ($P<0.05$; Figure 3C). In post hoc pairwise comparisons, HI+hypothermia at 29 hours increased ubiquitin levels to exceed that of HI+normothermia at 6 hours, HI+hypothermia at 6 hours, and HI+hypothermia at 20 hours ($P<0.05$ for all comparisons).

We assessed protein oxidative damage using levels of carbonyl-modified proteins. Carbonylated proteins were detected by western blotting in pig brain at 10 to 250 kD throughout the length of the gels. The most intense bands were in the range of 20 to 50 kD (Figure 3I). Carbonylated protein levels did not statistically differ among shams (Figure 3D), but protein oxidation did differ after HI ($P<0.05$; n=4; Figure 3E). In post hoc comparisons, HI followed by 29 hours of normothermia increased carbonylated protein levels to exceed that of HI+normothermia at 6 hours, HI+hypothermia at 6 hours, and HI+hypothermia at 20 hours ($P<0.05$ for all comparisons). Hypothermia for 29 hours after HI also increased carbonylated proteins to above that of HI+normothermia at 6 hours, HI+hypothermia at 6 hours, and HI+hypothermia at 20 hours ($P<0.05$ for all comparisons).

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Table. Pig Physiology

Parameter/Group	Baseline (Mean±SD)	n	Hypoxia 42 min (Mean±SD)	n	Asphyxia* (Mean±SD)	n	ROSC 1 h (Mean±SD)	n	ROSC 3 h (Mean±SD)	n	ROSC 20 h† (Mean±SD)	n	ROSC 28 h (Mean±SD)	n
Temperature, °C														
Sham normothermia	38.0±0.9	8					38.5±0.7	8	38.7±0.8	8	38.8±0.4	4	39.0±0.5	4
Sham hypothermia	37.2±1.5	17					38.7±0.8	17	34.8±1.3	17	33.9±0.2	13	33.4±0.1	4
Sham rewarming	38.6±0.9	4					39.0±1.2	4	34.9±0.4	4	33.9±0.2	4	38.2±0.2	4
HI normothermia	37.9±0.6	8	38.7±0.4	8	38.7±0.4	8	38.6±0.6	8	38.6±0.7	8	38.7±0.3	4	38.8±0.6	4
HI hypothermia	37.5±0.9	19	38.5±0.5	19	38.5±0.4	19	38.5±0.5	19	34.2±0.4	19	33.8±0.2	15	33.8±0.2	4
HI rewarming	38.6±0.6	4	38.7±0.3	4	38.5±0.3	4	38.6±0.5	4	34.2±0.9	4	34.0±0.8	4	38.1±0.1	4
pH														
Sham normothermia	7.34±0.07	8					7.39±0.05	8	7.34±0.08‡	8	7.30±0.11	4	7.32±0.09	4
Sham hypothermia	7.43±0.06§	17					7.45±0.05	17	7.44±0.04	17	7.40±0.03‖	13	7.37±0.08	4
Sham rewarming	7.45±0.07§	4					7.46±0.06	4	7.42±0.02	4	7.41±0.04	4	7.42±0.05	4
HI normothermia	7.39±0.08	8	7.41±0.06	8	6.91±0.17	8	7.34±0.13	8	7.38±0.10	8	7.41±0.02	4	7.39±0.07	4
HI hypothermia	7.43±0.05§	19	7.36±0.06	19	6.87±0.15	19	7.38±0.09	19	7.43±0.09	19	7.39±0.05	15	7.34±0.02	4
HI rewarming	7.34±0.05	4	7.38±0.11	4	6.87±0.03	4	7.34±0.16	4	7.45±0.03	4	7.44±0.04‖	4	7.38±0.04	4
$PaCO_2$, mm Hg														
Sham normothermia	33±8	8					39±6	8	37±5	8	41±7	4	42±9	4
Sham hypothermia	32±5	17					34±5	17	32±5	17	37±3	13	46±9	4
Sham rewarming	36±7	4					36±5	4	36±4	4	37±2	4	38±7	4
HI normothermia	35±7	8	36±3	8	101±22	8	39±9	8	38±5	8	37±6	4	41±6	4
HI hypothermia	32±5	19	36±6	19	101±25	19	35±6	19	35±7	19	35±5	15	38±2	4
HI rewarming	39±9	4	30±14	4	106±21	4	39±8	4	37±6	4	38±5	4	41±3	4
SaO_2, %														
Sham normothermia	95±11	8					100±0	8	100±0	8	100±1	4	100±0	4
Sham hypothermia	99±2	17					100±0	17	100±0	17	100±0	13	100±0	4
Sham rewarming	100±0	4					100±0	4	100±0	4	100±0	4	100±0	4
HI normothermia	99±1	8	37±7	8	15±34	8	100±1	8	100±0	8	100±0	4	100±0	4
HI hypothermia	100±0	19	37±9	19	10±22	19	100±1	19	100±1	19	100±0	15	100±0	4
HI rewarming	100±0	4	26±12	4	3±1	4	100±0	4	100±0	4	100±0	4	100±0	4

Continued

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Table. Continued

Parameter/Group	Baseline (Mean±SD)	n	Hypoxia 42 min (Mean±SD)	n	Asphyxia* (Mean±SD)	n	ROSC 1 h (Mean±SD)	n	ROSC 3 h (Mean±SD)	n	ROSC 20 h† (Mean±SD)	n	ROSC 28 h (Mean±SD)	n
Hemoglobin, g/dL														
Sham normothermia	6±0.8	8					7.2±22	8	7.3±1.8	8	8.2±0.7	4	7.5±0.6	4
Sham hypothermia	7.6±1.3	17					8.0±1.8	17	8.6±2.9	17	10.1±2.1	13	10.3±1.4	4
Sham rewarming	8.2±1.0	4					8.2±1.1	4	9.4±1.4	4	10.7±2.0	4	10.1±1.1	4
HI normothermia	7.2±1.3	8					8.2±1.5	8	8.5±1.8	8	7.2±2.3	4	8.1±1.7	4
HI hypothermia	7.1±1.5	19					8.2±1.7	19	9.7±1.9	19	9.9±1.7	15	9.0±2.3	4
HI rewarming	6.5±0.5	4					8.2±1.8	4	9.9±3.6	4	10.0±3.3	4	9.5±2.4	4
Sodium, mEq/dL														
Sham normothermia	149±12	8					141±4¶	8	142±5	8	140±4	4	138±5	4
Sham hypothermia	146±5	17					145±4	17	144±3	17	142±6	13	136±7	4
Sham rewarming	143±3	4					143±1	4	144±1	4	138±4	4	138±4	4
HI normothermia	151±15	8					151±14	8	146±3	8	151±5#	4	150±15	4
HI hypothermia	146±10	19					150±10	19	148±5	19	145±6	15	153±6	4
HI rewarming	153±13	4					149±1	4	146±1	4	141±6	4	142±2	4
Mean arterial blood pressure, mm Hg														
Sham normothermia	72±14	8					83±9	8	84±18	8	74±7	4	84±21	4
Sham hypothermia	82±13	17					75±9	17	84±12	17	79±14	13	85±13	4
Sham rewarming	84±13	4					73±4	4	84±11	4	73±8	4	66±10	4
HI normothermia	80±13	8	64±15	8	41±12	8	60±16**	8	66±12††	8	77±12	4	63±7	4
HI hypothermia	75±19	19	69±11	19	47±10	19	72±17	19	75±14	19	68±17	15	58±14	4
HI rewarming	90±20	4	63±17	4	45±16	4	67±17	4	79±7	4	62±19	4	52±10‡‡	4

"ROSC 1 h" refers to measurements 1 hour after ROSC. Sequential times are indicated. SaO_2, arterial oxyhemoglobin saturation. Normally distributed data were analyzed by 1-way ANOVA with post hoc Holm–Sidak pairwise comparisons, and non-normally distributed data were analyzed by Kruskal–Wallis ANOVA on ranks with post hoc Dunn's pairwise comparisons for data at each time point. HI indicates hypoxia-ischemia; ROSC, return of spontaneous circulation.
*Piglets evaluated by histology received asphyxia for 7 minutes, and we report data at 6-minute asphyxia. Piglets evaluated by western blot received asphyxia for 8 minutes, and we report data at 7-minute asphyxia.
†The temperature before rewarming is presented.
‡The pH at 3 hours after resuscitation differed among groups (P=0.043) without significant post hoc comparisons.
Baseline pH differed among groups (P=0.002). §P<0.05 vs sham normothermia in post hoc pairwise comparisons.
The pH at 20 hours after resuscitation also differed (P=0.013). ‖P<0.05 vs sham normothermia in post hoc comparisons.
¶Sodium levels differed among groups 1 hour after resuscitation (P=0.005) without significant post hoc pairwise comparisons.
Sodium levels were also different 20 hours after resuscitation (P=0.020). #P<0.050 vs sham rewarming in post hoc comparisons.
Mean arterial blood pressure differed 1 (P=0.026), 3 (P=0.018), and 28 hours (P=0.017) after resuscitation. In post hoc comparisons. **P<0.05 vs sham normothermia in post hoc comparisons.
††P<0.05 vs sham hypothermia in post hoc comparisons.
‡‡P<0.05 vs sham hypothermia in post hoc comparisons.

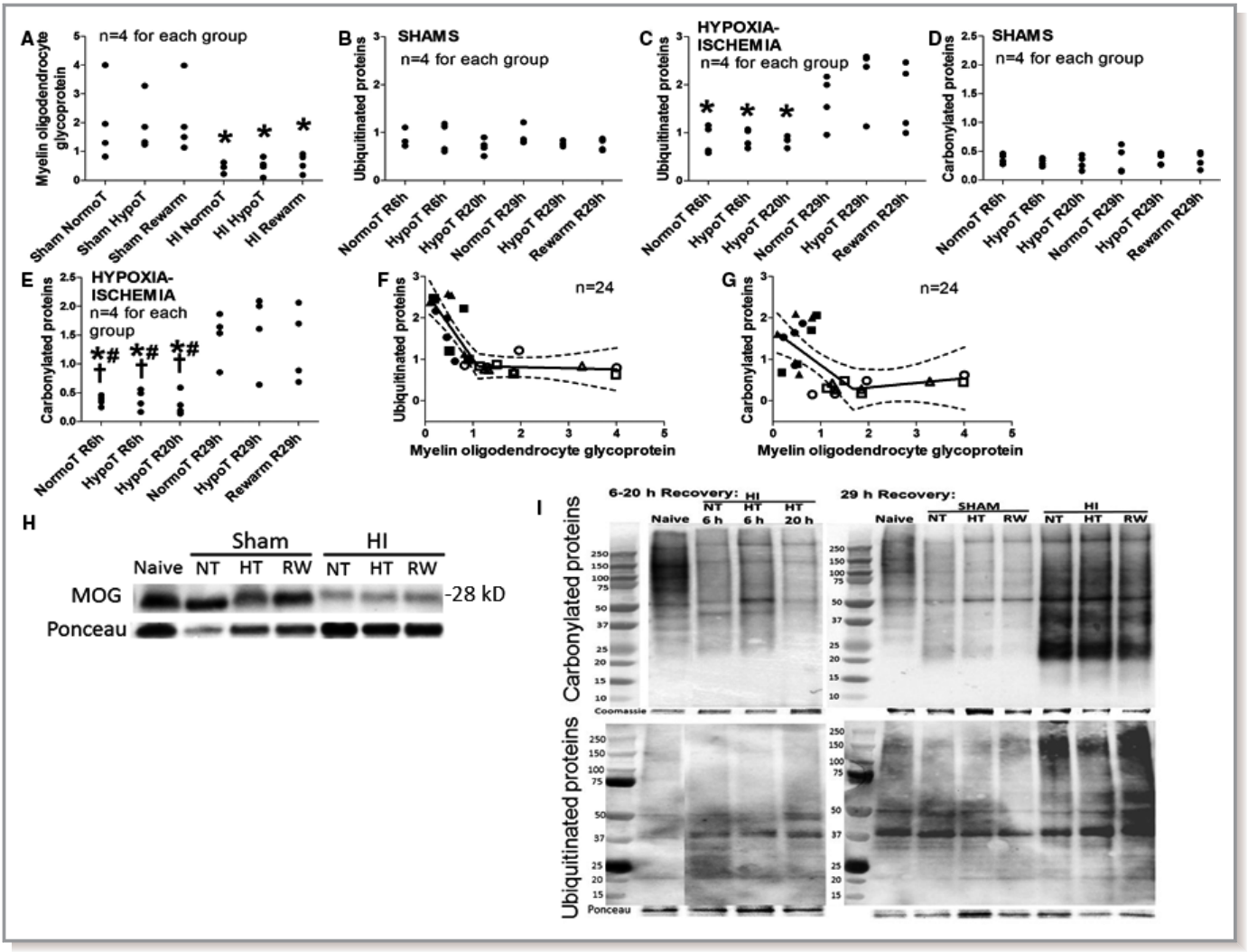

Figure 3. White matter changes in myelination and global protein modification by oxidative damage and ubiquintination after HI. A, Myelin oligodendrocyte glycoprotein (MOG), a component of the myelin sheath, was decreased by hypoxia-ischemia (HI; $P<0.05$) at 29 hours of recovery. In post hoc comparisons, all HI groups had lower MOG levels than each sham group ($^{*}P<0.05$). Hypothermia (hypoT) did not preserve MOG after HI. Each dot represents 1 pig. B and C, Ubiquitinated protein levels were similar among sham groups ($P=0.393$), but differed after HI ($P<0.05$). *$P<0.05$ vs HI+hypoT at 29 hours of recovery (R) in post hoc comparisons. D and E, Carbonylated protein levels were similar among sham groups ($P=0.944$), but differed after HI ($P<0.05$). *$P<0.05$ vs HI+normothermia (normoT) at 29 hours; $^{\#}P<0.05$ vs HI+hypothermia at 29 hours; $^{\dagger}P<0.05$ vs HI+rewarming at 29 hours. F, In segmented linear regression, ubiquitinated protein levels increased as MOG decreased when MOG was below the break point 1.104 (β=−1.637; 95% confidence interval [CI], −2.218, −1.056; $P<0.05$) at 29 hours of recovery. When MOG exceeded this break point, the change in ubiquitin relative to the change in MOG was not significant (β=−0.025; 95% CI, −0.252, 0.202; $P>0.05$). G, Carbonylated protein levels also increased as MOG decreased when MOG was below the break point 1.676 (β=−0.850; 95% CI, −1.381, −0.319; $P<0.05$) at 29 hours of recovery. MOG and carbonylated protein levels were not related above this break point (β=0.110; 95% CI, −0.302, 0.523; $P>0.05$). For panels F and G: circle=normothermia, triangle=hypothermia, square=rewarming, open symbol=sham, and solid symbol=HI. H and I, Representative MOG, oxyblot, and ubiquitinated protein western blots with Ponceau and Coomassie loading controls. Densities from all proteins of various molecular weights were analyzed for the oxyblot and ubiquitinated protein western blot. HT indicates hypothermia; NT, normothermia; RW, hypothermia+rewarming.

Moreover, protein carbonylation 29 hours after HI, hypothermia, and rewarming exceeded that of HI+normothermia at 6 hours, HI+hypothermia at 6 hours, and HI+hypothermia at 20 hours ($P<0.05$ for all).

When segmented linear regression was used to compare MOG to ubiquitinated protein levels, the best fit model had a breakpoint in MOG at 1.104. (Because the western blot data were normalized to immunoreactivity of naïve unanesthetized piglets, a value of 1 approximates that of the naïve group.) Ubiquitinated protein levels increased as MOG decreased when MOG was below 1.104 (β=−1.637; 95% CI, −2.218, −1.056; $P<0.05$; Figure 3F). MOG and ubiquitin levels were not related when MOG exceeded this breakpoint (β=−0.025; 95% CI, −0.252, 0.202; $P>0.05$).

Segmented linear regression also showed that carbonylated protein levels increased as MOG decreased when MOG was below 1.676 (β=−0.850; 95% CI, −1.381, −0.319; $P<0.05$; Figure 3G). MOG and carbonylated protein levels were not related above this break point (β=0.110; 95% CI, −0.302, 0.523; $P>0.05$). Based on these findings, we measured a proteasome protein to explore whether impaired protein clearance parallels the accumulation of ubiquitinated and carbonylated proteins after HI.

The Anesthetic Did Not Affect Glial P20S Immunoreactivity and Localization

We used P20S immunoreactivity as a proteasome marker. In white matter, P20S was mostly localized in the nucleus of macroglia (oligodendrocytes and astrocytes, Figures 2A, B), consistent with previous observations in mammalian cells.[39] The numbers of P20S-negative, -trace, -enriched, and highly enriched glia did not statistically differ in naïve unanesthetized and normothermic sham piglets at 29 hours in the motor and somatosensory subcortical white matter and in the internal capsule ($P>0.05$ for all comparisons; data not shown).

HI and Hypothermia Increased Proteasome Immunoreactivity at 20 Hours

Among groups that recovered for 20 hours with hypothermia, HI increased the number of P20S highly enriched glia to exceed that of shams in motor and somatosensory subcortical white matter and in the internal capsule ($P<0.05$ for all comparisons; Figure 4). HI also decreased the number of trace-positive glia to below that of shams in motor subcortical white matter ($P<0.05$), but not in the somatosensory subcortical white matter or internal capsule. P20S-negative and -enriched glia counts did not statistically differ in any region.

Sham Hypothermia+Rewarming Upregulated Proteasomes, Whereas Sustained Hypothermia Suppressed Proteasomes at 29 Hours

IHC in all white matter regions showed that rewarming increased P20S glial enrichment to above that of normothermia or sustained hypothermia at 29 hours in shams (no HI). Hypothermia for 29 hours decreased the number of P20S-enriched glia to below that at 20 hours in somatosensory subcortical white matter and internal capsule. More specifically, the number of P20S-enriched glia differed in the motor subcortical white matter ($P<0.05$; Figure 5A). In post hoc comparisons, rewarmed sham piglets had more P20S-enriched glia than did normothermic ($P<0.05$) and hypothermic ($P<0.05$) shams at 29 hours. P20S-enriched glia counts also differed in the somatosensory subcortical white matter ($P<0.05$; Figure 5B). Rewarming increased the number of P20S-enriched glia to exceed that of normothermia ($P<0.05$) and hypothermia ($P<0.05$) groups at 29 hours. Hypothermia at 29 hours suppressed P20S immunoreactivity to below that at 20 hours ($P<0.05$). Finally, P20S enrichment differed in the internal capsule ($P<0.05$; Figure 5C). Rewarming increased the number of P20S-enriched glia to above that of the normothermic group ($P<0.05$). Piglets that were hypothermic at 20 hours had more P20S-enriched glia than did normothermic ($P<0.05$) or hypothermic ($P<0.05$) piglets at 29 hours.

The number of P20S-negative glia in somatosensory subcortical white matter differed among sham groups ($P<0.05$; Figure 6A) without significant post hoc comparisons. P20S-negative glia counts were not statistically different in motor subcortical white matter and internal capsule. The number of P20S trace-positive or highly enriched glia did not statistically differ in any region. Total glial counts (sum of P20S-negative, -trace positive, -enriched, and highly enriched macroglia) were not different in the motor and somatosensory subcortical white matter and in the internal capsule among sham groups.

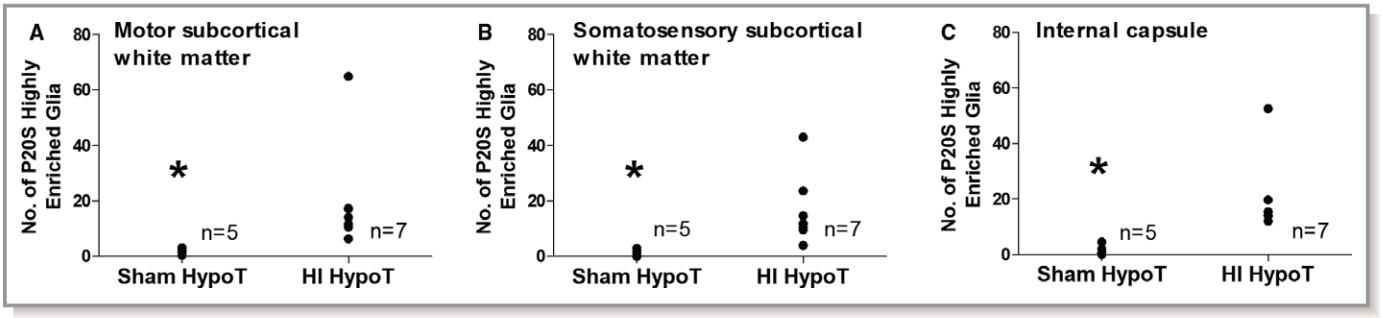

Figure 4. At 20 hours of hypothermia (hypoT), hypoxia-ischemia (HI) increased the number of P20S highly enriched glia in the motor (A, *$P<0.05$) and somatosensory (B, *$P<0.05$) subcortical white matter and in the internal capsule (C, *$P<0.05$) to exceed that of shams. P20S indicates proteasome 20S. See figure 2 for examples of P20S localization.

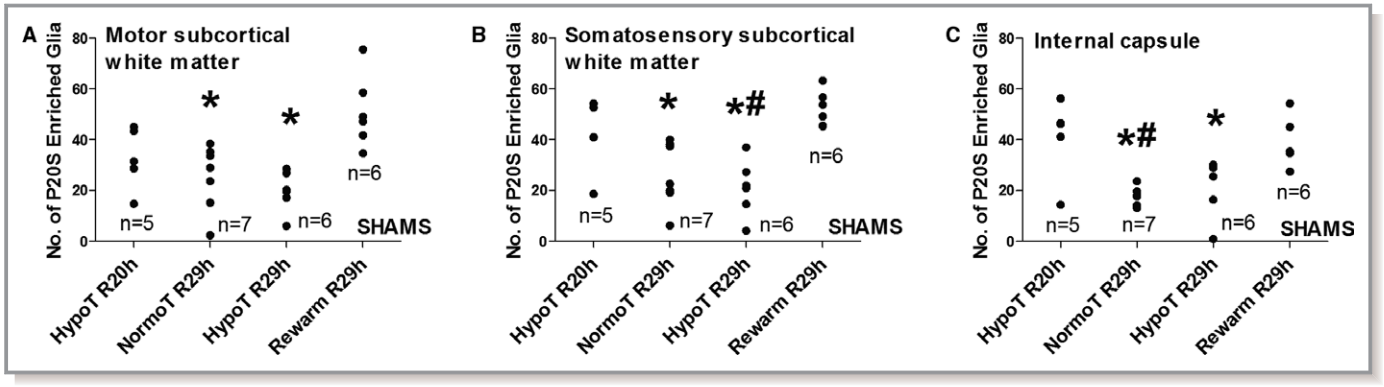

Figure 5. The number of proteasome 20S (P20S)-enriched glia in sham piglets. A, In motor subcortical white matter, the number of P20S-enriched glia differed ($P<0.05$) among groups. *$P<0.05$ vs rewarming in post hoc comparisons. B, The number of P20S-enriched glia also differed among groups in the somatosensory subcortical white matter ($P<0.05$). *$P<0.05$ vs rewarming; #$P<0.05$ vs hypothermia (hypoT) at 20 hours of recovery (R). C, Differences in the number of P20S-enriched glia were also observed in the internal capsule ($P<0.05$). *$P<0.05$ vs rewarming; #$P<0.05$ vs hypoT at 20 hours. NormoT indicates normothermia.

HI Suppressed Macroglial Proteasomes at 29 Hours

HI and hypothermia at 20 hours produced more P20S highly enriched glia than did HI+normothermia, HI+hypothermia, and HI+rewarming at 29 hours in all white matter regions (Figure 7). Specifically, high glial P20S enrichment differed in motor and somatosensory subcortical white matter and in the internal capsule ($P<0.05$ for all). HI+normothermia, HI+hypothermia, and HI+rewarming at 29 hours depressed P20S immunoreactivity to below that of HI+hypothermia at 20 hours in all regions ($P<0.05$ for all post hoc comparisons).

The number of trace-positive macroglia differed in the internal capsule after HI ($P<0.05$) without significant post hoc comparisons (Figure 6B). As with P20S-negative and -enriched glia counts in the internal capsule, the number of P20S-negative, -trace, and -enriched glia did not statistically differ in the motor and somatosensory subcortical white matter. No differences were observed in the total number of glia in the motor and somatosensory subcortical white matter and in the internal capsule among HI groups.

HI and Temperature Interactively Affect Subcortical White Matter Proteasomes, and Temperature Independently Affects Internal Capsule Proteasomes

HI and temperature interacted in their effects on subcortical white matter P20S immunoreactivity at 29 hours. In motor subcortical white matter, HI and temperature interactively affected the number of P20S-enriched glia ($P<0.05$) without independent effects (Figure 8A). Rewarming in the sham

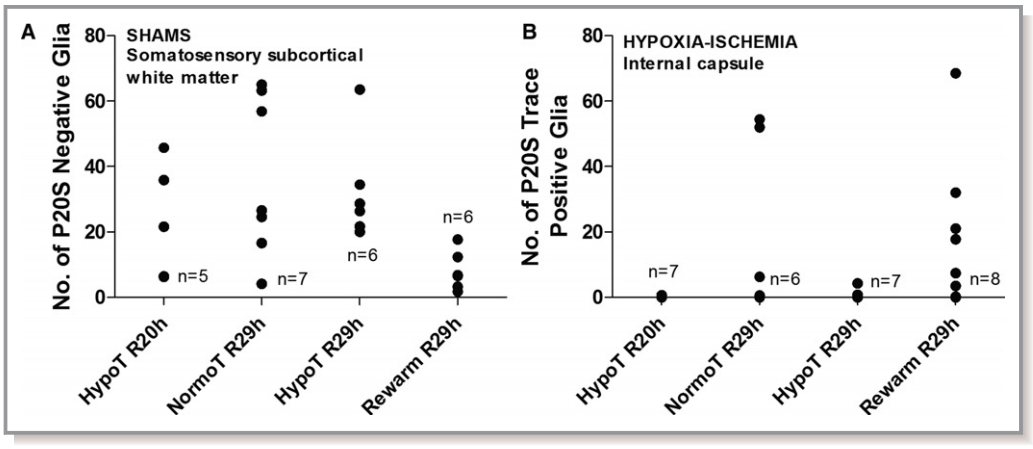

Figure 6. Counts of proteasome 20S (P20S)-negative and trace-positive glia. A, The number of P20S-negative glia differed among sham groups in somatosensory subcortical white matter ($P<0.05$). Post hoc pairwise comparisons were not statistically significant. B, The number of P20S trace-positive glia in the internal capsule differed among groups after hypoxia-ischemia ($P<0.05$) without significant post hoc comparisons. HypoT indicates hypothermia; NormoT, normothermia.

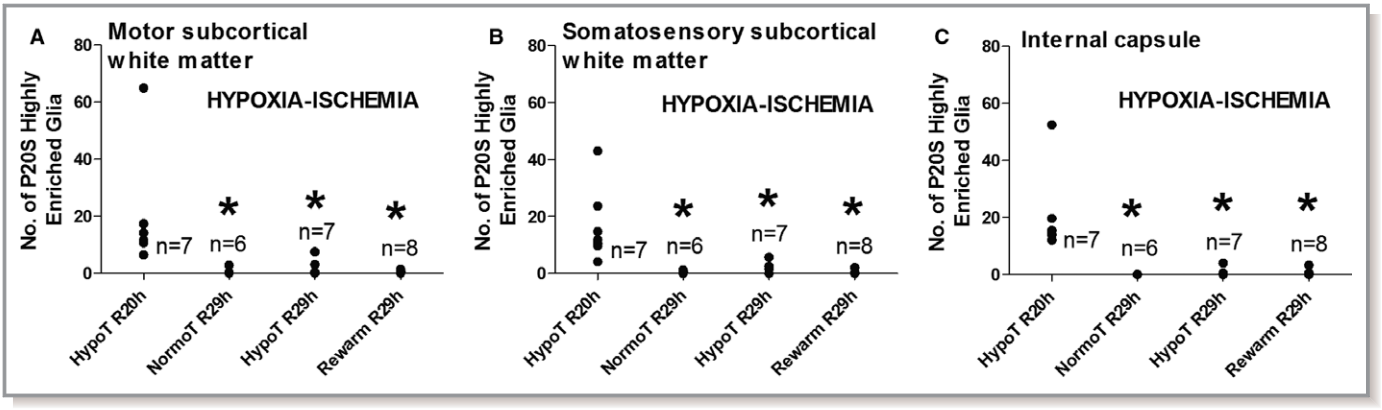

Figure 7. At 29 hours of recovery (R) after HI, the numbers of proteasome 20S (P20S) highly enriched glia were depressed in motor (A, $P<0.05$) and somatosensory (B, $P<0.05$) subcortical white matter and in the internal capsule (C, $P<0.05$) during normothermia (normoT), hypothermia (hypoT), and hypothermia+rewarming. *$P<0.05$ vs HI+hypothermia at 20 hours in post hoc comparisons. HI indicates hypoxia-ischemia.

group increased the number of P20S-enriched glia to exceed that of sham+normothermia ($P<0.05$) and sham+hypothermia ($P<0.05$) in post hoc comparisons. In contrast, HI and rewarming decreased P20S enrichment to below that of sham+rewarming ($P<0.05$).

HI and temperature also interactively affected macroglial P20S enrichment in somatosensory subcortical white matter ($P<0.05$) without independent effects (Figure 8B). Rewarmed shams had more P20S-enriched glia than did normothermic ($P<0.05$) or hypothermic ($P<0.05$) shams. HI and rewarming again decreased the number of P20S-enriched glia to below that of rewarmed shams ($P<0.05$).

In the internal capsule, a white matter structure that contains corticofugal and corticopetal axons forming corticospinal and corticobulbar tracks and thalamocortical pathways, temperature independently affected the number of P20S-enriched glia ($P<0.05$) whereas HI did not (Figure 8C). Temperature and HI did not interact. Sustained hypothermia and rewarming both increased P20S glial enrichment to above that of normothermia ($P<0.05$ for both).

The number of trace-positive glia, an indicator of low proteasome immunoreactivity, corroborated the P20S enrichment findings. In the motor subcortical white matter, HI and temperature interactively affected P20S trace glia counts ($P<0.05$) without independent effects (Figure 8D). After HI, rewarming increased the number of trace-positive glia to above that of hypothermia ($P<0.05$). Piglets in the HI+rewarming group had more P20S trace glia than those in the sham+rewarming group ($P<0.05$). (Cell counts for P20S trace glia without logarithmic transformation are in Table S1.)

HI and temperature also interactively affected the number of trace-positive glia in the somatosensory subcortical white matter ($P<0.05$) without independent effects (Figure 8E). Rewarmed HI piglets had more trace-positive glia than did rewarmed shams ($P<0.05$).

In the internal capsule, the interaction between HI and temperature was significant ($P<0.05$), but HI and temperature did not independently affect trace P20S glia counts (Figure 8F). As in the subcortical white matter, post-HI rewarming increased the number of P20S trace-positive glia to above that of the HI+hypothermia group ($P<0.05$) and the sham+rewarming group ($P<0.05$).

HI and temperature interactively affected the number of P20S-negative glia in the somatosensory subcortical white matter ($P<0.05$) without independent effects. Rewarming in the sham group decreased the negative glia count to below that of sham+normothermia and HI+rewarming ($P<0.05$ for both). The number of negative glia did not statistically differ in the motor subcortical white matter or internal capsule by 2-way ANOVA. The number of P20S highly enriched glia also did not statistically differ in all regions by 2-way ANOVA.

Confirmation of P20S Localization Changes in White Matter by Immunoblotting

Western blot analysis of somatosensory subcortical white matter confirmed our IHC findings after HI and rewarming. P20S levels differed after HI and 6, 20, or 29 hours of recovery ($P<0.05$; Figure 9). HI and rewarming at 29 hours suppressed P20S levels to below that of HI and hypothermia at 20 hours ($P<0.05$). We did not observe statistical differences in P20S levels among sham groups.

Putamen Neuronal Injury Did Not Influence Macroglial Proteasome Levels in Subcortical and Capsular White Matter

After HI, the percentage of viable putamen neurons relative to the mean of normothermic shams was 65% (SD: 19) after normothermia, 100% (SD: 19) after hypothermia, and

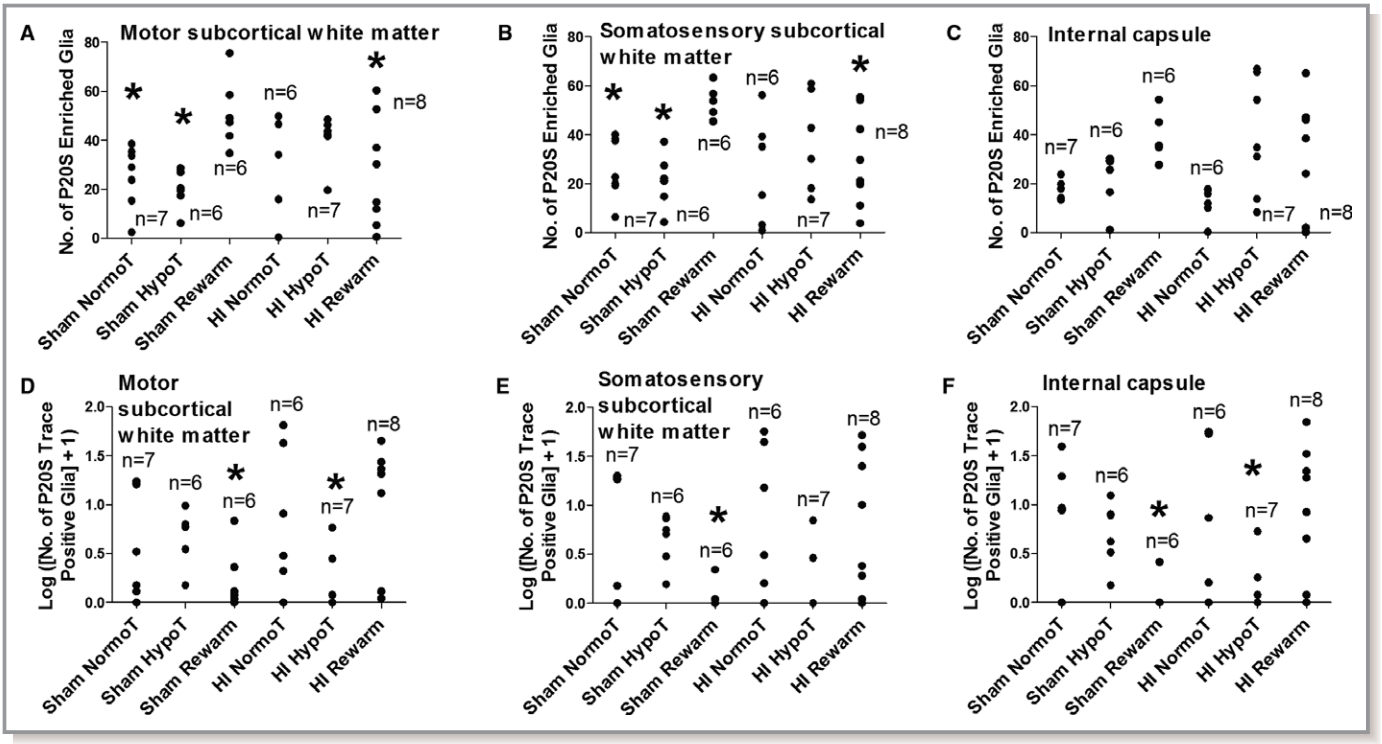

Figure 8. A through C, The number of proteasome 20S (P20S)-enriched glia differed among groups at 29 hours by 2-way ANOVA. HI and temperature interactively affected P20S glial enrichment in motor (A, $P<0.05$) and somatosensory (B, $P<0.05$) subcortical white matter. *$P<0.05$ vs sham rewarming in post hoc comparisons. In the internal capsule, temperature independently affected P20S enrichment (C, $P<0.05$). Sustained hypothermia (hypoT; $P<0.05$) and hypothermia+rewarming ($P<0.05$) both increased P20S enrichment to exceed that of normothermia (normoT). D through F, The number of P20S trace-positive glia, an indicator of low proteasome expression, corroborated the P20S enrichment findings. HI and temperature interacted in their effects on the number of trace-positive glia in motor (D, $P<0.05$) and somatosensory (E, $P<0.05$) subcortical white matter and in the internal capsule (F, $P<0.05$). *$P<0.05$ vs HI rewarming. HI indicates hypoxia-ischemia.

95% (SD: 17) after hypothermia+rewarming. The number of putamen viable neurons was associated with temperature ($P<0.05$). In post hoc comparisons, sustained hypothermia ($P<0.05$) and hypothermia with rewarming ($P<0.05$) both increased the viable neuron count to exceed that of normothermia after HI. The numbers of P20S-enriched glia and viable neurons were moderately correlated in the internal capsule ($r=0.495$; $P<0.05$), but not in subcortical white matter ($r\leq0.420$; $P>0.05$ for motor and subcortical). Temperature, viable neuron count, and their interaction did not affect the number of P20S-enriched glia in any region ($P>0.05$ for all comparisons).

OLE Protected the Myelin, and Proteasome Genetic Knockdown Blocked OLE-Mediated Protection

OLE increased P20S and MOG levels in the subcortical white matter after HI, hypothermia, and rewarming ($P<0.05$ for both; Figure 10), thereby confirming the proteasome activator activity of OLE. We then tested whether the mechanism of OLE's myelin protection after HI involves the proteasome. Median P20S expression on western blot was 2.6 (range, 2.4–3.7; n=4) and 3.6 (range, 2.1–5.1; n=2) for pigs that received only OLE or OLE and AAV-GFP, respectively. We combined these 6 piglets into 1 group to analyze OLE without proteasome knockdown. Five other piglets received OLE and AAV-shRNA to PA28γ.

AAV-shRNA-PA28γ reduced P20S, PA28γ, and MOG levels ($P<0.05$ for each; n=5) in the subcortical white matter to below that of OLE without proteasome knockdown (n=6; Figure 11A through 11C) after HI, hypothermia, and rewarming. Proteasome knockdown by AAV-shRNA-PA28γ also increased protein ubiquitination to exceed that of OLE ($P<0.05$; Figure 11D). Carbonylated protein levels were not affected by AAV-shRNA-PA28γ.

Proteostasis in Cerebral Cortex After HI

Cerebral cortical patterns of protein ubiquitination and carbonylation and P20S expression were distinct from that in the subcortical white matter at baseline. Ubiquitinated protein levels differed among the sham groups ($P<0.05$; Figure 12A). Post hoc comparisons showed that rewarmed sham piglets at 29 hours had more ubiquitinated proteins in somatosensory cortex than did shams that received normothermia or hypothermia for 6 hours ($P<0.05$ for both). In

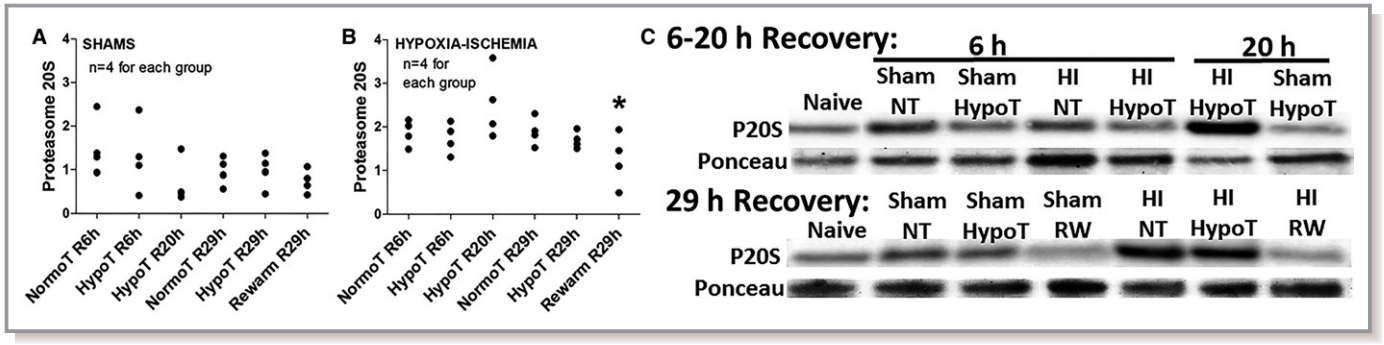

Figure 9. Proteasome 20S (P20S) western blots of somatosensory subcortical white matter. A, P20S levels were similar among sham groups with normothermia (NormoT) and hypothermia (HypoT) at 6, 20, and 29 hours (h) and hypoT with rewarming. Each dot represents 1 pig. B, P20S levels differed among hypoxia-ischemia (HI) groups ($P<0.05$). *$P<0.05$ vs HI+hypothermia at 20 hours of recovery (R). C, Representative western blots of sham and HI pig subcortical white matter showing P20S levels and corresponding lane protein loading in Ponceau S–stained membranes. HypoT indicates hypothermia; NT, normothermia; RW, hypothermia+rewarming.

contrast, subcortical white matter ubiquitination did not differ among the sham groups at 6 to 29 hours (Figure 3B).

After HI, cerebral cortical ubiquitinated protein levels also differed ($P<0.05$) with more ubiquitination at 20 hours of hypothermia than at 6 hours of normothermia, 6 hours of hypothermia, or 29 hours of hypothermia in post hoc comparisons ($P<0.05$ for all; Figure 12B). In contrast, protein ubiquitination in HI subcortical white matter through 20 hours remained close to the sham baseline and then increased at 29 hours of HI+hypothermia (Figure 3C).

Carbonylated protein levels in cortex were not statistically different among sham groups, but differed after HI ($P<0.05$; Figure 12C and 12D). In post hoc comparisons, only HI+rewarming at 29 hours increased protein carbonylation to exceed that of HI+hypothermia at 20 hours ($P<0.05$). White matter, by contrast, had greater protein carbonylation at 29 hours of normothermia, hypothermia, and hypothermia+rewarming compared with the 6 to 20 hours recovery period after HI (Figure 3E).

P20S levels differed after HI ($P<0.05$), but not after sham procedure (Figure 12E and 12F). HI+hypothermia at 29 hours reduced cortical P20S expression to below that of HI+hypothermia at 20 hours ($P<0.05$) in post hoc comparisons.

White Matter and Cerebral Cortical Proteasome Enzymatic Activity Levels Differ

We established that the assay was valid because it effectively distinguished proteasome activity in pig brain homogenate in the presence of the proteasome inhibitor, marizomib (Figure 13A). When the white matter and cortex were analyzed separately, treatment group and assay reaction time interactively affected proteasome activity in the subcortical white matter and cortex ($P<0.05$ for both; Figure 13B and 13C). Activity levels between treatment groups did not differ at any assay time point in post hoc comparisons.

We then analyzed the piglets that received HI and 20 hours of hypothermia. There was no statistical difference between the white matter or cortex and no interaction between brain region and assay reaction time on proteasome activity level (Figure 13D).

Among pigs that received 29 hours of hypothermia after HI, brain region and assay reaction time interactively affected proteasome activity ($P<0.05$). Mean white matter proteasome activity levels were lower than cortical levels, and white matter activity lagged behind that in cortex during the initial reaction (Figure 13E).

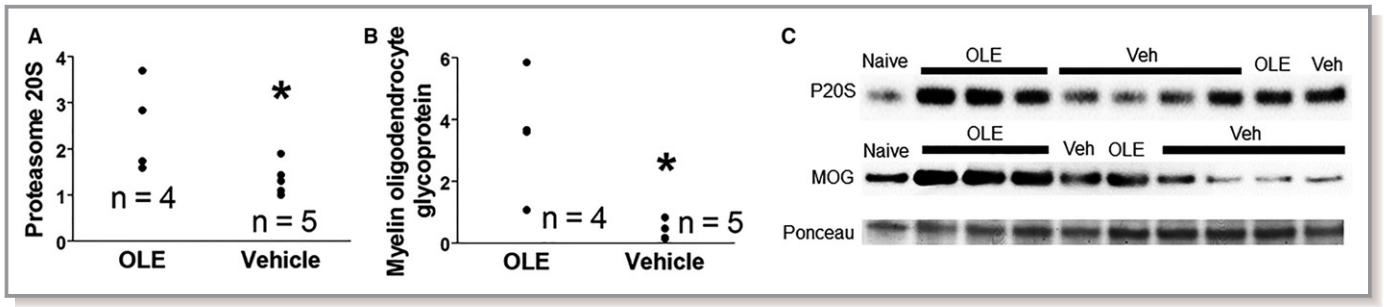

Figure 10. Western blots of subcortical white matter of piglets that received intravenous oleuropein (OLE) or vehicle (veh) after hypoxia-ischemia, hypothermia, and rewarming. A and B, OLE increased proteasome 20S (P20S) and myelin oligodendrocyte glycoprotein (MOG) levels (*$P<0.05$). C, Representative western blots show P20S and MOG levels with Ponceau S staining of the protein loading.

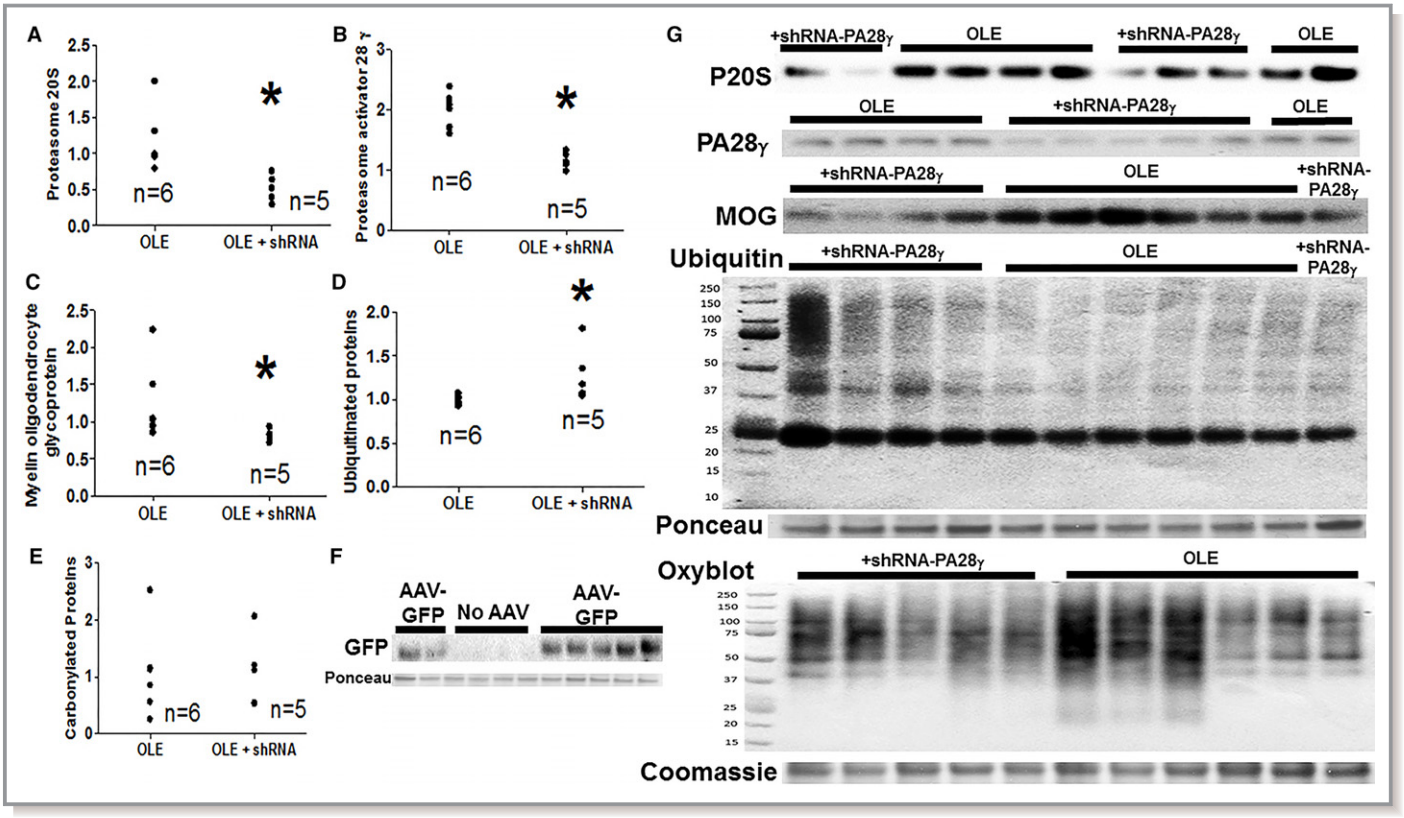

Figure 11. Western blots of subcortical white matter of piglets that received hypoxia-ischemia, hypothermia, and rewarming. Six piglets received proteasome activation by oleuropein (OLE) without proteasome knockdown, including 2 that received adeno-associated virus (AAV) with green fluorescent protein (GFP). Five piglets had AAV with short hairpin RNA (shRNA) to proteasome activator 28γ (PA28γ) in addition to oleuropein (OLE+shRNA). A and B, AAV-shRNA to PA28γ decreased proteasome 20S (P20S; **P*<0.05) and PA28γ (*P*<0.05) levels to below that of OLE. C, AAV-shRNA to PA28γ reduced myelin oligodendrocyte glycoprotein (MOG) levels to below that of OLE (**P*<0.05). D, AAV-shRNA to PA28γ increased protein ubiquitination to exceed that of OLE (**P*<0.05). E, Carbonylated protein levels did not differ between groups. F, Verification of white matter gene transduction with AAV-GFP. G, Representative western blots of subcortical white matter showing the levels of P20S, PA28γ, MOG, ubiquintinated proteins, and carbonylated proteins (Oxyblot) and the corresponding lane protein loading as seen by Ponceau S and Coomassie staining.

Discussion

Our study identified novel components of white matter injury that occur after HI despite the use of hypothermia. We found that white matter injury 1 day after HI is related to proteasome insufficiency and failure to clear carbonylated and ubiquitinated proteins. Hypothermia did not prevent myelin injury or preserve the proteasome. Accumulation of damaged proteins in the white matter contrasted with the low ubiquitinated and carbonylated levels found in cerebral cortex 1 day after HI and hypothermia. This may be related to the lag in proteasome enzyme activity that occurred in white matter, but not in cortex. Proteasome activation by OLE protected the myelin, and proteasome genetic knockdown blocked OLE's protection and, accordingly, ubiquitinated protein levels increased and MOG loss was exacerbated. Thus, the white matter protection afforded by OLE depends upon the proteasome. Proteasome insufficiency could be a novel mechanism for white matter injury after HI and hypothermia.

Damaged Proteins Accumulate in Injured White Matter After HI and Hypothermia

HI caused loss of MOG regardless of temperature treatment. Hypothermia did not prevent myelin loss 29 hours after HI. It is possible that this inefficacy is related to the timing of the hypothermia. We delayed the start of hypothermia induction by 2 hours to mimic clinical delays in cooling. Additionally, we limited hypothermia to 27 hours in our protocol, rather than the 72 hours used clinically, because of the potential for anesthesia toxicity (unpublished data; J.K.L., M.D., 2013). However, 72 hours of hypothermia in unanesthetized fetal sheep after HI also failed to fully protect white matter when hypothermia induction was delayed.[40,41]

Decreases in MOG were associated with increases in carbonylated and ubiquitinated protein levels. These post-translational protein modifications could partially reflect the accumulation of abnormal myelin proteins 1 day after HI. The decrease in MOG may also reflect a decline in protein synthesis

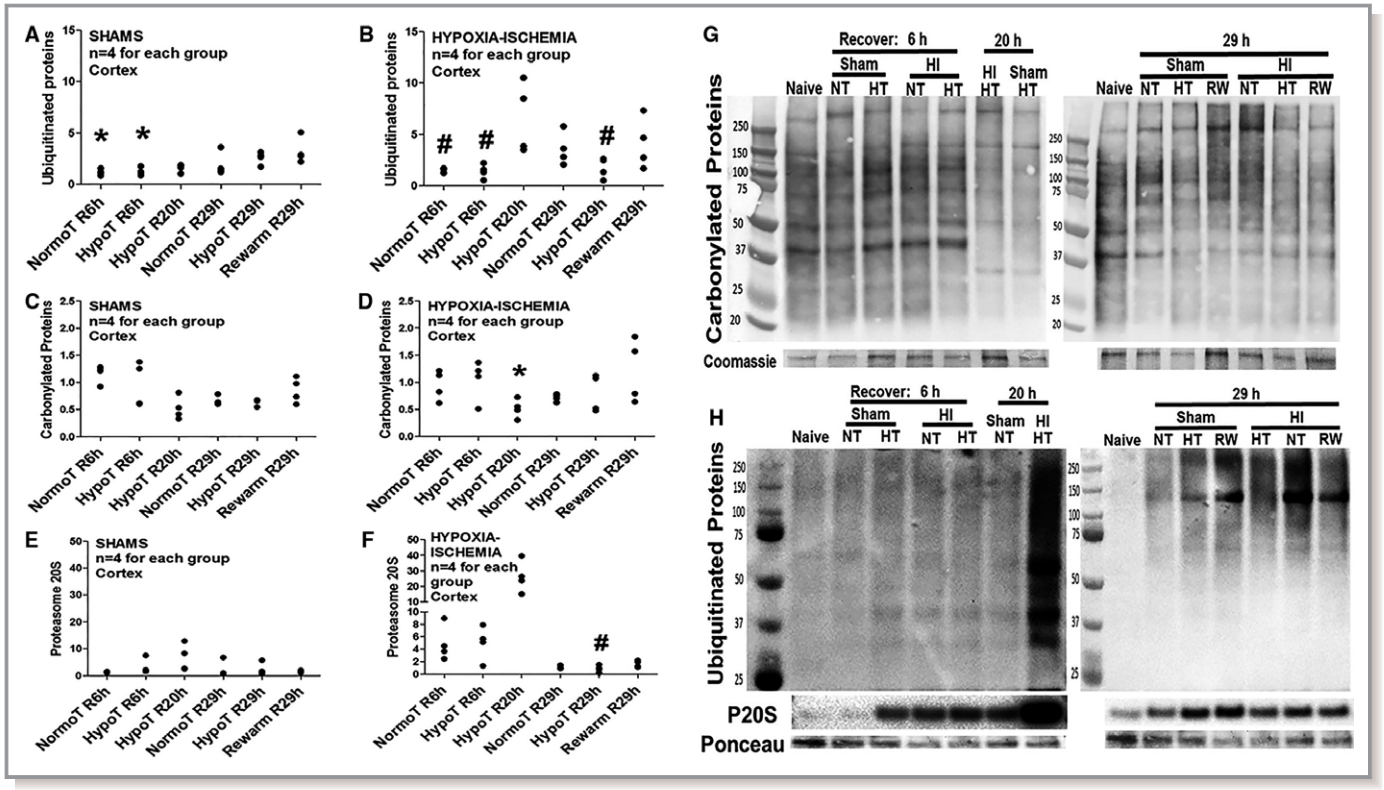

Figure 12. Western blots of primary somatosensory cortex in pigs with normothermia (NormoT) and hypothermia (HypoT) at 6, 20, and 29 hours (h) and hypoT with rewarming. A and B, Ubiquitinated protein levels differed among sham ($P<0.05$) and hypoxia-ischemia (HI; $P<0.05$) groups. $^{*}P<0.05$ vs sham rewarming at 29 hours of recovery (R); $^{\#}P<0.05$ vs HI hypothermia at 20 hours in post hoc comparisons. Each dot represents 1 pig. C and D, Carbonylated protein levels did not differ among shams ($P>0.05$), but did among HI groups ($P<0.05$). $^{*}P<0.05$ vs HI rewarming at 29 hours of recovery in post hoc comparisons. E and F, Proteasome 20S (P20S) levels were similar among shams ($P>0.05$) and differed after HI ($P<0.05$). $^{\#}P<0.05$ vs HI hypothermia at 20 hours in post hoc comparisons. G and H, Representative western blots of carbonyl- and ubiquitin-modified proteins and P20S with the corresponding protein loading as shown by Coomassie and Ponceau S staining. HT indicates hypothermia; NT, normothermia; RW, hypothermia+rewarming.

or myelin damage that is coupled to the accumulation of damaged proteins. Oxidative stress, protein misfolding, and inflammation[42] after HI induce protein oxidation and ubiquitination, the accumulation of which can trigger cell death.[22–24] Because most proteasomal proteinase activity is also ATP dependent, accumulation of damaged proteins could be related to delayed secondary failure in white matter bioenergetics. Indeed, we observed a lag in white matter proteasome enzyme activity at 29 hours of hypothermia after HI. Though multiple nonproteasome systems clear oxidized proteins, including lysosomes,[23] our data indicate that proteasome insufficiency plays a critical role in neonatal white matter injury after HI.

Proteasome Insufficiency Did Not Occur in Cerebral Cortex

In contrast to white matter, ubiquitinated protein levels declined in the cortex 29 hours after HI during hypothermia. Although P20S decreased in both cortex and white matter 29 hours after HI, higher cortical proteasome activity might improve damaged protein clearance in cerebral cortex to exceed that in white matter. We previously found that hypothermia's protective efficacy differs between white and gray matter in HI piglets. Hypothermia preserved cortical neurons after HI[31] whereas white matter apoptosis increased after HI and hypothermia.[18] This may be partially explained by the differential proteasome function in cortex and white matter after HI that we identified here. Oxidative stress also differs between gray and white matter in multiple sclerosis.[43] Further studies are needed on the varying effects of HI and hypothermia on gray and white matter in the developing brain.

OLE Protected the White Matter and Proteasome Knockdown Blocked OLE-Mediated Protection

OLE increased P20S and MOG levels to exceed that in the vehicle group after HI, hypothermia, and rewarming. We verified the proteasome's role in OLE's white matter protection using conditional gene expression knockdown of the proteasome with AAV encoding shRNA to PA28γ, a predominant proteasome subunit in the brain.[44] Proteasome knockdown promoted myelin sheath injury with loss of MOG and

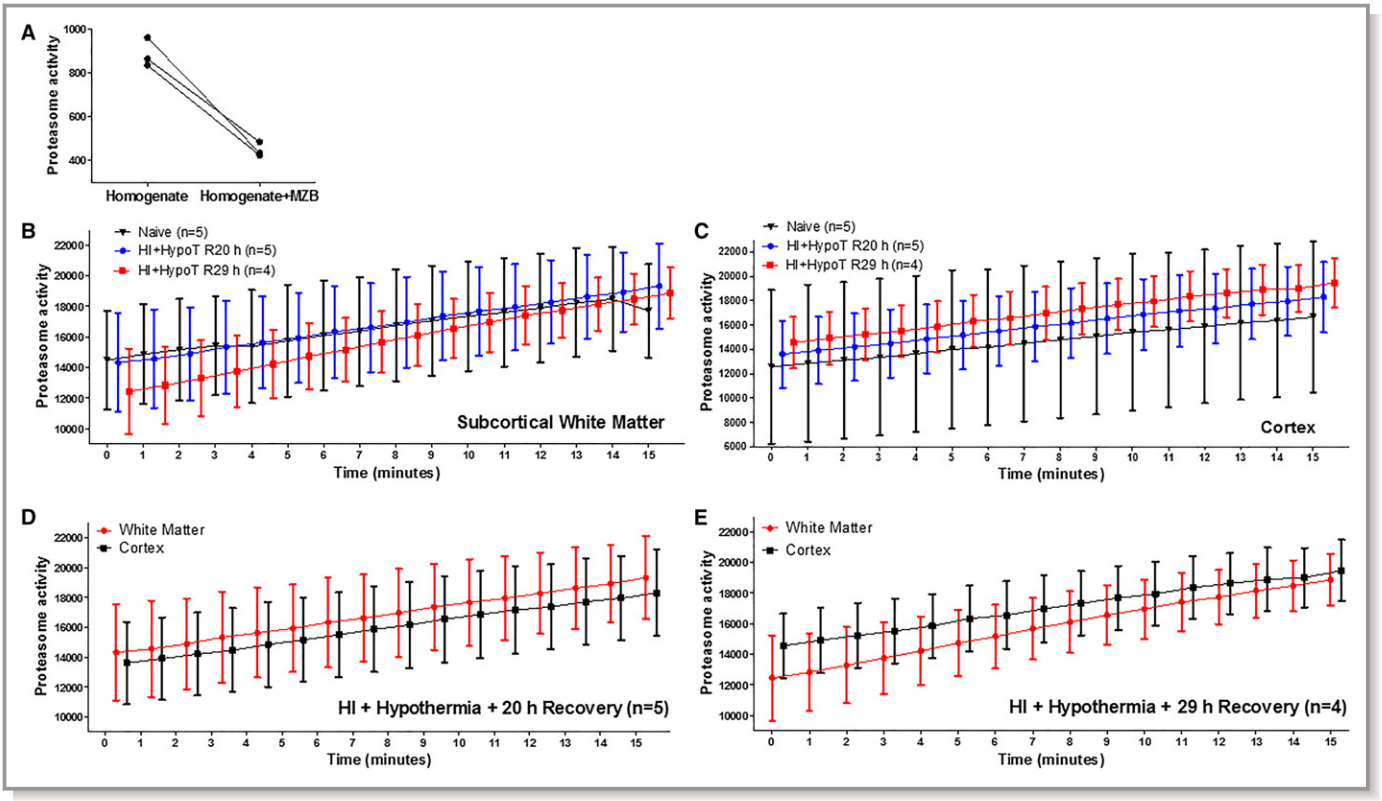

Figure 13. Proteasome enzyme activity in sensorimotor subcortical white matter and sensorimtor cortex. A, We verified that the assay detects a change in piglet proteasome activity by adding marizomib (MZB), a proteasome inhibitor, to brain homogenate. B and C, The treatment group and assay time interactively affected proteasome activity in the white matter ($P<0.05$) and cortex ($P<0.05$). D, At 20 hours (h) of HI+hypothermia, brain region ($P>0.05$) and interaction between brain region and assay reaction time ($P>0.05$) did not affect proteasome activity level. E, At 29 hours of HI+hypothermia, brain region and assay reaction time interactively affected proteasome activity level ($P<0.05$). HI indicates hypoxia-ischemia; HypoT, hypothermia.

exacerbated ubiquitinated protein accumulation. We study OLE as a potential therapeutic for HI because it is a proteasome activator[30] that had inconsequential side effects in clinical trials for cardiovascular disease.[45–47] However, OLE has other mechanisms of action, including modulation of oxidative stress[48] and autophagy.[49] Our findings in this pilot study indicate that OLE has potential to improve myelin protection, as indicated by OLE's preservation of MOG after HI, hypothermia, and rewarming. Selective proteasome knockdown blocked OLE's ability to protect white matter. Although the roles of OLE-mediated modulation of oxidative stress and autophagy need to be explored as well, our data indicate that proteasome activation is a key mechanism of OLE-mediated white matter protection.

White Matter Proteasome Insufficiency Develops Between 20 and 29 Hours of Hypothermia After HI

To study the cellular localization of the proteasome in piglet white matter, we developed a 4-tier, histological classification system to characterize the range of macroglial P20S localization. The number of P20S-enriched glia was corroborated by P20S-negative or trace-positive glia counts because shifts were observed in frequency distributions. P20S enrichment was highest at 20 hours of hypothermia, with the greatest enrichment occurring after HI. Carbonylated and ubiquitinated proteins levels remained low at 6 and 20 hours of hypothermia, suggesting normal proteasome degradation of these proteins in response to HI-induced oxidative stress. Accordingly, proteasome activity at 20 hours of HI+hypothermia did not vary across the proteasome activity assay reaction time, which indicates rapid proteasome docking and degradation of the target substrate.

Then, proteasome insufficiency manifested 29 hours after HI with a significant decline in the number of P20S highly enriched glia. We observed this proteasomal decline even in the presence of hypothermia. This change occurred in the absence of major changes in total number of white matter macroglial cells after HI. Though some oligodendrocytes undergo apoptosis, our model exhibits no evidence of white matter necrosis.[18] Thus, the P20S highly enriched glia did not appear to be dying off. Moreover, cytoplasmic-to-nuclear transport appeared to be

intact because P20S immunoreactivity did not accumulate in the cytoplasm. This white matter proteasome suppression corresponded with significant accumulation of carbonylated and ubiquitinated proteins. Moreover, white matter proteasome activity levels lagged behind that in cortex at 29 hours of hypothermia. This suggests a slowed proteasome enzymatic start with potential impairments in proteasome recognition and docking of target proteins in the white matter.

HI and Temperature Interactively Affect White Matter Proteasomes

Rewarming in the sham group increased the number of P20S-enriched glia, but HI suppressed rewarming-induced proteasome upregulation. This interaction was regional because sustained hypothermia and hypothermia with rewarming both independently increased P20S in the internal capsule. We previously reported that hypothermia and hypothermia with rewarming promote white matter apoptosis in sham-operated piglets.[18] Cold-induced apoptosis in liver endothelial cells may involve the proteasome,[50] and proteasome activation occurs early in thymocyte apoptosis.[51] The proteasome's role in hypothermia and rewarming-induced apoptosis requires further investigation in the developing brain.

Western blots of somatosensory subcortical white matter confirmed the histologically detected decrease in P20S after HI and rewarming. In shams, however, we did not observe the P20S differences by western blot that we found by IHC. Our IHC analysis used a 4-tier system of categorizing glial P20S with precise glia counts and identification of P20S-positive foci at high resolution. We excluded neurons, blood-borne cells, and vascular cells. In contrast, western blots of crude homogenates include all of these cell types. It is possible that the difference in P20S levels was too small to be detected in shams by a crude homogenate assay.

White Matter Proteasome Insufficiency Is Independent of HI Injury Severity in Putamen

Damage to the basal ganglia, specifically the putamen, is a reliable hallmark of HIE in piglet as it is in human HIE.[35] We sought to identify possible relationships between individual injury severity and white matter proteasome integrity. We used the number of viable neurons in putamen to estimate individual HI injury severity because the putamen is injured 1 day after HI in our model.[34] We conducted this exploratory analysis because individual variability in the response to HI may affect white matter injury.[52] Because neuron viability and temperature are related, we could not test the independent effect of viable neuron counts on white matter P20S. Nonetheless, we found no interactive effect between the number of viable putamen neurons and temperature on glial P20S. White matter proteasome suppression thus appears to be independent of individual HI injury severity when temperature is taken into account in this cohort of piglets.

Methodological Considerations

We used MOG protein level as a metric for white matter integrity. MOG loss could be attributed to impaired protein synthesis, myelin damage, or a combination. We did not assess other myelin markers or determine whether decreased protein synthesis preceded the accumulation of carbonylated or ubiquitinated proteins and reduction in P20S levels. Decreases in MOG might also reflect oligodendrocyte cell death that was not measured here; however, total glial counts were similar among groups. We focused only on P20S and PA28γ, but the proteasome is a complex structure composed of many subunits. Future studies of other proteasome subunits are needed. Our model had a delayed start of hypothermia induction by 2 hours, but immediate hypothermia or longer delays may affect the proteasomes and protein clearance differently. Last, nonproteasome methods for clearing abnormal proteins, such as by lysosomes, also need to be tested.

Conclusion

Myelin injury is related to proteasomal failure to clear damaged proteins after HI and hypothermia. Proteasome insufficiency with accumulation of ubiquitinated and carbonylated proteins occurs in white matter, but not the cortex, 1 day after HI. Hypothermia does not protect the myelin, preserve the proteasome, or support clearance of damaged proteins in white matter after HI. Proteasome activation by OLE protects the myelin after HI, and proteasome genetic knockdown blocks OLE's white matter protection. The concept of proteasome failure in HI-induced white matter injury and the potential use of proteasome-targeted therapies in HIE are novel and deserve further study.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Table S1. The number of proteasome 20S (P20S) trace-positive glia at 29 hours of recovery.

Brain region	n	Number of P20S trace-positive glia (median, IQR)
Motor subcortical white matter		
Sham normothermia	7	2 (0, 15)
Sham hypothermia	6	5 (3, 5)
Sham rewarming	6	0.3 (0, 1)
HI normothermia	6	5 (1, 33)
HI hypothermia	7	0.2 (0, 1)
HI rewarming	8	16 (0, 23)
Somatosensory subcortical white matter		
Sham normothermia	7	17 (0, 18)
Sham hypothermia	6	4 (3, 6)
Sham rewarming	6	0 (0, 1)
HI normothermia	6	8 (1, 43)
HI hypothermia	7	0 (0, 0)
HI rewarming	8	5 (1, 17)
Internal capsule		
Sham normothermia	7	8 (0, 13)
Sham hypothermia	6	3 (2, 7)
Sham rewarming	6	0 (0, 0)
HI normothermia	6	4 (0, 41)
HI hypothermia	7	0.2 (0, 1)
HI rewarming	8	13 (3, 24)

IQR, interquartile range; HI, hypoxia-ischemia.