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Bax Shuttling After Neonatal Hypoxia–Ischemia: Hyperoxia Effects

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Abstract

Perinatal hypoxia–ischemia (HI) occurs in 0.2%–0.4% of all live births, with 100% O₂ resuscitation (HHI) remaining a standard clinical treatment. HI produces a broad spectrum of neuronal death phenotypes ranging from a more noninflammatory apoptotic death to a more inflammatory necrotic cell death that may be responsible for the broad spectrum of reported dysfunctional outcomes. However, the mechanisms that would account for this phenotypic spectrum of cell death are not fully understood. Here, we provide evidence that Bcl-2-associated X protein (Bax) can shuttle to different subcellular compartments in response to HI, thus triggering the different organelle-associated cell death signaling cascades resulting in cell death phenotype diversity. There was an early increase in intranuclear and total nuclear Bax protein levels followed by a later Bax redistribution to the mitochondria and endoplasmic reticulum (ER). Associated with the organelle-specific Bax shuttling time course, there was an increase in nuclear phosphorylated p53, cytosolic cleaved caspase-3, and caspase-12. When HI-treated P7 rats were resuscitated with 100% O₂ (HHI), there were increased lesion volumes as determined by T2-weighted magnetic resonance imaging with no change in cortical apoptotic signaling compared with HI treatment alone. There was, however, increased inflammatory (cytosolic-cleaved interleukin-1 β) and necrotic (increased nuclear 55-kDa-cleaved PARP-1 [poly-ADP-ribose 1] and decreased nuclear HMGB1 [nuclear high-mobility group box 1]) after HHI. Furthermore, HHI increased ER calpain activation and ER Bax protein levels compared with HI alone. These data suggest that 100% O₂ resuscitation increases Bax-mediated activation of ER cell death signaling, inflammation, and lesion volume by increasing necrotic-like cell death. In light of these findings, the use of 100% O₂ treatment for neonatal HI should be reevaluated.

Keywords

Bax; hypoxia-ischemia; inflammation; necrosis; apoptosis; hyperoxia

Perinatal hypoxic-ischemia (HI) occurs in 0.2%–0.4% of all live births (Odding et al., 2006; Platt et al., 2007), with 100% O₂ resuscitation (hyperoxia, HHI) remaining a standard clinical treatment (Bissinger and Ohning, 2006) despite both clinical and animal model reports that there may be detrimental consequences associated with hyperoxia (Grafe et al., 2008; Smith et al., 2008). HI produces a cell death phenotype spectrum ranging from necrosis to apoptosis (Cole and Perez-Polo, 2004; Edinger and Thompson, 2004; Nakajima et al., 2000). However,

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the mechanisms that explain the broad spectrum of resulting cell death phenotypes are not well understood.

By means of the the Rice-Vannucci model (Vannucci et al., 1988), we have shown that there are significant decreases in the antiapoptotic Bcl-xL and corresponding minor increases in total cellular Bax protein levels after HI or hypoxia alone that correlate with increased DNA damage and repair processes (Englander et al., 1999; Hu et al, 2003; Martin et al., 2005), cortical and hippocampal cell death (Hu et al., 2005), neuronal losses, and subsequent developmental events associated with neuronal migration and neurotransmitter function (Hu et al., 2006; Louzoun-Kaplan et al., 2008). The transcriptional components of the observed cell death were found to include interleukin (IL)-1 β stimulation of NF- κ B induction of the free radical generating enzymes COX-2 and iNOS and could be blocked at both the IL-1 receptor and NF- κ B activation stage (Qiu et al., 2004; Hu et al., 2005; Fabian et al., 2007).

Cell death signaling can also include cascades at multiple subcellular sites: nuclei, mitochondria, and endoplasmic reticulum (ER) (Sanges and Marigo, 2006) acting via p53-mediated transcription (She et al., 2000), differential necrotic and apoptotic cleavage of poly-ADP-ribose 1 (PARP-1) (Gobeil et al., 2001), and necrosis-induced release of nuclear high-mobility group box 1 (HMGB1) (Kim et al., 2006b; Ditsworth et al., 2007); and activation of downstream effector caspases (Kalai et al., 2003; Reed, 2006) and anti-apoptosis inducing factor (AIF)-mediated caspase-independent mechanisms (Culmsee et al., 2005; Zhu et al., 2007). HI has also been shown to increase cell death signaling at the nucleus (Walton et al., 1999), mitochondria (Zhu et al., 2000), and ER (Larner et al., 2006; also see Noda and Farquhar, 1992; Clarkson et al., 2007; Pal et al., 2007).

Although the precise mechanisms of proapoptotic Bax activation are not fully characterized (Kim et al., 2006a), the Bax-mediated initiation of mitochondrial apoptosis remains canonical in the programmed cell death literature (Wolter et al., 1997; Korsmeyer et al., 1999). However, recent cancer cell biology studies have shown that chemotreatment treatments shift Bax protein to the nucleus and ER in addition to mitochondria (Salah-Eldin et al., 2000; Gajkowska et al., 2001, 2004), with subsequent cell death via disruption of Ca²⁺ homeostasis (Oakes et al., 2005). Therefore, we sought to characterize the coordinating role of Bax intracellular shuttling in neonatal HI cell death signaling and cell death phenotype diversity.

We asked whether the well-known Bax cell death signaling activator was restricted to a single cellular membrane site and whether the increased oxidative stress associated with HHI would alter Bax intracellular localization and organelle-related cell death signaling. We observed a novel organelle-specific time course for increased Bax protein levels, with an early increase in nuclear Bax levels followed by Bax shifts to mitochondria and ER, which correlated with cognate organelle cell death signaling activation. We also showed that HHI increased lesion volumes, as detected by T2-weighted magnetic resonance imaging (MRI), increased inflammation and subsequent necrotic-like cell death; that it had no effect on cortical apoptosis compared with HI treatment alone; but that it increased ER calpain activation and ER Bax protein levels. Hence, the use of 100% O₂ treatment for neonatal HI should be reevaluated.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich (Saint Louis, MO). N,N-bisacrylamide was from Bio-Rad Laboratories (Hercules, CA). Surgical instruments were from Roboz Surgical Instrument Company (Gaithersburg, MD), and 5-0 P3 surgical silk was from Ethicon (Somerville, NJ).

Animal Care

E15 pregnant Wistar rat dams (Charles Rivers Laboratories, Wilmington, MA) were housed in a 12-hr light–dark cycle with access to food and water ad libitum. The day of birth was designated P0. On P1, litters were culled to 10 pups. On P7, the day of HI insult, all pups were removed from the dam, sexed, weighed, and randomly assigned to sham, HI, or HHI groups with various survival times. Time-course analyses required multiple dams, and after culling to 10 pups on P1, pups were mixed among dams to minimize potential confounding litter effects.

Surgical Protocols (HI and HHI)

Hypoxia–ischemia (HI) involving a unilateral, permanent ligation of the common carotid was performed as described elsewhere (Vannucci et al., 1988) with minor modifications. Briefly, P7 rat pups were anesthetized by 7-min incubation in 5% isoflurane, balanced with blood-gas grade 100% O₂, in a 37°C E-Z anesthesia chamber (Euthanex Corp., Palmer, PA) with maintenance of anesthesia at 2.0% isoflurane. After a midneckline incision was made, the left common carotid was isolated and ischemia induced by electrocauterization at two points, one rostral and one caudal, along the isolated artery with a ball-end cauterizer. Electrocauterization severed the left common carotid in the majority of surgeries (>95%). In those cases in which the blood vessel did not sever, the artery was cut between the two cauterization points with microspring scissors to prevent reperfusion across cauterization points. Care was taken not to damage the vagal nerve, which runs parallel and dorsal (underneath) the common carotid artery. After cauterization, the midneckline incision was sutured with 5-0 P3 surgical silk, and the pup was returned to a normoxic (20.8% O₂) 37°C chamber until the effects of the anesthesia dissipated (5–10 min). Sham-operated pups were anesthetized, given the midneckline incision, and then sutured. Isolation of the left common carotid was not performed on sham-treated pups because isolation of the artery can produce minor (<1–2 sec) ischemia–reperfusion events (unreported observational finding). The total time for performing ischemia surgery on a litter of pups was approximately 1 hr. After the last pup recovered from anesthesia, the pups were returned to the dam for a 2.5-hr recovery period to prevent any potential isoflurane-mediated respiratory complications during the hypoxia component of the HI injury.

After the 2.5-hr recovery period, all pups were removed from the dam and placed either in a normoxic 37°C chamber (sham) or hypoxic (8.0% O₂ balanced with blood-gas-grade nitrogen) 37°C chamber for 1.5 hr. A small pet intensive care unit (Harvard Apparatus, Boston, MA) was used for the hypoxia chamber because its larger volume promoted greater stability of oxygen levels and its heated internal water bath reduced temperature variations commonly present in small chambers wrapped in heated water pads (unreported observational finding). After the 1.5-hr period, designated 0-hr survival, pups were returned to the dam until they were killed. Cortices were collected from sham-operated (0 hr) and HI-injured pups at 0.5-, 1-, 2-, 3-, 6-, 12-, 24-, 48-, and 72-hr survival times after HI (*n* = 3 per time point).

For both HI and HHI treatments, HI was performed as above except that immediately after the 1.5-hr hypoxic period, the groups receiving 100% oxygen were incubated in 100% blood-gas grade O₂ for 2 hr, while the sham-operated pups and HI-alone pups remained in a normoxic 37°C chamber. After the 2-hr period, all pups were returned to dams until they were killed. The end of the hypoxic component remained the designated 0-hr survival point. Cortices were collected from sham-operated (*n* = 3), HI-injured (HI) (*n* = 3), and HI-injured + hyperoxia treatment pups (HHI) (*n* = 4) at 3 hr after injury.

Subcellular Fractionation of P7 Wistar Rat Pup Cortices

At the designated survival time point after HI or HHI, pups were deeply anesthetized in an isoflurane-filled gas chamber and then decapitated. Whole brains were removed, and both contralateral and ipsilateral cortices, from the anterior tuber cinereum to anterior of the occipital

cortex (encompassing the parietal cortex), were isolated and flash frozen in liquid nitrogen. Cortical tissue was stored at -80°C until processed. All of the following fractionation steps were carried on ice or a 4°C cold room. Each isolated cortex was suspended in ice-cold fractionation buffer (FB) (in mM: 10 HEPES, 0.5 EGTA, 2 EDTA, 10 DTT, 250 sucrose) containing 1 Roche Complete Protease Inhibitor Tablet (Roche Applied Sciences, Indianapolis, IN) and 100 μL Phosphatase Inhibitor Cocktail II (Sigma-Aldrich, Saint Louis) per every 10 mL of FB. Cortices were disrupted by 30 strokes in a tight-fitting dounce homogenizer (Wheaton Science, Millville, NJ). Homogenates were centrifuged for 20 min at $1,000g$ to pellet nuclei and unbroken cells. To aid in the enrichment of the fractionation procedure, we used a 50–80 supernatant removal process. Briefly, 50% of the supernatant was removed to a new chilled tube, labeled supernatant 1 (S1), and FB roughly equivalent to half of the supernatant volume taken off was added to the pellet. The nuclear pellet was resuspended, and the homogenate was again centrifuged for 20 min at $1,000g$ to repellet nuclei and any remaining unbroken cells. Now 80% of the supernatant was removed and placed in the S1 tube. The remaining supernatant was discarded and the nuclear pellet was resuspended in pellet buffer (PB) (in mM: 20 HEPES, 400 NaCl, 1 EDTA, 1 EGTA, 1 DTT, 1 PMSF) containing 1 Roche Complete Protease Inhibitor Tablet and 100 μL Phosphatase Inhibitor Cocktail II per every 10 mL of PB. The suspended nuclear pellet was shaken at 1,400 rpm on an orbital shaker for 30 min and then centrifuged at 14,000 rpm for 10 min. To minimize repetitive freeze–thaw cycles, the supernatant (nuclear fraction) was stored in aliquots at -80°C until further analysis.

While processing the nuclear fraction, the supernatant in the S1 tube was centrifuged at $3,300g$ for 10 min to pellet any remaining nuclei and unbroken cells. The supernatant was removed to a new chilled tube (M), and the pellet was discarded. The supernatant in the M tube was centrifuged at $10,000g$ for 20 min to pellet heavy and light mitochondria. As with the original $1,000g$ step, which used the 50–80 supernatant removal steps, 50% of the supernatant was removed to a new chilled tube (S2), and the pellet was resuspended and centrifuged again at $10,000g$ for 20 min. Eighty percent of the supernatant was removed to the S2 tube, and the remaining supernatant was discarded. The mitochondrial pellet was resuspended in PB and then was briefly pulse-sonicated (30 kHz, <2 sec). After sonication, the sonicated resuspension (mitochondrial fraction) was stored in aliquots at -80°C until further analysis.

While processing the mitochondrial fraction, the supernatant in the S2 tube was centrifuged at $15,000g$ for 10 min to pellet any remaining light mitochondria. The supernatant was then removed to a new chilled tube (E), and the pellet was discarded. The supernatant in the E tube was centrifuged at $100,000g$ for 1 hr to pellet rough and smooth ER. The supernatant (cytosolic fraction) was removed and stored in aliquots at -80°C until further analysis. The ER pellet was resuspended in PB and then briefly pulse sonicated (30 kHz, <2 sec). After sonication, the sonicated resuspension (ER fraction) was stored in aliquots at -80°C until further analysis. By using lamin-B₁, IkB- α , cytochrome *c* oxidase IV (COXIV), and protein disulfide isomerase (PDI) as respective nuclear, cytosolic, mitochondrial, and ER markers, we observed that this subcellular fractionation protocol yielded highly enriched nuclear, cytosolic, mitochondrial, and ER fractions (Fig. 1A).

Western Blot Analysis

Protein concentration of subcellular fractions was determined via the bicinchoninic acid assay (Pierce, Rockford, IL). A total of 50 μg of protein were separated via electrophoresis on a 10% or 12% sodium dodecyl sulfate–polyacrylamide gel, depending on the main protein of interest for analysis. Protein was transferred overnight at 4°C in transfer buffer (in mM: 25 Tris base, 192 glycine, 10% [v/v] methanol) to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Protein-containing PVDF membranes were blocked for 1 hr at room temperature in blocking buffer (4% bovine serum albumin [BSA], 0.2% Tween-20 in Tris-

buffered saline [TBS, pH 7.4]). After blocking, membranes were washed two times in wash buffer (WB) (0.2% Tween-20 in TBS [pH 7.4]). Next, membranes were incubated overnight (or 1 hr at room temperature) with primary antibody diluted in primary antibody buffer (1% BSA, 0.2% Tween-20 in TBS [pH 7.4]). The following primary antibodies (and their dilution) were examined: mouse anti-Bax (6A7 clone) (1:1,000), rabbit anti-IL-1 β (1:500), mouse anti-PARP-1 (clone C-2-10) (1:500), mouse anti-lamin B₁ (1:1,000) (Invitrogen, Carlsbad, CA), rabbit anti-I κ B- α (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-COXIV (1:4000) (Abcam, Cambridge, MA), rabbit anti-high mobility group protein 1 (HMG1/HMGB1) (1:1,000), rabbit anti-caspase 12 (C-terminus) (1:1,000) (Millipore, Billerica, MA), mouse anti-PDI (1:1,000) (Assay Designs, Ann Arbor, MI), mouse anti-alpha fodrin (1:1,000) (Biomol International, Plymouth Meeting, PA), rabbit anti-phospho-p53 (Ser15) (1:1,000), rabbit anti-cleaved caspase-3 (1:1,000), rabbit AIF (1:500) (Cell Signaling Technologies, Danvers, MA), and mouse anti- β -actin (1:50,000) (Sigma-Aldrich, Saint Louis, MO).

After incubation with the primary antibody, membranes were washed six times for 10 min in WB. After these washes, the membranes were incubated 1 hr at room temperature with the appropriate secondary antibody diluted in secondary antibody buffer (0.5% BSA, 0.2% Tween-20 in TBS [pH 7.4]). The following secondary antibodies were used: goat anti-mouse-POD, rat adsorbed, and goat anti-rabbit F(ab')₂-POD, human and mouse adsorbed (Southern Biotech, Birmingham, AL). All secondary antibodies were diluted 1:3,000, except for mouse anti- β -actin, for which the secondary antibody was diluted 1:15,000. After incubation with the secondary antibody, membranes were washed six times for 10 min in WB and then developed with the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ). Films were scanned and densitometric analyses were performed by AlphaEase software (Alpha Innotech, San Leandro, CA). Samples were normalized first to β -actin values and then presented as fold values relative to sham-treated animals.

Enzyme-linked Immunosorbent Assay Detection of Cytosolic Oligonucleosome Formation

As a measure of apoptotic cell death, the presence of cytosolic oligonucleosomes was detected with the Cell Death Detection enzyme-linked immunosorbent assay (Roche Applied Sciences, Indianapolis, IN) following the manufacturer's protocol. All steps were carried out at room temperature. Briefly, a 96-well microplate was coated for 1.5 hr with a mouse monoclonal antibody diluted in supplied coating buffer (clone H11-4 to the histones H2A, H2B, H3, and H4). The plate was then incubated in the supplied incubation buffer for 0.5 hr. A total of 50 μ g of cytosolic protein from each sample loaded in triplicate wells was diluted in incubation buffer. The cytosolic samples were incubated for 1.5 hr. After incubation, the microplate was washed three times with supplied wash buffer and then incubated for 1.5 hr in a peroxidase-conjugated antibody to DNA diluted in incubation buffer. The microplate was then washed three times in wash buffer and then incubated for 5 min to 1 hr in ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) substrate solution on a rotary shaker (250 rpm). During this incubation, multiple readings at 405 nm were recorded with a Dynex MRX microplate reader (Dynex Technologies, Chantilly, VA). Absorbance values at 405 nm were represented as fold change relative to sham-treated animals.

Tissue Preparation for Immunofluorescent Staining

Survival time points after HI, which showed maximal Bax protein levels in each fraction, were selected for confocal immunohistochemical analysis. At these time points, pups were given an overdose of Nembutal (120 mg/kg, i.p.) and perfused transcardially with 0.22 μ m-filtered 0.9% (w/v) saline. Pups were then fixed with 0.22 μ m-filtered 4% (w/v) paraformaldehyde/phosphate-buffered saline (PBS). Whole brains were removed and fixed for an additional 24 hr in 0.22 μ m-filtered 4% (w/v) paraformaldehyde/PBS at 4°C. Fixed, whole brains were trimmed from the anterior tuber cinereum to anterior of the occipital cortex (encompassing the

parietal cortex) and incubated in 30% (w/v) sucrose/PBS at 4°C until the sections sank (roughly 72 hr). Trimmed brains were given a nick on the ipsilateral hemisphere (for distinguishing the hemispheres), encased in OCT compound (Sakura Finetek, Torrance, CA) on dry ice, and then stored at -20°C until frozen solid. Trimmed brains were sectioned with a frozen microtome into 20 µm slices onto poly-L-lysine-coated glass slides and stored at -20°C until immunohistochemical staining.

For immunofluorescence staining, sections were post-fixed with ice cold acetone for 5 min and then allowed to air dry for 30 min. Sections were washed three times for 7 min in immunohistochemistry wash buffer (IWB) (50 mM Tris base, 0.9% (w/v) NaCl, 0.45 µm filter-sterilized, pH 7.6). Then sections were permeabilized three times for 7 min with IWB + 0.1% Triton X-100. After permeabilization, sections were blocked for 45 min with IWB + 5% (v/v) normal goat serum + 0.3% (w/v) BSA + 0.1% Triton X-100. After removal of the blocking solution, sections were incubated overnight in a humidified chamber with the primary antibodies diluted in IWB + 1% (v/v) normal goat serum + 0.3% (w/v) BSA + 0.1% Triton X-100. The following primary antibodies (and their dilutions) were examined: rabbit anti-Bax (N-terminus) (1:200) (Millipore, Billerica, MA), mouse anti-PDI (1:100) (Assay Designs, Inc., Ann Arbor, MI), and mouse anti-ATP synthase β (1:100) (Abcam, Cambridge, MA). After overnight incubation, the sections were washed three times for 7 min in IWB + 0.05% (v/v) Tween-20. Then the sections were incubated for 1 hr with appropriate fluorescent secondary antibodies diluted in IWB. The following secondary antibodies (and their dilutions) were used: AlexaFluor 568 goat anti-rabbit (1:1,000) and AlexaFluor 488 goat anti-mouse (1:1,000) (Invitrogen, Carlsbad, CA). Again, sections were washed three times for 7 min in IWB + 0.05% (v/v) Tween-20. After these wash steps, the sections were incubated with Hoechst 33258 (0.5 µg/mL) for 5 min to stain nuclei. Vecta-shield mounting medium (Vector Labs, Burlingame, CA) was applied to the sections and Assistant glass coverslips (No. 1.5 thickness) (Harvard Apparatus, Holliston, MA) were placed on top and sealed with clear nail polish. Fluorescent-stained slides were stored in the dark at -20°C until viewing. Sets of slides to be used for comparison studies using the same primary antibodies were performed on the same day to eliminate interex-perimental variance.

Confocal Microscopy

Fluorescent-stained slides were viewed by using the Bio-Rad Radiance 2100 laser scanning system (Carl Zeiss, Thornwood, NY) fitted onto a Nikon Eclipse E800 microscope (Nikon, Melville, NY). Images were collected by Laser-Sharp 2000 software (Carl Zeiss, Thornwood, NY). Fluorescence was detected by the following laser and emission filter sets: for cyan fluorophores, a 405-nm laser with an HQ442/45 emission filter, for green fluorophores, a 488-nm laser with an HQ515/30 emission filter, and for red fluorophores, a 568-nm laser with an E600LP emission filter. Two dichroic mirrors were placed along the optical path to prevent fluorescence bleedthrough: a 500DCLPXR mirror for the cyan and green fluorophores, and a 560DCLPXR for the green and red fluorophores. A third 100% deflection mirror was used when imaging the cyan fluorophore.

Sham-treated animals were used to set the baseline parameters for laser intensity, gain, and offset for slides stained with the same primary antibodies. By means of the SETCOL lookup table, the three parameters were chosen that maximized the width of the gray scale with few red (high intensity) pixels and few green (low intensity) pixels. Optimal pinhole size was computer generated for each laser at each objective. For the Fluor 40× oil (NA 1.3), the pinhole settings were as follows: for 405 nm, 0.9 mm; for 488 nm, 1.1 mm; and for 568, 1.3 mm. For the Plan Apo 60× oil (NA 1.4), the pinhole settings were as follows: for 405 nm, 1.3 mm; for 488 nm, 1.5 mm; and for 568 nm, 1.8 mm. After the parameters had been selected, the settings

were maintained for all images taken involving the same primary antibodies, and the entire image set was collected on the same day.

Images were scanned by using the sequential scan setting for the three lasers set at a scan rate of 50 lines per second and by using Kalman averaging ($n = 6$) to minimize background signal. A 60 $\times$ oil objective (NA 1.4) with a computer-driven 2.6 $\times$ zoom was used to acquire images for analyzing the presence of Bax colocalization with particular subcellular organelles, and a Fluor 40 $\times$ oil objective (NA 1.3) was used to acquire images for the blind counting of the cells within regions of interests (ROIs) that exhibited Bax colocalization with a particular subcellular organelle. Sets of 40 $\times$ confocal images for each primary antibody combination were counted by the same blind reviewers to minimize reviewer-to-reviewer variability. Before analysis, collected images were deconvolved with a 2D iterative deconvolution plug-in that was based on the DAMAS algorithm (Brooks and Humphreys, 2004; Dougherty, 2005) for NIH ImageJ shareware (<http://rsb.info.nih.gov/ij/>).

Detecting Intranuclear Bax and Nuclear Bax Fluorescence Intensity by LineProfile Analysis

To study whether Bax translocated into the nucleus and the changes in nuclear Bax fluorescence as a result of HI, LineProfile analyses of z-stacks of cells from sham and HI-injured cortices were performed. Briefly, random ROIs within the ipsilateral cortex from both sham-treated and HI-injured stained slides were used to generate z-stack images. Having established the baseline parameters as described above, multiple scans at 0.7- μ m steps in the z-direction of a ROI were taken. Then, using the LineProfile program within LaserSharp 2000 software, a hemisecting line 1 pixel in diameter was drawn across the nuclei of cells within the ROI (Fig. 9A). By using the Hoechst 33258 (blue) pixel intensity values along the hemisecting line to define the boundary of the nucleus, we defined intranuclear Bax as the presence of ascending and descending peaks in Bax (red) pixel intensity along the same hemisecting line within the boundaries set by the Hoechst 33258 pixel intensity (Fig. 9B). By using this same hemisecting line but increasing its diameter to equal the size of the nucleus at a particular 0.7 μ m step, we measured the average nuclear Bax pixel intensity at this z-step. Then we defined the average nuclear Bax pixel intensity of a cell within the ROI as the average of the individual 0.7- μ m step averages of Bax nuclear pixel intensity. We performed these measurements on multiple cells from multiple ROI for comparison of nuclear Bax fluorescence intensities from within sham and injured cortices (Fig. 9C) (three ROIs per hemisphere, $n = 15$ cells per hemisphere, three sham hemispheres and three 1-hr survival post-HI hemispheres analyzed). Colocalization was quantitated by blind counting of green (organelle) or red (Bax) and orange (colocalization) in six randomly selected parietal cortex sections for each treatment. After scoring, data were collated and analyzed as described and percentage of cells with colocalized expression calculated.

MRI

T2-weighted MRI were acquired with a Bruker 7-T MRI scanner using a quadrature surface coil. For MRI scans, P7 and P8 Wistar rat pups, anesthetized with 2.5% isoflurane, were placed on a custom-built Plexiglas bed. Respiratory rate, electrocardiogram, oxygen saturation, and body temperature were continuously monitored during the MRI scans, and a feedback-controlled warm air heating system maintained body temperature at $37.0 \pm 0.1^\circ\text{C}$. T2-weighted MRI data were acquired as dual echo fast-spin echo images; the following imaging parameters were used: TR (repetition time) = 5 sec, TE (echo time) = 10.096 msec, acquisition matrix = 256 pixels $\times$ 256 pixels, field of view = 35 mm (resulting spatial resolution = 0.1367 mm $\times$ 0.1367 mm), and slice thickness = 0.5 mm. Each brain scan consisted of 25–30 contiguous slice locations, with each resulting slice image equaling the average of four images at that location. Total scan time for each brain was 16 min.

Lesion volume analysis of T2-weighted MRI scans were analyzed by the MeasureStack plug-in for NIH ImageJ share-ware (<http://rsb.info.nih.gov/ij/>). Briefly, cortices of MRI brain scans were blocked around the parietal cortex from just posterior to the hippocampal formation until just anterior to the caudal occipital cortex. ROIs at each slice were placed around lesions within the cortex, and then the lesion volumes for each blocked cortical region were calculated. MRI lesion volume rendering was performed by the 360° 3D Stack Macro plug-in for NIH ImageJ shareware.

Statistical Analysis

Statistical analyses were performed by GraphPad Prism 5 software (GraphPad Software, San Diego, CA). For analyses involving two groups, a Student's *t*-test was performed, and a *P* value of less than 0.05 was considered significant. If variances for the two groups were deemed significantly different, a Welch correction was added to the Student's *t*-test. For analyses involving two or more groups, a one-way analysis of variance was performed with a Tukey-Kramer post hoc test to compare groups. A *P* value of less than 0.05 was deemed significant. A χ^2 test, including calculation of relative risk, was performed for MRI lesion volume analyses.

RESULTS

Bax in Sham-treated Animals Is Mostly in Cytosol

To determine the levels of Bax in the cytosol, mitochondria, ER, and nucleus after HI, we carried out subcellular fractionations of P7 Wistar rat pup cortices that yield highly enriched cytosolic, mitochondrial, ER, and nuclear fractions. We modified the centrifugation and the supernatant removal steps in a commonly used sucrose fractionation protocol to optimize the conditions for organelle enrichment (Boehning et al., 2003). By using lamin-B₁, PDI, COXIV, and I κ B- α as respective nuclear, ER, mitochondrial, and cytosolic markers in Western blot tests, we confirmed that the P7 rat pup cortex fractionation protocol used gave highly enriched nuclear, ER, mitochondrial, and cytosolic fractions (Fig. 1A). Because I κ B- α has been shown to translocate into the nucleus after cellular perturbations, we also confirmed the level of our cytosolic enrichment with another cytosolic marker, β -tubulin (data not shown).

To determine the distribution of Bax among the four cell fractions in sham-treated animals as a frame of reference, we measured Bax levels in each cell organelle-enriched fraction by Western blot analysis. Although the major portion of Bax was in the cytosolic fraction (85.8% $\pm$ 1.8%), the remaining Bax was mostly in the nuclear (6.45% $\pm$ 1.7%) and ER (5.35% $\pm$ 1.5%) fractions, and a lesser amount was in the mitochondrial fraction (2.3% $\pm$ 0.7%; *P* < 0.05 compared with nuclear; Fig. 1B).

HI Induces Cell Death in the Ipsilateral Cortex Via Activation of Mitochondrial- and ER-mediated Cell Death

We asked whether HI induces apoptotic cell death in the ipsilateral cortex via activation of mitochondrial and/or ER cell death. We assessed the extent of apoptosis over time by measuring cytosolic oligonucleosome levels (a measure of apoptotic cell death on the basis of the appearance of histone/DNA complexes resulting from their cleavage at the interlinker regions between histones and DNA). We measured caspase-3 cleavage as a measure of mitochondria-dependent apoptotic cell death and caspase-12 cleavage in the ER fraction as a measure of ER-dependent cell death over time. We also measured mitochondrial AIF levels, which are indicative of caspase-independent mitochondrial cell death. HI increased cytosolic oligonucleosome at all times measured (Fig. 2A). HI also increased both cytosolic caspase-3 cleavage (Fig. 2B) and ER caspase-12 cleavage (Fig. 2C) but decreased mitochondrial AIF levels over time (Fig. 2D), consistent with its extrusion from mitochondria, an apoptotic event.

HI Shifts Bax Sequentially to the Nucleus and Then to Mitochondria and ER

Having established that HI increased apoptotic cell death in the ipsilateral cortex, in part through activation of mitochondrial and ER cell death cascades, we asked whether the time course of the HI-induced cell death cascades correlated with the HI-induced shifts of Bax to the different organelles. Thus, we did a time-course analysis of Bax levels in cytosolic, mitochondrial, ER, and nuclear fractions after HI. Cytosolic Bax levels decreased steadily and returned to sham levels 3 hr after HI.

Mitochondrial Bax levels increased at 3 and 6 hr and then returned promptly to levels below those in sham-treated animals by 72 hr after HI (Fig. 3B). HI also increased ER Bax levels after 6 hr (Fig. 3C). Interestingly, there was an HI-induced increase in nuclear Bax levels as early as 0.5 hr after HI that persisted for at least 3 hr (Fig. 3D). Thus, there was an organelle-specific pattern of Bax localization over time after HI consisting of an early, abrupt increase in nuclear Bax levels, followed by a transient increase in mitochondrial Bax and a later steady increase in ER Bax levels. Furthermore, the increases in mitochondrial and ER Bax levels correlated temporally with the initiation of mitochondrial and ER cell death cascades.

HI-induced Mitochondrial Bax

To confirm the HI-induced increases in mitochondrial Bax levels as determined by Western blot analyses of mitochondrial fractions, we performed confocal immunofluorescence analysis on sham- and HI-treated cortices with ATP-synthase β used as a mitochondrial marker. There were maximal levels of Bax in mitochondria as assessed by colocalization of Bax with ATP-synthase β at 3 and 6 hr after HI in the parietal cortex (Fig. 4A–D and Fig. 5C).

To quantitate the level of mitochondrial Bax, we performed blind cell counts in randomly selected confocal images (Fig. 5A) from within the selected parietal cortical region (Fig. 5C) for both sham- and HI-treated animals at 3 hr for colocalization of Bax and ATP-synthase β . There were significantly more cells labeled with both Bax and ATP-synthase β 3 hr after HI in the parietal cortex compared with sham-treated parietal cortex, in agreement with the mitochondrial fraction Western blot data (Fig. 5B).

HI-induced ER Bax

To confirm the HI-induced increases in ER Bax levels as determined by Western blot analyses of ER fractions, we performed confocal immunofluorescence analysis on sham- and HI-treated cortices with PDI used as an ER marker. There were maximal levels of Bax in ER as assessed by colocalization of Bax with PDI at 6 hr after HI in the parietal cortex (Fig. 6A–D; 7C).

To quantitate the level of ER Bax, we performed blind cell counts in randomly selected confocal images (Fig. 7A) from within the selected parietal cortical region (Fig. 7C) for both sham- and HI-treated animals at 6 hr for colocalization of Bax and PDI. There were significantly more cells labeled with both Bax and PDI 6 hr after HI in the parietal cortex compared with sham-treated parietal cortex, in agreement with the ER fraction Western blot data (Fig. 7B).

HI Induces Bax Translocation Into Nuclei and Nuclear p53 Phosphorylation

To confirm the observation of HI-induced increases in Bax in nuclear fractions by Western blot assays, we performed multiple z-series confocal immunofluorescence analyses on sham- and HI-injured cortices for both Bax and Hoechst 33258, a nuclear stain. There was maximal Bax colocalization with Hoechst 33258 1 hr after HI (Fig. 8A). To determine whether the colocalized nuclear Bax was intranuclear or perinuclear, i.e., associated with the nuclear envelope, we performed LineProfile analyses (see Materials and Methods) to determine whether increasing and decreasing peaks of Bax fluorescence were present within the Hoechst 33258-defined nuclear boundaries moving through the z-series of 60 $\times$ ROI images. There were

peaks of high-intensity Bax fluorescence within the Hoechst 33258–defined nuclear boundaries present in the ipsilateral cortex of 1-hr post-HI-injured pups (Fig. 8B). To quantify this finding, we calculated the intranuclear Bax fluorescence average for cells within the parietal cortex at every step of the z-series. There was a significantly increased intranuclear Bax fluorescence intensity in the ipsilateral cortex 1 hr after HI (Fig. 8C), consistent with the Western blot data and confirming that HI triggers intranuclear Bax translocation.

As with ER- and mitochondria-mediated cell death, we hypothesized that HI-induced increases in nuclear Bax would accompany activation of nuclear cell death signaling cascades. We measured nuclear phospho-p53^{Ser15} levels after HI by Western blot analysis because phosphorylation of p53 at serine 15 activates p53-mediated proapoptotic transcription. There was a significant HI-induced increase in nuclear phospho-p53^{Ser15} levels in the ipsilateral cortices of the HI-treated pups shortly after Bax nuclear translocation (Fig. 8D). Thus, as was the case for the mitochondria and ER, nuclear Bax localization increases were associated with one component of nuclear cell death signaling.

At 24 hr After HI, Hyperoxia Treatment Increases HI-induced Lesion Volume Size

A clinical treatment currently in practice for pre-term or term newborns suspected of experiencing an HI insult is placement in a warmed neonatal intensive care unit supplemented with 100% O₂ until they have “pinked up” (pulse oxymetry saturations of 92%–96% in term infants and 88%–92% in preterm infants). Variable FiO₂ levels are permitted during resuscitation, but if the infant’s condition does not improve after 90 sec, then a FiO₂ of 1 (100% O₂) is recommended because it effectively increases the infant’s Apgar (Activity, Pulse, Grimace, Appearance, Respiration) score (Bissinger and Ohning, 2006; de Menezes and Shaw, 2006).

To assess the benefit of 100% oxygen treatment on HI-injured cortices, we added to the Rice-Vannucci model of neonatal HI a 2-hr exposure to 100% oxygen, HHI. Immediately after the hypoxia component, we saturated the 37°C hypoxia chamber with 100% oxygen until the chamber oxymeter read 100% (<2 min) and then incubated the HI-injured pups for 2 hr in the hyperoxic chamber (see Materials and Methods). We asked whether 2-hr hyperoxia treatment would decrease the HI-induced cortical lesion volume in 24-hr post-HI-injured pups, as measured by T2-weighted MRI. We used noninvasive MRI to minimize confounding and to use a measure compatible with future clinical studies. As expected, 24-hr post-HI cortices showed dramatic injury in the ipsilateral parietal cortex (Fig. 9A). However, hyperoxia treatment did not decrease but rather increased ipsilateral T2-weighted MRI cortical lesion volume at 24 hr after HI compared with 24-hr post-HI-injured cortices with no hyperoxia exposure (Fig. 9B,C).

Hyperoxia Treatment Does Not Decrease Apoptotic Cell Death Signaling, but Does Increase Inflammatory Signaling After HI

To characterize the exacerbating effects of hyperoxia on HI-mediated cell death, we selected a 3-hr post-HI time point because it is at this time after HI that there was significant activation of apoptotic cell death cascades (Fig. 2). First, we hypothesized that hyperoxia treatment increased ipsilateral cortical lesion volume via increased apoptosis relative to treatment with HI alone. To test this hypothesis, we measured the levels of cytosolic caspase-3 cleavage and cytosolic oligonucleosome levels. Interestingly, at 3 hr after HI, hyperoxia did not increase either cytosolic cleaved caspase-3 or cytosolic oligonucleosome levels in ipsilateral cortices of the HHI pups compared with pups treated with HI alone, although both groups did show increased cytosolic cleaved caspase-3 and cytosolic oligonucleosome levels relative to sham-treated cortices (Fig. 10A,B). We also measured the nuclear levels of cleaved 85-kDa PARP-1 fragments, another marker for apoptotic signaling. Again, hyperoxia did not significantly

increase nuclear 85-kDa PARP-1 levels above the levels of HI alone, although both HHI and HI had increased 85-kDa PARP-1 fragment levels compared with sham-treated animals (Fig. 11C).

Because the HHI treatment had no apparent effect on apoptotic markers, we hypothesized that the hyperoxia-induced increases in lesion volume observed by MRI could be due to increased necrosis. To test this hypothesis, we asked whether the nuclear levels of cleaved 55-kDa PARP-1 fragment would increase while the levels of HMGB1 protein would decrease after HHI, both changes widely associated with necrotic cell death.

Hyperoxia treatment of HI significantly increased ipsilateral levels of nuclear 55-kDa PARP-1 at 3 hr, and both groups had increased nuclear 55-kDa PARP-1 levels relative to sham-treated pups (Fig. 10C). Also, nuclear HMGB1 levels in the hyperoxia-treated, ipsilateral cortices decreased compared with pups treated with HI alone (Fig. 11A). The HHI-induced increase in necrotic markers would also be consistent with increased inflammatory signaling. Therefore, we measured the levels of cytosolic cleaved interleukin 1-beta (IL-1 β), a known marker for cellular inflammation. HHI increased ipsilateral cortical cleaved IL-1 β levels at 3 hr compared with pups exposed to HI alone; again, both groups had higher levels of cleaved IL-1 β levels compared with sham-treated pups (Fig. 10D).

Hyperoxia Treatment Increases Calpain-mediated but not Caspase-mediated Cleavage of Cytosolic α -Fodrin

Early in vitro studies of activation of ER-mediated cell death described the cell death phenotype as necrotic-like (Kalai et al., 2003). If hyperoxia treatment increases HI-induced ipsilateral cortical lesion volume through increased inflammation, we hypothesized that ER-mediated cell death pathways could be involved.

To measure the contributions of both apoptosis and necrosis to the hyperoxia-mediated increases in lesion size volume, we measured the cleavage of the cytosolic cytoskeletal protein, α -fodrin, which has been widely reported to yield protease-specific cleavage fragments: a 145-kDa fragment resulting from ER calpain-mediated cleavage alone (contributing to necrosis) and a 120-kDa fragment resulting from cytosolic caspase-3-mediated cleavage (contributing to apoptosis alone) (Blomgren et al., 2001). The hyperoxia treatment increased cytosolic-cleaved 145-kDa α -fodrin levels but had no effect on cytosolic-cleaved 120-kDa α -fodrin ipsilateral cortical levels compared with the nonhyperoxia treated cortices at 3 hr after HI, and both the hyperoxia-treated and nontreated HI groups had increased levels of 145-kDa and 120-kDa α -fodrin compared with sham-treated pups (Fig. 12A–C). Thus, hyperoxia increased activation of ER calpain-mediated cell death relative to mitochondria-mediated cell death, indicative of increased cell death with necrotic characteristics.

Hyperoxia Treatment Increases ER Bax Levels but Not Nuclear and Mitochondrial Bax Levels

ER Bax is known to promote a greater release of Ca²⁺ and activation of ER calpain-mediated cell death (Oakes et al., 2005). Thus, because hyperoxia treatment selectively increased calpain-mediated cleavage but not caspase-3-mediated cleavage, we hypothesized that if hyperoxia treatment increased ER-mediated cell death, then there should be a selective increase in ER Bax levels but not mitochondrial Bax levels in the hyperoxia-treated ipsilateral cortices of HI-injured pups compared with their cohorts treated with HI alone.

When we measured Bax levels in each subcellular fraction, hyperoxia treatment of HI did not significantly increase mitochondrial or nuclear Bax levels but did significantly increase ER Bax levels compared with HI exposure alone, although both hyperoxia-treated and untreated pups exposed to HI showed higher Bax levels in mitochondria, nuclei, and ER compared with

the sham-treated pups (Fig. 13A–C). Thus, hyperoxia treatment increases HI-induced cortical lesion volume through increased Bax-mediated activation of ER calpain-mediated cell death, which also increases inflammation and cell death with necrotic features.

DISCUSSION

Bax Organelle Occupancy Coordinates Cell Death Cascades After HI

We used the Rice-Vannucci neonatal HI model to demonstrate that HI shifts in cortical Bax organelle occupancy with Bax shuttling to the nucleus as early as 30 min after HI, then to mitochondria and finally to the ER (Figs. 3–8). Together with the appearance of Bax at the different organelles, there were increases in each organelle's cognate cell death cascade (Fig. 2). This is the first evidence that Bax acts to coordinate the nuclear, mitochondrial, and ER cell death signaling cascades in the cortex after HI.

In agreement with previous reports (Hu et al., 2005b; Martin et al., 2005), we observed a significant increase in cortical HI-induced apoptosis, as cytosolic oligonucleosome levels increased over time. In addition, there was a significant increase in mitochondrial Bax levels that correlated with the increase in cleaved caspase-3 (Figs. 2, 3). Interestingly, mitochondrial apoptotic activity would not account for the observed increased oligonucleosome cytosolic presence at later time points, suggesting that other cell death mechanisms were also involved, i.e., caspase-independent mechanisms. As cleaved-caspase-3 levels returned to sham basal levels, there was an increase in AIF (a DNase capable of large scale DNA fragmentation) mitochondrial extrusion, concurrent with the later increases in cytosolic oligonucleosome levels (Fig. 2), with both parameters returning to sham basal levels by 72 hr after HI. Therefore, the AIF mitochondrial fluxes could account for the late increases in cytosolic oligonucleosome levels.

Gajkowska et al. (2004) have reported that chemotherapeutic treatment of MCF-7 cells shifts Bcl-2 family proteins to locales outside of their canonical mitochondrial site. Also, Oakes et al. (2005) have shown that targeting antiapoptotic Bcl-2 to the ER reduces resting ER Ca^{2+} levels such that increased ER stressors decrease ER Ca^{2+} mobilization and cell death; here, Bax targeting to ER had the opposite effect, increasing a cell death, which could not be inhibited by apoptotic antagonists.

In support of these reports, we observed a delayed increase in ER Bax levels beginning 6 hr after HI (Fig. 3) that, together with the observed increased cleavage of ER caspase-12 (Fig. 2), suggested that HI stimulates ER Bax-mediated activation of apoptotic signaling. Interestingly, hyperoxia treatment accelerated the HI-induced increases (3 hr after HI) in ER Bax levels and calpain activity, together with increased necrotic-like signaling relative to HI treatment alone (Figs. 11, 12, 13). These results suggest that Bax-mediated activation of ER caspase-12 vs. calpain-mediated cell death contributes to the resulting apoptotic vs. necrotic cell death phenotypes, respectively.

In agreement with reports of cross-talk between ER and caspase-independent mitochondrial cell death signaling cascades (Polster et al., 2005; Sanges and Marigo, 2006), we observed that after HI, there is mitochondrial extrusion of AIF concurrent with the observed increases in cleaved caspase-12 (Fig. 2). A recent report states that calpain activation occurs before mitochondrial Bax translocation, suggesting that Bax activation is downstream of calpain activation (Moubarak et al., 2007). However, this report did not determine whether Bax localizes to the ER concurrently with calpain activation. In addition, whether mitochondrial-mediated cell death precedes its ER-mediated counterpart, or the reverse, is likely to depend on the specific cell death stimulus given (Polster et al., 2005).

There are reports of nuclear Bax localization in the cancer literature (Godlewski et al., 2001) that suggest a role for Bax in p53 nuclear activity (Raffo et al., 2000). In addition, Bax-overexpression-transformed SK-SH-SY5Y cells results in the presence of full-length Bax at both the nuclear envelope and the interior of the nucleus, with N-terminal Bax cleavage trapping Bax in the nucleus (Karlsson et al., 2007). Furthermore, regulation of nuclear Ca^{2+} fluxes by nuclear-envelope-associated Bax has been reported for porcine models of global fetal hypoxia (Ashraf et al., 2001), suggesting that Bax regulation of Ca^{2+} homeostasis in response to oxidative stress is common in all subcellular organelles.

We observed Bax colocalization with the perinuclear envelope and intranuclear space before shifts to the ER and mitochondria (Fig. 3, 8). Furthermore, there was a significant increase in p53^{ser15} phosphorylation concurrent with the observed increases in nuclear Bax (Figs. 8, 13), in agreement with reports linking Bax and p53 in cell death processes. Not surprisingly in a P5 cortical neuronal culture model, there were early increases in nuclear Bax after exposure to rotenone (Gill et al., unpublished observations), suggesting that nuclear Bax localization may be an early promoter of cell death across many paradigms. We did not observe the presence of cleaved Bax after HI. However, the Bax antibody used for the Western blot tests in these studies was raised against the N-terminal sequence, the portion of Bax reported to be cleaved (Karlsson et al., 2007). Therefore, it remains to be determined whether nuclear Bax cleavage occurs in vivo after HI.

Hyperoxia Treatment Increases HI-induced Lesion Volume Via ER Stimulation of Necrosis and Inflammation

Mitochondria mediated apoptosis via Bcl-2-mediated processes (Wei et al., 2001); however, necrosis has been classically defined as a passive event (Cole and Perez-Polo, 2004), although Edinger and Thompson (2004) have suggested that in addition to passive necrosis, there is programmed necrosis, which can play a developmental role as an alternative to apoptotic programmed cell death. There are markers for programmed necrosis, such as the nuclear release of HMGB1, the differential cleavage of PARP-1, and ER Ca^{2+} -activated calpain activation, in addition to mitochondrial AIF extrusion, itself an outcome of calpain-mediated cleavage (Gobeil et al., 2001; Polster et al., 2005; Ditsworth et al., 2007; Moubarak et al., 2007). Furthermore, chronic treatment of animals undergoing central nervous system trauma with antiapoptotic agents has been shown not to inhibit cell death, but to shift cell death phenotypes to a more necrotic-like phenotype that increases inflammation (Cittelly et al., 2007; Gobeil et al., 2001).

We modified the Rice-Vannucci model with a 2-hr isobaric 100% O_2 intervention after HI to determine whether 100% O_2 decreased HI-induced cortical lesion volumes via decreased Bax-mediated activation of nuclear-, mitochondrial-, and/or ER-mediated cell death cascades. Surprisingly, the 100% O_2 treatment did not decrease but rather increased HI-induced cortical lesion volume at 24 hr after HI (Fig. 9). Furthermore, although there was no increase in cortical apoptosis, there was an increase in necrotic cell death signaling and inflammation (Fig. 10). In agreement with there being no increase in HI-induced apoptosis, there was no increase in mitochondrial Bax levels after HHI. Instead, HHI further augmented the HI-induced increases in ER Bax levels concurrent with increased calpain activity (Figs. 11–13), suggesting that HHI increased HI-induced Bax-mediated activation of ER cell death signaling and subsequent inflammation and injury by increasing necrotic-like cell death (Fig. 14). We have also shown that there is diminished long-term locomotor recovery in a rodent model of spinal cord injury due to increases in inflammation (Cittelly et al., 2007). Furthermore, neonatal infection has been linked to multiple central nervous system disorders, ranging from schizophrenia to autism (Fatemi et al., 2005). Thus, this report supports the concept that early triggering of cell death

signaling may produce injuries with detrimental outcomes that persist long after the initial cell death and inflammatory events have subsided.

Conclusion

There is a distinct time course for Bax intracellular shuttling in the HI-injured cortex, a novel finding. Bax protein first shifts to the nucleus after HI, then to mitochondria and the ER. The intracellular shifts in Bax protein correlate with the differential activation of cognate organelle-specific cell death cascades. In addition, 100% O₂ treatment does not abate HI-induced cortical cell death but rather increases HI-induced cortical lesion volume by increased Bax-mediated activation of ER cell death signaling, leading to increased injury and inflammation via increased necrotic-like cell death. These observations suggest that early cell death signaling produces injury with detrimental outcomes that can persist long after the cell death cascades have subsided. Also, the use of 100% O₂ resuscitation of infants at risk at birth should be reexamined.

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Fig. 1.

The majority of Bax in sham cortices is found in cytosol compared with subcellular fractions enriched for the different membrane organelles. **A:** Representative Western blot tests for lamin-B₁, PDI, COXIV, and IκB-α illustrate high enrichment of the nuclear, ER, mitochondrial, and ER fractions obtained by using the cell fractionation protocol described in Materials and Methods. **B:** Western blot analyses of Bax distribution among the four subcellular fractions in sham ipsilateral cortices. Graph represents densitometric values for Bax protein levels in the four fractions. **Inset:** Enlargement of the mitochondrial, ER, and nuclear Bax values. All values are normalized to β-actin and presented as mean percentage of total Bax ± SD. **P* < 0.05 compared with mitochondrial Bax levels in sham-treated animals.

Fig. 2.

HI increases cell death markers. Densitometric values for cytosolic cleaved caspase-3 and -12 and AIF levels in sham and HI over time. Ipsilateral cortex values after HI normalized to β -actin and presented as mean fold change relative to sham $\pm$ SD. *P* values compared with sham. **A:** Cytosolic oligonucleosome levels. **B:** Cleaved caspase-3 levels. **C:** ER-cleaved caspase-12 levels. **D:** AIF levels.

Fig. 3.

HI shifts Bax to the nucleus followed by Bax redistribution to mitochondria and ER. Densitometric values for (A) cytosolic, (B) mitochondrial, (C) ER, and (D) nuclear Bax levels over time in sham and HI ipsilateral cortices with representative Western blot tests of Bax and actin. Values after HI normalized to β -actin and presented as mean fold change relative to sham $\pm$ SD. *P* values compared with sham. Values normalized to β -actin and are represented as mean fold change of sham $\pm$ SD.

Fig. 4.

HI induces maximal colocalization of Bax with mitochondria at 3 hr after HI; 60× (2.6× zoom) confocal fluorescent images from sham-operated and HI-treated ipsilateral cortices 3 hr after HI. Zoom Merge is a digital zoom of the boxed portion of the Merge frame. Hoechst 33258 is a nuclear fluorescent dye; ATP-synthase β is a mitochondrial marker. (A) Sham-treated animals, (B) 3 hr after HI, (C) 6 hr after HI, (D) 24 hr after HI. Scale bars = 20 μ m.

Fig. 5.

Number of cells with mitochondrial colocalization of Bax 3 hr after HI; 40 $\times$ confocal fluorescent images from sham-operated and HI-treated ipsilateral cortices after HI. Hoechst 33258 is a nuclear fluorescent dye; ATP-synthase β is a mitochondrial marker. **(A)** Sham-treated animals, **(B)** HI-treated animals, and **(C)** blind cell counts of representative fields from sham and HI 3 hr after HI. Values are percentage of cells colocalizing Bax with ATP-synthase β presented as mean percentage of cells expressing colocalization $\pm$ SD. **D:** Depiction of ipsilateral region sampled. Scale bars = 50 μ m.

Fig. 6.

HI induces maximal colocalization of Bax with ER at 6 hr after HI; 60 $\times$ (2.6 $\times$ zoom) confocal fluorescent images from sham-operated and HI-treated ipsilateral cortices 3 hr after HI. Zoom Merge is a digital zoom of the boxed portion of the Merge frame. Hoechst 33258 is a nuclear fluorescent dye; protein disulfide isomerase (PDI) is an ER marker. (A) Sham-treated animals, (B) 3 hr after HI, (C) 6 hr after HI, (D) 24 hr after HI. Scale bars = 20 μ m.

Fig. 7.

Number of cells with ER colocalization of Bax 6 hr after HI; 40× confocal fluorescent images from sham-operated and HI-treated ipsilateral cortices after HI. Hoechst 33258 is a nuclear fluorescent dye; protein disulfide isomerase (PDI) is an ER marker. **(A)** Sham-treated animals, **(B)** HI-treated animals, and **(C)** blind cell counts of representative fields from sham and HI 6 hr after HI. Values are percentage of cells colocalizing Bax with PDI presented as mean percentage of cells expressing colocalization ± SD. **D:** Depiction of ipsilateral region sampled. Scale bars = 50 μm.

Fig. 8.

HI induces Bax translocation into nuclei and increased nuclear p53 phosphorylation. **A:** Representative 60 $\times$ (2.6 $\times$ zoom) confocal fluorescent micrograph from a 1-hr post-HI ipsilateral cortex. Zoom Merge is a digital zoom of the boxed portion of the Merge frame. Hoechst 33258 is a nuclear fluorescent dye. Line in the Zoom Merge frame represents the location of the hemisecting line used in (B) for LineProfile analysis of the fluorescence pixel intensity of Bax and Hoechst 33258. Scale bars = 20 μ m. **B:** HI induces increasing and decreasing peaks of Bax fluorescence within the Hoechst 33258-defined nuclear boundary. Representative fluorescence pixel intensity (FPI) data gathered by the LineProfile analysis (see Materials and Methods) to determine the presence of intranuclear Bax 1 hr after HI. For each 0.7 μ m step (**top to bottom**), a FPI plot for Hoechst 33258 (**left**) and a FPI plot for Bax (**right**) was gathered by the same 1-pixel-diameter hemisecting line across a nucleus. On the x - y graph of FPI data generated, the abscissa is the pixel location along the hemisecting line for which the ordinate contains the fluorescence intensity at the pixel. Sequential increasing and decreasing peaks of fluorescence intensity within the Hoechst 33258-defined nuclear boundary were considered evidence for the presence of intranuclear Bax. **C:** Average intranuclear Bax fluorescence intensity quantified as described in Materials and Methods. Data are presented as mean fold change relative to average sham intranuclear Bax fluorescence $\pm$ SD. **D:** Densitometric values for nuclear phospho-p53^{Ser15} in sham- and HI-treated cortices after HI. Values normalized to β -actin and presented as fold change relative to sham $\pm$ SD. P values compared with sham.

Fig. 9.

Hyperoxia treatment increases HI-induced lesion volume size 24 hr after HI. **A:** HI increases injury lesion volume within the ipsilateral cortices of 24-hr post-HI-injured animals.

Representative T2-weighted magnetic resonance image (T2-MRI) of an HI-injured rat pup brain at 24 hr after HI. **B:** Hyperoxia treatment increases lesion volume within the ipsilateral cortices of 24-hr post-HI-injured animals compared with untreated 24-hr post-HI-injured animals. Representative T2-MRI of a HI-injured + 2 hr 100% hyperoxia-treated rat pup brain at 24 hr after HI.

C: Hyperoxia treatment does not change the HI-induced injury localization but rather enhances the degree of the injury. 3D lesion volume rendering comparing hyperoxia-treated and untreated HI-injured rat pup brains at 24 hr after HI. XYZ coordinate graphs are included for orientation purposes. D, dorsal; I, ipsilateral; R, rostral. 3D renderings were blocked around the parietal cortex, eliminating brain regions anterior to the hippocampal formation.

D: Hyperoxia treatment (24 hr + post-HI = HHI) increases lesion volume within the ipsilateral cortices at 24 hr compared with HI alone (24 hr post-HI = HI). Quantification of lesion volume size from T2-weighted MRIs of male (M) and female (F) pups. Lesion volume values presented as mean fold change relative to sham-treated animals $\pm$ SD.

Fig. 10.

Hyperoxia treatment does not affect apoptotic cell death signaling, but does increase inflammatory signaling. Densitometric values 3 hr after sham treatment, HI treatment, and HHI treatment for ipsilateral cortices for (A) cytosolic cleaved caspase-3, (B) cytosolic oligonucleosome, (C) nuclear 55-kDa-cleaved PARP-1 levels, and (D) cytosolic-cleaved interleukin 1-beta (IL-1β) levels. Also shown are representative Western blot tests. Values are presented as mean fold change relative to sham-treated animals ± SD. * $P < 0.05$ compared with HI alone.

Fig. 11.

Hyperoxia treatment decreases nuclear HMGB1 levels in HHI ipsilateral cortices compared with HI alone pups. Densitometric values for nuclear HMGB1, Bax, and nuclear 85-kDa-cleaved PARP-1 levels at 3 hr in sham-, HI-, and HHI-treated cortices. **Bottom:** Representative Western blot for nuclear HMGB1, Bax, nuclear 85-kDa-cleaved PARP-1 and β -actin. Densitometric values normalized to β -actin and presented as fold change relative to sham $\pm$ SD. Bax nuclear levels in sham-, HI-, and HHI-treated ipsilateral cortices. P value compared with sham and $*P < 0.05$ compared with HI alone. **A:** Nuclear HMGB1 levels in sham, HI, and HHI ipsilateral cortices. **B:** Time course of nuclear HMGB1 levels in the ipsilateral cortex after HI. **C:** Nuclear 85-kDa-cleaved PARP-1 levels in sham-, HI-, and HHI-treated ipsilateral cortices. **D:** Bax nuclear levels in sham-, HI-, and HHI-treated ipsilateral cortices.

Fig. 12.

Hyperoxia treatment increases calpain-mediated but not caspase-mediated cleavage of cytosolic α -fodrin. Western blot of cytosolic cleaved α -fodrin levels at 3 hr in sham-, HI-, and HHI-treated ipsilateral cortices relative to β -actin. Densitometric values for cytosolic (A) 120-kDa- and 145-kDa-cleaved α -fodrin fragment levels, (B) 120-kDa-cleaved α -fodrin fragment levels, and (C) 145-kDa-cleaved α -fodrin levels. Values normalized to β -actin and presented as fold change relative to sham-treated animals $\pm$ SD. *P* value compared with sham and **P* < 0.05 compared with HI alone.

Fig. 13.

Hyperoxia treatment increases ER but not nuclear and mitochondrial Bax levels. Densitometric values for (A) cytosolic, (B) mitochondrial, (C) ER, and (D) nuclear Bax levels at 3 hr in sham-, HI-, and HHI-treated ipsilateral cortices. **Bottom:** Representative Western blot for cytosolic Bax and β -actin. Densitometric values normalized to β -actin and presented as fold change relative to sham-treated animals $\pm$ SD. P value compared with sham and $**P < 0.01$ compared with HI alone.

Fig. 14.

Bax localization in sham-, HI-, and HHI-treated cortices. **A:** Cytosolic localization of Bax sham cortical cells with little Bax associated with nucleus, mitochondria, or ER. **B:** After HI, Bax shifts to the nucleus, mitochondria, and ER and activates each organelle's cognate cell death signaling cascade and determining the cell death phenotype after HI, with increased mitochondria-mediated apoptotic and ER-mediated necrotic-like cell death. **C:** HHI treatment increases lesion volume via selectively increasing Bax-mediated activation of ER cell death signaling, resulting in increased necrosis and inflammation.