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Additive neuroprotection of a 20-HETE inhibitor with delayed therapeutic hypothermia after hypoxia-ischemia in neonatal piglets

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Abstract

The severity of perinatal hypoxia-ischemia and the delay in initiating therapeutic hypothermia limit the efficacy of hypothermia. After hypoxia-ischemia in neonatal piglets, the arachidonic acid metabolite, 20-hydroxyeicosatetraenoic acid (20-HETE), has been found to contribute to oxidative stress at 3 hours of reoxygenation and to eventual neurodegeneration. We tested whether early administration of a 20-HETE-synthesis inhibitor after reoxygenation augments neuroprotection with 3-hour delayed hypothermia. In two hypothermic groups, whole body cooling from 38.5 to 34°C was initiated 3 hours after hypoxia-ischemia. Rewarming occurred from 20 to 24 hours; then anesthesia was discontinued. One hypothermic group received a 20-HETE inhibitor at 5 minutes after reoxygenation. A sham-operated group and another hypoxia-ischemia group remained normothermic. At 10 days of recovery, resuscitated piglets with delayed hypothermia alone had significantly greater viable neuronal density in putamen, caudate nucleus, sensorimotor cortex, CA3 hippocampus, and thalamus than did piglets with normothermic recovery, but the values remained less than those in the sham-operated group. In piglets administered the 20-HETE inhibitor before hypothermia, the density of viable neurons in putamen, cortex, and thalamus was significantly greater than in the group with hypothermia alone. Cytochrome P450 4A, which can synthesize 20-HETE, was expressed in piglet neurons in these regions. We conclude that early treatment with a 20-HETE inhibitor enhances the therapeutic benefit of delayed hypothermia in protecting neurons in brain regions known to be particularly vulnerable to hypoxia-ischemia in term newborns.

Keywords

asphyxia; cerebral ischemia; cytochrome P450; heart arrest; 20-HETE; hypoxic-ischemic encephalopathy; neonate; neuroprotection

INTRODUCTION

Therapeutic hypothermia has been shown to protect the brain after cardiac arrest in adults and after hypoxia-ischemia (HI) in term newborns [1–3], and it is currently undergoing a multicenter trial for children after cardiac arrest [4]. Despite reductions in mortality and improvements in early neurologic outcome in neonates, significant neurologic deficits and learning disabilities persist into childhood [5,6]. Thus, investigations continue to seek therapies directed at the mechanisms of injury that are proceeding before the brain is cooled [7].

The time to reach the target temperature with external cooling can be 2–6 hours in adults [8], and the delay in initiating hypothermia in most neonates enrolled in clinical HI encephalopathic trials has been 3–6 hours [2,9,10]. Delays of this duration have diminished efficacy of hypothermia in perinatal animal models of HI [11–13]. In a neonatal piglet model of hypoxia plus complete asphyxia, severe ultrastructural damage and ischemic morphology become evident at 3–6 hours after reoxygenation [14,15]. One strategy is to administer an agent soon after reoxygenation that will slow the progression of the cell death signaling cascade until hypothermia is initiated [7].

One deleterious effect of cerebral ischemia is increased intracellular calcium influx, which can stimulate phospholipase A₂ and cause release of arachidonic acid. In addition to metabolism by cyclooxygenases and lipoxygenases, arachidonic acid can undergo ω -hydroxylation by cytochrome P450 (CYP) 4A and other CYPs and generate 20-hydroxyeicosatetraenoic acid (20-HETE) [16]. CYP 4A has been well studied in vascular smooth muscle [16], and it appears to contribute to vasospasm after subarachnoid hemorrhage [17]. However, recent evidence indicates that CYP 4A is also expressed in rat hippocampal neurons [18] and that 20-HETE synthesis inhibitors reduce infarction from focal cerebral ischemia [19] independent of vascular effects [20,21]. Such inhibitors have been shown to directly protect hippocampal slice cultures from oxygen-glucose deprivation [18]. CYP 4A is also expressed in striatal neurons of neonatal piglets, and early administration of the 20-HETE synthesis inhibitor *N*-hydroxy-*N'*-(4-*n*-butyl-2-methylphenyl)formamidine (HET0016) [22] after reoxygenation in a piglet model of hypoxia followed by complete asphyxia partially protected striatal neurons without affecting the recovery of cerebral blood flow [23]. Direct effects of 20-HETE on neuronal signaling are supported by the observed increases in phosphorylation of N-methyl-D-aspartate receptors and Na,K-ATPase during infusion of 20-HETE into piglet putamen and by attenuation of phosphorylation at these same sites by HET0016 administration after HI [23]. Furthermore, early administration of HET0016 improved Na,K-ATPase activity and decreased oxidative stress markers at 3 hours of recovery. Therefore, early administration of a 20-HETE inhibitor may suppress the ongoing injury process sufficiently such that it would

result in additive neuroprotection with hypothermia when hypothermia is delayed by 3 hours.

We tested the hypothesis that early administration of HET0016 before induction of hypothermia at 3 hours after reoxygenation from HI in neonatal piglets provides greater neuronal survival than hypothermia alone. Results were also compared to a sham-operated group and to a HI group without hypothermia in order to assess the relative degree of neuroprotection. Term newborns with HI encephalopathy often display selective injury in basal ganglia, thalamus, and peri-Rolandic cortex representing primary sensorimotor cortex [24,25]. Because a similar pattern of selective vulnerability is present in the piglet model of hypoxia plus asphyxia [26], we assessed the density of viable neurons in these regions as the primary endpoints.

METHODS

Animal Preparation

All procedures on piglets were approved by the Animal Care and Use Committee of the Johns Hopkins University and conformed to the National Institutes of Health guidelines for the care of animals. Male piglets were transported from a USDA-approved farm to the laboratory housing at least one day before the procedure to allow them to become accustomed to their new housing environment and to learn to drink formula milk from a bowl. They were housed in groups of 2–3 piglets per cage and studied at 3–5 days of age. Anesthesia was induced with 5% isoflurane in 50% N₂O and 50% O₂ via face mask. The trachea was orally intubated and mechanically ventilated to maintain end-tidal CO₂ at approximately 35–40 mm Hg. After intubation, the lungs were ventilated with 1.5–2% isoflurane in 70% N₂O and 30% O₂. Rectal temperature was maintained at 38.5–39.0°C (normal for piglets) with a warming blanket and heat lamp. Under aseptic conditions, an incision was made in the groin, a femoral artery and vein were isolated, and sterilized catheters were advanced into the thoracic aorta and vena cava. The catheters were routed subcutaneously to the back of the piglet and incisions were closed with suture. If baseline hemoglobin concentration was less than 6 g/dL, the piglet was excluded and euthanized. Cephalothin (20 mg/kg) was administered intravenously after catheterization and intramuscularly on the first three days of recovery as a prophylactic antibiotic.

Experimental protocol

After surgery was complete, fentanyl was injected intravenously (10 µg/kg) and a continuous infusion of 10 µg·kg⁻¹·h⁻¹ was started. Isoflurane inhalation was stopped, and piglets were maintained on 70% N₂O and 30% O₂. The combination of fentanyl and N₂O was chosen for sedation because it blocks limb withdrawal to hoof pinch in newborn lambs with minimal effects on cerebral blood flow and oxygen consumption [27], and 24-h sedation with fentanyl does not enhance neuroapoptosis in piglets [28]. The N₂O-fentanyl combination also blocked the limb withdrawal response to hoof pinch and the cough reflex in piglets. At approximately 10 minutes after administration of fentanyl, vecuronium (0.2 mg/kg plus 0.2 mg·kg⁻¹·h⁻¹) was infused intravenously to prevent ventilatory efforts during subsequent hypoxia and asphyxia.

Isocapnic hypoxemia was induced by decreasing the inspired O₂ to approximately 10% for 45 minutes. Asphyxia was produced by occluding the endotracheal tube for 7 minutes. A 5-minute period of ventilation with 21% O₂ was interposed between the hypoxia and asphyxia periods because previous experience showed that the rate of success of subsequent cardiac resuscitation was low without a brief period of partial reoxygenation. The infusion of fentanyl and vecuronium was stopped at the onset of asphyxia.

At 7 minutes of asphyxia, the clamp on the endotracheal tube was removed, and ventilation commenced with 50% O₂. Circumferential manual chest compressions were performed at a rate of approximately 100/min. Epinephrine (300 µg) was injected intravenously if return of spontaneous circulation (ROSC) with a mean arterial blood pressure above 60 mmHg did not occur. If arterial pressure remained below 60 mmHg, a second dose of epinephrine was injected. If ROSC was not established by 3 minutes of cardiopulmonary resuscitation, chest compressions were stopped and the piglet was excluded. After ROSC, inspired O₂ was gradually reduced to 30% when the pulse oximetry indicated greater than 95% saturation. Inspired N₂O was restored to 70% when inspired O₂ was restored to 30% (usually by 5 minutes after ROSC), and intravenous infusion of fentanyl (10 µg·kg⁻¹·h⁻¹) and vecuronium (0.2 mg·kg⁻¹·h⁻¹) was resumed. Vecuronium was used to prevent shivering and consequent reductions in glucose stores. Ventilation was adjusted to maintain end-tidal CO₂ at approximately 35–40 mm Hg throughout recovery.

Arterial blood was sampled for measurement of PO₂, PCO₂, pH, hemoglobin concentration, oxyhemoglobin saturation, and glucose concentration (ABL 825 Flex blood gas analyzer, Radiometer, Copenhagen, Denmark) at baseline, at 5, 15, 27, and 42 minutes of hypoxia, at 4 minutes of 21% O₂ ventilation, at 6 minutes of asphyxia, at 5, 15, 30, 60, 120, 180, and 240 minutes after reoxygenation, and at 4-hour intervals thereafter through 24 hours. Values were corrected to body temperature. After the blood sample at 5 minutes after reoxygenation, sodium bicarbonate was infused intravenously to correct the base deficit. The infusion of fentanyl was increased in 5 µg·kg⁻¹·h⁻¹ increments up to a maximum of 40 µg·kg⁻¹·h⁻¹ as needed to maintain heart rate at less than 200 beats/min. The infusion of vecuronium was increased to 0.3 mg·kg⁻¹·h⁻¹ if piglets showed signs of shivering. Throughout the first 24 hours of recovery, the eyes were lubricated at 4-hour intervals, the endotracheal tube was suctioned, the body was rotated onto the left side, right side, or back at 2-hour intervals, and the piglets received a continuous intravenous solution of 5% dextrose in 0.45% NaCl at 10 ml/h. Additional dextrose was infused if arterial glucose concentration decreased below 70 mg/dL.

On a single day, 2–3 piglets were subjected to sham surgery or to HI followed by normothermia, delayed hypothermia, or HET0016 injection plus delayed hypothermia. Because some variability may occur among litters, each piglet on a particular day was usually assigned to a different experimental group to reduce possible bias between litters. Because of potential seasonal variability, experimental group assignments were generally alternated on consecutive weeks so that one group would not be over-represented at a particular time of the year. Thus, we purposely did not perform a true randomized study in order that biological variability and the required number of animals would be minimized.

The allocation was determined by filling the sample size of each group in a balanced fashion and replacing non-survivors as needed.

One resuscitated group remained normothermic, and a second was made hypothermic beginning at 3 hours after reoxygenation. The third group was treated at 5 minutes after reoxygenation with a 5-minute infusion of 1 mg/kg HET0016 (Cayman Chemical Company, Ann Arbor, Michigan, USA) dissolved in 10% (2-hydroxypropyl)- β -cyclodextrin and 0.9% saline and then had hypothermia induced at 3 hours after reoxygenation. This dose of HET0016 was previously shown to provide significant neuroprotection, whereas a higher dose of 10 mg/kg provided no additional neuroprotection and the vehicle alone had no effect [23]. Because the vehicle had no effect, vehicle was not administered to other groups. For hypothermic groups, whole body cooling was induced by lowering the temperature of a water-perfused blanket underneath the piglet. Cooling was accelerated by placement of ice packs on the sides of the head and torso. The ice packs were removed from the head when rectal temperature dropped to 36°C and from the torso when rectal temperature dropped to 35°C. The target rectal temperature of 34°C was achieved within 30 minutes and was maintained by adjusting the temperature of the circulating-water blanket. Previous work demonstrated that brain temperature was similar to rectal temperature during this procedure [15]. Rewarming was started at 20 hours after reoxygenation at 1°C per hour to return rectal temperature to approximately 38.5°C.

A sham-operated group had the same anesthetic regimen, surgery, and timing of measurements in the experimental protocol but without hypoxia and asphyxia. For all four groups, the infusions of fentanyl and vecuronium were decreased at 21, 22, 23, and 24 hours to 75%, 50%, 25%, and 0%, respectively, of whatever the rate was just prior to 21 hours. Ventilation with 70% N₂O was stopped at 24 hours, and ventilation with 30% O₂ continued until a cough reflex returned and the piglet could be safely extubated. Piglets continued to receive intravenous 5% dextrose in 0.45% NaCl until they accepted swine formula milk by syringe feeding. The time to feed independently from a bowl was recorded. Rectal temperature was monitored daily. Piglets were housed in a cage with padding, usually in groups of 2–3.

After 1–2 days of recovery, some piglets displayed aggressive demeanor with glassy eyes before the onset of clinical seizures. If aggressive demeanor with glassy eyes persisted, 5 mg/kg phenobarbital was injected intravenously to help prevent seizures. If seizures with running movements developed, a second dose of 5 mg/kg phenobarbital was injected. If seizures were severe and refractory to phenobarbital treatment, the piglet was euthanized.

Immunohistochemistry

Anesthetized naïve piglets were perfused transcardially with ice-cold phosphate-buffered saline (PBS) and 4% paraformaldehyde. The right forebrain was cryoprotected in 20% glycerol-PBS for 24 hours, frozen, and cut into serial 60- μ m sections.

Immunohistochemistry was completed on free-floating sections to detect CYP 4A in putamen, primary sensorimotor cortex, hippocampus, and ventral posterolateral (VPL) thalamus. Endogenous peroxidase was quenched with 30-minute incubation of 1% H₂O₂ in methanol. Sections were blocked in normal serum and incubated with rabbit anti-CYP 4A

(1:250; Abcam ab3573, Cambridge, UK) overnight at 4°C. After rinsing in PBS, sections were incubated with biotinylated anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA, USA) and VECTASTAIN Elite ABC reagent (Vector). Immunostaining was developed with diaminobenzidine as a chromogen (Vector). Negative controls were treated without primary antibodies and showed no positive signals.

Histologic Assessment

At 10 days of recovery, piglets were injected intraperitoneally with 50 mg/kg pentobarbital and 6.4 mg/kg phenytoin. The body was perfused transcardially with cold PBS followed by cold 4% paraformaldehyde. After overnight post-fixation, the brain was bisected mid-sagittally and cut into a 14-mm coronal slab between +4 and +18 mm from bregma to include basal ganglia and VPL thalamus. The entire slab was then cut into seven 2-mm slices and embedded with paraffin. Profile counting was performed on 10- μ m sections stained with hematoxylin and eosin under oil immersion at 1000 $\times$ power by an investigator blinded to treatment. On each of five equally spaced slides from five blocks covering the entire length of putamen, the number of viable neurons was counted in seven non-overlapping fields in each of putamen, caudate nucleus, and parasagittal cortical gyrus layer V. On two other slides from two more caudal blocks, the number of viable neurons was counted in seven non-overlapping fields in VPL thalamus and in the dentate gyrus, CA1, and CA3 region of hippocampus. For each brain region, the data are expressed as a percent of the mean value in the sham group.

Statistical analysis

In a previous study with hypothermic treatment after HI, the standard deviation (SD) of neuronal counts in the various brain regions averaged 16.8% of the mean regional values in the sham group [29]. Based on this SD, a sample of 12 would provide 80% power for detecting 20% changes for the primary comparison of neuronal counts between the HI-hypothermia group and the HI-HET0016 plus hypothermia group. The sample size in the sham group and in the HI-normothermia group was reduced to 6 because 1) a comparison between the hypothermia group and these groups was not the primary comparison, and the sham group and normothermia group were used to determine the relative changes in improvement with treatment, 2) counts in the sham groups have been highly reproducible among studies, and 3) the effect size between normothermia and hypothermia HI groups was shown previously to be more than 20%. Thus, the study was designed to minimize animal usage while providing adequate statistical power for the primary comparison.

The density of viable neurons in each region and intensive care-related variables (Table 1) were analyzed with one-way analysis of variance (ANOVA); if the *F*-value was significant at the 0.05 significance level, multiple comparisons were made with the Sidak procedure. Arterial blood gases, pH, glucose, mean arterial blood pressure, and rectal temperature were analyzed at individual time points with one-way ANOVA followed by the Sidak procedure when the *F*-value was significant. The family-wise error rate for all multiple comparisons with the Sidak procedure was set at $P < 0.05$. The incidence of clinical seizures at any time during the 10 days of recovery was analyzed among the four groups by analysis of frequencies.

RESULTS

Mortality, intensive care, and physiologic data

Sixty piglets were studied, and 50 of these were subjected HI. Successful resuscitation was achieved in 42 of the 50 piglets (86.7%). Two piglets required intravenous lidocaine for ventricular arrhythmias. Ten piglets (4 sham, 2 HI-normothermia, 2 HI-hypothermia, 2 HI-HET0016 plus hypothermia) were euthanized prematurely because of severe diarrhea. Six piglets (4 HI-normothermia, 2 HI-hypothermia) died during the first 4 days as a result of severe seizures. Long-term survivors with histologic analysis included 6 piglets in the sham-operated group, 6 piglets in the HI-normothermia group, 12 piglets in the HI-hypothermia group, and 12 piglets in the HI-HET0016 plus hypothermia group. The dose of administered epinephrine and the duration of cardiopulmonary resuscitation required to restore spontaneous circulation were well matched among the three HI groups (Table 1).

In all HI groups, arterial PO₂ decreased to approximately 20–25 mmHg during the 45 minutes of ventilation with 10% O₂, increased to approximately 80 mmHg during the 5 minutes of ventilation with 21% O₂, and then decreased to <10 mmHg by 6 minutes of asphyxia (Fig. 1A). The corresponding arterial oxyhemoglobin saturations were approximately 25–35% during hypoxia, 95% during 21% O₂ ventilation, and <5% by 6 minutes of asphyxia (data not shown). During the early recovery period, arterial PO₂ was purposely kept above the pre-arrest levels to counter the acidemia-produced decrease in O₂ affinity of hemoglobin and thereby ensure that oxyhemoglobin saturation was in the 95–99% range.

Arterial glucose concentration increased from baseline during hypoxia and increased further during asphyxia (Fig. 1B). The glucose concentration in the three HI groups was significantly greater than that in the sham group during asphyxia and at 5 minutes of recovery. The level of hyperglycemia during HI did not reach the 350 mg/dL level shown by others to decrease recovery of high energy phosphates in piglets [30].

Arterial pH decreased modestly during the 45 minutes of hypoxia but remained >7.30 (Fig. 2A). Arterial PCO₂ remained unchanged during hypoxia (Fig. 2B). By 6 minutes of asphyxia, arterial pH profoundly decreased to 6.9–7.0, and arterial PCO₂ increased to approximately 90 mmHg. With the administration of sodium bicarbonate and rapid normalization of arterial PCO₂ after resuscitation, arterial pH largely recovered by 15–30 minutes and remained stable near a value of 7.40 through the subsequent 24 hours of monitoring.

Mean arterial blood pressure increased slightly during hypoxia and decreased markedly during the 7 minutes of asphyxia (Fig. 3). After resuscitation, arterial pressure briefly increased above the baseline levels. However, arterial pressure in the three HI groups was not significantly different from pressure in the sham group between 15 minutes and 24 hours of recovery.

Rectal temperature was maintained at near the normal piglet temperature of 38.5°C during HI and early recovery in all groups (Fig. 4). In both hypothermia groups, whole body

cooling started at 3 hours of recovery; the target temperature of 34°C was achieved within 30 minutes and was well maintained through 20 hours of recovery. Rectal temperature recovered to 38.5°C by 24.5 hours.

Hence, the level of hypoxia and the severity of acidemia, hypercapnia, hyperglycemia, and hypotension during asphyxia were well matched among the HI groups before the therapeutic interventions. Neither HET0016 administration nor induction of hypothermia affected recovery of arterial pressure. Previous work demonstrated that cerebral blood flow fully recovers to pre-arrest levels in this model and HET0016 does not significantly affect this recovery of blood flow [23]. Although hypothermia does decrease cerebral blood flow in this model, it does not impair cerebrovascular autoregulation [31].

Postoperative recovery

At 24 hours, ventilation with 70% N₂O and infusion of fentanyl and vecuronium were stopped in all groups. The additional time required to wean the piglets off of the ventilator was similar in the sham and HI groups (Table 1). However, the time for the piglets to drink formula milk independently was greater in the HI groups than in the sham group (Table 1). Piglets in the sham group began to gain weight 2 days after recovery, whereas those in the three HI groups did not begin to gain weight until after 3 days. At 10 days, weight gain remained significantly smaller in the three HI groups than in the sham group, with no significant differences among the HI groups (Table 1). No evidence of hyperthermia was seen over the 10 days of recovery in any of the groups (Fig. 4).

The incidence of clinical seizures in piglets (including those that died before 4 days of recovery and were not included in the histological analysis) was 0 of 6 in the sham group, 8 of 10 in the HI-normothermia group, 4 of 14 in the HI- hypothermia group, and 0 of 12 in the HI-HET0016 plus hypothermia group. Analysis of frequencies indicated an overall treatment effect ($P < 0.001$), and individual comparisons by the Fisher Exact Test revealed that the incidence in the HI-normothermia group was significantly greater than that in the sham group ($P = 0.007$), HI-hypothermia group ($P = 0.036$), and HI-HET0016 plus hypothermia group ($P < 0.001$). The difference in seizure incidence between the HI-hypothermia group and the HI-HET0016 plus hypothermia group was of marginal significance ($P < 0.10$) with these sample sizes. None of the piglets in the HI-HET0016 plus hypothermia group received phenobarbital (Table 1).

Immunohistochemistry

Immunohistochemistry for CYP 4A revealed staining in cells in naive piglet brain in putamen, primary sensorimotor cortex, CA3 region of hippocampus, and VPL thalamus (Fig. 5). Staining was positive in pyramidal-shaped cells with large nuclei, consistent with expression in neurons.

Histopathology

At 10 days of recovery from HI without hypothermia, extensive neuronal damage consisting of cytoplasmic microvacuolation, eosinophilia, nuclear pyknosis, and cell homogenization was evident in putamen, sensorimotor cortex, hippocampal CA3 region, and VPL thalamic

nucleus (Fig. 6). With delayed hypothermic treatment or with HET0016 plus delayed hypothermic treatment, more viable neurons survived, although some neurons with ischemic morphology remained.

The density of viable neurons in the HI-normothermia group was significantly less than that in the sham group in putamen (Fig. 7A), caudate nucleus (Fig. 7B), sensorimotor cortex (Fig. 7C), VPL thalamus (Fig. 7D), and CA3 hippocampus (Fig. 7E; $P < 0.001$ for all regions, Sidak procedure). The putamen was the most severely damaged region. Compared to the HI-normothermia group, the density of viable neurons was significantly increased in the HI-hypothermia group in putamen ($P < 0.001$), caudate nucleus ($P < 0.001$), sensorimotor cortex ($P = 0.026$), VPL thalamus ($P = 0.001$), and CA3 hippocampus ($P < 0.001$). Compared to the HI-hypothermia group, the density of viable neurons was significantly increased in the HI-HET0016 plus hypothermia group in putamen ($P = 0.003$), somatosensory cortex ($P < 0.001$), and VPL thalamus ($P < 0.001$); however, the differences in caudate nucleus ($P = 0.23$) and CA3 hippocampus ($P = 0.29$) were not significant. In these five regions, the density of viable neurons in both the HI-hypothermia group and HI-HET0016 plus hypothermia group remained less than the values in the sham group ($P < 0.001$). However, ANOVA did not indicate any significant differences among the sham and the three HI groups in the CA1 region ($P = 0.75$) or dentate gyrus ($P = 0.25$).

DISCUSSION

Initiation of therapeutic hypothermia is often delayed after reoxygenation from cerebral ischemia. In the case of HI during labor and delivery, hypothermia was generally started at 3–6 hours after birth in many neonatal clinical trials [2,9,10]. In the current model of hypoxia plus complete asphyxia in neonatal piglets, the major finding is that administration of a 20-HETE inhibitor soon after reoxygenation augmented the partial neuroprotection seen with a 3-hour delay in the commencement of hypothermia.

Term neonates with HI encephalopathy often display selective injury in basal ganglia, perirolandic cerebral cortex, hippocampus, and thalamus [24,25]. A similar topology of selective vulnerability has been described in the neonatal piglet model of hypoxia plus asphyxia [26]. The most severely injured region was putamen, in which density of surviving viable neurons at 10 days of recovery without hypothermia was only 23% of that in the sham-operated group. Here, hypothermia initiated at 3 hours after reoxygenation resulted in substantial improvement to 63% of the control density of surviving neurons. This value is intermediate between the approximately 100% value obtained when hypothermia commenced at 5 minutes after resuscitation [15] and the 46% value seen when hypothermia was delayed by 4 hours [29]. These comparisons are congruent with previous temporal data showing oxidative and nitrative stress in proteins and nucleic acids and disruption of organelles appearing in putamen at 3–6 hours after reoxygenation [14,23,32,33]. Together, these results emphasize the importance of early initiation of therapeutic hypothermia after global ischemia in developing brain.

Whereas some models of perinatal HI restrict the insult to the brain and can generate severe brain injury, our model induces HI throughout the body as occurs clinically with global HI

and thus permits interactions among organs. The whole body hypothermia may influence such interactions among organs. However, one limitation of the model is that the duration of complete asphyxia is restricted to 7 minutes in order to obtain a high success rate of cardiac resuscitation. To enhance the neuronal injury, we first exposed the body to hypoxia with an arterial O₂ saturation of about 30%, which does not cause arterial hypotension but which augments the injury from the subsequent asphyxia. The 45-minute duration of hypoxia was extended from 30-minute [15] and 40-minute [29] durations previously used and was chosen to increase the severity and reduce the variability of histologic injury. Because the fetus is likely to experience progressive hypoxia during difficult labor and delivery, the model likely has relevance to clinical HI encephalopathy. Furthermore, neonates and infants may experience periods of hypoxia before suffering from asphyxic cardiac arrest.

Another limitation is that we studied the piglets at 3–5 days of age rather than as true newborns because younger piglets often experience diarrhea after the insult, presumably because the severe asphyxia and associated hypotension cause intestinal ischemia. However, we have observed similar patterns of neuropathology in 1- and 7-day old piglets, and we assume that the results obtained in 3–5-day-old piglets will resemble that of a newborn piglet. Nevertheless, we cannot discount the possibility that the additive effect of 20-HETE inhibition and hypothermia might be different in a piglet immediately after birth. It should be noted that the MRI-determined volume of the vulnerable brain structures in 2-week-old piglets is reported to be approximately 40–45% of the corresponding volume in sexually mature 24-week-old pigs [34]. The smaller piglet brain at 3–5 days compared to 14 days likely has rough correspondence to that of a human between term and the first several months after birth.

Previous work demonstrated that administration of 1 mg/kg HET0016 in rat inhibited 20-HETE formation in brain by greater than 90% [35], thereby suggesting that this dose permits adequate entry into the brain. Other studies also report entry of HET0016 in brain sufficient to inhibit 20-HETE formation [36,37]. In piglets, administration of the 1 mg/kg dose at 5 minutes after reoxygenation improved viable neuronal density in putamen from 21% in control piglets to 52% in treated piglets [23]. Treatment also reduced the HI-induced increase in phosphorylation at protein kinase C-dependent sites on the NR1 subunit of N-methyl-D-aspartate receptors and on the α -subunit of Na,K-ATPase, partially restored Na,K-ATPase activity, and reduced protein carbonyl formation and tyrosine nitration at 3 hours after reoxygenation. HET0016 also reduced superoxide generation after oxygen-glucose deprivation in cultured hippocampal slices [18]. Thus, early administration of HET0016 likely attenuates post-ischemic excitotoxicity and oxidative stress for this critical 3-hour period until induction of hypothermia, which is capable of ameliorating oxidative stress in this model [32].

Delayed hypothermia also significantly increased the density of viable neurons in the caudate nucleus. However, early treatment with HET0016 did not significantly augment the hypothermic protection. Treatment with HET0016 alone was previously found to produce a trend for neuroprotection (increase in viable neurons from 69% to 87%) in normothermic piglets [23]. Injury was less severe in caudate nucleus than in putamen in the normothermic

group both in the present and previous work [29], and a ceiling effect may exist in caudate nucleus with the severity of the insult obtained in this experimental model.

In contrast to the rapid neurodegeneration in striatum, the neurodegeneration in primary sensorimotor cortex and thalamus is delayed in this model. The majority of cortical neurons display ischemic morphology between 1 and 2 days, whereas apoptotic morphology is often seen in thalamic sensory nuclei between 2 and 4 days. Because of this relatively delayed neurodegeneration, we anticipated that initiating hypothermia at 3 hours of reoxygenation would be highly effective in protecting these regions. However, the neuroprotection from hypothermia alone was incomplete, and early administration of HET0016 was capable of augmenting hypothermic neuroprotection in these regions. These findings suggest that 20-HETE-dependent signaling pathways are already being recruited in the first 3 hours after reoxygenation and that hypothermia can then continue to suppress these upstream pathways or perhaps blunt engagement of downstream effectors of caspase-dependent and independent cell death [38]. Alternatively, one day of hypothermia may temporarily arrest cell death signaling, which then resumes in some neurons after rewarming. Thus, a limitation of the present study is that we cannot exclude the possibility that 3 days of therapeutic hypothermia, as typically used clinically [1,2], would have provided more robust neuroprotection.

Whereas delayed neurodegeneration in the CA1 region of hippocampus after global ischemia is a characteristic feature in adult brain, this phenomenon is not prominent in developing rodent or piglet brain [39,40]. The present study confirmed greater neuronal preservation in the CA1 region and dentate gyrus than in the CA3 region. Moreover, we found that delayed hypothermia partially protected the CA3 region but that early treatment with HET0016 did not significantly augment the hypothermic protection. Likewise, early treatment with an N-methyl-D-aspartate receptor antagonist failed to augment neuroprotection afforded by delayed hypothermia in the CA3 region in preterm fetal sheep [41]. HET0016 is capable of partially protecting rodent hippocampal slice cultures from oxygen-glucose deprivation [18]. If cell death signaling is sufficiently delayed in hippocampus, then the 3-hour delay in hypothermia might suppress the 20-HETE-dependent cell death signaling cascade and thereby account for the lack of an additive effect of HET0016 with hypothermia.

Hippocampal injury in vivo may also be sensitive to delayed occurrence of seizure activity. Although HET0016 plus hypothermia prevented the appearance of clinical seizures, we cannot exclude the presence of subclinical electrical seizures that could contribute to the residual CA3 damage after rewarming. Nevertheless, because clinical neonatal seizures are often refractory to anti-epileptic medications, the robust reduction in the incidence of clinical seizures with the combination of HET0016 and hypothermia could have important clinical significance. Because piglets treated with HET0016 plus hypothermia did not require phenobarbital treatment, barbiturate neuroprotection does not explain the greater number of surviving neurons in this treatment group. The 5–10 mg/kg cumulative doses of phenobarbital used in the other HI groups were relatively modest and unlikely to cause significant depression of cardiac function or arterial pressure.

Limitations of the study

This study has other limitations in addition to those discussed. The dose of 1 mg/kg of HET0016 was based on previous work that increasing the dose to 10 mg/kg provided no additional benefit [23]. Conceivably, the optimal dose for augmenting hypothermic protection may differ. Moreover, the time window of opportunity of HET0016 administration was not assessed with different delays in drug administration and in hypothermia onset. The additional benefit of HET0016 administration compared to hypothermia alone was not large in some brain regions; this additional benefit could become non-significant with modest delays in HET0016 administration or could become augmented with longer delays in hypothermia onset. These issues were not addressed because the purpose of the study was to provide a proof of concept that early administration of a 20-HETE inhibitor could suppress the injury process until hypothermia was instituted at a clinically relevant delay and thereby provide additional neuroprotection. Because neurologic and cognitive deficits can persist into childhood despite treatment with hypothermia [5,6], another key issue is the impact of the combination of drug therapy with hypothermia on functional outcome later in life. Whether the improved neuronal viability seen at 10 days in piglets with combination therapy translates into better motor and cognitive function later in development was not assessed in our study.

At high concentrations, HET0016 may exert inhibitory effects on epoxigenase activity [22], although this effect was not observed in brain with the 1 mg/kg dose [35]. Another limitation is that we did not measure changes in 20-HETE concentrations in brain after HI. Because we cannot exclude that hypothermia itself would decrease 20-HETE, the additive effect of HET0016 with hypothermia does not necessarily imply that HET0016 and hypothermia act by independent mechanisms. For example, early administration of HET0016 could reduce 20-HETE formation that is then sustained by prolonged hypothermia. In addition, the study was limited to males, and possible sex-dependent differences were not explored.

Relation to other combination therapies with hypothermia

Other strategies have been used to augment the efficacy of hypothermia in other large animal perinatal HI models. Inhalation of 50% xenon for 18 hours starting at 30 minutes of recovery provided additive neuroprotection with 12 or 24 hours of hypothermia initiated soon after resuscitation [42]. Melatonin is protective in a fetal sheep model [43], and infusion of melatonin starting at 10 minutes after reoxygenation from HI in piglets provided additional neuroprotection with 24 hours of hypothermia initiated at 2 hours of recovery [44]. Erythropoietin, which has long-term benefits in a rodent model of neonatal stroke [45], was found to improve neurologic outcome in non-human primates when treatment at 30 min after umbilical cord compression and delivery was combined with hypothermia compared to hypothermia alone [46]. In contrast, infusion of the antioxidant EUK-134, which by itself provided partial neuroprotection when infused 30 minutes after resuscitation from hypoxia plus asphyxia [47], had no additive effect with hypothermia initiated at 4 hours after reoxygenation [29]. The present study is unique in demonstrating a pharmacological agent that renders an additive neuroprotective effect when hypothermia is delayed as long as 3 hours after an HI insult. Although combined therapy did not completely protect all neurons

and the additional protection was modest or non-significant in some regions, the results do provide a proof of concept that additional neuroprotection is feasible when hypothermia is delayed by 3 hours after the insult. Thus, our results suggest that early use of a 20-HETE inhibitor might offer one strategy to improve the clinical efficacy of delayed hypothermia after global ischemic insults in the developing brain.

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Figure 1.

Arterial PO₂ (A) and glucose concentration (B) at baseline, during 45 minutes of hypoxia (inspired O₂ of 10%), at 5 minutes of ventilation with 21% O₂, at 6 minutes of the 7-minute period of asphyxia (Asph), and during 24 hours of recovery in groups with normothermia (n = 6), hypothermia started at 3 hours (n = 12), and HET0016 injected at 5 minutes plus hypothermia started at 3 hours (n = 12). Values are also shown at equivalent times in the sham-operated group (n = 6). Note x-axis break with change in the time scale after 30 minutes of recovery. Increase in PO₂ at 5 minutes of recovery is related to brief increase in inspired O₂ to 50% before returning to 30%. Values are means ± SD. No significant differences were present among the three hypoxia-ischemia (HI) groups. Compared to sham values, glucose was significantly increased in the three HI groups during asphyxia and 5 minutes of recovery and in the HET0016-treated group through 30 minutes of recovery.

Figure 2.

Arterial pH (**A**) and PCO₂ (**B**) at baseline, during 45 min of hypoxia (inspired O₂ of 10%), at 5 min of ventilation with 21% O₂, at 6 min of the 7-min period of asphyxia (Asph), and during 24 h of recovery in groups with normothermia (n = 6), hypothermia started at 3 h (n = 12), and HET0016 injected at 5 min plus hypothermia started at 3 h (n = 12). Values are also shown at equivalent times in the sham-operated group (n = 6). Note x-axis break with change in the time scale after 30 min of recovery. Values are means ± SD. No significant differences were present among the three HI groups.

Figure 3.

Mean arterial blood pressure at baseline, during 45 min of hypoxia (inspired O₂ of 10%), at 5 min of ventilation with 21% O₂, 7 min of asphyxia (Asph), and 24 h of recovery in groups with normothermia (n = 6), hypothermia started at 3 h (n = 12), and HET0016 injected at 5 min plus hypothermia started at 3 h (n = 12). Values are also shown at equivalent times in the sham-operated group (n = 6). Note x-axis break with change in the time scale after 30 min of recovery. Values are means $\pm$ SD. Arterial pressure decreased during asphyxia and briefly increased after resuscitation to similar extents in the three HI groups.

Figure 4.

Rectal temperature at baseline and during hypoxia, asphyxia, and 10 days of recovery in groups with normothermia ($n = 6$), hypothermia started at 3 h ($n = 12$), and HET0016 injected at 5 min plus hypothermia started at 3 h ($n = 12$). Values are also shown at equivalent times in the sham-operated group ($n = 6$). Note x-axis break with change in the time scale after 25 h of recovery. Temperature in hypothermic groups was decreased to approximately 34°C after 3 h and remained stable until rewarming started at 20 h. Values are means $\pm$ SD.

Figure 5.

Immunohistochemical staining for cytochrome P450 4A with diaminobenzidine chromogen shows staining of cells with neuronal morphology in putamen, cerebral cortex, CA3 region of hippocampus, and ventral posterolateral (VPL) thalamus. Scale bar = 40 μ m.

Figure 6.

Representative photomicrographs of hematoxylin-and-eosin–stained sections of putamen, sensorimotor cerebral cortex, the hippocampal CA3 area (CA3), and the ventral posterolateral nucleus (VPL) of the thalamus in piglets subjected to sham surgery or hypoxia-ischemia (HI) and recovery with normothermia (NT), hypothermia (HT), or HT plus HET0016 treatment. Arrows point to representative ischemia-damaged neurons with shrunken nuclei, condensed chromatin, and eosinophilic cytoplasm. Arrowheads show viable neurons with large nuclei and small nucleolus. HT and HT plus HET0016 treatment preserved many viable neurons in each region. Scale bar = 40 μ m.

Figure 7.

Density of viable neurons in putamen, caudate nucleus, sensorimotor cortex, ventral posterolateral nucleus (VPL) of thalamus, and CA3, CA1, and dentate gyrus region of hippocampus after sham surgery ($n = 6$) or HI followed by normothermia ($n = 6$), hypothermia ($n = 12$), or HET0016 plus hypothermia ($n = 12$). Values are means $\pm$ SD. * $P < 0.05$ compared to the sham group; † $P < 0.05$ compared to the HI-normothermia group; ‡ $P < 0.05$ compared to the HI-hypothermia group.

Table 1

Intensive care management variables

Parameter	Sham	HI+ Normothermia	HI+ Hypothermia	HI+HET0016+ Hypothermia
Cumulative epinephrine dose during resuscitation ($\mu\text{g/kg}$)	NA	187 $\pm$ 62	180 $\pm$ 44	178 $\pm$ 51
Duration of cardiopulmonary resuscitation (s)	NA	55 $\pm$ 7	48 $\pm$ 13	52 $\pm$ 10
Duration of mechanical ventilation after cessation of vecuronium (h)	2.1 $\pm$ 0.5	2.3 $\pm$ 0.7	2.3 $\pm$ 0.6	2.5 $\pm$ 0.7
Time to feed independently after extubation (h)	5.3 $\pm$ 2.4	14.3 $\pm$ 3.7*	19.2 $\pm$ 9.0*	19.8 $\pm$ 7.6*
Pre-arrest body weight (kg)	1.8 $\pm$ 0.3	2.1 $\pm$ 0.3	2.0 $\pm$ 0.3	2.2 $\pm$ 0.4
Change in body weight at 10 days (kg)	2.2 $\pm$ 0.1	1.1 $\pm$ 0.1*	1.3 $\pm$ 0.5*	1.4 $\pm$ 0.2*
Cumulative dose of phenobarbital (mg/kg)	0 $\pm$ 0	5.7 $\pm$ 6.5	3.4 $\pm$ 5.0	0 $\pm$ 0
Number of long-term survivors/total treated	6/10	6/12	12/16	12/14

Values are means $\pm$ SD. NA = not applicable.

* $P < 0.05$ compared to sham; no significant differences were present among hypoxia-ischemia (HI) groups.