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Rapid Alterations in Cerebral White Matter Lipid Profiles After Ischemic-Reperfusion Brain Injury in Fetal Sheep as Demonstrated by MALDI-Mass Spectrometry

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Abstract

Background—Perinatal ischemia-reperfusion (I/R) injury of cerebral white matter causes long-term cognitive and motor disabilities in children. I/R damages or kills highly metabolic immature oligodendroglia via oxidative stress, excitotoxicity, inflammation, and mitochondrial dysfunction, impairing their capacity to generate and maintain mature myelin. However, the consequences of I/R on myelin lipid composition have not been characterized.

Objective—This study utilized matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) to assess alterations in cerebral supraventricular white matter myelin lipid profiles in a fetal sheep model of perinatal I/R.

Methods—Fetal sheep (127 days gestation) were studied after 30 minutes of bilateral carotid artery occlusion followed by 4 (n = 5), 24 (n = 7), 48 (n = 3), or 72 (n = 5) hours of reperfusion, or sham treatment (n = 5). White matter lipids were analyzed by negative ion mode MALDI-MS.

Results—Striking I/R-associated shifts in phospholipid and sphingolipid expression occurred over the 72-hour time course with most responses detected within 4 hours of reperfusion and

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Author Contributions

Gina M Gallucci processed the brain samples for MALDI, performed bioinformatics analysis of the results, helped prepare the tables and graphs, and reviewed the manuscript. Ming Tong organized and guided the brain sample processing for MALDI and reviewed the manuscript. Xiaodi Chen generated the experimental model, provided critical results about the experimental model, and reviewed the manuscript. Barbara S Stonestreet designed the in vivo experimental model and critically reviewed the manuscript. Suzanne M de la Monte conceived of the collaborative research, directed and supervised the collection of primary data including sample analysis, analyzed the data, and wrote the manuscript.

Declaration of Conflicting Interests

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Supplementary Material

Supplementary material for this article is available Online.

progressing at the 48- and 72-hour points. I/R decreased expression of phosphatidic acid and phosphatidylethanol amine and increased phosphatidylinositol, sulfatide, and lactosylceramide.

Conclusions—Cerebral I/R in mid-gestation fetal sheep causes rapid shifts in white matter myelin lipid composition that may reflect injury, proliferation, or recovery of immature oligodendroglia.

Keywords

ischemia reperfusion; white matter; fetal brain; sheep; lipidomics; mass spectrometry

Introduction

Perinatal hypoxic-ischemic cerebral injury in infants is the leading cause of long-term neurocognitive, motor, and neurosensory impairments and cerebral palsy in children.¹ The 2 sequential stages of hypoxic-ischemic injury are ischemia and reperfusion. Ischemic injury with attendant necrosis is caused by critical reductions in blood flow leading to inadequate delivery of oxygen and nutrients to support cellular energy metabolism and viability. Reperfusion injury is mediated by flooding of injured tissue with oxygenated blood and nutrients vis-à-vis local tissue breakdown and increased oxidative stress.² Mitochondrial dysfunction intensifies reperfusion injury because the associated calcium dyshomeostasis triggers mitochondrial permeability transition and overproduction of reactive oxygen species (ROS).³ Increased generation of ROS contributes to lipid and protein damage, activates astrocyte and microglial derived inflammatory mediators,⁴ and transduces proapoptosis cascades in neurons, glia, and vascular elements.²

Repeated and sustained hypoxic-ischemic insults cause laminar necrosis within the cerebral cortex, and central white matter injury ranging from myelin loss to coagulative necrosis.⁴ In extreme cases, white matter injury in premature neonates may advance to periventricular leukomalacia.¹ Immaturity or pathophysiological damage to mechanisms needed to autoregulate cerebral blood flow contribute to the pathogenesis of periventricular white matter reperfusion injury.⁵ Significant reductions in morbidity from perinatal white matter hypoxic-ischemic-reperfusion injury will require approaches that support energy metabolism and optimize neuroprotection.⁴ Refinements in our understanding of target cell responses to hypoxic-ischemic injury and subsequent long-lasting effects on the structural and functional integrity of developing white matter are needed and could be aided by new diagnostic and therapeutic strategies.

White matter is composed of axons, oligodendrocytes, astrocytes, microglia, and blood vessels. Oligodendrocytes, the myelin-producing cells of the central nervous system (CNS), are especially vulnerable to hypoxic, ischemic, and reperfusion injury. Ischemic injury to white matter causes progressive loss of oligodendrocytes and degeneration of myelin. Loss of myelin impairs nerve impulse conductivity and renders exposed axons susceptible to ischemic, metabolic, and reperfusion injury. Immature oligodendrocytes are highly susceptible to ischemia-reperfusion (I/R) injury due to high metabolic demands. Combined effects of oxidative stress, excitotoxicity, inflammation, and mitochondrial dysfunction impair oligodendrocyte viability, proliferation, myelin synthesis, maturation, and myelin

maintenance. Although oligodendrocyte precursor cells (OPCs) proliferate in response to hypoxic-ischemic injury and could potentially replace lost oligodendrocytes, OPCs may fail to mature and adequately remyelinate axons.⁶ However, remyelination and restoration of function can be achieved via recovery of surviving oligodendrocytes.⁷

Preventing long-term neurological sequelae of perinatal hypoxic-ischemic white matter injury will require restoration of oligodendrocyte functions, including myelin maintenance. Although progress has been made toward understanding effects of I/R on immature white matter oligodendroglia,⁶ little is known about the nature and degree of white matter lipid abnormalities, or the functional modifications that would be needed to restore proper myelination of white matter axons in the developing brain. Since myelin is composed of 70% lipid, and recent studies demonstrated that myelin lipid abundance and composition shift with white matter injury,⁸ we hypothesized that I/R in the immature brain would be marked by altered myelin lipid composition, which could reflect injury to oligodendroglia. In this study, we utilized matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to characterize alterations in cerebral white matter lipids using an established mid-gestation fetal sheep model of I/R.⁹

Methods

Experimental Model

Surgery was performed on mixed breed pregnant ewes at 118 to 122 days of gestation under 1% to 2% isoflurane anesthesia as previously described.^{9,10} At 125 to 130 days of gestation, 20 fetal sheep were subjected to cerebral I/R for 30 minutes followed by 4 (I/R-4; n = 5), 24 (I/R-24; n = 7), 48 (I/R-48; n = 3), or 72 (I/R-72; n = 5) hours of reperfusion (Table 1). Five additional fetal sheep served as sham-instrumented controls.

Immediately before sacrifice by intravenous injection of pentobarbital (100–200 mg/kg), fetal mean arterial blood pressure was measured and arterial blood was obtained to measure standard physiological parameters (Supplementary Table 1). Postmortem samples of supraventricular and intragyral cerebral white matter were frozen in liquid nitrogen and stored at –80°C. At the time of sacrifice, the fetal sheep were 80% to 85% of full gestation, that is, similar to near-term human infants.¹¹ This research was approved by the Institutional Animal Care and Use Committees of the Alpert Medical School of Brown University and the Women & Infants Hospital of Rhode Island.

MALDI Plate Assay

Lipids extracted from fresh frozen white matter (50 ± 5 mg) by the Folch method¹² were solubilized in methanol, mixed with 2,5-dihydroxybenzoic acid (DHB; Sigma Aldrich, St. Louis, MO) as matrix,¹³ and spotted in duplicate into a 384-well ground steel MALDI target plate (Bruker Daltonics, Bremen, Germany) along with mass calibration standards (Peptide Calibration Standard II, Bruker Daltonics). The samples were analyzed in the negative ion mode with an Ultraflextreme MALDI-time-of-flight (TOF)/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer as described.^{13,14}

Data Analysis

MALDI data were processed using FlexAnalysis v3.4 (Bruker Daltonics, Billerica, MA) and visualized with FlexImaging software v4.0 (Bruker Daltonics, Billerica, MA). Results were analyzed using ClinProTools v3.0 (Bruker Daltonics, Billerica, MA). Lipids were identified using LIPID MAPS (<http://www.lipidmaps.org/tools/index.html>) and confirmed by tandem mass spectrometry (MS/MS) in the LIFT-TOF/TOF mode.¹⁵ The multiplot function from the R software scatter package (Version 3.2.2) was used to align plots across experimental groups. Intergroup comparisons were displayed using data bar plots (Microsoft Excel 2016 Conditional Formatting) and a heatmap. For the data bar plots, intergroup differences were analyzed using T tests with a 5% false discovery rate (Graphpad Prism 7, La Jolla, CA). For the heatmap, lipid profiles were analyzed using Cluster 3.0.¹⁶ Hierarchical clustering was applied, and the dendrogram was displayed using Java TreeView.^{16–18} Intergroup differences were compared by 1-way analysis of variance (ANOVA) and the post hoc Tukey repeated measures test. χ^2 tests assessed proportional alterations in lipid subtypes after different durations of I/R (Graphpad Prism 7, La Jolla, CA).

Results

Characteristics of the Experimental Model

Pregnancy in sheep lasts 150 days or 5 months. Most of the fetal growth occurs within the final 60 days of gestation. Therefore, the ischemia-reperfusion (I/R or IRRI) models were generated at the mid-point of the third trimester of pregnancy. The mean gestational ages, fetal body weights, and brain weights of the control and experimental groups did not differ significantly by 1-way ANOVA tests (Table 1). In addition, mean arterial PO₂, PCO₂, base excess, blood pressure, hematocrit, and glucose did not differ significantly among the groups (Supplementary Table 1). Therefore, the groups were well-matched for subsequent comparisons.

Lipid Ion Profiles

The Peak Statistic report identified 68 fetal sheep white matter lipids that had mass/charge (m/z) ratios between 627.6 and 914.7 Daltons. The lipids were identified using the LIPID MAPS database and published literature and are listed categorically as well as in ascending m/z order in Supplementary Table 2(A) and (B). The lipids were categorized as (1) sphingolipids (n = 15; 22.1%), including 13 (19.1%) sulfatides (STs) and 2 (2.9%) glycosphingolipids (lactoceramides); (2) phospholipids (n = 45; 66.2%), including 6 (8.8%) phosphatidic acids (PAs), 1 (1.5%) phosphatidylcholine (PC), 12 (17.6%) phosphatidylethanolamines (PEs), 3 (4.4%) phosphatidylglycerols (PGs), 16 (23.5%) phosphatidylinositides, and 9 (13.2%) phosphatidylserines (PS); (3) 1 (1.5%) diacylglycerol (DG); or (4) unknown/unidentified (n = 5; 7.4%). C13-isotopes of STs and phospholipids were also detected and grouped accordingly. Overall, phosphatidylinositol (PI) and ST (~20%) were most abundantly expressed, followed by PS (~14%) (Supplementary Figure 2). PE, PA, other uncharacterized phospholipids, and unknown lipids comprised the middle group in which each comprised 8% to 11% of the expressed lipids. Ceramides, plasmalogen ethanolamine (PlsEtn), and PG each represented ~3% of the lipid population. In general, ceramides, PCs, sphingomyelins, and cholesterol were not detected because in

general, they are accessible via positive ionization mode mass spectrometry.¹⁹ To avoid redundant representation of lipid data, the C13 isotopes were excluded from the statistical analyses.

I/R Effects on White Matter Lipid Profiles Demonstrated by PCA and χ^2 Tests

To determine how I/R injury altered white matter lipid profiles, that is, distributions, the groups were compared based on the percentages of each lipid subtype expressed (Supplementary Table 3, Supplementary Figure 3) and principal component analysis (PCA) plots (Figure 1). With the lipids subcategorized broadly, there were no differences in the expression profiles associated with I/R injury relative to control, regardless of the duration of reperfusion (Supplementary Table 3).

Next, we determined if I/R caused significant loss or de novo expression of specific lipids by calculating the percentages of cases/group that had detectable (above threshold) expression of each of the 68 lipids and depicted the results with a heatmap (Supplementary Figure 3). Twenty-five lipids (36.8%) were detected in all samples, irrespective of I/R duration. Two (2.9%) were detected only in the I/R samples, that is, they were expressed de novo. Of the remaining 41 (60.3%) lipids, 14 (20.6%) were expressed at lower frequencies, 22 (32.4%) at higher frequencies, and 5 (7.4%) at similar frequencies in the I/R groups relative to control. Furthermore, 9 of the lipids that were expressed at reduced frequencies immediately after I/R, increased and normalized or were expressed in higher proportions of I/R relative to control brains, whereas 5 remained low in frequency of detection. Among the 22 lipids expressed at higher rates in I/R samples, 17 continued to be expressed in higher percentages of the I/R cases relative to control, and 5 declined in frequency over the time course of I/R. χ^2 tests demonstrated significant intergroup differences in the percentages of brains that expressed DG(40:2), PA(36:2), and PC(28:2), and trend effects ($.05 < P < .10$) for PE(33:3), PE(31:3), PG(28:2), ST(36:1)(2OH), and 1 unidentified lipid (m/z 940.69). Therefore, I/R led to either de novo or abolished expression of relatively few lipid ions and mainly after 48 or 72 hours of reperfusion (Supplementary Figure 3).

In contrast, the 3-dimensional and 2-dimensional PCA plots, which compared the full spectra of white matter lipids expressed in each group, revealed striking effects of I/R relative to sham-manipulated controls (Figure 1). Irrespective of I/R duration, the lipid profile clusters were distinct and exhibited only modest overlap with controls. Therefore, the intergroup differences detected by PCA suggested that I/R mainly altered the levels of lipid ion expression rather than the presence or absence.

Heatmap Analysis of Lipids Expressed in Relation to I/R Duration

A Java TreeView-generated heatmap with hierarchical clustering was used to demonstrate time-dependent effects of I/R on the relative levels of expressed white matter lipids (Figure 2). Lipids that were not detected in all groups, that is, de novo expressed or lost due to I/R, were excluded from the heatmap. Three main clustered responses were identified in control white matter: (1) the uppermost cluster was associated with abundant lipid expression; (2) the middle cluster had intermediate levels of lipid expressed; and (3) the lowest cluster had very low levels of lipid expression. I/R resulted in immediate and sharp reductions in the

expression of lipids in cluster A and variable degrees of recovery over time. For cluster B, lipid ion expression was only weakly modulated relative to control over the first 24 hours of reperfusion, but at the 48- and 72-hour time points, lipid expression increased and reached peak levels at 72 hours. For cluster C, lipid expression progressively increased over the time course of reperfusion, with peak levels reached mainly at the 48- or 72-hour time points. These findings indicate that I/R dynamically altered the relative abundances of lipid subtypes in white matter. One-way ANOVA tests revealed significant ($P < .05$ or better) or trend-wise ($.10 < P < .05$) I/R-associated differences in the mean levels of 21 lipids. Further comparisons were made using data bar plots.

Data Bar Displays of the Relative Effects of I/R on Lipid Ion Abundance

To assess the effects of I/R on lipid expression, mean peak intensities (reflecting lipid abundance) were compared by *T* test analysis with a 5% false discovery rate. However, to compare effects of I/R duration across the full spectrum of lipids expressed, the calculated percentage differences in mean expression relative to control are displayed with bar plots in which results are sorted from lowest to highest *m/z* (Supplementary Figure 4). Bars to the left of the median axis reflect I/R-associated reductions in lipid expression, and those to the right indicate I/R-mediated increases in lipid expression. Based on this overall analysis, the most striking effects of I/R were to decrease expression of a high proportion of lipids with *m/z*'s under 775 and increase expression of most lipids with *m/z*'s above 800. However, those responses varied with the time course of I/R. At 4 hours, 95% of the low *m/z* lipids were reduced relative to control, whereas at the 24-hour point, only 40% were reduced, and at the 48- and 72-hour time points, 50% to 60% of the lipids were increased relative to control. With respect to the higher *m/z* lipids, the responses were more consistent over the time course such that I/R increased most lipids, and the responses were greater with longer durations of I/R. To gain a better appreciation for how different lipid subtypes were modulated by I/R duration, the bar plots were clustered by lipid class and then sorted by ascending *m/z* (Figure 3).

Among the 5 PA lipids identified, 4 were reduced by at least 20% relative to control in the I/R-4, I/R-48, and I/R-72 groups. However, in the I/R-24 group, the responses were relatively modest in that 2 lipids modulated and 1 other that was unchanged by I/R-4, I/R-48, or I/R-72 were just modestly (less than 12%) reduced. Nonetheless, the dominant overall of I/R was to inhibit PA expression.

Among the 6 PE lipids, all were reduced in expression by I/R-4, 5 were reduced by I/R-48, and 4 by I/R-72. As observed for PA, the I/R-24 responses were heterogeneous in that 2 lipids were reduced, 2 were unchanged, and 2 were increased relative to control. In regard to the 2 plasmalogen ethanolamine species detected, PlsEtn(36:1) progressively declined while PlsEtn(40:7) progressively increased in expression with longer durations of I/R.

Just 2 PG lipids were identified, and neither was significantly modulated by I/R except for an approximately 40% increase in PG(27:1) in the I/R-72 group.

Fourteen PIs, including 2 C-13 isotopes, were detected. The main effect of I/R was to progressively increase PI expression with longer durations of reperfusion. In the I/R-4

samples, 11 PIs were increased by 6% to 25% and 3 were reduced by 6% to 16% relative to control. For I/R-24, 10 PIs were increased by 7% to 27% and 4 were unchanged relative to control. For I/R-48, 11 PIs were increased by 7% to 39% and 3 were unchanged relative to control and in the I/R-72 group, 12 PIs were increased by 10% to 69% and 2 were unchanged relative to control.

The effects of I/R on PS expression were heterogenous compared with PA, PE, PG, and PI. Consistent trend responses occurred with respect to PS(38:5) and PS(40:6) which progressively increased over the I/R time course, and PS(38:4), which was reduced at all I/R time points relative to control. In contrast, mixed directional and generally modest alterations in PS expression were observed for the other 6 PS lipids.

Seven phospholipids that could not be specifically classified due to isobaric m/z assignments also showed mixed or modest to nil I/R-associated responses. Exceptions included phospholipid m/z 884.7 which progressively increased in expression with I/R duration and m/z 697.4 which was consistently reduced in all I/R groups relative to control. However, 4 of the phospholipids in this group were expressed at higher levels in the I/R-4 and I/R-24 groups, but their levels declined at the 48- and 72-hour time points, suggesting early transient responses that subsequently normalized.

Two lactosylceramides were detected. Both were expressed at near-control levels at the 4- and 24-hour time points but increased progressively in I/R-48 and I/R-72 brain samples.

Thirteen STs, including 1 C-13 isotope, were detected. In the I/R-4 group, 8 STs were increased by at least 10%, 2 were inhibited by 8% or 13%, and 3 were unchanged relative to control. In the I/R-24 samples, 8 STs were increased, 3 were inhibited, and 2 were unchanged. In the I/R-48 and I/R-72 samples, the effects of I/R were nearly identical in that the 9 STs were increased, 2 were inhibited, and 2 were unchanged relative to control. Overall, 7 STs were globally increased in expression throughout the time course of study.

Finally, among the 6 lipids that could not be identified from the Lipid Maps database or published literature, only 1 (m/z 940.7) exhibited consistently reduced expression, whereas the others varied in their responses to I/R over the time course of investigation.

The data bar results were further consolidated and summarized using χ^2 tests to demonstrate significant differential effects of I/R on lipid ion expression at each time point (Figure 4). The solid bars show the percentages of each lipid subtype that increased, and the striped bars show the percentages that decreased after 4 (Figure 4(A)), 24 (Figure 4(B)), 48 (Figure 4(C)), or 72 (Figure 4(D)) hours or reperfusion. The main overall effects were that (1) the expression of 50% or more of the specific lipids identified among 7 of the 10 lipid classes declined after 30 minutes of ischemia followed by 4 hours of reperfusion, and increased in 50% or more of the PG, PI and ST lipids; (2) over time, the proportions of each lipid subtype that increased in expression also increased, whereas the proportions of lipids that declined in expression relative to control, decreased sharply between I/R-4 and I/R-24, but then increased to similar degrees at the I/R-48 and I/R-72 time points; and (3) the overall trend was to increase expression of most lipid subtypes with longer durations of I/R.

Discussion

The application of MALDI-TOF lipidomics to study experimental and human diseases is relatively new. Recent technological advances that enable high throughput sample processing have expanded access and interest in using MALDI as an investigational and diagnostic tool. In contrast to proteins, the molecular regulation of specific lipids for biosynthesis, structure, function, and turnover is not well understood, and despite detailed knowledge of their structures, for the most part, functional assignments have not been made. However, it is likely that lipids within a given class share similar functions and therefore modulations in their expression levels would likely be clustered as observed in this study. Unlike proteins and mRNA transcripts, disease-linked fold changes in the expression of specific lipids is seldom observed. However, fold differences in the clustered or aggregate responses of 5 to 20 related lipids occur frequently. Altogether, such responses should be regarded as important if they consistently mark effects of an experimental or human disease state. More research is needed to better understand the functional roles of specific lipids and the consequences of their changes in the expression in relation to disease.

During the perinatal period, cerebral white matter in preterm and full term infants is especially vulnerable to injury due to high metabolic demands of oligodendroglia undergoing maturation and myelin synthesis. In the vast majority of cases, perinatal white matter injury is triggered by hypoxic, ischemic, and reperfusion-related insults. The long-term consequences include deficits in cognitive and motor functions, often attributed to cerebral palsy. Mechanistically, damage to oligodendrocytes impairs myelin homeostasis and the integrity of lipid-rich myelin sheaths that enable rapid and efficient neuroconductivity. To better understand the effects of acute I/R injury on myelin lipid composition and ultimately myelin integrity, we used MALDI mass spectrometry to examine shifts in myelin lipid profiles that occur over a 72-hour period after ischemia and reperfusion in fetal sheep.^{9–11} We hypothesized that both injury to oligodendrocytes and the proliferation of immature oligodendroglia following I/R⁶ mediate substantial alterations in white matter myelin lipid composition. The characterization of I/R-related abnormalities may help direct future research for therapeutic targeting in humans.

The major CNS white matter lipids include cholesterol, glycosphingolipids, ST, gangliosides, and phospholipids which consist of glycerophospholipids (PA, PC, PE, PG, PI, PS, and plasmalogens) and sphingomyelin.²⁰ Sphingomyelin is composed of ceramide plus a phosphocholine or phosphoethanolamine polar head group.²⁰ Although a broad range of CNS diseases have been linked to abnormal metabolism and expression of phospholipids and STs,²¹ the mechanisms and consequences of their altered expressions are not well understood. Long-term outcomes of dysregulated lipid metabolism may correspond with functional impairments in the affected lipid. This study employed negative ionization mode MALDI-MS which is primarily suited for detecting glycosphingolipids, ST, PA, PE, PG, PI, and PS, rather than ceramide and PC. Therefore, the data analysis was focused on I/R duration effects on myelin lipids that could be detected and quantified in fetal cerebral white matter with those technical limitations.

Review of the histopathological sections obtained from earlier identical studies (Supplementary Figure 1) demonstrated reductions in Luxol fast blue (LFB) staining of white matter, indicating reduced myelin content within 48 hours of I/R. Recovery of LFB staining was not detected over the 72-hour time course.⁹ Instead, reactive inflammatory and astrocytic cellular responses associated with injury but also needed for recovery increased from 48 to 72 hours after I/R. The MALDI MS studies were performed with lipids extracted from white matter, the majority of which likely originated from oligodendrocyte membranes because oligodendrocytes are the most abundant cell type in white matter. Correspondingly, the percentages of most lipid subtypes did not change over the time course of study, and just a few lipids ceased to be expressed or arose de novo after different durations of I/R. De novo lipid expression may have been due to early tissue infiltration by inflammatory cells, activation of astrocytes, or proliferation of immature oligodendroglia. Loss of specific lipids may have reflected injury responses in maturing oligodendrocytes.

Despite modest overall changes in white matter lipid composition, the PCA plots demonstrated substantial I/R-associated shifts in lipid expression. Therefore, further studies assessed I/R-mediated changes in the expression levels of lipids according to their subtypes and masses using heatmap and data bar plots. The heatmap revealed clustered responses to I/R, such that the expression levels of some lipids were downregulated, whereas others were upregulated, either progressively or mainly within the last 48 to 72 hours of reperfusion. Alignments of the data bar plots showed broadly reduced expression of low m/z lipids after short durations of I/R and partial reversal or normalization of lipid expression with longer durations of I/R. In contrast, the expression higher m/z lipids tended to increase with duration of I/R. Further analysis of the lipid subtypes that were altered by I/R was necessary to understand the significance of these findings. Known functions of the lipid subtypes detected in sheep white matter by MALDI-MS in the negative ion mode are summarized in Supplementary Table 4.

Most of the PAs detected were inhibited by I/R such that their expression levels were similarly reduced at the 4-, 48-, and 72-hour time points. The immediate responses at I/R-4 could have been due to declines in PA levels in previously intact membranes, whereas later responses may have been mediated by shifts in gene expression, or limited substrate availability. PAs are important intermediates in lipid metabolism and precursors of all membrane glycerophospholipids.²² PAs are synthesized by phospholipase D hydrolysis of PC, phosphorylation of DG, and acylation of lysophosphatidic acid. In addition, PAs are used in the synthesis of PEs and PCs via DG, and they serve as essential substrates for enzymes that promote synthesis of triacylglycerols and glycerophospholipids.²³ In addition, PAs regulate signal transduction, membrane dynamics,²⁴ glycerophospholipid synthesis,²² and activation of the mammalian target of rapamycin complex 1 for promoting cell growth.²⁵

The pronounced inhibition of PA could account for the concomitant reductions on PE, particularly with longer durations of I/R. PEs comprise 45% of all brain white matter phospholipids. Beyond the contribution of PA as a substrate, in mitochondrial membranes, PE is generated by decarboxylation of PS, and in the cytosol and Endoplasmic reticulum (ER), PE is generated from ethanolamine. However, PE is transported throughout the cell for

varied functions in other membranes. Importantly, in mature white matter, over 70% of PEs exist as plasmalogens²⁶ in lipid rafts, and they mediate membrane dynamics, vesicle fusion (fluidity), myelin membrane formation, or maintenance.²⁶ Therefore, I/R inhibition of PE could have profound adverse effects on myelin integrity and intracellular signaling needed to maintain normal oligodendrocyte functions.

In contrast to the predominantly inhibitory effects I/R had on PA and PE, PIs were mainly upregulated, and the magnitudes of those responses tended to increase with duration of I/R. PIs are membrane structural lipids that serve as substrates for lipid kinases that phosphorylate hydroxyl groups and phospholipases, and they generate second messengers for regulating cellular and lipid signaling, and membrane fluidity, trafficking, and permeability.²⁷ Therefore, the striking I/R-associated increases in white matter PI content could impact broad biological functions, including those needed for cell survival, plasticity, growth, and energy metabolism. Conceivably, the I/R-associated increases in PI expression may reflect compensatory responses linked to reactive proliferation and activation of PI3 kinase pathways in oligoprogenitor cells following I/R.⁶

PSs had the most varied responses to I/R in that 2 PS lipids were upregulated, 1 was downregulated and 6 were unchanged by I/R. Although the physiological or pathophysiological consequences of these responses are not known, these major acidic phospholipids are enriched in the inner leaflet of plasma membranes and form parts of protein docking sites needed for the activation of pro-survival and pro-growth networks²⁸ via insulin-like growth factor, type 1 signaling.²⁹ Therefore, I/R-associated changes in PS expression could impact growth and survival of oligodendrocytes. Furthermore, sustained alterations in PS expression, beyond the period of study could affect regulation of neurotransmitter release, white matter myelination,³⁰ and cognitive function.²⁸

Between 61% and 69% of the white matter STs detected were increased by I/R, whereas 15% to 23% of STs were either inhibited or unaffected by I/R. STs are localized on the extracellular leaflet of myelin plasma membranes³¹ and have important roles in neuronal plasticity, memory, myelin maintenance and stability, protein trafficking, adhesion, glial-axonal signaling, insulin secretion, and oligodendrocyte survival.^{21,32} In the mature brain, reductions in membrane ST disrupt myelin sheath structure and function and compromise neuronal conductivity.³³ However, since STs also negatively regulate oligodendrocyte differentiation,²¹ their increased expression could reflect proliferation of immature oligodendroglia, and also may contribute to the impairments in oligodendroglial maturation that occur with white matter I/R.⁶ Furthermore, ST degradation yields ceramides^{31,34} which promote neuroinflammation, ROS formation, apoptosis, and dysregulate signaling through cell survival and metabolic pathways.^{33,35} The progressively upregulated expression of 2 lactosylceramides with duration of I/R may have contributed to the neuroinflammatory and parenchymal destructive responses observed with prolonged I/R.

Conclusions

In conclusion, these studies have identified major rapid shifts in lipid composition of fetal sheep cerebral white matter as a function of reperfusion duration after isolated in utero brain

ischemia. Ischemia/reperfusion in fetal sheep is thought to cause pathophysiological changes in oligodendrocytes, similar to those observed in white matter of preterm infants.^{6,36–38} The timing of injury in preterm infants is difficult to ascertain in clinical settings and often the impact of the injury cannot be determined until weeks or months after the initial insult when cystic or diffuse white matter injury becomes apparent.³⁹ Based upon the rapid shifts in lipid composition that we observed in fetal sheep brains, we speculate that I/R-related white matter injury in neonates begins long before its detection is possible by ultrasound or magnetic resonance imaging, highlighting the need for sensitive noninvasive biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Principal component analysis of white matter lipid profiles. MALDI-IMS (negative ion mode) lipid data (600–1000 Da mass range) were compared between controls (green) and fetal sheep subjected to I/R (red) for 4, 24, 48, or 72 hours. Note the separation of control and I/R clusters at all time points.

Figure 2.

Heatmap illustrating I/R duration effects on lipid ion levels in white matter. Intensities are displayed using a 6-color palette corresponding to z-scores scaled to a mean of 0 and standard deviation of 1.5. Hierarchical clustering dendrograms are shown. Three main clusters (A–C) demonstrate broad and progressive effects of I/R. Significant (red arrows: $P < .05$ or better) or trend (blue arrows: $.10 < P < .05$) effects of I/R relative to control were detected for 21 lipids.

Figure 3.

Data bar plots depict paired comparisons of the mean percentage directional shifts in the expression of 68 lipids in from fetal sheep white matter after 30 minutes of ischemia and 4 (I/R-4), 24 (I/R-24), 48 (I/R-48), and 72 (I/R-72) hours of reperfusion. Blue bars indicate I/R-associated reductions in lipid expression and red bars show increased lipid expression relative to control. Results are grouped by lipid subtype and sorted by ascending m/z. Lipid identities and m/z values are listed to the left. Intergroup comparisons were made using *T* tests with 5% false discovery rates (Yellow = $0.10 < P < 0.05$; Green = $P < 0.05$; Pink = $P < 0.01$; Red = $P < 0.005$; Gray = $P < 0.001$; Navy Blue = $P < 0.0001$). CER, ceramide; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PlsEtn, plasmenylethanolamine; PS, phosphatidylserine; ST sulfatide; UNK, unknown.

Figure 4.

Summary effects of I/R duration on lipid ion expression. I/Rs aggregate effects on the levels of different lipid subclasses relative to control were evaluated using 2 tests. The percentages of PA, PE, PG, PI, PIE, PS, Ph CER, ST, and UNK lipids that were increased (solid bars) or decreased (striped bars) by 5% or more after 30 minutes of ischemia and (A) 4, (B) 24, (C) 48, or (D) 72 hours of reperfusion are depicted graphically. Significant P values are shown in the panels. CER, ceramide; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PlsEtn, plasmeneylethanolamine; PS, phosphatidylserine; ST sulfatide; UNK, unknown.

Table 1.

Characteristics of Fetal Sheep Included in the Cerebral Ischemia/Reperfusion Study.

Study Group	Number	Gestation (Days)	Sex		Fetal Body Weight (kg)	Fetal Brain Weight (g)
			Male	Female		
Control	5	129 ± 2	3	2	3.3 ± 0.8	42.7 ± 9.6
I/R-4	5	126 ± 1	1	4	3.0 ± 0.5	40.6 ± 1.8
I/R-24	7	128 ± 2	4	3	3.0 ± 0.5	39.7 ± 5.7
I/R-48	3	129 ± 1	2	1	3.0 ± 0.4	39.1 ± 5.5
I/R-72	5	129 ± 1	3	ND	3.2 ± 0.3	41.6 ± 6.3

Abbreviations: I/R, ischemia/reperfusion; ND, not determined; SD, standard deviation.

Values are mean SD. The I/R groups were subjected to cerebral ischemia for 30 minutes followed by reperfusion for 4, 24, 48, or 72 hours. Control sheep had sham procedures.