Potential Adverse Effects of Prolonged Sevoflurane Exposure on Developing Monkey Brain: From Abnormal Lipid Metabolism to Neuronal Damage

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ABSTRACT

Sevoflurane is a volatile anesthetic that has been widely used in general anesthesia, yet its safety in pediatric use is a public concern. This study sought to evaluate whether prolonged exposure of infant monkeys to a clinically relevant concentration of sevoflurane is associated with any adverse effects on the developing brain. Infant monkeys were exposed to 2.5% sevoflurane for 9 h, and frontal cortical tissues were harvested for DNA microarray, lipidomics, Luminex protein, and histological assays. DNA microarray analysis showed that sevoflurane exposure resulted in a broad identification of differentially expressed genes (DEGs) in the monkey brain. In general, these genes were associated with nervous system development, function, and neural cell viability. Notably, a number of DEGs were closely related to lipid metabolism. Lipidomic analysis demonstrated that critical lipid components, (eg, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol) were significantly downregulated by prolonged exposure of sevoflurane. Luminex protein analysis indicated abnormal levels of cytokines in sevoflurane-exposed brains. Consistently, Fluoro-Jade C staining revealed more degenerating neurons after sevoflurane exposure. These data demonstrate that a clinically relevant concentration of sevoflurane (2.5%) is capable of inducing and maintaining an effective surgical plane of anesthesia in the developing nonhuman primate and that a prolonged exposure of 9 h resulted in profound changes in gene expression, cytokine levels, lipid metabolism, and subsequently, neuronal damage. Generally, sevoflurane-induced neuronal damage was also associated with changes in lipid content, composition, or both; and specific lipid changes could provide insights into the molecular mechanism(s) underlying anesthetic-induced neurotoxicity and may be sensitive biomarkers for the early detection of anesthetic-induced neuronal damage.

Key words: sevoflurane; development; cytokines; lipid metabolism; neuronal degeneration

Millions of children undergo general anesthesia for surgery each year. Numerous studies have demonstrated the adverse effects of general anesthetics on the developing brain using both *in vitro* and *in vivo* preclinical models (Ikonomidou et al., 1999; Jevtovic-Todorovic et al., 2003; Kahraman et al., 2008; Paule et al., 2011; Scallet et al., 2004; Slikker et al., 2007), thus, raising public concern regarding the safety of anesthetics in children. Although verification of the pre-clinical findings is yet to be accomplished in children, researchers have been making efforts to test anesthetics in nonhuman primate models (Paule et al., 2011; Slikker et al., 2007; Zou et al., 2011) that more closely mimic the pediatric population.

Sevoflurane is a volatile anesthetic of the ether group, which has various advantages over other intravenous (i.v.) or inhalation anesthetics including less discomfort and a lower blood:gas solubility that facilitates rapid induction and recovery. Sevoflurane is also less irritating to airways, has a more pleasant smell, and provides more hemodynamic stability than other volatile anesthetics such as isoflurane or halothane (Smith et al., 1996). All these features make it favorable for use in children.

Although sevoflurane may have shown less severe effects on the developing brains of animal models compared with those of isoflurane (a structurally related inhaled anesthetic (Liang et al., 2010)), there is ample evidence that sevoflurane can cause neuronal apoptosis and behavioral dysfunctions (Lu et al., 2010; Shen et al., 2013; Takaenoki et al., 2014; Wang et al., 2012b,c).

It is known that the primate brain as an organ contains the largest diversity of lipid classes and molecular species and the largest lipid mass relative to proteins in comparison to other organs except adipose tissue. The literature on the frequently used general anesthetics has detailed their effects on synaptogenesis, synaptic networking, neurogenesis, neural cell death, and behavioral deficits. However, there has been no research evaluating whether and/or how these agents might affect lipids, the most abundant component in the brain other than water. Although there are several advantages to using sevoflurane for pediatric anesthesia, its potential adverse effects on the developing brain merit a thorough evaluation. The utilization of a highly relevant preclinical model for assessing the effects of sevoflurane is critical to decrease the uncertainty associated with extrapolating the preclinical findings to humans. Moreover, identifying biomarkers, especially from the samples of brain and blood plasma may assist in the early detection of the neurotoxic effects associated with exposure to general anesthetics and help with their safety evaluation.

Due to the close relevance in anatomical, physiological and developmental features of nonhuman primates to humans, postnatal day (PND) 5 or 6 rhesus monkeys were used as subjects in the current studies. It is observed that prolonged exposure of the monkey infant to a clinically relevant concentration of sevoflurane caused significant changes in cytokine levels, lipid content, and composition, as well as enhanced neuronal damage.

MATERIALS AND METHODS

Animals. Eight PND 5 or PND 6 rhesus monkeys were used in this study. All monkeys were born and housed at the FDA's National Center for Toxicological Research (NCTR) nonhuman primate research facility. All animal procedures were approved by NCTR's Institutional Animal Care and Use Committee and conducted in full accordance with the public health service Policy on Humane Care and Use of Laboratory Animals. Animal procedures were designed to minimize the number of animals

required and any pain or distress they might experience. Infant monkeys (2 males and 6 females) were randomly assigned to control (n=4, 1 male and 3 females) and sevoflurane-exposure (n=4, 1 male and 3 females) groups. Immediately prior to the initiation of anesthesia or sequestration, the infant monkeys (both sevoflurane-exposed and control animals) were separated from their mothers, removed from their home cage and hand carried to a procedure room.

Anesthetic exposure. Sevoflurane was delivered using an agent-specific vaporizer (Tec 7, Baxter, Dallas, Texas) with oxygen into an anesthetic chamber at a concentration of 2.5% and a rate of 1l/min. Monkeys were kept in the chamber with a circulating water heating pad to maintain body temperature at approximately 37°C for the duration of the 9-h experiment. Controls were kept at environmental conditions in the procedure room. A relief valve on the anesthesia chamber allowed continuous escape of gases to avoid accumulation of carbon dioxide. Waste anesthetic gas was scavenged using an attached canister containing activated charcoal. During the exposure period, dextrose (5%) was administered by stomach tube (5 ml) every 2 h to both anesthetized and control monkeys to maintain blood glucose levels. Glycopyrrolate (0.01 mg/kg, American Reagent, Shirley, New York) was administered intramuscularly prior to anesthesia or room air exposure to both anesthetized and control monkeys to reduce airway secretions. A 4-h postexposure period preceded animal sacrifice for both control and anesthetic-exposed infants. The animals were then deeply anesthetized with ketamine (20 mg/kg, i.p.) and sacrificed. Brains were harvested quickly for collection of fresh tissue and/or fixation in 4% paraformaldehyde in 0.1 M phosphate buffer.

Physiological measurements. The status of experimental subjects was closely monitored during anesthesia as previously described (Hotchkiss et al., 2007; Slikker et al., 2007). Briefly, pulse oximetry (N-395 Pulse Oximeter, Nellcor, Pleasanton, California), noninvasive sphygmomanometry (Critikon Dynamap Vital Signs Monitor, GE Healthcare, Waukesha, Wisconsin), and a rectal temperature probe were used to monitor body temperature, oxygen saturation of hemoglobin, expired CO₂ concentrations, and blood pressures, at an interval of 2 h. Blood (0.25 ml) was collected every 2 h for the measurement of plasma glucose (Ascensia Elite XL Blood Glucose Meter, Bayer Diagnostics, Tarrytown, New York) and determination of venous blood gases (Rapidlab, East Walpole, Massachusetts).

Protein and RNA extraction from brain tissue. Total RNA from frontal cortex of the brain was isolated using RNeasy Lipid Tissue Mini Kits (Qiagen Inc, Valencia, California). The yielded RNA concentration was measured spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, Delaware), and the integrity of the total RNA was evaluated using the RNA 6000 LabChip kit and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California). High-quality RNA with RNA integrity numbers (RINs) greater than 8.5 was used for the microarray experiments.

Protein was extracted from the frontal cortex of the brain using lysis buffer containing protease inhibitor cocktail (Sigma-Aldrich). The extracts were centrifuged at 4°C at 13 000 × g for 20 min. The supernatant was taken for assessment using a Lumines protein assay.

Microarray analysis. RNA samples from the monkey brains were subjected to microarray analysis. Gene expression profiling was

performed using the Agilent M. Mulatta (Rhesus) Oligo Microarray platform (Agilent Technologies) containing 43 663 probes. The gene expression data from 4 sevoflurane-treated monkeys and 4 controls were analyzed using GeneSpring GX (Agilent Technologies) with quantile normalization. Pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, California; <http://www.ingenuity.com/>).

To identify differentially expressed genes (DEGs), 2 sets of criteria were adopted for comparison. First, inclusion criteria of a *p*-value less than .05 and a fold change greater than 1.5 (up or down) suggested by the MicroArray Quality Control Consortium (Shi *et al.*, 2006) were applied. The combination of a *p*-value cutoff and a fold-change ranking (metric) has been demonstrated to be a practical way for selecting DEGs with good sensitivity, specificity, and reproducibility. The cutoff at the *p*-value minimizes the possibility of false selections of genes with no change in expression; and then ranking the genes which meet the *p*-value cutoff by their fold-changes serves to generate a list of DEGs with minimal discrepancy when the microarray analysis is performed by other researchers using different microarray platforms (Shi *et al.*, 2008). Since differences between groups are usually smaller for brain tissues (Liu *et al.*, 2011, 2012; Shi *et al.*, 2010), the previously used standard cut-off for fold-change was chosen to maximize the number of DEGs that could be identified. Second, more stringent criteria (fold-change greater than 2.0 and *p*-value less than .05) were adopted to identify a more specific and shorter list of DEGs for comparison of pathway analysis with those DEGs recognized by the 1.5-fold change criteria.

Preparation of lipid extracts. Approximately 20 mg of brain tissue from the frontal cortex was homogenized in PBS, and a protein assay was performed following homogenization using a bicinchoninic acid protein assay kit (Pierce, Rockford, Illinois) with bovine serum albumin as a standard. The protein content was used to normalize the determined lipid levels for the individual samples. Individual homogenates of the brain samples were carefully transferred to a glass culture test tube, and a premixed solution of internal standards was included for global lipid analysis. Lipid extraction was performed using a modified Bligh and Dyer method as previously described (Christie, 2010). The organic layers were collected and evaporated under a nitrogen stream and subsequently reconstituted using 1:1 (vol/vol) chloroform/methanol to a final concentration of 200 µl/mg protein. Samples were then flushed with nitrogen, capped, and stored at -20°C for electrospray ionization-mass spectrometry (ESI-MS) analysis. In addition, 4-hydroxyalkenal species were derivatized using carnosine, as previously described (Wang *et al.*, 2012a). To confirm the identification of the subclass of phosphatidylethanolamine (PE) species, a portion of the lipid extract was derivatized using fluorenylmethoxycarbonyl (Fmoc) chloride, as previously described (Han *et al.*, 2005), and exposed to acidic vapors (concentrated hydrochloric acid) for 3 h, which allowed for differentiation between isobaric alkyl-acyl and alkenyl-acyl (plasmalogen) PE species, as previously shown (Han *et al.*, 2001). Precursor ion scanning was performed to monitor the fatty acid fragments in order to identify the aliphatic chains in each of the PE species, as previously described (Yang *et al.*, 2009).

Lipidomics analysis. Lipid analyses were performed on a Thermo triple-quadrupole mass spectrometer (Thermo Scientific TSQ Vantage, San Jose, California) equipped with a Nanomate automated nanospray device (Advion Bioscience Ltd, Ithaca, New York) using Xcalibur software as previously described (Han

et al., 2008). Diluted lipid extract was directly infused through the Nanomate device (Han *et al.*, 2008). For each mass spectrum, typically a 1-min period of signal averaging in the profile mode was employed. For tandem MS experiments, the collision gas (argon) pressure was set at 1.0 mTorr, and the collision energy was varied depending on the class of lipids being analyzed, as previously described (Han and Gross, 2005; Yang *et al.*, 2009), and a 2- to 5-min period of signal averaging was used. Data processing including ion peak selection, baseline correction, data transfer, peak intensity comparison, ¹³C de-isotoping, and quantitation were performed using an in-house programmed Microsoft Excel (Microsoft, Redmond, Washington) macro, described previously (Yang *et al.*, 2009), after taking into consideration the principles of lipidomics (Yang and Han, 2011).

Luminex protein assay. A cytokine Monkey Magnetic 28-Plex panel kit was purchased from Life Technologies (Grand Island). The entire assay was performed according to the instructions provided with the kit. Brain tissue lysates were thawed on ice and centrifuged at 13 000 × *g* for 10 min at 4°C to remove any particulates. Manufacturer supplied polystyrene beads conjugated to protein specific capture antibodies were added to the wells of the microplate. Eight working standards were prepared in duplicates by serially diluting the reconstituted standard. This contained 28 different cytokines. The samples were diluted 1:2 with supplied assay buffer. The beads were washed with the wash buffer followed by the addition of incubation buffer. After reconstitution, 100 µl of standard and samples were added into the wells of the microplate containing the beads. This reaction mixture was allowed to incubate with agitation for a period of 2 h during which the proteins bind to the capture antibodies. After incubation, the beads were washed with the wash buffer followed by addition of protein-specific biotinylated detector antibodies and incubated with the beads for 1 h. After incubation, the bead mixture was washed thoroughly again to remove excess antibodies. This was followed by the addition of streptavidin conjugated to the fluorescent protein R phycoerythrin (Streptavidin RPE) and a 30-min incubation, during which a solid phase sandwich was formed. After washing to remove unbound streptavidin, the beads were analyzed using the Luminex detection system.

Fluoro-Jade C staining. Because both RNA and protein were extracted from the frontal cortex, the frontal lobe was chosen for pathological analysis. Coronal sections (50 µm) of the frontal cortex were cut with a vibratome for Fluoro-Jade C staining, as described previously (Schmued *et al.*, 2005). Briefly, prior to staining, sections were mounted onto subgelatinized slides. The mounted sections were bathed in a solution of 1% sodium hydroxide in 80% ethanol for 5 min, and washed in 70% ethanol and distilled water for 2 min, respectively, followed by an incubation in 0.06% potassium permanganate solution for 10 min, and another incubation in a 0.0001% solution of Fluoro-Jade C (Histo-Chem, Inc, Jefferson, Arkansas) dissolved in 0.1% acetic acid vehicle for 10 min. A stock solution of 0.01% Fluoro-Jade C was prepared before being diluted to the 0.0001% working solution. The slides were rinsed through 3 changes of distilled water for 1 min per change. The air-dried slides were cleaned in xylene and a coverslip was applied with DPX [A mixture of distyrene, a plasticiser, and xylene, called DPX] nonfluorescent mounting media (Sigma).

Statistical analysis. Statistical analyses on physiological parameters, cytokine levels, and lipidomics data between control and

sevoflurane-exposed monkeys were performed, and graphs were produced using GraphPad Prism. Data are expressed as mean $\pm$ SD. Parameter comparisons between the control and treated group was performed using student t test. All analyses were considered statistically different with a p value $< .05$.

RESULTS

Physiological Responses to Sevoflurane

During the experiments, infant monkey physiological parameters, such as percent oxygen saturation, body temperature, heart rate, blood pressure (systolic and diastolic), and glucose, were monitored. Physiological conditions for both control and sevoflurane exposed animals were maintained at stable conditions through the entire experimental procedure. Table 1 summarizes the physiological measurements for both control and sevoflurane-exposed monkeys. All important physiological values such as body temperature, blood glucose, and O₂ saturation levels remained within normal ranges for all animals and none differed significantly between groups.

Sevoflurane-Induced Changes in Gene Expression and Pathway Analysis

Applying the previously used inclusion criteria (fold-change greater than 1.5, up or down, and p -value $< .05$), a list of 576 DEGs was generated: 303 genes were up-regulated and 273 genes

were downregulated in the sevoflurane-exposed monkey brain (Supplementary Table 1). These genes were loaded into IPA database for pathway, disease and biological function, and network analysis. The identified DEGs contributed significantly to nervous system development and functions (Table 2), neurological diseases (Supplementary Table 2); and cell death/survival (Supplementary Table 3). Importantly, a considerable number of identified DEGs were associated with networks of lipid metabolisms, such as fatty acid metabolism, steroid metabolism, and concentrations of lipid, cholesterol, triacylglycerol, etc. (Table 3), suggesting effects of the sevoflurane on cytoarchitecture of the developing brain.

When the stringent criteria (fold change greater than 2.0, and p -value $< .05$) were applied, a total of 87 DEGs were identified; of which 47 genes were upregulated and 40 genes were downregulated. Pathway analysis was conducted using IPA (Supplementary Tables 5-1-5-4). Although fewer pathways were located, the relevant canonical pathways identified under the stringent criteria (Supplementary Table 5-4) had 80% overlap, including the pathway of fatty acid α -oxidation, with the pathways (Supplementary Table 4) identified by the criteria of fold-change greater than 1.5 and p -value $< .05$, indicating reproducible results even using different criteria. Both sets of criteria identified networks related to cell death (Supplementary Table 5-3 and Table 3), though the stringent criteria had excluded a significant number of DEGs. Because the IPA screening networks are based on the percentage of molecules falling into the networks, the 87 DEGs generated by the stringent criteria substantially reduce the identified diseases and biological functions (Supplementary Table 5-2, and Table 2). Due to the same reasons, the specific networks and biological functions identified by IPA varied in the 2 sets of DEGs.

Elevated Cytokine Levels in Sevoflurane-Exposed Brain Tissue Lysates

In this study, the involvement of cytokines and chemokines in sevoflurane-induced brain damage was examined. Luminex protein analysis showed that among the 28 analyzed cytokines, interleukin (IL)-17 (control vs sevoflurane: 17 pg/ml vs 36 pg/ml), macrophage inflammatory protein-1 α (MIP-1 α , control vs

TABLE 1. Physiological Parameters of Control and Sevoflurane-Treated Monkeys

	PND 5/6 Monkeys	
	Control	Sevoflurane
O ₂ saturation (%)	98 $\pm$ 1.5	98 $\pm$ 1.8
Temperature (°C)	36.7 $\pm$ 0.45	35.4 $\pm$ 1.6
Systolic blood pressure (mmHg)	120 $\pm$ 15	124 $\pm$ 6
Diastolic blood pressure (mmHg)	89 $\pm$ 13	93 $\pm$ 11
Glucose (mg/dl)	63 $\pm$ 14	73 $\pm$ 28

TABLE 2. DEGs Associated With Nervous System Development and Functions (Selected)

Diseases or Functions Annotation	p -Value	Molecules
Abnormal morphology of nervous system	3.52E-04	ACP2,AKAP12,CACNA1F,CAMKK1,CASP3,CEP290,DLL3,FOXA2,GAD1,GRIN3A,IL1RAPL1,JAG2,KALRN,KCNJ10,KIF1B,KLF7,CLK8,LGI1,LMNA,MEOX1,MET,MTHFR,NPAS4,NTRK2,PPT2,PROM1,RAX,RELA,RIMS1,SCN2A,SEMA5B,SIX1,SPP1,ST8SIA2,TP53,TYRO3,VPS13A
Neurotransmission	5.40E-04	GABRG1,GABRG2,GAD1,GAD2,GRIN3A,GRM1,GRM8,HAA,KALRN,KIF1B,CLK8,LGI1,MET,NBEA,NRXN2,NTRK2,PTGS2,RIMS1,SCN2A,SCN3A,SLC8A3,UNC13C
Morphology of nervous system	9.90E-04	ACP2,AKAP12,CACNA1F,CAMKK1,CASP3,CEP290,DLL3,FOXA2,GAD1,GRIN3A,IL1RAPL1,JAG2,KALRN,KCNJ10,KIF1B,KLF7,CLK8,LGI1,LMNA,MEOX1,MET,MTHFR,NPAS4,NTRK2,PPT2,PROM1,RAX,RELA,RGS7,RIMS1,SCN2A,SEMA5B,SIX1,SPP1,ST8SIA2,TP53,TYRO3,VPS13A
Synaptic transmission	3.65E-03	GABRG1,GABRG2,GAD1,GAD2,GRIN3A,GRM1,GRM8,HAA,KIF1B,LGI1,MET,NBEA,NRXN2,PTGS2,RIMS1,SLC8A3,UNC13C
Morphology of nerves	4.65E-03	DLL3,KCNJ10,KLF7,LMNA,MEOX1,MET,NTRK2,RELA,TP53
Abnormal morphology of brain	1.26E-02	AKAP12,CASP3,CEP290,FOXA2,IL1RAPL1,KALRN,KIF1B,KLF7,CLK8,LGI1,MTHFR,NTRK2,PPT2,RAX,RIMS1,SCN2A,SPP1,ST8SIA2,TP53,TYRO3,VPS13A
Abnormal morphology of central nervous system	1.39E-02	AKAP12,CASP3,CEP290,FOXA2,IL1RAPL1,KALRN,KCNJ10,KIF1B,KLF7,CLK8,LGI1,MTHFR,NTRK2,PPT2,RAX,RIMS1,SCN2A,SPP1,ST8SIA2,TP53,TYRO3,VPS13A
Synaptic transmission of cells	1.46E-02	GABRG1,GABRG2,GAD1,GAD2,GRIN3A,GRM1,GRM8,LGI1,NBEA,NRXN2,RIMS1,UNC13C

TABLE 3. Selected Networks Associated With Lipid Metabolism

DEG-Involved Network Functions Annotation	p-Value	Molecules Involved
Metabolism of cholesterol	1.08E – 03	NPC1L1, SEC14L2, STAR
Fatty acid metabolism	1.54E – 03	ACACB, CADM1, MSMO1, NPC1L1, ST8SIA2, STAR
Steroid metabolism	2.21E – 03	MSMO1, NPC1L1, SEC14L2, STAR
Concentration of lipid	3.85E – 03	ACACB, AQP3, DIO2, NPC1L1, SEC14L2, STAR, VGF
Concentration of cholesterol	5.19E – 03	ACACB, NPC1L1, SEC14L2, STAR
Concentration of triacylglycerol	5.78E – 03	ACACB, AQP3, DIO2, NPC1L1
Uptake of lipid	6.02E – 03	NPC1L1, SEC14L2, STAR
Quantity of steroid	6.30E – 03	ACACB, NPC1L1, SEC14L2, STAR, VGF
Synthesis of lipid	6.74E – 03	ACACB, CADM1, NPC1L1, SEC14L2, ST8SIA2, STAR
Metabolism of membrane lipid derivative	8.47E – 03	NPC1L1, SEC14L2, ST8SIA2, STAR
Synthesis of steroid	1.10E – 02	NPC1L1, SEC14L2
Synthesis of steroid	2.41E – 02	NPC1L1, SEC14L2, STAR
Transport of lipid	4.45E – 02	NPC1L1, STAR

sevoflurane: 7.06 pg/ml vs 9.75 pg/ml), epidermal growth factor (EGF, control vs sevoflurane: 29 pg/ml vs 35 pg/ml), monokine induced by gamma interferon (MIG, control vs sevoflurane: 26 pg/ml vs 43 pg/ml) were all significantly elevated in sevoflurane-exposed animals (Fig. 1).

Assessment of Sevoflurane-Induced Neurotoxicity by Fluoro-Jade C Staining

Sevoflurane-induced neurotoxicity was assessed using Fluoro-Jade C staining. Fluoro-Jade C is a neuronal stain that was found to have a good affinity for degenerating neurons, regardless of specific insult or mechanism of cell death (Schmued *et al.*, 2005). Fluoro-Jade C stained neurons are characterized by high resolution and great contrast, and therefore, the degenerating neurons can be precisely localized (Schmued *et al.*, 2005). The 9-h exposure to a clinically relevant concentration of sevoflurane produced extensive neural damage as indicated by increased Fluoro-Jade C-positive neuronal cells in the frontal cortex (Fig. 2).

Reduced Phospholipid Levels and Increased Lipid Oxidation-Caused by Sevoflurane Exposure

Following the DNA microarray findings on changes in the expression of lipid-relevant genes, we performed ESI-MS to measure the contents of many lipid classes in infant monkey brains. Mass spectral analyses demonstrated substantial lipid alterations in brain tissue (frontal cortex) in monkeys exposed to sevoflurane. Lipid extracts of the frontal cerebral cortex of both control and sevoflurane-exposed animals were prepared using a modified Bligh-Dyer extraction and individual lipid classes were analyzed. It was found that broad classes of lipids were changed substantially (most reduced, but a few increased) after sevoflurane exposure. For example, PE (Fig. 3) species decreased in the sevoflurane group. Several of these reduced species belong to the plasmalogen subclass, which is thought to have antioxidant properties in the body and is more susceptible to reactive oxygen species (ROS) (Lessig and Fuchs, 2009). Lyso-PE was shown to be nearly significantly increased, with the observed species being consistent with those formed from the dissociation of the reduced PE species. Figures 4 and 5 show representative graphs indicating reduced levels of phosphatidylserine (PS) and phosphatidylglycerol (PG) in the sevoflurane group. 4-Hydroxynonenal (HNE), a known product of lipid peroxidation and oxidative stress, was found to be significantly increased, indicating the increased oxidative stress after sevoflurane exposure.

FIG. 1. Luminex protein analysis showed that IL-17, MIP-1 α , EGF, and MIG were significantly elevated in sevoflurane-treated brain tissue. *: $p < .05$; ***: $p < .001$.

Figure 5 summarizes the mass levels of the altered lipid classes induced by exposure to sevoflurane. The mass spectral analyses demonstrated that PE (control vs treated: 131.4 ± 1.89 vs 110.61 ± 11.40 nmol/mg protein), PS (control vs treated: 75.84 ± 3.96 vs 61.22 ± 5.73 nmol/mg protein), and PG (control vs treated: 4.49 ± 0.33 vs 3.15 ± 0.09 nmol/mg protein) were significantly lower in sevoflurane-treated animals, while the level of HNE control vs treated: 5.40 ± 2.02 vs 9.67 ± 2.75 nmol/mg protein) was significantly elevated (Fig. 5).

DISCUSSION

In this study, it has been demonstrated that at clinically relevant concentrations (2.5%) sevoflurane, the most commonly used gaseous anesthetic agent for human infants and children, can effectively induce and maintain a light surgical plane of anesthesia in developing nonhuman primates: the anesthetized monkeys lost voluntary movement with reduced muscle tone and minimal reaction to physical stimulation.

The selection of exposure duration (9 h) was based on our previous *in vivo* data from sevoflurane-treated rodents (Liu *et al.*, 2013, 2014). The data indicated that sevoflurane-induced neurodegenerative effects were dependent on delivered concentrations and exposure duration. In addition, sevoflurane-induced apoptosis could only be detected in rat pups subjected to prolonged sevoflurane exposure at 2.5 %, eg, 9 h; but not from a short duration exposure, eg, 3 h. It should be mentioned that some surgeries require long durations of

Fluoro-Jade C Staining (*Frontal Lobe*)

FIG. 2. Representative examples of Fluoro-Jade C staining of the frontal cortex of the developing monkey brain. Only a few Fluoro-Jade C-positive neuronal profiles were observed in a control monkey (A). Enhancement of neurotoxicity as evidenced by an increased number of Fluoro-Jade C-positive neuronal cells in sevoflurane-exposed monkey brain (B).

FIG. 3. Representative graphs of mass spectral analyses for phosphatidylethanolamine (PE). The lipidomic analysis data indicated that the PE was substantially lower in sevoflurane-exposed brain (B) compared with the control (A). Each paired mass spectra are displayed after normalization to the internal standard (IS) peaks (ie, the peaks corresponding to the IS are equally intense in each paired spectra) for direct comparisons.

anesthesia. For example, liver transplants usually require 6–10 h (http://kidshealth.org/parent/system/surgery/liver_transplant.html#). According to the Organ Procurement and Transplant Network, from January 1998 to January 2015, there were 133 463 liver transplant recipients in the United States. Among them, 4150 recipients were below 1 year of age, and 5505 recipients were 1–5 years of age.

During general anesthesia the procedures followed for the maintenance and monitoring of experimental subjects were similar to those previously detailed (Slikker *et al.*, 2007) and to those used in clinical settings. Physiological parameters including percent oxygen saturation, body temperature, blood pressure, and glucose were all monitored and maintained within normal limits. None of these parameters were significantly

affected by sevoflurane exposure, and all monkeys tolerated the procedures well and recovered from anesthesia uneventfully.

The potential adverse effects of sevoflurane on the developing monkey brain were evaluated at transcriptional, molecular, and cellular levels, using DNA microarrays, lipidomics analyses, Lumindex protein assays, and histological observations, providing a relatively complete picture of sevoflurane's effects. The DNA microarray analysis provided a general gene expression profile for the developing monkey brain after 9 h of sevoflurane exposure. In this study, to emphasize the specificity and reproducibility, additional stringent criteria were also adopted. Consistently, the identified DEGs (total 87 genes) from more stringent criteria were closely relevant to neural cell death, neurological diseases, and nervous system development and

FIG. 4. Representative graphs of mass spectral analyses for phosphatidylserine (PS). The lipidomic analysis data indicated that PS was significantly lower in sevoflurane-exposed brain (B), compared with the control (A). The paired mass spectra are displayed after normalization to the internal standard (IS) peaks (ie, the peaks corresponding to IS are equally intense in each paired spectra) for direct comparisons.

FIG. 5. Phospholipid and HNE content was evaluated by lipidomic analysis. Phosphatidylserine (PS), PE, and phosphatidylglycerol (PG) levels were significantly lowered by prolonged sevoflurane exposure, whereas the level of 4-hydroxynonenal (HNE) was markedly increased by sevoflurane. *: $p < .05$; **: $p < .01$; ***: $p < .001$.

function. Related pathways of these genes overlap with most of the pathways identified by previously used inclusion criteria (Supplementary Table 5-1-5-4).

Using DNA microarray analysis, a variety of effects of sevoflurane have been identified including effects on synaptic function and neurotransmission. The fact that numerous DEGs were relevant to the morphology of central nervous system (CNS) (Table 2) also suggests possible alterations in brain cytoarchitecture, synaptogenesis, and CNS networking. Notably, the IPA analysis indicated that a group of DEGs are directly involved in lipid metabolism, falling into networks that are involved with synthesis, metabolism, and transport of lipids (Table 3). Fatty acids are basic components in many complex lipids found in cell membranes, such as glycerophospholipids, triacylglycerols, and sphingolipids. It has been reported that glycerophospholipids account for 20.3% of the dry weight of the grey matter in the 10-month old human brain (O'Brien and Sampson, 1965),

making glycerophospholipids the most abundant lipids in cell membranes. Changes in the quantity or quality of fatty acids may affect the structure and function of glycerophospholipids in cell membranes, and subsequently, cell functions and viability. The widespread recognition that cellular lipids play vital regulatory roles in cellular signaling and metabolism has sparked the emergence of genetic- and chemistry-based research into this topic and MS (Han and Gross, 2003; Hsu and Turk, 2009; Pulfer and Murphy, 2003). Meanwhile, altered lipid composition has been found at the earliest clinical diagnostic stage of degenerative diseases, suggesting aberrant lipid metabolism may be one of the determinants of neuronal damage. However, currently there has been no research evaluating whether and/or how anesthetic agents might affect lipids, the most abundant component in the brain other than water. Based on this information, we hypothesized that prolonged exposure of developing brains to anesthetics will result in perturbations of the lipid homeostasis in the brain and blood plasma, and identification of these altered lipids may serve as biomarkers and aid in determining the underlying mechanisms of anesthesia-induced neurotoxicity.

Physiologically, PE and PS are located in the inner leaflet of plasma membrane. Functionally, PS is involved in the activation of several signaling pathways that are associated with neuronal survival, neurite growth, and synaptogenesis (Akbar et al., 2005; Huang et al., 2011; Kim, 2007; Kim et al., 2000, 2010). We observed that the species that significantly decreased correspond to the docosahexaenoic acid (22:6, DHA) fatty acid species, loss of which is of indicative inflammation since the oxidative product of DHA (i.e., docosanoids) is believed to be neuroprotective. PE is also abundantly found in the brain and PE is a precursor of anandamide (N-arachidonylethanolamine), the ligand for cannabinoid receptors in the brain (Devane et al., 1992; Jin et al., 2007; Vance and Tasseva, 2013). The lipidomics analyses in this study showed that PE and PS were significantly decreased in the brains of sevoflurane-exposed monkey infants, suggesting altered membrane phospholipid metabolism and content, a finding that

is also consistent with the DNA microarray data. Alterations of lipids in monkey brain plasma membranes are likely to disrupt phospholipid homeostasis and change the integrity, orientation, permeability, and functions of plasma membranes, which in turn, could lead to subsequent neural dysfunction and/or degeneration. Interestingly, species belonging to a subclass of PE, known as plasmalogens and those containing polyunsaturated fatty acids such as DHA were significantly decreased between the sevoflurane-exposed and control frontal cortical nonhuman primate brains (Fig. 3). Plasmalogens contain a vinyl-ether linkage that is susceptible to oxidative stress, and therefore, are believed to act as natural antioxidants in the body (Lessig and Fuchs, 2009). Although membrane lipid metabolism has never been explored with respect to anesthetic-induced developmental neurotoxicity, its roles in neurodegenerative diseases such as Alzheimer's has been extensively studied (Han, 2010; Klein, 2000; Ryan et al., 2009; Sanchez-Mejia et al., 2008; Sweet et al., 2002). Such studies have revealed that changes in membrane composition dynamically regulate neuronal functions. The DNA microarray and lipidomic analyses in this study demonstrated altered lipid metabolism and decreased phospholipids in sevoflurane-exposed monkey brains, shedding light on the possible mechanisms involved in sevoflurane-induced developmental neurotoxicity. This study also showed that PG decreased substantially after sevoflurane exposure. Since PG is exclusively present in mitochondrial membranes (Benjamins et al., 2012), this finding suggests that mitochondria are significant targets of sevoflurane exposure. In addition to PG, the other major phospholipids in mitochondrial membranes are similar to those in plasma membranes, and PE is one of the most abundant mitochondrial phospholipids (Colbeau et al., 1971). It has been reported that changes in mitochondrial membrane phospholipids can affect mitochondrial function (Ohtsuka et al., 1993). Mitochondria are the "power houses" of a cell and since they are involved in various critical cellular functions, numerous diseases have been linked to their dysfunction. Mitochondria are also important generators of ROS. The imbalance of ROS and antioxidants leads to oxidative stress that can damage proteins, lipids and DNA. Meanwhile, among the regulated lipid species, HNE is an aldehydic product of membrane lipid peroxidation (Kruman et al., 1997). Its negative effects on brain mitochondrial complexes II and III have been reported (Picklo et al., 1999), and it has been found to mediate oxidative stress-induced neuronal apoptosis (Kruman et al., 1997). Our lipidomic analysis showed significantly higher levels of HNE in sevoflurane-exposed monkey brain, suggesting that sevoflurane causes elevated ROS generation in mitochondria leading to lipid peroxidation and HNE production. Although it is not yet known how general anesthetics induce apoptosis, impairment of mitochondrial function has been generally recognized as a pathology central to the processes leading to apoptosis (Wang, 2001). These lipidomic signatures identified in this study not only may serve to elucidate the causal biochemical mechanisms underpinning cytoplasmic and mitochondrial lipid changes, but may also allow the identification of biomarker candidates for the early detection of neuronal damage induced by general anesthetics.

Lipids play many roles in cellular functions, from membrane structural components to second messengers. Thus, it is likely that perturbations of the nervous system should be reflected in changes in lipid content, composition, or both. It has been reported that disruption of phospholipid integrity in neural membranes caused cytokine secretion from microglia and exacerbated inflammation and neuronal damage in neurodegenerative diseases (Dickson et al., 1993). Cytokines are signaling molecules that play critical roles in many biological processes.

Moreover, neurons express chemokines and the receptors (Boutet et al., 2001; Coughlan et al., 2000; Guo et al., 2003). Therefore, we measured the levels of 28 cytokines in brain tissue lysate using a Luminex protein assay and demonstrated that the levels of IL-17, MIP-1 α , EGF, and MIG were significantly elevated in sevoflurane-exposed monkey brain. Both IL-17 and MIP have been found to contribute to neuronal damage in various situations (Swardfager et al., 2013; Wang et al., 2008, 2009). MIG is thought to contribute to inflammatory pathologies in the CNS (Glabinski et al., 1997; Liu et al., 2001). The elevated levels of IL-17, MIP, and MIG suggest that sevoflurane exposure may have stimulated an inflammatory reaction in the CNS. Meanwhile, increased EGF in brain tissue may indicate a compensatory mechanism of the brain, since EGF has been found to have neurotrophic effects on different types of neurons. In this study, elevated ROS production and cytokine secretions could be critical for the development of neuronal damage induced by sevoflurane, as evidenced by increased number of Fluoro-Jade C-positive neurons in frontal cortex (Fig. 2). Importantly, sevoflurane-induced neuronal damage in frontal cortex in the monkey infant is consistent with that observed from our previous rodent studies, indicating sevoflurane-induced neurodegenerative effects are dependent on delivered concentrations and exposure duration (Liu et al., 2014). Thus, it is quite possible that the presence and severity of sevoflurane-induced neurotoxicity could effectively be reflected in specific alterations in cytokine/chemokine secretion and the levels of ROS-mediated polyunsaturated fatty acid peroxidative products, such as HNE in brain tissue, plasma (blood), and cerebral spinal fluid.

In summary, this study undertook a general evaluation of sevoflurane's effects on the developing monkey brain and identified a number of changes at the genetic, protein, and cellular levels, induced by sevoflurane exposure. The findings verified observations made by other groups and provided novel findings that merit further studies. The brain consists of more lipids than any other organ, and subtle changes in lipid quality, quantity, or composition may, thus, result in disturbance of brain function, and vice versa. Both the DNA microarray and lipidomic analyses carried out in this study demonstrated that lipids seem to be especially sensitive indicators of the brain status after anesthetic exposure. This is the first time that lipidomics has been used to find potential biomarkers and identify underlying mechanisms of anesthesia-induced neurotoxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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