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Anesthetic Preconditioning Inhibits Isoflurane-Mediated Apoptosis in the Developing Rat Brain

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Attestation: Jun Peng has seen the original study data, reviewed the analysis of the data and approved the final manuscript.

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Abstract

Background—We hypothesized that preconditioning with a short exposure to isoflurane (ISO) would reduce neurodegeneration induced by prolonged exposure to ISO in neonatal rats, as previously shown in neuronal cell culture.

Methods—We randomly divided 7-day-old Sprague-Dawley rats into 3 groups: control, 1.5% ISO, and preconditioning (PC) + 1.5% ISO. The control group was exposed to carrier gas (30% oxygen balanced in nitrogen) for 30 min and then carrier gas again for 6 h the following day. The 1.5% ISO group was exposed to carrier gas for 30 min and then 1.5% ISO for 6 h the following day. The PC + 1.5% ISO group was preconditioned with a 30 min 1.5% ISO exposure and then exposed to 1.5% ISO for 6 h the following day. Blood and brain samples were collected 2 h after the exposures for determination of neurodegenerative biomarkers, including caspase-3, S100 β , caspase-12, and an autophagy biomarker Beclin-1.

Results—Prolonged exposure to ISO significantly increased cleaved caspase-3 expression in the cerebral cortex of 7-day old rats compared to the group preconditioned with ISO and the controls using Western blot assays. However, significant differences were not detected for other markers of neuronal injury.

Conclusion—The ISO-mediated increase in cleaved caspase-3 in the postnatal day 7 rat brain is ameliorated by preconditioning with a brief anesthetic exposure, and differences were not detected in other markers of neuronal injury.

Introduction

Isoflurane (ISO) is a widely used general anesthetic for both adult and pediatric surgeries. Many studies have been performed to elucidate the harms and benefits of ISO on neurons^{1–9} and in the developing brain.^{4,10–12} Depending on the circumstances, ISO has been reported to have both neurotoxic and neuroprotective effects.

A large number of *in vivo* studies have shown that ISO causes apoptosis in the developing brain of various species of animals^{4,10,11,13–15} and that subsequent learning and memory are impaired.^{10,11} Furthermore, ISO has also been shown to be toxic in various cell culture models.^{2,4,16,17}

Other studies have also shown that ISO can have neuroprotective effects. In particular, when ISO is used as a preconditioning drug, it can provide neuroprotection against various hypoxic-ischemic insults to the developing rodent brain.^{12,18–21} Previous *in vitro* work from our laboratory, and others, has shown that preconditioning with ISO has a protective effect on neuronal cell cultures subsequently exposed to ISO for a longer duration.² However, inhibition of ISO-induced neuronal apoptosis during brain development by preconditioning has not yet been examined.

Given that ISO has been a successful preconditioning drug against subsequent anesthetic exposure for developing neurons *in vitro*² and also against brain infarction induced by hypoxia and/or ischemia *in vivo*,^{12,18,20,22,23} we hypothesized that preconditioning with a short exposure to ISO would reduce neurodegeneration induced by a prolonged exposure to ISO in an animal model. We assessed the effects of 1.5% ISO exposure for 6 hours on apoptotic biomarkers in 7-day old rats, and then determined whether preconditioning with a short exposure to 1.5% ISO for 30 minutes changed the apoptotic response.

Methods

Animals

The experimental procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. All efforts were made to minimize the number of animals used and their suffering. Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were housed with a 12-hour light-dark cycle at 22°C, with food and water provided *ad libitum*. Thirty-eight postnatal day 7 (P7) rats were used for the ELISA and Western blots and 11 for immunohistochemistry, with approximately equal numbers of male and female rat pups randomly assigned to each condition.

Anesthesia exposure

The groups of rats were exposed to treatments in parallel. The minimum number of rats in each group was determined by a power analysis. We anticipated a large effect size would be clinically significant and chose an effect size of 1.3, and using the desired statistical level of 0.8 and probability level of 0.05, determined a minimum sample size per group (2-tailed hypothesis) of 11 animals. P7 rats were placed in plexiglass chambers resting in a 37°C

water bath to maintain a constant environmental temperature. The rat pups were exposed in these chambers to carrier gas (30% oxygen balanced in nitrogen) for 30 min and then 1.5% ISO for 6 h the following day (1.5% ISO), or preconditioned (PC) with a 30 min 1.5% ISO exposure and then exposed to 1.5% ISO for 6 h the following day (PC + 1.5% ISO). The control animals were exposed to carrier gas (30% oxygen balanced in nitrogen) for 30 min and then carrier gas again for 6 h the following day in the plexiglass chambers but not in the water bath. Exposure to ISO for 30 min alone at P7 has been shown not to be detrimental¹² and thus this control group was not included. In order to maintain a steady state of anesthetic gas and to prevent accumulation of expired carbon dioxide within the chamber, we used 6 liters of total gas flow throughout the experiments. The ISO, oxygen and carbon dioxide levels in the chamber were monitored using IR absorbance (Ohmeda 5330, Datex-Ohmeda, Louisville, CO) as described in our previous studies.^{4,15,24} Two rats died during exposure to 1.5% ISO for 6 hrs, 1 from the ISO alone group and the other from the PC plus ISO group.

Determination of plasma S100 β

Two hours after the completion of the anesthetic treatment, P7 rats from the control, 1.5% ISO and PC+1.5%ISO groups were deeply anesthetized with 2–3% ISO. Blood (0.1 ml) was collected from the left ventricle and centrifuged to separate the plasma. We measured levels of S100 β , a neuronal injury marker, using Sangtec 100 ELISA kits (DiaSorinInc, Stillwater, MN) following the manufacturer's protocol and as we described previously.²⁵ Briefly, 50 μ l of plasma from each rat was placed in each well of a 96-well-plate and mixed with 150 μ l of tracer from the kit, and incubated for 2 hours. Afterwards, 3,3',5,5'-tetramethylbenzidine substrate and stop solution were added to each well. The optical density was read at 450 nm. The sensitivity was determined by plotting the standard curve and then measuring concentrations of the samples from the standard curve.

Western Blot Assays

Western blots were performed as we described previously.^{15,24} Two hours after the ISO exposure, after the mice were anesthetized and blood samples collected from the heart (see above), the mice were perfused with ice-cold saline through the heart and the parietal cortex dissected, frozen in liquid nitrogen and stored at -80°C . At the time of the assay, the brain tissue from the P7 rat cortical tissue was thawed and homogenized and the total protein concentrations were quantified. The proteins were then separated by 12% gel electrophoresis and were transferred to a nitrocellulose membrane. The blots were incubated with an antibody against cleaved caspase-3 (Cell Signaling #9664), caspase-12 (Cell Signaling #2202), or Beclin-1 (Cell Signaling #3495). The density was measured by Quantity One software (BIO-RAD version 4.5.0) and GS-800 Densitometer (BIO-RAD, Hercules, CA) and the data are expressed as the percent of control of the means from 1 blot per animal per group.

Immunohistochemistry

Immunohistochemical localization of caspase-3 was performed in a separate group of P7 rats, as previously described.¹⁵ Briefly, 2 hours after the ISO exposure, P7 pups were deeply anesthetized with ISO and transcardially perfused with ice cold saline before the brains were removed, fixed with 4% paraformaldehyde, cryoprotected in 30% sucrose, frozen in

isopentane and stored at -80°C . Coronal cryosections ($10\mu\text{m}$) were incubated in 3% hydrogen peroxide, 10% normal goat serum and cleaved caspase-3 antibody (1:400; Cell Signaling Technology, #9664) overnight at room temperature. The next day, the sections were incubated with Alexa Fluor® 594 goat anti-rabbit IgG and coverslipped using ProLong® Gold Antifade Reagent containing the nuclear stain, DAPI (Invitrogen). Quantitative imaging was conducted on an Olympus IX70 microscope equipped with a Cooke SensiCam camera (Applied Scientific Instrumentation, Eugene, OR) and IP lab 4.0 software (Biovision Technologies, Exton, PA). Caspase-positive and total number of cells were counted in the CA1 region of the hippocampus and the adjacent parietal cortex at $20\times$ magnification. The brain sampled and analyzed in parietal cortex was the same region used in the Western blot from the opposite brain hemisphere. The mean number of cells was calculated from 3 sections per animal and the data expressed as the percentage of caspase-3 positive cells in each region.

Statistical analysis

All data were analyzed using the Mann-Whitney U test to determine between-group differences and exact p-values using STATA statistical software. Differences were considered statistically significant at $p<0.01$.

Results

Preconditioning significantly reduces ISO's apoptotic effect

This study tested the effects of PC, with a short exposure to ISO before a prolonged exposure to ISO, on apoptotic neurodegeneration in postnatal rats. The level of apoptosis was evaluated by determining cleaved caspase-3 levels in the cerebral cortex using Western blot immunoassays (Fig. 1A). The cleaved caspase-3 levels, expressed as percent of control, in the P7 rats exposed to ISO alone were significantly higher than the PC group ($P=0.0009$) or the controls ($P=0.0004$) (Fig. 1B) (Tables 1–2). In addition, the animals that were PC with ISO were also significantly different than controls ($P=0.0007$) (Fig. 1B) (Tables 1–2). When the P7 cortex and hippocampus were quantitatively analyzed for the immunohistochemical localization of caspase-3 (Fig. 2), significant differences were not detected in the number of caspase-3 positive-cells in the ISO-exposed group compared to controls or the PC group in the cortex or the hippocampus (Fig. 2 B, C) (Tables 1–2). These data support the claim that PC, with a short exposure to ISO, before a prolonged ISO exposure, significantly reduces the ISO-mediated apoptotic neurodegeneration in the developing rat brain using Western blot assays.

The level of neurodegeneration was further studied by determining the plasma levels of S100 β , a marker of neuronal injury, in P7 rats exposed to 1.5% ISO for 6 h, with and without ISO PC (Fig. 3). The ELISA assay did not reveal significant differences in plasma S100 β levels between these groups (Tables 1–2).

The effect of PC on the apoptotic pathway was also examined by caspase-12 activation in the developing brain. Western blot analysis of caspase-12 levels in the P7 cerebral cortex

(Fig. 4A) after either a 6h exposure to 1.5% ISO or PC with ISO before the prolonged exposure, were not significantly different from controls (Fig. 4B) (Tables 1–2).

Effect of ISO treatment on Autophagy

Autophagy, after anesthetic exposures, with and without PC, was examined in the P7 developing brain by measuring Beclin-1 levels. Western blot analysis (Fig. 5A) showed that a 6 h ISO exposure did not significantly reduce Beclin-1 levels (Fig. 5B) (Tables 1–2).

Discussion

This study provides new evidence that PC with ISO, before a long ISO exposure, significantly decreases ISO-mediated apoptosis in the developing brain. This finding is based on a significant reduction in the caspase-3 levels using Western blot assays. However, significant differences were not detected in caspase-3 levels in the cerebral cortex and hippocampus after either ISO exposure or PC. Other markers of neuronal injury, S100 β , caspase-12 and Beclin-1 were not significantly affected by either the prolonged ISO exposure or PC. While we have been able to exclude a large effect of these markers, it is possible that we could not detect significant effects due to our small sample size (e.g., Figures 2 and 3).

Caspase-3 is one of the final mediators of the apoptotic pathway and is a well-established biomarker of apoptosis. The most important aspect of our results is that ISO PC decreased caspase-3 levels induced by a prolonged ISO exposure in the developing brain. In a similar study, Shu *et al*²⁶ showed that xenon pretreatment before a combined ISO/nitrous oxide exposure decreased apoptosis, while nitrous oxide PC had no effect. Furthermore, we have previously shown sevoflurane PC can also inhibit neuronal cell death induced by prolonged exposure to ISO.² Thus, more studies are necessary to determine the mechanism for the protective effect of PC so that novel approaches can be developed to mimic this effect.

Anesthetics have been shown to be both neuroprotective and neurotoxic in the developing brain and thus concentrations and durations must be considered in pediatric anesthetic practice. Our previous study suggested that prolonged exposure to sevoflurane can induce neuronal damage *in vitro*.²⁷ The IV anesthetic, propofol, has also been shown to be both neurotoxic^{28,29} and neuroprotective against brain damage induced by ischemia and other stress factors.^{30,31} Therefore, it is important to investigate the dose and time responses of anesthetic-induced effects in the developing brain to use their neuroprotective features but minimize their neurotoxic effects.

S100 β , a dimeric cytosolic calcium binding protein released by glial cells, is a biomarker of blood-brain barrier dysfunction³² and overall brain distress.³³ It has been studied clinically as a biomarker for traumatic brain injury and hypoxic-ischemic brain injury.^{34,35} We have previously studied S100 β in the developing fetal rat brain and found that an *in utero* exposure to 3% ISO for 1 hour resulted in higher levels of S100 β in the plasma of fetal rats when compared to controls.²⁵ Furthermore, we showed an increase in plasma S100 β after exposure to a subclinical concentration of ISO in neonatal mice.¹⁵ Though the current study did not show a significant difference in S100 β , S100 β may be a useful biomarker to detect

anesthetic-mediated damage in the developing brain as indicated above, although further studies are needed to investigate its role in pediatric patients.

Caspase-12, part of the apoptotic pathway, is activated by disruption of the calcium homeostasis in the endoplasmic reticulum (ER).³⁶ Anesthetics have been shown to cause calcium dysregulation in the ER via multiple mechanisms.³⁷ In immature hippocampal neurons, ISO exposure was shown to enhance gamma-aminobutyric acid-induced intracellular calcium increase, which was blocked by dantrolene, indicating that ISO exposure causes ryanodine receptor-dependent calcium release from the ER,¹⁷ which is consistent with our previous studies in different types of neurons.⁵ Other studies also suggest that ISO exposure during brain development causes increased activation of inositol triphosphate receptors (InsP3R) resulting in increased calcium release from the ER leading to cell damage and neurodegeneration.^{3,4,27,38} Furthermore, caspase-12 positive neurons in the hippocampus were significantly increased in fetal rats exposed to 1.3% ISO for 4 hours.³⁹ Caspase-12 has also been shown to indirectly activate caspase-3 in the neuronal apoptotic pathway.⁴⁰ Although a previous study suggested that ISO-induced neuroapoptosis during brain development in rodents involved both intrinsic and extrinsic pathways,⁴¹ it is not clear whether the caspase-12-dependent pathway is also involved. In this study, we did not find significant caspase-12 activation after the ISO exposures, possibly because our ISO concentration may not have been high enough to cause ER stress and caspase-12-dependent neuroapoptosis. Further dose-dependent and exposure duration studies are needed to clarify this question.

Beclin-1, a protein required for autophagosome formation, is an important regulator and biomarker of autophagy activity.^{42–44} Interestingly, autophagy may have both beneficial and harmful effects on the brain, depending on the experimental conditions. Autophagy appears to be essential to both ischemic and hyperbaric oxygen PC before cerebral ischemia.⁴⁵ Furthermore, reducing Beclin-1 levels has been shown to exacerbate neurodegeneration in Alzheimer disease models, while overexpression of Beclin-1 can prevent neuronal cell death.⁴⁶ Autophagy activity can be regulated by InsP3R activity, while ISO has been shown to activate InsP3R and cause cell apoptosis by overactivation of InsP3R.^{47,48} In addition, autophagy activity may be an upstream regulator of apoptosis, and excessive autophagy may lead to cell death by apoptosis. The effects of InsP3R activity on autophagy depend on the level of InsP3R activation, which then determines whether the effect will be protective or toxic. While this study did not find an effect on Beclin-1 expression at P7 after exposure to 1 concentration of ISO, further studies are needed to investigate the effects of general anesthetics on cell autophagy, and therefore neuroprotection or neurotoxicity, especially in the developing brain.

While we have not yet discovered the mechanism by which ISO PC prevents ISO-induced apoptosis, other groups have studied ISO PC before cerebral ischemia and have linked several mechanisms to this process. One study found that ISO PC caused a decrease in glutamate receptor activation,⁴⁹ and another found that it decreased protein aggregation.⁵⁰ Changes in the expression of various genes have also been discovered, but the significance of these genetic changes has not been fully determined.^{51–53} Furthermore, ISO may provide PC neuroprotection by causing a moderate increase of cytosolic calcium concentrations via

adequate activation of the InsP3R calcium channel.^{54,55} While we recognize that ISO PC for brain ischemia is not the same as for a subsequent anesthetic exposure, it is possible that there may be similarities in the cascade of events and consequences. One of the limitations of the current study is that we only investigated a few of the potential mechanisms underlying the dual effects of neuroprotection and neurotoxicity caused by ISO. Future experiments could continue to address the many other mechanisms that are likely involved in this process.

The long-term behavioral adaptations to the effects of pre-adolescent drug exposures are more permanent compared to the same exposures later in life,⁵⁶ with the peak period of anesthetic-induced apoptosis occurring during synaptogenesis.⁵⁷ This vulnerable period during rat development is between approximately postnatal days 2 and 14 and between post-conception day 153 to postnatal 288 days in the human, based on a species prediction model recently developed to correlate the timing of neural events between species.⁵⁸ Translating exact developmental milestones between rats and humans is complicated. Postnatal brain maturation in the rat encompasses many developmental events, such as neurogenesis, neuronal migration, synaptogenesis and apoptosis, the extent of which varies greatly depending on the brain region of interest.^{59,60}

The present study tested the hypothesis that PC with ISO can prevent apoptosis caused by a prolonged exposure to ISO, and our results with caspase-3, but not other markers of neuronal injury, support this hypothesis and indicate that *in vivo* ISO PC is neuroprotective, while a prolonged exposure to ISO is neurotoxic during early postnatal brain development. It is important to determine the optimal concentration and duration range for anesthetic exposures during postnatal brain development, which has implications for our pediatric patients.

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A **B**

Figure 1. Preconditioning inhibits the increase of cleaved caspase-3 induced by isoflurane
 A) Representative Western blot of cleaved caspase-3 in the cerebral cortex after 6 h exposure to isoflurane (1.5%ISO) or preconditioning (PC) with 1.5% isoflurane before the same 6 h isoflurane exposure (PC+1.5%ISO) at postnatal day 7. B) Quantitative analysis of the Western blots of caspase-3, normalized to β-actin as percent of control, showed that prolonged exposure to 1.5% isoflurane (1.5%ISO) significantly increased cleaved caspase-3 compared to controls ($P=0.0004$), while PC (PC+1.5% ISO) significantly ameliorated the increase compared to 1.5% ISO ($P=0.0009$). The PC+1.5% ISO group was also significantly

different from controls ($P=0.0007$). $N=16$ for all groups, $**P<0.001$. Data are presented as box plots of the means with whiskers (min to max).

Figure 2. Isoflurane preconditioning significantly inhibits neuronal apoptosis induced by prolonged exposure to isoflurane in the cerebral cortex and hippocampus

A) Representative images of immunostaining for cleaved caspase-3 in the postnatal day 7 (P7) rat cerebral cortex (left panels) and hippocampus (right panels) of controls (top panels), after isoflurane exposure (middle panels), and preconditioning (PC) with a brief anesthetic exposure before the isoflurane exposure (PC + Isoflurane) (bottom panels). Apoptotic cells are stained for caspase-3 (red) and total cells are stained with DAPI (blue). The insets indicate the areas sampled in the cortex and hippocampus for quantitation. Scale bar, 25 μ m.

B) Quantitative analysis of the percentage of cleaved caspase-3 positive cells in the cerebral

cortex and C) the hippocampal CA 1 region. No significant differences were found in the number of caspase-3 positive cells in the cortex in the isoflurane ($P=0.034$) or PC + isoflurane (0.049) groups compared to controls or between the isoflurane and PC groups ($P=0.021$), or in the hippocampus ($P=0.034$, $P=0.21$, $P=0.021$ respectively). Data represent the mean of 3 adjacent brain sections per animal, $n=4$ animals for both experimental groups and $n=3$ for controls. Data are presented as box plots of the means with whiskers (min to max).

Figure 3. Effects of isoflurane preconditioning on S100 β in plasma

No significant differences were found in plasma S100 β levels with ELISA assays from postnatal day 7 (P7) rats after exposure to 1.5% isoflurane (1.5%ISO) for 6 hours with (P=0.34) or without preconditioning (PC) (P=0.19) or with PC (PC+1.5%ISO) compared to controls (P=0.049). Controls, n=9; 1.5%ISO, n=13; PC+1.5%ISO, n=13. Data are presented as box plots of the means with whiskers (min to max).

A **B**

Figure 4. Effects of isoflurane on cleaved caspase-12 in the cerebral cortex

A) Representative Western blot of cleaved caspase-12 in the cerebral cortex after 6 h exposure to isoflurane (1.5% ISO) or preconditioning (PC) with 1.5% isoflurane before the same 6 h isoflurane exposure (PC+1.5% ISO). B) Quantitation of caspase-12 levels, normalized to β-actin as percent of control, showed no significant differences in the levels of cleaved caspase-12 in the postnatal day 7 (P7) brain between groups exposed to 1.5% isoflurane for 6 hours compared to controls ($P=0.96$) or compared with isoflurane PC

($P=0.17$) or the PC group compared to controls ($P=0.27$). Controls, $n=9$; 1.5%ISO, $n=9$; PC +1.5%ISO, $n=9$. Data are presented as box plots of the means with whiskers (min to max).

A **B**

Figure 5. Effects of isoflurane exposure on Beclin-1 in the cerebral cortex

A) Representative Western blot of Beclin-1 levels in the cerebral cortex after a 6 h exposure to isoflurane (1.5%ISO) or preconditioning (PC) with 1.5% isoflurane before the same 6 h isoflurane exposure (PC+1.5%ISO). B) Quantitative analysis of Beclin-1, normalized to β-actin as percent of control, showed that 1.5% isoflurane for 6 hours, with ($P=0.53$) or without isoflurane PC ($P=0.71$), or PC compared to controls ($P=0.57$) did not significantly affect the levels of Beclin-1 in the cerebral cortex at postnatal day 7 (P7). Controls, $n=10$;

1.5%ISO, n=10; PC+1.5%ISO, n=11. Data are presented as box plots of the means with whiskers (min to max).

Table 1

P values (95% confidence intervals) of the Western blot and immunohistochemical analyses

Western blots	Control vs Isoflurane	Con vs Preconditioning + Isoflurane	Isoflurane vs Preconditioning + Isoflurane
Caspase-3	0.0004 (−1310 to −428.7)	0.0007 (−777.8 to 103.8)	0.0009 (91.69 to 973.2)
S100β	0.19 (−147.4 to 7.044)	0.49 (−101.0 to 53.43)	0.34 (−23.46 to 116.2)
Caspase-12	0.96 (−79.41 to 48.46)	0.27 (−44.11 to 83.76)	0.17 (−28.64 to 99.24)
Beclin-1	0.71 (−59.91 to 44.18)	0.57 (−38.61 to 63.09)	0.53 (−30.75 to 70.96)
Immunohistochemistry			
Caspase-3 Cortex	0.034 (−7.155 to −1.245)	0.049 (−3.880 to 2.030)	0.021 (0.539 to 6.011)
Caspase-3 Hippocampus	0.034 (−4.346 to −1.804)	0.21 (−1.696 to 0.846)	0.021 (1.474 to 3.826)

Table 2Means $\pm$ standard errors of the Western blot and immunohistochemical analyses

Western blots	Control vs Isoflurane	Con vs Preconditioning + Isoflurane	Isoflurane vs Preconditioning + Isoflurane
Caspase-3	100 $\pm$ 36.03	1106 $\pm$ 219.7	489.9 $\pm$ 116.3
S100 β	100 $\pm$ 6.26	170 $\pm$ 30.03	123.8 $\pm$ 9.97
Caspase-12	100 $\pm$ 13.78	115.5 $\pm$ 22.06	80.17 $\pm$ 15.79
Beclin-1	100 $\pm$ 16.63	107 $\pm$ 15.40	87.76 $\pm$ 10.55
Immunohistochemistry			
Caspase-3 Cortex	0.4 $\pm$ 0.058	4.600 $\pm$ 0.984	1.325 $\pm$ 0.357
Caspase-3 Hippocampus	0.2 $\pm$ 0.116	3.275 $\pm$ 0.335	0.625 $\pm$ 0.290