Both JNK and P38 MAPK pathways participate in the protection by dexmedetomidine against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats

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Abstract
Dexmedetomidine, a highly selective α2-adrenergic agonist, has been reported to attenuate isoflurane-induced cognitive impairment and neuroapoptosis. However, the underlying molecular mechanisms remain poorly understood. The aim of this study was to investigate whether mitogen-activated protein kinase (MAPK) pathway was involved in dexmedetomidine-induced neuroprotection against isoflurane effects. Seven-day-old (P7) neonatal Sprague-Dawley rats were pretreated with various concentrations of dexmedetomidine, and then exposed to 0.75% isoflurane or air for 6 h. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was used to detect neuronal apoptosis in their hippocampus. Activated caspase-3, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), p38, phospho-ERK1/2, phospho-JNK and phospho-p38 proteins were detected by Western blotting in the hippocampus at the end of exposure. Also, P7 rats were pretreated with 75 μg/kg dexmedetomidine alone, or given the ERK inhibitor U0126 before dexmedetomidine pretreatment, or pretreated with the p38 MAPK inhibitor SB203580 or JNK inhibitor SP600125 alone, and then exposed to 0.75% isoflurane for 6 h. Isoflurane induced significant neuroapoptosis, increased the protein expression of phospho-JNK, phospho-c-Jun, phospho-p38 and phospho-nuclear factor-κB (NF-κB), decreased the level of phospho-ERK1/2 protein and reduced the ratio of Bcl-2/Bax in the hippocampus. Dexmedetomidine pretreatment inhibited isoflurane-induced neuroapoptosis and restored protein expression of MAPK pathways and the Bcl-2/Bax ratio after isoflurane exposure. Moreover, SB203580 and SP600125 also partly attenuated the isoflurane-induced protein changes. However, U0126 did not reverse dexmedetomidine-induced neuroprotection. Our results indicate that the JNK and p38 pathways, not the ERK pathway are involved in dexmedetomidine-induced neuroprotection against isoflurane effects.

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1. Introduction
Recent studies have demonstrated that prolonged exposure to volatile anesthetics causes apoptotic neurodegeneration in the developing animal brains and persistent learning deficits (Brambrink et al., 2010; Jevtovic-Todorovic et al., 2003; Kong et al., 2011; Li et al., 2013a,b; Satomoto et al., 2009). Some retrospective studies have found that children younger than 4 years old exposed to surgery under general anesthesia for more than once have a higher risk of developing disabilities in reading and learning (DiMaggio et al., 2011; Ing et al., 2012). Therefore, it is important to identify the mechanisms for anesthetics-induced neurotoxicity in animals so that potential protective strategies can be developed should the effects are confirmed in humans.

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Xenon, a noble anesthetic gas, has been reported to completely reverse isoflurane-induced neurototoxicity in neonatal mouse brain (Ma et al., 2007; Shu et al., 2010). However, xenon is only registered for anesthesia in adults currently and is not available widely because of high price, which limit its use in clinic. Besides, recent research shows xenon causes neuronal cell death in an in vitro model of the developing rodent brain at 1 MAC, as does isoflurane and sevoflurane at similarly potent concentrations (Brosnan and Bickler, 2013). Isoflurane preconditioning is also a protective strategy that attenuates isoflurane-induced neurotoxicity (Wei et al., 2007), but it is difficult to perform in pediatric anesthesia. Subclinical carbon monoxide limits apoptosis in the developing mouse brain after isoflurane exposure (Cheng and Levy, 2014). However, this method may not be practical due to the toxicity of CO. Recently, Sanders and co-authors report dexmedetomidine, a highly selective α2-adrenergic agonist, attenuates isoflurane-induced neuroapoptosis and neurocognitive impairment in neonatal rats (Sanders et al., 2009, 2010). However, the mechanisms of this effect in the developing brain are still not clear.

Dexmedetomidine can increase the expression of phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) in astrocytes and mouse brain (Du et al., 2009; Li et al., 2008) and inhibit increase of pro-inflammatory cytokines in lipopolysaccharide-stimulated astrocytes by suppressing c-Jun NH2-terminal kinases (JNK) (Zhang et al., 2014), which suggests mitogen-activated protein kinase (MAPK) pathways may be related to dexmedetomidine–induced neuroprotection.

MAPKs are a family of serine-threonine protein kinases that consists of three major members: ERK, p38 MAPK, and JNK. MAPK signaling cascades play crucial cellular roles under normal and pathological conditions, such as nervous system development (Mousa and Bakhiet, 2013), neurodegeneration (Harper and Wilkie, 2003), pain (Ji et al., 2009) and brain inflammation (Kaminska et al., 2009). Recent studies also showed MAPK pathways are associated with anesthetics-induced neurotoxicity. N-arachidonoylthanolamine (AEA) analog N-stearoyl-l-tyrosine protects developing brain against sevoflurane-induced neurotoxicity through ERK1/2 signaling pathway (Wang et al., 2013). Our previous study has demonstrated JNK pathway is involved in isoflurane-induced neuronal apoptosis in the hippocampus of neonatal rats (Li et al., 2013b). Moreover, Sanders et al. have found dexmedetomidine reduces the decrease of phosphorylated ERK1/2 induced by isoflurane in neonatal rat brain (Sanders et al., 2010). However, whether ERK, JNK and P38 pathways are involved in the dexmedetomidine–induced neuroprotection against isoflurane is still undetermined. We hypothesize dexmedetomidine pretreatment provides neuroprotection against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats through preserving ERK1/2 pathway activity as well as inhibiting JNK and P38 pathway activity.

2. Materials and methods

2.1. Animals

This study was approved by the animal care committee at Sun Yat-sen University and performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. Seven-day-old (P7) Sprague-Dawley rat pups (Guangdong Medical Laboratory Animal Co., China, permission number: SCXK 2011–0029) weighting 13–16 g were used. Rats were exposed to 0.75% isoflurane for 6 h (approximately 0.3 MAC in P7 rats as determined by Orliguet et al. (2001)) in 30% oxygen or air in a temperature-controlled chamber as we described before (Li et al., 2013b).

2.2. Experimental protocol

Two experiments were performed. In experiment one, three doses of dexmedetomidine (25, 50, or 75 µg/kg) or saline in 150 µl were administered by intraperitoneal injection 20 min before the exposure to isoflurane. One group received 25 µg/kg dexmedetomidine in 3 doses during the 6-h isoflurane exposure (at 0, 2, and 4 h after the onset of isoflurane exposure), which was used as a different method for administration. Additionally, the dose of 75 µg/kg dexmedetomidine was given alone to determine whether a high dose of dexmedetomidine could induce apoptosis. Rats after these various treatment were used for Western blot study (n = 6). Rats of 75 µg/kg dexmedetomidine pretreatment were used for TUNEL (n = 6) as we described before (Li et al., 2013b).

In experiment two, some rats received intracerebroventricular (i.c.v.) injection of the ERK inhibitor U0126 (Selleck Chemicals LLC, Houston, TX, USA) 10 nmol 20 min before the pretreatment with 75 µg/kg dexmedetomidine and isoflurane exposure. Other rats received the p38MAPK inhibitor SB203580 20 nmol (i.c.v.) or JNK inhibitor SP600125 30 µg (i.c.v.) 20 min before isoflurane exposure. The i.c.v. injection was performed as we described before (Li et al., 2013b). Vehicle (5 µl 10% DMSO) or the three MAPK inhibitors were separately injected into the left lateral ventricle (stereotaxic coordinates: 2.0 mm rostral and 1.5 mm lateral to the lambda and 2.0 mm deep to the skull surface) using a 5 µl Hamilton syringe at a constant rate of 2.5 µl/min. Rats were sacrificed at the end of gas exposure. Their hippocampi were used for Western blot study (n = 6) to determine whether and which MAPK pathways are functionally important in mediating dexmedetomidine–induced neuroprotection against isoflurane effects.

2.3. TUNEL fluorescent assay

TUNEL studies were performed as we described before (Li et al., 2013b). Briefly, rat pups were anesthetized with isoflurane and perfused transcardially with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Their brains were post-fixed for 48 h at 4 °C and paraffin embedded and sectioned at 5 µm thickness. At least three sections (100 µm apart among them) corresponding to Figures 95–97 in the Atlas of the Developing Rat Brain (Paxinos et al., 1990) for each animal were chosen for TUNEL. TUNEL fluorescent assay was performed using the Dead End TM fluorometric TUNEL system kit (Promega, Madison, WI, USA). The slides were protected from direct light during experiment and Hoechst was used to stain nuclei. TUNEL positive cells in the hippocampal CA1, CA3 and DG regions were analyzed with NIH-Elements BR imaging processing and analysis software (Nikon Corporation, Japan). The density of TUNEL positive cells in CA1 region was calculated by dividing the number of TUNEL positive cells by the area of that brain region.

2.4. Immunoblotting

For Western blot studies, rat pups were sacrificed by decapitation at the end of the exposure. Hippocampi were isolated immediately on ice and then stored at −80 °C until used. Western blotting was performed as we have described previously (Li et al., 2013a,b). In brief, protein concentrations of samples were determined using the BCA protein assay (Bio-Rad, Hemel Hempstead, Herts, UK). Forty micrograms of each protein sample were subjected to Western blot analysis using the following primary antibodies: anti-cleaved caspase-3 at 1:1000 dilution, anti-Bax at 1:1000 dilution, anti-Bcl-2 at 1:1000 dilution, anti-phospho-ERK1/2 at 1:1000 dilution, anti-ERK1/2 at 1:1000 dilution, anti-phospho-JNK at 1:1000 dilution, anti-JNK at 1:1000 dilution, anti-phospho-c-Jun at 1:1000 dilution, anti-phospho-P38 at 1:1000 dilution, anti-phospho-ERK1/2 at 1:1000 dilution, anti-ERK1/2 at 1:1000 dilution, anti-phospho-JNK at 1:1000 dilution, anti-JNK at 1:1000 dilution, anti-phospho-c-Jun at 1:1000 dilution, anti-phospho-P38 at 1:1000 dilution.
dilution, anti-P38 at 1:1000 dilution, anti-phospho-nuclear factor-kB (NF-kB) at 1:1000 dilution, and anti-β-actin at 1:2000 dilution. All antibodies were purchased from Cell Signaling Technology, Beverly, MA, USA. Images were scanned by an Image Master II scanner (GE Healthcare, Milwaukee, WI, USA) and were analyzed using ImageQuant™ TL software v2003.03 (GE Healthcare, Milwaukee, WI, USA). The protein expression of phospho-ERK1/2, phospho-JNK or phospho-P38 was normalized to the total ERK1/2, JNK or P38, respectively. The band signals of other interesting proteins were normalized to those of β-actin from the same samples.

2.5. Statistical analysis

Sample size was calculated by using the PASS 11 software to achieve an 80% power at a significance level of 0.05. All data were normally distributed as tested using the Shapiro–Wilks test and had no significant heterogeneity of variance as detected by Levene’s test. Data were presented as means ± SD and analyzed by one-way ANOVA with Tukey’s multiple comparisons. The GraphPad Prism 6.0 software was used to conduct the statistical analyses. Statistical significance was accepted at P < 0.05.

3. Results

3.1. Dexmedetomidine pretreatment decreased isoflurane-induced neuroapoptosis in hippocampus

The number of TUNEL positive cells induced by isoflurane was increased by 43.5% (P < 0.001), 390% (P = 0.001), 285% (P < 0.001), respectively, when compared with control group in hippocampal CA1, CA3 and DG areas. Pretreatment with 75 µg/kg dexmedetomidine significantly inhibited the increase of isoflurane-induced TUNEL positive cells in the hippocampal CA1 area (Fig. 1A and C) and DG area (Fig. 1B and E), (P = 0.029, P = 0.011, respectively), but have no significant difference in CA3 area. Application of one dose of 75 µg/kg dexmedetomidine alone did not increase neuronal apoptosis compared with control (P = 0.54). Quantification of the expression of cleaved caspase-3 protein in whole hippocampus by western blots also revealed that isoflurane significantly induced caspase-3 activation (P < 0.001) (Fig. 2A and B), while pretreatment with 50 µg/kg or 75 µg/kg but not 25 µg/kg dexmedetomidine or administration of three doses of 25 µg/kg dexmedetomidine during isoflurane exposure significantly reduced isoflurane-induced cleaved caspase-3 activation (P = 0.003, P < 0.001, P = 0.228, P < 0.001, respectively). Furthermore, pretreatment with 75 µg/kg dexmedetomidine or administration of three doses of 25 µg/kg dexmedetomidine provided similar and the most potent protection. Dexmedetomidine alone did not induce cleaved caspase-3 activation.

3.2. Dexmedetomidine reversed isoflurane-induced protein changes in MAPK pathways

Isoflurane at 0.75% significantly decreased the protein expression of phospho-ERK1/2 at 44 kDa (P = 0.03, Fig. 2C) and increased the proteins expression of phospho-JNK at 54 kDa (P < 0.001, Fig. 2D) and phospho-p38 (P < 0.001, Fig. 2E). However, pretreatment with 75 µg/kg but not 25 µg/kg or 50 µg/kg dexmedetomidine or administration of three doses of 25 µg/kg dexmedetomidine increased the expression of phospho-ERK1/2 at 44 kDa compared with the isoflurane-treated rats (P = 0.044, P = 0.058, P = 0.061, P = 0.037, Fig. 2A and C). Pretreatment with dexmedetomidine (25, 50 or
Fig. 2. Dexmedetomidine reversed isoflurane-induced inhibition of phospho-ERK1/2 and increase of cleaved caspase-3, phospho-JNK and phospho-p38 in the hippocampus of P7 rats (n = 6 in each group). (A) Representative Western blot of caspase-3, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38 and p38; (B–E) the quantitative analysis of cleaved caspase-3 (B), phospho-ERK1/2 (C), phospho-JNK (D), phospho-p38 (E) by one-way ANOVA with Tukey’s multiple comparisons. Results are the means ± SD. NS: normal saline, Dex: dexmedetomidine, Iso: isoflurane, Dex75: Dex 75 μg/kg, Dex25: Dex 25 μg/kg for three times, Dex50: Dex 50 μg/kg. *P < 0.05, ***P < 0.0001 versus Air + NS; #P < 0.05, ##P < 0.01, ###P < 0.0001 versus Iso + NS; ▲P < 0.05 versus Iso + Dex25.

75 μg/kg) or administration of three doses of 25 μg/kg dexmedetomidine dose-dependently reduced isoflurane-induced increase of phospho-JNK proteins at 54 kD (P = 0.008, P = 0.002, P = 0.001, P < 0.001, respectively, Fig. 2A and D) and phospho-p38 proteins (P = 0.023, P = 0.009, P = 0.002, P < 0.001, respectively, Fig. 2A and E). Three doses of 25 μg/kg dexmedetomidine provided a similar protective effect on preventing isoflurane-induced changes of the expression of above proteins compared with pretreatment with 75 μg/kg dexmedetomidine. Dexmedetomidine alone did not cause a significant change of the phosphorylation of ERK1/2, JNK and p38.
3.3. ERK1/2 pathway did not participate in the neuroprotection of dexmedetomidine

The ERK inhibitor U0126 was used to investigate whether the ERK1/2 pathway participated in the neuroprotection of dexmedetomidine. Consistent with the data described above, isoflurane significantly induced capase-3 activity and decreased the protein expression of phospho-ERK1/2. Pretreatment with dexmedetomidine 75 µg/kg reversed isoflurane-induced proteins change above. Moreover, dexmedetomidine also reduced isoflurane-induced decrease of Bcl-2 proteins (P < 0.007, Fig. 3A and C) and increase of Bax proteins (P < 0.001, Fig. 3A and D) to recover the ratio of Bcl-2/Bax (P = 0.038, Fig. 3A and E). While U0126 did not reverse dexmedetomidine pretreatment-induced neuroprotection, U0126 combined with dexmedetomidine pretreatment did not cause significant changes of proteins expression of capase-3, phospho-ERK1/2, Bcl-2, Bax and the ratio of Bcl-2/Bax when compared with dexmedetomidine alone pretreated rats after isoflurane exposure (Fig. 3).

3.4. JNK pathway may participate in the neuroprotection of dexmedetomidine

SP600125, a JNK inhibitor, was used to investigate whether JNK pathway participated in the neuroprotection of dexmedetomidine against isoflurane. Consistent with the data described above, isoflurane significantly induced capase-3 activity and increased the protein expression of phospho-JNK. Pretreatment with dexmedetomidine 75 µg/kg also reversed isoflurane-induced protein expression change. Moreover, dexmedetomidine reduced isoflurane-induced increase of phospho-c-Jun (P < 0.001) and Bax proteins (P < 0.001) and decrease of Bcl-2 proteins (P = 0.012) as well as recovered the ratio of Bcl-2/Bax (P = 0.001, Fig. 4). Pretreatment of the JNK inhibitor SP600125 provided a similar protective effect on preventing isoflurane-induced protein changes by significantly decreasing cleaved capase-3 (P = 0.012, Fig. 4A and B) and Bax protein expression (P < 0.001, Fig. 4A and D), reducing phospho-JNK (P < 0.05, Fig. 4A and F) and phospho-c-Jun protein expression (P = 0.002, Fig. 4A and G), as well as increasing Bcl-2 protein expression (P = 0.048 Fig. 4A and C). Thus, the ratio of Bcl-2/Bax was increased (P = 0.002, Fig. 4A and E). SP600125 alone did not inhibit the expression of these proteins when compared with controls.

3.5. P38 MAPK pathway may participate in the neuroprotection of dexmedetomidine

The P38 MAPK inhibitor SB203580 was used to investigate whether p38 MAPK pathway participated in the neuroprotection of dexmedetomidine against isoflurane. Pretreatment with dexmedetomidine 75 µg/kg not only reversed isoflurane-induced increase of cleaved capase-3 (P = 0.002) and phospho-p38 protein (P = 0.015), but also reduced isoflurane-induced increase of phospho-NF-κ-B (P = 0.019) and Bax proteins (P < 0.001) and decrease of Bcl-2 proteins (P = 0.035) as well as recovered the ratio of Bcl-2/Bax (P = 0.011, Fig. 5). Pretreatment with the p38 inhibitor SB203580 provided a similar protective effect to prevent isoflurane-induced protein changes by significantly decreasing cleaved capase-3 (P < 0.001, Fig. 5A and B) and Bax protein expression (P < 0.001, Fig. 5A and D), reducing phospho-p38 (P = 0.019, Fig. 5A and F) and phospho-NF-κB protein expression (P = 0.049, Fig. 5A and G), as well as increasing Bcl-2 protein expression (P < 0.001, Fig. 5A and C). Thus, the ratio of Bcl-2/Bax was increased (P = 0.019, Fig. 5A and E). SB203580 alone did not inhibit the expression of these proteins when compared with controls.

4. Discussion

The present study demonstrated that dexmedetomidine pretreatment provided neuroprotection against isoflurane-induced neuroapoptosis in a dose-dependent manner. Moreover, dexmedetomidine pretreatment reversed isoflurane-induced phosphorylation increase of JNK, c-Jun, p38 and NF-κB as well as increased expression of Bax, decreased Bcl-2 expression and Bcl-2/Bax ratio. Further, the p38 MAPK inhibitor SB203580 and JNK inhibitor SP600125 attenuated isoflurane-induced increase of cleaved caspase-3 and Bcl-2/Bax ratio. Although dexmedetomidine pretreatment increased ERK1/2 phosphorylation, which was inhibited during isoflurane exposure, the ERK1/2 inhibitor U0126 did not reverse the dexmedetomidine-induced neuroprotection. These results indicate that the JNK and p38 MAPK pathways, but not the ERK pathway, may be involved in the mechanisms for dexmedetomidine neuroprotection.

Isoflurane have been reported to induce neurodegeneration in the developing brain (Brambink et al., 2010; Jevtovic-Todorovic et al., 2003; Kong et al., 2011; Li et al., 2013b). Hippocampus is the most sensitive region to isoflurane-induce neurotoxicity because isoflurane-treated neonatal rats show normal short-term memory, a function predominantly involving the prefrontal cortex, but have an abnormal response to contextual fear conditioning, indicating a severe hippocampal lesion (Sanders et al., 2009). Besides, dexmedetomidine reduces isoflurane-induced neuroapoptosis in the hippocampus, but not in the cortex, and also prevents hippocampus-dependent neurocognitive impairment after isoflurane exposure (Sanders et al., 2009). In this study, we used hippocampus to further study the mechanism of the neuroprotection induced by dexmedetomidine.

Dexmedetomidine is commonly used in pediatric patients for anesthesia and sedation. Unlike other commonly used anesthetics, neonatal administration with dexmedetomidine does not impair rat hippocampal synaptic plasticity later in adulthood (Tachibana et al., 2012). Moreover, the study by Sanders et al. (2009) has identified that three doses of 25 µg/kg dexmedetomidine provide the maximal neuroprotection compared with three doses of 1 µg/kg or 10 µg/kg by reducing neuroapoptosis induced by isoflurane in the developing rat brain. Our recent study (Li et al., 2014) has found that pretreatment with dexmedetomidine also provides neuroprotection against long-duration isoflurane exposure in a dose-dependent manner. Pretreatment with 75 µg/kg dexmedetomidine provides a protection similar to that of applying 25 µg/kg for three times. Consistent with previous study, our results showed pretreatment with 75 µg/kg dexmedetomidine significantly reversed isoflurane-induced neuronal apoptosis in the hippocampus of neonatal rats by preserving PI3K/Akt pathway activity (Li et al., 2014). However, either α2-adrenoceptor antagonist atipamezole or PII3K inhibitor LY294002 partly reversed neuroprotection of dexmedetomidine. Other mechanisms may be involved. ERK signaling pathway plays an important role in the neuroprotection of dexmedetomidine in both neuropathic pain and traumatic brain injury models (Liu et al., 2012; Schoeler et al., 2012). Dexmedetomidine can activate phosphorylation of ERK1/2 in astrocytes (Guan et al., 2005). Epidermal growth factor (EGF) receptor transactivation in astrocytes in the mature brain in vivo is an important process in response to dexmedetomidine stimulation and may lead to phosphorylation of ERK(1/2) both in astrocytes.
Fig. 3. The inhibition of ERK pathway had no effect on dexmedetomidine’s neuroprotection against isoflurane. P7 rats were injected intracerebroventricularly with ERK inhibitor U0126 (10 nmol) before dexmedetomidine (intraperitoneally, 75 μg/kg). After treated with 0.75% isoflurane for 6 h, the hippocampal tissues were collected for detection of P-ERK1/2, total ERK1/2 (A), cleaved Caspase-3 (B), Bcl-2 (C) and Bax (D) by western blot. The histogram represented the quantification of p-ERK1/2, cleaved Caspase-3, Bcl-2 and Bax, respectively. *P<0.05, **P<0.01, ***P<0.001, vs. group Air + NS; #P<0.05, ##P<0.01, ###P<0.001 vs. Group Iso + NS; NS, no significance, vs. Group Iso + Dex75. Error bar represent the means ± SD, n=6/group.
Fig. 4. Both dexmedetomidine and SB203580 attenuated isoflurane-induced neurotoxicity in the hippocampus of P7 rats (n = 6 in each group). (A) Representative Western blot of cleaved caspase-3, phospho-p38, p38, phospho-NF-κB, Bcl-2, Bax; (B–G) The quantitative analysis of cleaved caspase-3 (B), Bcl-2 (C), Bax (D), and the ratio of Bcl-2 to Bax (E), phospho-p38 (F), phospho-NF-κB (G) by one-way ANOVA with Tukey’s multiple comparisons. Results are the means ± SD. NS: normal saline, Dex: dexmedetomidine, Iso: isoflurane, Dex75: Dex 75 μg/kg, *P < 0.05, **P < 0.01, ***P < 0.0001 versus Air + NS; *P < 0.05, **P < 0.01, ***P < 0.0001 versus Iso + NS.
Fig. 5. Both dexmedetomidine and SP600125 attenuated isoflurane-induced neurotoxicity in the hippocampus of P7 rats (n=6 in each group). (A) Representative Western blot of cleaved caspase-3, phospho-JNK, JNK, phospho-c-Jun, Bcl-2, Bax; (B–G) the quantitative analysis of cleaved caspase-3 (B), Bcl-2 (C), Bax (D), and the ratio of Bcl-2 to Bax (E), phospho-JNK (F), phospho-c-Jun (G) by one-way ANOVA with Tukey’s multiple comparisons. Results are the means ± SD. NS: normal saline, Dex: dexmedetomidine, Iso: isoflurane, Dex75: Dex 75 μg/kg. *P<0.05, **P<0.01, ***P<0.0001 versus Air + NS; *P<0.05, **P<0.01, ***P<0.0001 versus Iso + NS.
themselves and in adjacent neurons (Du et al., 2009; Li et al., 2013c). Sanders et al. (2009) have found that dexamethasone reversed isoflurane-induced downregulation of ERK1/2 phosphorylation in the cortex of neonatal rats. Consisting with their results, our study in the hippocampus of neonatal rats also showed that pretreatment with 75 μg/kg dexamethasone once or 25 μg/kg three times reversed isoflurane-induced downregulation of ERK1/2 phosphorylation. While ERK1/2 U0126 inhibitor did not reverse dexamethasone-induced increase of Bcl-2 and inhibition of cleaved caspase-3 and Bax during isoflurane exposure, which suggests that increase of ERK1/2 phosphorylation may not be involved in the neuroprotection of dexamethasone against isoflurane effects. The JNK signaling pathway is implicated in neuronal apoptosis triggered by several brain injury stimuli, such as ischemia/reperfusion and ethanol (Guan et al., 2005, 2008; Han et al., 2008). The JNK pathways include nuclear pathway and non-nuclear pathway (Han et al., 2008; Li et al., 2013c). Activated JNK phosphorylates a nuclear substrate, the transcription factor c-Jun, which leads to increase of activator protein-1 transcription activity to modulate transcription of genes related to apoptosis. On the other hand, activated JNK regulates the activation of non-nuclear substrates including Bcl-2 family members (Guan et al., 2005, 2008). Our previous study has demonstrated that JNK signaling activation was involved in isoflurane-induced neuronal apoptosis (Li et al., 2013b). The present results showed that dexamethasone pretreatment inactivated JNK nuclear pathway by preventing isoflurane-induced increase of phosphorylation of JNK and transcription factor c-Jun, as well as inhibited JNK non-nuclear pathway by preventing isoflurane-induced increase of Bax and downregulation of Bcl-2 expression, thus reversed isoflurane-induced increase of caspase-3. JNK inhibition with SP600125 also prevented both phosphorylation of c-Jun and neuroapoptosis induced by isoflurane. These results suggest that JNK signaling is involved in the neuroprotection of dexamethasone against isoflurane effects. Much recent evidence has demonstrated that neuroinflammation contributes to volatile anesthetic-induced neuroapoptosis and cognitive deficits (Li et al., 2013c; Cao et al., 2012; Lin and Zuo, 2011; Shu et al., 2012; Wu et al., 2012). P38 MAPK–NF-κB signaling pathway plays an important effect in inflammatory process (Kim et al., 2006). Isoflurane can activate p38 MAPK by itself in rat brain (Zheng and Zuo, 2004). Moreover, isoflurane can increase pro-inflammatory cytokine interleukin (IL)-6 levels in the plasma of patients undergoing minimally invasive surgery (Mazotti et al., 2013) and in neuroglioma cells (Zhang et al., 2013), as well as increase the expression of TNF-α, IL-1β, IL-6, and caspase 3 in the brain of adult and aged rodents (Cao et al., 2012; Lin and Zuo, 2011; Wu et al., 2012). More importantly, IL-1β mediates isoflurane-induced cognitive impairment (Cao et al., 2012; Lin and Zuo, 2011). Activation of the NF-κB pathway is involved in isoflurane-induced hippocampal IL-1β and IL-6 elevation (Li et al., 2013c; Zhang et al., 2013). Our results showed that isoflurane activated p38 MAPK pathway by increasing phosphorylation of p38 and NF-κB in the hippocampus of neonatal rats. Both dexamethasone and the p38 MAPK inhibitor SB203580 pretreatment prevented isoflurane-induced increase of phosphorylated P38 and NF-κB, as well as increase of Bax expression and downregulation of Bcl-2 expression. Both agents also reversed isoflurane-induced increase of caspase-3. These results suggested P38 MAPK signaling pathway was involved in both isoflurane-induced neuronal apoptosis and neuroprotection of dexamethasone against isoflurane effects. It is possible that isoflurane induces neuroapoptosis by neuroinflammation via activation of P38 MAPK and NF-κB pathway and dexamethasone ameliorated isoflurane-induced neuroapoptosis by its anti-inflammatory effects. Consisting with our presumption, Shen et al. have found exposure to sevoflurane for 2h daily for 3 days induced cognitive impairment and neuroinflammation in neonatal mice, while anti-inflammatory treatment ameliorated the sevoflurane-induced cognitive impairment (Shen et al., 2013), neuroinflammation also further augmented neuroapoptosis and cognitive impairment induced by isoflurane in neonatal rats. In addition to the p38 pathway, JNK pathway activation also mediates neuroinflammation in several brain injury models such as Alzheimer’s disease (Banjii-Mirza et al., 2014), ischemic brain damage (Nijboer et al., 2013) and white matter injury (Wang et al., 2012). Dexamethasone also exert anti-inflammatory effects on astrocytes stimulated by lipopolysaccharide by suppressing the JNK pathway (Zhang et al., 2014). Thus, we presume that dexamethasone may attenuate isoflurane-induced proinflammatory response and neuroapoptosis via suppressing both P38 MAPK and JNK signaling pathways. There are limitations in our study. First, we did not detect the expression of inflammatory cytokines in the neonatal rat brain. Whether dexamethasone is effective to reduce isoflurane-induced neuroinflammation needs further study. Also, we only studied acute effects in hippocampus of neonatal rats. Additional experiments are needed to study the long-term effects and to test the impact of dexamethasone pretreatment on isoflurane-induced injury in other brain regions and cognitive dysfunction. In conclusion, isoflurane-induced neurotoxicity by activating both JNK and p38 MAPK pathway. Dexamethasone pretreatment provided neuroprotection against isoflurane-induced neuroapoptosis by inhibiting both JNK and p38 MAPK pathways. Since dexamethasone is commonly used clinically for analgesia, sedation and anesthetic sparing, this effect of dexamethasone may have a great translational potential if a clinical injury from anesthetics is proven in children.

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