Dexmedetomidine attenuates propofol-induced neuroapoptosis partly via the activation of the PI3k/Akt/GSK3β pathway in the hippocampus of neonatal rats

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ABSTRACT

Recent studies have demonstrated that propofol causes neurodegeneration in developing brains. Evidence has shown that dexmedetomidine has neuroprotective effects. However, whether dexmedetomidine can reduce propofol-induced neuroapoptosis and by what mechanisms it acts remain unclear. We investigated whether dexmedetomidine can attenuate propofol-induced neuroapoptosis by disturbing the PI3K/Akt/GSK3β pathway during brain development. Seven-day-old rats were randomly exposed to 100 mg/kg propofol and 100 mg/kg propofol plus different doses of dexmedetomidine or 100 mg/kg propofol and 75 μg/kg dexmedetomidine plus PI3K inhibitor LY294002 or GSK3β inhibitor TDZD-8. TEM and TUNEL were used to detect neuronal structure changes and apoptosis. The expression of phospho-Akt, phospho-GSK3β, Akt and GSK3β were quantified using western blots and immunofluorescence. Pretreatment with different doses of dexmedetomidine protected against propofol-induced neuroapoptosis. Furthermore, propofol decreased the levels of phospho-Akt and phospho-GSK3β, whereas dexmedetomidine partially reversed this inhibition. In addition, treatment with LY294002 inhibited the neuroprotection of dexmedetomidine, whereas TDZD-8 enhanced neuroprotection. Our results indicate that dexmedetomidine prevents propofol-induced neuroapoptosis by increasing the levels of phospho-Akt and phospho-GSK3β.

1. Introduction

Propofol has been used safely and widely in pediatric anesthetic practice for many years (Istaphanous and Loepke, 2009) due to its attractive pharmacokinetic profile, rapid onset and short recovery period (Miner and Burton, 2007; Srivastava et al., 2014). However, increasing evidence from animal studies suggest that high doses of propofol or prolonged exposure may induce neuronal cell apoptosis in the developing brain (Cattano et al., 2008; Fredriksson et al., 2007). Thus, we aimed to investigate the mechanism underlying propofol-induced neurodegeneration and the protective measures.

Dexmedetomidine, an α2-adrenergic agonist, has been developed for human clinical use as an anesthetic and sedative. In developing rat brains, dexmedetomidine has been shown to attenuate anesthesia-induced neuroapoptosis, particularly ketamine- and isoflurane-induced neuroapoptosis (Duan et al., 2014; Li et al., 2014). The current study showed that both the PI3K/Akt and the ERK1/2 pathways participate in this neuroprotection by dexmedetomidine against transient focal cerebral ischemia/reperfusion injury in rats (Zhu et al., 2013), and the JNK, P38 MAPK and PI3K/Akt pathways have been shown to participate in dexmedetomidine-mediated neuroprotection against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats (Li et al., 2014; Liao et al., 2014). Whether dexmedetomidine can protect against propofol-induced neuroapoptosis in the developing rat brain remains unclear.

The PI3K/Akt/GSK3β signaling pathway is widely known for its pivotal roles in neuronal development, survival and death (Arboleda et al., 2010; Bijur and Jope, 2003). Glycogen synthase kinase-3 (GSK3β) is involved in fundamental cellular processes that can determine cell fate, tumorigenesis, and cell death (Frame and Cohen, 2001). GSK3β is involved in the regulation of the growth and development of neural tissues in Drosophila and in vertebrates (Leroy and Brion, 1999). Studies have demonstrated that the PI3K/Akt/GSK3β signaling pathway plays an important role in the neuroprotective effects of dexmedetomidine (Xia et al., 2008; Ye et al., 2015). Previous studies have shown that propofol induces neuroapoptosis by decreasing Akt phos-
phosphorylation (Huang et al., 2016; Twaroski et al., 2014), and that dexmedetomidine dramatically attenuates isoflurane-induced neuroapoptosis by increasing Akt phosphorylation (Li et al., 2014). More recent analyses have demonstrated that GSK3β plays an important role in ketamine-induced neuroapoptosis in developing rats, in which ketamine downregulates pAkt and pGSK3β (Liu et al., 2013). The role of GSK3β, a downstream mediator of Akt, in the neuroapoptosis of propofol and the neuroprotection of dexmedetomidine needs to be investigated. We hypothesized that propofol induces neuroapoptosis via the Akt/GSK3β pathway and that dexmedetomidine reverses these propofol-induced protein changes to promote neuroprotection.

2. Materials and methods

All animals were treated in strict accordance with the guidelines of Guangxi Medical University, and the animal protocols were approved by the Animal Care and Use Committee of the university. Seven-day-old (P7) Sprague-Dawley (male and female) rat pups (average body weight, 10–15 g) were used in this study. The rats were maintained in a 12-h light/dark cycle with free access to food and water, and the temperature of the room was maintained at 22 °C ± 1 °C. The animals were randomly divided into 10 groups (n = 6 per group) as follows: saline group (NS); intralipid group representing the fat emulsion vehicle control (F); 100 mg/kg propofol group (P) treated with two intraperitoneal injections (50 mg/kg each); propofol and dexmedetomidine groups (PD25, PD50, PD75) treated with 100 mg/kg propofol and 25 μg/kg (PD25), 50 μg/kg (PD50), or 75 μg/kg (PD75) dexmedetomidine administered 30 min prior to propofol (Li et al., 2014); DMSO groups (D1, D2) treated with 10% dimethyl sulfoxide via intracerebroventricular injection (D1) or intraperitoneal injection (D2); LYPD group, treated with intracerebroventricularly injected LY294002 (PI3K inhibitor, 25 μg/kg dissolved in 10% dimethyl sulfoxide) administered 30 min prior to propofol and dexmedetomidine as in the PD75 group; and TDPD group, treated with intraperitoneally injected TDZD-8 (GSK3-β inhibitor, 1 mg/kg dissolved in 10% dimethyl sulfoxide) following the protocol for LYPD (Fig. 1). The hippocampi of the rats in all groups were obtained 2 h after the animals were fully awake and used for electron microscopy, western blot (WB) and immunofluorescence.

2.1. Tissue preparation

The rat pups were sacrificed by decapitation after the rat fully awake. Hippocampus was isolated immediately on ice and then stored at −80 °C until used. For immunohistochemistry and TUNEL studies, the rat pups were anesthetized with chloral hydrate (350 mg/kg), then perfused with icecold saline followed by 4% paraformaldehyde, then their brain was isolated immediately and post-fixed for 48 h at 4 °C and paraffin embedded until used.

2.2. Western blot analysis

Variations in protein levels were analyzed in all experimental groups. After treatment, the protein was extracted and measured using bicinchoninic acid protein assays (Biotype Biotech Co., Shanghai, China). Equal amounts of protein were loaded onto 12% SDS-polyacrylamide gels, and were transferred onto polyvinylidene difluoride membranes (0.22-μm pore size, Millipore, Bedford, USA) after electrophoretic separation. The membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) at room temperature (RT) for 2 h, followed by incubation with primary antibody (Akt, p-Akt, GSK3β and p-GSK3β (1:1000, Cell Signaling Technology, USA); GAPDH (1:10000, Santa Cruz Biotechnology, Inc., USA)) overnight at 4 °C. The membranes were washed three times with TBST, followed by incubation with secondary antibody (1:5000; Santa Cruz Biotechnology, Inc., USA) at RT for 30 min. The blots were developed using the Odyssey infrared imaging system (LI-COR, USA) and evaluated using densitometric analysis.

2.3. Immunofluorescence

The hippocampal expression of p-Akt and p-GSK3β was also measured using immunofluorescence. The rat pups were deeply anesthetized with 10% chloral hydrate (3.5 mL/kg, intraperitoneal

![Fig. 1. The experimental design of this investigation.](image-url)
injection) and intracardially perfused with 20 ml saline followed by 50 ml 4% ice-cold paraformaldehyde, and paraaffin-embedded sections were prepared for immunofluorescence. Paraffin sections were deparaffinized and permeabilized with 0.2% Triton X-100. The sections were then incubated with 10% normal goat serum to block nonspecific binding at RT for 30 min, followed by incubation at 4 °C overnight with the primary antibody (1:1000, Cell Signaling Technology, USA) and incubation with FITC/TRITC-conjugated secondary antibodies at RT for 3 h (Santa Cruz Biotechnology, Inc., USA). For nuclear staining, 4′-diamidino-2-phenylindole (DAPI) (500 ng/ml, Sigma, USA) was applied for 5 min. After the sections were rinsed, the sections were observed under a fluorescence microscope.

2.4. Transmission electron microscope (TEM)

Transmission electron microscopy (TEM) was used to verify the neuronal ultrastructure following propofol-induced neurodegeneration and the neuroprotective effects of dexmedetomidine. All of the groups of pups were sacrificed via transcardiac perfusion of 0.9% saline and 4% ice-cold paraformaldehyde, and the hippocampal tissue was removed. The hippocampus was fixed in 1% osmium tetroxide, washed with 25% and 50% ethanol plus 5% uranyl acetate, dehydrated in an ascending ethanol series and embedded in epon-araldite. The hippocampus was cut in semithin sections (1 um). The sections were examined using electron microscopy.

2.5. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay

We detected cell apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays (Millipore, Serological Corporation, Norcross, GA, USA). Brain hippocampal sections were deparaaffinized and rehydrated. After treatment with proteinase K (Roche Applied Science, Indianapolis, IN, USA) and quenching with 3.0% hydrogen peroxide, sections were incubated in a terminal deoxynucleotidyl transferase (TdT) reaction mix for 1 h at 37 °C. Sections were washed, incubated for 30 min in a solution of antidigoxigenin conjugate, and colorized with DAB. All sections were counterstained with hematoxylin. The TdT reaction mix was omitted for control sections. The density of TUNEL-positive cells was counted in randomly selected sampling areas.

2.6. Statistical analysis

Data were expressed and graphed as the mean ± standard error of the mean and analyzed using SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA) and Origin version 7.5 software (OriginLab Co., Northampton, MA, USA). Multiple comparisons were performed with a one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test as appropriate. P values < 0.05 were considered significant.

3. Result

3.1. Propofol induced neuroapoptosis and decreased akt and GSK3β phosphorylation

To determine the role of propofol in neuroapoposis, we detected the effects of propofol by TEM and TUNEL. We examined the F group to exclude the effects of the vehicle and the route of administration, and the results indicated that there were no significant differences between the intralipid group and the NS group. Compared with the NS group, propofol (P group) induced hippocampal neuroapoptosis; electron microscopy images (P group) revealed neuronal cell swelling and nuclear fragmentation. Furthermore, chromatin density decreased, apoptotic bodies were observed, the synaptic cleft widened, and the postsynaptic densities became thinner (Fig. 2). The number of TUNEL-positive cells in the propofol-treated animals was 4.1-fold higher than in the NS group (Fig. 3). To investigate whether propofol acted on the PI3K/Akt/GSK3β signaling pathway, we measured the expression of pAkt (Ser473), pGSK3β (Ser9), Akt and GSK3β by western blot analysis. In the propofol group, the expression levels of pAkt and pGSK3β were 0.19-fold and 0.18-fold, respectively, the levels of the NS group (Fig. 4). The immunofluorescence results were consistent with the western blot analysis; compared with NS group, the fluorescence intensities of pAkt and pGSKβ were 0.83-fold and 0.68-fold, respectively, in the P group (Fig. 5). These results suggested that propofol specifically induced neuroapoptosis and decreased Akt and GSK3β phosphorylation.

3.2. Dexmedetomidine attenuated propofol-induced neuroapoptosis and increased akt and GSK3β phosphorylation

Transmission electron microscope results and TUNEL are shown in Figs. 2 and 3. To determine the role of dexmedetomidine in neuroapoptosis, we also detected the effects of propofol by TEM and TUNEL. Compared with the P group, the dexmedetomidine pretreatment groups (PD25, PD50, and PD75) exhibited attenuated hippocampal neuron apoptosis; the electron microscopy image of the dexmedetomidine-treated groups (PD25, PD50, and PD75) revealed that the changes in the neuronal cells and the synaptic cleft can be subtle (Fig. 2). The number of TUNEL-positive cells was 0.48-fold less than in the P group. The western blot analysis and immunofluorescence revealed that, when the pups were pretreated with dexmedetomidine, the expression of pAkt and pGSK3β were significantly increased compared with the P group (Fig. 4 and 5). Dexmedetomidine dose escalation substantially increased the expression of neuroprotection and the expression of pAkt and pGSK3β. However, dexmedetomidine was unable to completely reverse neuroapoptosis and restore pAkt and pGSK3β expression to the NS group levels. These results suggest that dexmedetomidine attenuates propofol-induced neuroapoptosis and increases Akt and GSK3β phosphorylation.

3.3. PI3K inhibition attenuated the neuroprotective effect of dexmedetomidine

To elucidate the role of Akt in dexmedetomidine-attenuated propofol-induced neuroapoptosis, we pretreated the rats with a PI3K inhibitor, LY294002, LY294002 treatment substantially abrogated detectable Akt phosphorylation. Compared with the PD75 group, the number of apoptotic cells after LY294002 treatment was significantly increased (Fig. 2). A 1.85-fold increase in the number of TUNEL-positive cells was observed in the LYPD group (Fig. 3). The expression of pAkt and pGSK3β decreased 0.69-fold and 0.53-fold, respectively, in the LYPD group (Fig. 4). The immunofluorescence results were similar to the WB results; relative to the NS group, the fluorescence intensity of pAkt and pGSKβ in the LYPD group were 0.64-fold and 0.66-fold, respectively (Fig. 5). The results reveal that pAkt and pGSKβ participate in dexmedetomidine-induced neuroprotection and that the PI3K inhibitor attenuates the neuroprotective effect of dexmedetomidine. We examined group D1 to exclude the effects of the vehicle and the route of administration, as our result revealed that there were no significant differences between the D1 group and the NS group.

3.4. GSK3β inhibition enhanced the neuroprotective effect of dexmedetomidine

To further elucidate the role of GSK3β in dexmedetomidine-attenuated propofol-induced neuroapoptosis, we administered a GSK3β inhibitor (TDZD-8), which resulted in a significant decrease in hippocampal neuron apoptosis (Fig. 2 and 3). The expression of pGSK3β increased 1.3-fold by both western blot analysis (WB) and immunofluorescence. The results further suggest that the expression of pGSK3β plays a role in dexmedetomidine-mediated neuroprotection.
We examined group D2 to exclude the effects of the vehicle and the route of administration, as our result showed that there were no significant differences between the D1 group and the NS group.

4. Discussion

Our data and previous studies (Zhong et al., 2014) demonstrate that propofol increases neuroapoptosis. When rat pups were pretreated with dexmedetomidine, we observed attenuated neuroapoptosis in a dose-dependent manner. The results strikingly suggest that dexmedetomidine exerts direct neuroprotective effects to prevent propofol-induced neuronal apoptosis. Therefore, we suggest that dexmedetomidine attenuates propofol-induced neuroapoptosis by altering the expression levels of pAkt and pGSK3β.

Recent studies have suggested that propofol exhibits significant negative effects on the development of neural cells (Briner et al., 2011; Huang et al., 2016), including the induction of neuronal apoptosis in different species (Guo et al., 2015). In our previous in vitro study, propofol induced neuroapoptosis in hippocampal neurons in vitro through the downregulation of NF-κB p65 and Bcl-2 and the upregulation of caspase-3 (Zhong et al., 2014). Whether other medications can attenuate propofol-induced neurotoxicity requires further investigation.

Dexmedetomidine is a widely used drug for sedation, hypnosis, and anesthesia. Several studies have demonstrated that dexmedetomidine exhibits neuroprotective effects and the literature suggests that dexmedetomidine dramatically attenuates developmental neuroapoptosis, protects against the neurotoxic effects of anesthetics (Li et al., 2014; Sanders et al., 2010) and reduces brain injury after brain hypoxia-ischemia (Ren et al., 2016). In our study, we found that dexmedetomidine pretreatment provided neuroprotection against propofol-induced neuroapoptosis in a dose-dependent manner. These data are consistent with previous studies by Li et al. (2014).

Hippocampal cells are widely used in neuroapoptosis studies, and neuroapoptosis is frequently measured by cleaved caspase-3 and caspase-9 expression (Yon et al., 2005; Zhong et al., 2014). In the present study, we provide morphologic evidence of neuroapoptosis using transmission electron microscopy (TEM) to directly observe cell morphological changes. TUNEL staining has been used extensively for detecting apoptosis and measuring hippocampal cell death (Kodama et al., 2011). Both TEM and TUNEL demonstrated that dexmedetomidine attenuated propofol-induced neuroapoptosis in a dose-dependent manner.

Several signaling pathways have been extensively investigated and shown to be involved in apoptosis induced by propofol and inhalational...
Fig. 3. Dexmedetomidine attenuated propofol-induced neuroapoptosis in the hippocampus of P7 rats (n = 6 in each group). (A) Representative TUNEL images in the hippocampus; green staining indicates TUNEL-positive cells, and blue staining indicates nuclei. (B) The quantitative analysis of the ratio of TUNEL-positive cells by one-way ANOVA and post hoc Bonferroni’s correction. The results are expressed as means ± SD. *P < 0.05 versus NS group; #P < 0.05 versus P group; ^P < 0.05 versus PD75 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
anesthetics in the developing brain (Pesic et al., 2009; Yi et al., 2016), although the underlying mechanisms remain obscure. A previous study demonstrated that dexmedetomidine reduces isoflurane-induced neuroapoptosis partly via the PI3K/Akt pathway in the hippocampus of neonatal rats (Li et al., 2014). The Akt/GSK3β signaling pathway is widely known for its pivotal roles in cell death, cell development, survival (Xia et al., 2008; Ye et al. 2015), and apoptosis (Arboleda et al., 2010). GSK3β is a downstream target of Akt and one of a small number of protein kinases that is inactivated by phosphorylation (Liu et al., 2013). Phospho-Akt promotes the phosphorylation of GSK3β, thereby downregulating the activity of GSK-3β to promote cell survival (Bhat et al., 2000). Phospho-Akt promotes the phosphorylation of GSK3β, thereby downregulating the activity of GSK-3β to promote cell survival (Bhat et al., 2000).

In our present study, we observed that propofol decreased Akt and GSK3β phosphorylation and that dexmedetomidine pretreatment partially attenuated these effects; these findings were consistent with the TEM and TUNEL results. Together, our data indicate that dexmedetomidine may confer considerable protection via the PI3 K/Akt/GSK3β pathway.

To further elucidate the mechanisms underlying dexmedetomidine-attenuated propofol-induced neuroapoptosis, we employed pharmacologic inhibition of the Akt/GSK-3β signaling pathway. pAkt inhibition by LY294002 (a selective inhibitor of PI3K/Akt) in the PD75 group reversed dexmedetomidine-induced neuroprotection and decreased the levels of both pAkt and pGSK3β. The levels of pAkt were decreased due to LY294002 directly, whereas the decrease in pGSK3β may have been decreased due to the decrease in pAkt. To explore the role of GSK3β, we inhibited GSK3β using 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) (Collino et al., 2008). Decreased pGSK3β resulted in the attenuation of neuroapoptosis, suggesting that decreased GSK3β activity was related to dexmedetomidine-induced neuroprotection, consistent with our previous study. Both propofol and dexmedetomidine directly affected Akt and GSK3β phosphorylation. However, dexmedetomidine was unable to completely inhibit the effects of propofol, suggesting that other factors may be involved. The underlying mechanisms require further study.

In summary, propofol induces neurotoxicity by inhibiting Akt and GSK3β phosphorylation. Dexmedetomidine increases Akt and GSK3β phosphorylation to promote neuroprotection and attenuate propofol-induced neuroapoptosis. Thus, the activation of the Akt/GSK3β signaling pathway may mediate the survival of hippocampus neurons. Dexmedetomidine, as a neuroprotectant, may be a useful adjuvant in propofol anesthesia, especially in pediatric populations.

**Conflict of interest**

The authors have no conflicts of interest.
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