Original Article Propofol inhibits growth of neurons through regulating insulin receptor and insulin-like growth factor-1 receptor

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Received January 11, 2016; Accepted March 23, 2016; Epub July 1, 2016; Published July 15, 2016

Abstract: Neurotoxicity is a common side-effect of anesthetics. Propofol, as an anesthetic, is reported to induce apoptosis of neurons and lead to cognition and learning deficits. In our present study, we explored the effect of propofol on growth of neurons as well as the underlying mechanism in vivo and in vitro. We found that propofol inhibited the growth of neurons and influenced the protein level of the insulin receptor (IR) and Insulin-like growth factor-1 receptor (IGF-1R). Further mechanism study showed that, besides the reduction in receptor level of IR and IGF-1R, propofol activated PI3K-AKT signal and decreased the sensitivity of receptor, thus reducing glucose transporters (GLUTs) and inhibiting the growth of neuronal cells. Our study demonstrated that propofol inhibited the growth of neurons through the regulation of IR and IGF-1R. This study lays foundation for the exploration of propofol function as well as the employment of propofol in clinic.

Keywords: Propofol, neurotoxicity, insulin receptor, insulin-like growth factor-1 receptor, PI3K, AKT

Introduction

In recent years, along with the improving operation, increasing anesthetics are used in the clinic. However, the use of anesthetics may cause different sequelae, especially in pediatrics and obstetrics.

Propofol (2,6-diisopropylphenol) is a clinical common general anesthetic. Propofol can induce anesthesia through N-methyl-D-aspartic acid (NMDA) receptor and y-aminobutyric acid-A (GABA,) receptor [1, 2]. Propofol is widely used in intensive care practice and pediatric anesthesia because of its short half-time and quick induction and revival. However, in recent years, propofol is reported to have neurotoxicity [2-4]. Evidences show that propofol can inhibit the growth of neurons and induce apoptosis of neuronal cells [3, 5-7]. Propofol can cause widespread neuroapoptosis in the neonate and led to a persistent decrease in dendritic growth in cultured GABA neurons even at a sub-anesthetic dose [8]. In the meanwhile, in vivo animal experiments also show that exposing to propofol causes long-term cognitive deficits in immature rats as well as behavioral deficits in offspring rats [3, 9].

Insulin and insulin receptor (IR) play important roles in glycolmetabolism. IR belongs to tyrosine kinase receptors. Binding by insulin, IR undergoes rapid auto-phosphorylation, followed by tyrosine phosphorylation of insulin receptor substrate (IRS). Then the downstream signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)-AKT and mitogen-activated protein kinase (MAPK), are activated to regulate cell growth, gene expression and glycogen synthesis. Insulin-like growth factor-1 receptor (IGF-1R) is also a receptor bound by insulin, and participates in cell growth and glucose utilization in the hippocampus [10]. IR is shown to be reduced in the brains of Alzheimer and Parkinson patients [11]. Down-regulation of IGF-1R is also discovered in aged mouse hippocampus and somatosensory cortex [10] and is associated with axonal regeneration [12]. Both IR and IGF-1R are believed to play important roles in the nervous system. However, whether propofol has effect on IR and IGF-1R in hippocampus is still unclear.

Propofol was reported to cause insulin resistance and reduce the uptake of glucose [13-15]. We hypothesized that the neurotoxicity of propofol might be associated with changes in IR and IGF-1R signal in hippocampus. In the present study, we explored whether propofol has influence on IR and IGF-1R signal in hippocampus. Results of our study laid foundation for the further exploration of propofol function.

Materials and methods

Propofol treatment for animal experiments

Male SD rats weighing 180-220 g were maintained in an environment with a constant temperature of 23 \pm 2°C, relative humidity of 50 \pm 5% and 12 h-light-dark cycles. Rats were randomly divided into two groups (n=6/group): the Propofol group and Control group. Rats in Propofol group received intravenous injection with 10 mg/kg of propofol followed by intravenous infusion with 40 mg/kg/h of propofol for 2 h. Rats in Control group received equal volume of normal saline with the same operation. After treatment with propofol, hippocampuses of rats in each group were collected for western blot or made into coronal sections for Nissl staining and immunofluorescence. All animal experiments in this study were performed according to the Guide for Care and Use of Laboratory Animals and approved by Institutional Animal Care and Use Committee of the Jilin University.

Nissl staining

Hippocampuses coronal sections were dewaxed in xylene, rehydrated with decreasing concentrations of ethanol. Then the sections were stained with Lauth's violet (Solarbio, Beijing, China) for 10 min at room temperature. After dehydration and cover slipping with neutral balsam, histological changes of hippocampus was observed under an optical microscope and the number of neuronal cells in the CA3 region of hippocampus was counted. Only intact neuronal cells with a clearly defined cell body and nucleus were counted.

Isolation of neural cells and cell culture

The hippocampuses of neonatal rats were obtained in a sterile environment. After removal of the meningeal tissues, the hippocampuses were cut into small pieces and digested in 0.25% trypsin solution (Beyotime, Shanghai, China) at 37°C for 30 min. After washing with phosphate buffered saline (PBS), hippocampus tissues were resuspended with neurbasal medium (Gibco, Grand Island, NY, USA) and mixed gently. After passing through 200-mesh sieves, the cells were washed with PBS, cultured in neurbasal medium with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and maintained in a humidified atmosphere at 37° C with 5% CO₂.

MTT assay

Isolated neural cells were seeded in 96-well plates (4×10^3 cells/well). After treatment with propofol (0.01, 0.1, 1 mg/ml) or normal saline for 3, 6, 12, 24 and 48 h, the cell viability was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT, Sigma, St. Louis, USA). MTT at a final concentration of 0.2 mg/ml was added into each well and incubated at 37°C for additional 4 h. After removal of supernatant, 200 µl of dimethyl sulfoxide (DMSO, Sigma) was added into each well. The absorbance was measured at 490 nm and a growth curve was drawn.

Propofol treatment for cell experiment

The isolated cells were divided into four groups and treated with propofol and/or insulin: (1) cells without treatment; (2) cells were treated with 0.1 mg/ml propofol for 24 h; (3) cells were treated with 100 nM insulin for 15 min; (4) cells were treated with 100 nM insulin for 15 min and then treated with 0.1 mg/ml propofol for 24 h. Then cells from each group were harvested for western blot and immunofluorescence.

Western blot

Hippocampuses of rats in each group were collected and lysed with RIPA lysis buffer (Beyotime) with 1% PMSF, and protein in hippocampus was extracted by centrifugation. After different treatment, the cultured cells were harvested and lysed in RIPA lysis buffer with 1% PMSF. Then total protein was extracted by centrifugation. Membrane protein and cytosol protein were extracted using a Membrane and Cytosol Protein Extraction Kit (Beyotime). Concentration of protein was measured using an Enhanced BCA Protein Assay Kit (Beyotime).



Figure 1. Propofol decreased population of neuronal cells in CA3 region of hippocampus. A. Nissl staining. After treatment with propofol or equal amount of normal saline, the brains of rats were obtained and subjected to Nissl staining. Scale bar=100 μ m. B. The number of neuronal cells in CA3 region. Only intact neuronal cells with a clearly defined cell body and nucleus were counted. Each experiment was repeated three times. Typical results are presented. The results are presented as mean ± SD. ***P* < 0.01 compared with Control.

Equal amount of protein from each group was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for electrophoresis. Then the separated protein was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The PVDF membranes were blocked with 5% skim

milk or 1% bovine serum albumin (BSA). After washing with Tris buffered saline with tween (TBST), the membranes were incubated with corresponding primary antibody against IR_β (1: 1000, Abcam, Cambridge, UK), IRS-1, p-IRS1^{s-} er307, glucose transporter (GLUT) 3, GLUT4, GLUT8 (1:500, Bioss, Beijing, China), IGF-1R (1:1000, Cell Signaling Technology, Beverly, MA, USA), PI3K, AKT (1: 400, Boster, Wuhan, China), p-AKT^{Ser473} (1:200, Santa Cruz, Dallas, TX, USA), β-actin (1:1000, Santa Cruz), Na+/ K+-ATPase (1:500, Santa Cruz) at 4°C overnight. Then the membrane was incubated with corresponding horseradish peroxidase (HRP)conjugated secondary antibody (1:5000, Beyotime) at 37°C for 45 min after washing with TBST. The signal of protein was detected with ECL Detection System.

Immunofluorescence

For hippocampuses coronal sections, after dewaxing and rehydration, sections were subjected to antigen retrieval in sodium citrate buffer for 10 min. After blockade with goat serum, the sections were incubated with primary antibody against neuronal nuclei antigen (NeuN) (1:200, Abcam) and IR α (1:50, Santa Cruz) overnight at 4°C. Then the sections were incubated with FITC or Cy3-conjugate secondary antibody (1:200, Beyotime) at room temperature for 90 min. The sections were incubated with 4'.6-diamidino-2-phenylindole (DAPI) for nucleus staining and observed under a fluorescence microscope. For isolated cells, cells were made into cell climbing after different treatment and fixed with 4% paraformaldehyde. After permeabilization with 0.1% TritonX-100. the cell climbing were blocked with goat serum and then incubated with primary antibody against IRa (1:50, Santa Cruz) at 4°C overnight. Then the cell climbing was incubated with Cy3-conjugate secondary antibody for 60 min at room temperature. After incubation with DAPI, the cell climbing was observed under fluorescence.

Statistical analysis

All experiments were repeated three times. The results were presented as mean \pm standard deviation (SD). Student's t test was performed for comparisons between two groups. P < 0.05 was considered to be significant.



Figure 2. IR and IGF-1R signal was influenced by propofol exposure. A, B. The expression level of IR β and IGF-1R in hippocampus was detected by western blot after exposure to propofol. The relative expression level of protein was calculated using β -actin as internal reference. C. After exposure to propofol, hippocampuseds of rats in each group were collected and subjected to immunofluorescence with antibodies against IR α and NeuN. Green fluorescence: IR α ; red fluorescence: NeuN; blue fluorescence: DAPI. Scale bar=50 µm. D, E. After exposure to propofol, the protein level of GLUT3, GLUT4 and GLUT8 was detected by western blot using β -actin as reference. Each experiment was repeated three times. Typical results are presented. The results are presented as mean ± SD. ***P < 0.001 compared with Control.

Results

Propofol reduced neurons in CA3 region of hippocampus

The growth of neurons in hippocampus is very important to the capability of cognition and study. Neuronal density in CA3 region of hippocampus was assessed by Nissl staining. As shown in **Figure 1A**, hippocampus of Control group showed intact neuronal cells, and the cell body and nucleus was clearly defined. However, neurons in hippocampus of Propofol group showed fuzzy edges (**Figure 1A**) and the population of neuronal cells was decreased significantly (**Figure 1B**, P < 0.01). This result demonstrated that neurons in hippocampus were reduced after exposure to propofol.



Figure 3. Propofol inhibited growth of neuronal cells in vitro. A. After treatment with 0.01, 0.1 and 1 mg/ml propofol, the cell viability of neuronal cells was detected by MTT assay. B. Inhibition ratio of propofol at 24 h and 48 h. All experiments were repeated three times. The results are presented as mean \pm SD. ***P* < 0.01, ****P* < 0.001 compared with Control.



Figure 4. Propofol influenced IR and IGF-1R in vitro. A, B. After treatment with propofol and/or insulin, the peotein level of IR β and IGF-1R was detected by western blot. Relative protein level was calculated using β -actin as internal reference. C. After treatment with propofol and/or insulin, the level of IR α was detected by immunofluorescence. Red fluorescence: IR α ; blue fluorescence: DAPI. Scale bar=50 µm. All experiments were repeated three times. Typical results are presented. The results are presented as mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with Insulin.



Figure 5. Influence of propofol on GLUT3, GLUT4 and GLUT8. A. The protein level of GLUT3, GLUT8, cytosol GLUT4 and membrane GLUT4 was detected by western blot after treatment with propofol and/or insulin. β -actin was used as internal reference for total protein and cytosol protein. Na+/K+-ATPase was used as internal reference for membran protein. B. Quantitative analysis for western blot. Each experiment was repeated three times. Typical results are presented. Results are presented as mean ± SD. ***P* < 0.01, ****P* < 0.001 compared with Control, #*P* < 0.05, ##*P* < 0.01 compared with Insulin.

Propofol influenced IR and IGF-1R signal in vivo

To explore whether IR and IGF-1R were influenced by propofol exposure, the expression level of IRB and IGF-1R was detected by western blot. After exposure to propofol, the expression level of IR β was decreased to 60 ± 13% and the expression level of IGF-1R was decreased to 49 ± 11% (Figure 2A and 2B). Immunofluorescence was used to detect the expression of IR α in hippocampus. As shown in Figure 2C, hippocampus in Control group showed a normal IR α expression, stronger in hippocampus neuronal cells than that in adjacent tissues. Whereas, hippocampus in Propofol group showed a decreased IRa expression and no obvious difference was observed between hippocampus neuronal cells and adjacent tissues. Changes in expression of IR and IGF-1R suggested that IR and IGF-1R were influenced by propofol exposure. After exposure to propofol, the fluorescence of NeuN was also reduced (Figure 2C). This result suggested the neuron injury role of propofol, which was consistent with our above results.

To further verify the influence of propofol on IR and IGF-1R signal, the protein expression of GLUT3, GLUT4 and GLUT8, which are important effector molecules downstream of IR and IGF-1R, was detected by western blot. After exposure to propofol, the relative expression level of GLUT3, GLUT4 and GLUT8 in hippocampus was decreased to $45 \pm 9\%$, $53 \pm 11\%$ and $46 \pm 9\%$ respectively (**Figure 2D** and **2E**). These results demonstrated that the IR and IGF-1R signal was influenced by propofol exposure.

Propofol influenced IR and IGF-1R signal in vitro

To further explore how propofol injured neuronal cells in hippocampus, neural cells in hippocampus were isolated and in vitro study was carried out. Then MTT assay was performed to evaluate the cytotoxicity of propofol. As shown in Figure 3A, after treatment with 0.1 and 1 mg/ml propofol, the cell viability of neuronal cells was decreased (Figure 3A). The inhibition ration was 34.74 ± 7.78% and 35.3 ± 8.31% respectively at 24 h and 41.55 ± 6.06% and $45.10 \pm 6.13\%$ respectively at 48 h (Figure 3B). However, treatment with 0.01 mg/ ml propofol has no significant influence on the cell viability of neuronal cells. This result demonstrated that propofol inhibited the growth of neuronal cells in vitro, which was consistent with its function in vivo. According to the result of MTT assay, 0.1 mg/ml propofol and was selected for the subsequent experiment.

To further explore the influence of propofol on IR and I GF-1R signal, insulin was introduced in



Figure 6. Propofol decreased the receptor sensitivity. A, B. After treatment with propofol, the protein level of IRS-1 and phosphorylation level of IRS (Ser³⁰⁷) was detected by western blot using β -actin as internal reference. C, D. The level of PI3K and phosphorylated AKT was detected by western blot. β -actin was used as internal reference. All experiments were repeated three times. Typical results are presented. Results are presented as mean ± SD. ***P* < 0.01, ****P* < 0.001 compared with Control.

our present study, and then the expression of IRα, IRβ, and IGF-1R was detected. After treatment with propofol, the expression level of IRB, IGF-1R (Figure 4A and 4B) and IR α (Figure 4C) in neuronal cells was decreased, which was consistent with our results of experiments in vivo. The protein level of GLUT3, GLUT8, GLUT4 in cytosol and GLUT4 on membrane was also detected by western blot. After treatment with propofol, the level of GLUT3, GLUT8 and membrane GLUT4 was decreased, and the level of cytosol GLUT4 increased (Figure 5A and 5B). These results indicated that propofol down-regulated the level of GLUT3 and GLUT8, and promoted GLUT4 translocation to cytoplasm.

After treatment with insulin, opposite effect to propofol was discovered. After treatment with insulin, the protein level of IR α , IR β , and IGF-1R was increased (**Figure 4**), the level of GLUT3, GLUT8 and membrane GLUT4 was increased, and the level of cytosol GLUT4 was decreased (**Figure 5**). Meanwhile, treatment with propofol was found to reverse the effect of insulin (**Figures 4** and **5**, propofol + insulin vs. insulin). All the above results indicated that propofol influenced IR and IGF-1R signal.

Propofol decreased receptor sensitivity

IRS-1, which is a main substrate of IR and IGF-1R, was detected in the present study. Results of western blot showed that the phosphorylation level of IRS-1 on Ser³⁰⁷ was increased after treatment with propofol (**Figure 6A** and **6B**), which indicated that the receptor sensitivity was decreased by propofol exposure. As a main reason of insulin resistance, the activation of PI3K-AKT signal was also detected in our study. Results of western blot showed that, after exposure to propofol, the protein level of PI3K was increased and the phosphorylation level of AKT was also increased, which indicated the activation of PI3K-AKT signal. These results demonstrated that propofol decreased receptor sensitivity of IR and IGF-1R.

Discussion

In the present study, the neurotoxicity of propofol as well as the underlying mechanism was explored. Propofol was found to injure neuronal cells in hippocampus. IR and IGF-1R signal was reduced after exposure to propofol. The receptor sensitivity of IR and IGF-1R was also decreased after exposure to propofol. Results of the present study indicated that propofol may injure neurons through regulating IR and IGF-1R signal.

In recent years, propofol, as an anesthetic, showed neurotoxicity leading to cognition and learning deficits [9]. In our present study, propofol was found to perform a neuron damaging effect to inhibit the growth of neuron in vivo and in vitro. Propofol was reported to inhibit the proliferation of neuronal cells and promote cell apoptosis of neuron [6]. Creeley et al also showed that propofol induced apoptosis of neurons in fetal and neonatal rhesus macaque brain [5]. Neurotoxicity is a common side-effect of anesthetics. A number of general anesthetics, such as isoflurane, sevoflurane and ketamine, also cause functional changes in the hippocampus of the neonates, affecting behavior in juveniles or adults [16-20].

Anesthesia with propofol is reported to induce insulin resistance [13]. Insulin plays a crucial role in glycometabolism and development of the nervous system, and is reported to have a neuroprotective effect [21, 22]. IR and IGF-1R also play important roles in neurodevelopment [23-25]. IR is found to be expressed at low level in the brains of patients with Alzheimers or Parkinson [11]. IGF-1R is also found to be expressed at low level in hippocampus of aged

mouse and may be associated with neurodegenerative disease [10]. In our study, the protein level of IR and IGF-1R in hippocampus was reduced after exposure to propofol. The protein level of GLUTs was also influenced by propofol. These results prompt us to the hypothesis that the neurotoxicity of propofol might be through the regulation of IR and IGF-1R signal. Report of Lou et al also shows that propofol exacerbates insulin resistance through GLUT4 trafficking [26]. Propofol reduces insulin-induced glucose uptake accompanied by lower GLUT4 trafficking to the sarcolemma, and promotes synthesis of glycogen [26]. In the meanwhile, propofol inactivates GSK3ß but activates AMPK and ERK1/2 in diabetic hearts [26]. The effect of insulin was also found to be antagonized by propofol in our study. These results provide stronger evidence for our hypothesis that propofol injured neurons through regulation of IR and IGF-1R signal.

IRS-1 is an important substrate of IR and IFG-1R. IRS-1 transfers insulin signaling to downstream molecules such as PI3K and MAPK. Activated IR and IGF-1R promote phosphorylation of IRS1-1 on Tyr which is necessary for normal insulin responses. Whereas, activated PI3K-AKT signaling also promotes phosphorylation of IRS-1 on Ser. Phosphorylation of IRS-1 on Ser has a dual role, either enhance or terminate the insulin effects [27]. Phosphorylation of IRS-1 on Ser³⁰⁷ will decrease insulin receptor sensitivity to insulin and is regarded as a marker of insulin resistance [28]. Results of our study showed increased activation of PI3K-AKT signal and increased phosphorylation of IRS-1 on Ser³⁰⁷ after treatment of propofol. These results suggested that propofol activated PI3K-AKT signal and decreased the receptor sensitivity.

In our study, we found that propofol can reduce the amount of IR and IGF-1R, decrease the receptor sensitivity, decrease the expression of GLUT3 and GLUT8, and influence GLUT4 translocation to the cytoplasm, thus influencing the growth of neurons. However, how exactly propofol influences the amount and sensitivity of IR and IGF-1R is still not clear and needs our further exploration. Propofol is commonly used in pediatric anesthesia because of its excellent anesthetic effect. Neurotoxicity of propofol will lead to seriously impacts in life quality in the further of infants who received anesthesia with propofol. Demonstration of the mechanism underlying the neurotoxicity of propofol will be beneficial to the employment of propofol in clinic.

Disclosure of conflict of interest

None.

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