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Propofol inhibits invasion and proliferation of C6 glioma cells by regulating the Ca^{2+} permeable AMPA receptor-system x_c^- pathway

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ABSTRACT

Anesthetics are documented to affect tumors; therefore, we studied the antiglioma effect of propofol on proliferation and invasiveness of glioma cells and explored the underlying mechanism. C6 glioma cells were cultured and treated with propofol, and cell viability, invasiveness, and migration were measured. Glutamate release was measured using an enzyme-catalyzed kinetic reaction. xCT protein and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor GluR2 subunit protein expression was assessed with Western blot analysis and immunofluorescent staining. We observed that propofol significantly inhibited C6 glioma cell viability, invasiveness, and migration and decreased glutamate release. An agonist of the cystine/glutamate antiporter system (system x_c^-), *N*-acetylcysteine (NAC), reversed propofol's effects, and propofol could inhibit C6 glioma cell proliferation by adding excess exogenous glutamate (100 μM). Finally, propofol increased the surface expression of GluR2, but decreased surface expression of xCT. The effects of propofol on surface expression of GluR2 and xCT could be rescued by (R, S)-AMPA, an agonist of Ca^{2+} permeable AMPA receptor (CPAR). Thus, propofol can inhibit cell viability, invasiveness, and migration of C6 glioma cells, and the CPAR-system x_c^- pathway contributes to these events.

1. Introduction

Glioma, a devastating glial-derived brain tumor, has a poor prognosis and represents a significant health burden. Epidemiology data suggest that 70% of all primary brain tumors are gliomas, and 5-year survival occurs in fewer than 10% of patients (Parsons et al., 2008). Although various therapies are available, surgery remains the most common option for managing gliomas (Ohgaki and Kleihues, 2005; Stummer et al., 2011). For patients with malignant glioma, removing the greatest amount of tumor possible is critical for improving prognosis (Eyupoglu et al., 2013). Because brain tumor patients are exposed to perioperative sedatives and anesthetics, the influence of anesthetics on tumor recurrence is of interest to scientists (Moss and Israel, 2009) and data may show that selection of optimal anesthetics is required to reduce potential patient risks.

Propofol (2,6-diisopropylphenol) is a commonly used intravenous sedative hypnotic for induction and maintenance of anesthesia and for sedation in the intensive care unit. Numerous previous studies indicate that propofol has an antitumor effect on several tumor types such as hepatocellular carcinoma (Zhang et al., 2014), ovarian cancer (Wang

Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CPAR, Ca^{2+} permeable AMPA receptor; System x_c^-, cystine/glutamate antiporter system; Glu, glutamate; Cys-Cys, cystine; GSH, glutathione; GSSG, glutathione disulfide; ROS, reactive oxygen species

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et al., 2013), and prostate cancer (Huang et al., 2014) via different mechanisms. Our work suggests that propofol may be neuroprotective by inhibiting the internalization of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor GluR2 subunit (Wang et al., 2011). AMPA receptors are vital glutamate (Glu) receptors that mediate fast synaptic transmission at excitatory synapses in the central nervous system. Functional AMPA receptors are tetramers consisting of four subunits (GluR1–4) (Hollmann and Heinemann, 1994; Liu et al., n.d.). AMPA receptors are Ca^{2+}-impermeable when they contain the GluR2 subunit, but AMPA receptors lacking GluR2 are Ca^{2+}-permeable AMPA receptor (CPARs), and binding of Glu allows calcium influx through CPARs (Lomeli et al., 1994). Most gliomas do not express GluR2, which may indicate that glioma cells express CPAR (Colman et al., 2010; Maas et al., 2001). This is intriguing because CPAR activation can enhance glioma invasion (Lyons et al., 2007).

High extracellular Glu release may be key to enhancing glioma invasiveness (de Groot and Sontheimer, 2011). Glu clearance is primarily achieved via the Na^+-dependent excitatory amino acid transporters, whereas Glu release in glial cells occurs via several ways, but predominantly through the cystine/glutamate antiporter system (system x_c^-) (Blecic et al., 2013; Montana et al., 2006), which is highly expressed in glioma cells (Ye et al., 1999; Ye and Sontheimer, 1999). The Na^+-independent electroneutral transport system x_c^- imports cystine (Cys-Cys) in exchange for Glu release at a 1:1 ratio (Bannai, 1986). Increased extracellular Glu promotes tumor expansion by promoting glioma proliferation and invasiveness (Sontheimer, 2003; Ye and Sontheimer, 1999). Meanwhile, transporters in system x_c^- increase intracellular Cys-Cys, which is an important precursor for glutathione (GSH) synthesis. GSH is required to reduce reactive oxygen species (ROS) (Yin et al., 2007). In glioma cells, Ca^{2+} influx through CPAR increases intracellular ROS and induces expression of system x_c^- (Deneke and Fanburg, 1989). However, Glu release increased with the upregulation of system x_c^-, and high extracellular Glu causes glioma enlargement. It is unclear whether propofol increases gliomal invasiveness by regulating expression of CPAR and system x_c; hence, we studied the effect of propofol on CPAR and system x_c^- and discussed any potential relationship between CPAR and system x_c^-, which are pivotal aspects of invasiveness of glioma cells.

2. Materials and methods

2.1. Materials

Propofol was purchased from AstraZeneca (MK364, Macclesfield, Cheshire). Dulbecco modified Eagle medium (DMEM) cell culture medium was purchased from HyClone Laboratories (Logan, UT). Fetal bovine serum (FBS) was purchased from Biological Industries (Israel), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St Louis, MO). *N*-acetylcysteine (NAC) and 1-naphthyl acetyl spermine (NAS) were both purchased from MedChem Express (Monmouth Junction, NJ). (R, S)-AMPA was purchased from Abcam (Cambridge, UK).

2.2. Cell culture

A rat C6 glioma cell line was graciously provided by Professor Yang, Medical College, Nankai University. Cells were cultured with DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cell density was approximately 5×10^5 cells/mL. The cells were cultured for 24 h and most cells reached 50% to 70% confluence before drug treatment for 6 h as follows: propofol (6.7, 22.4, 69.6 μM); L-glutamate (100 μM); NAC (100 μM), an agonist of system x_c^-; NAS (100 μM), a specific inhibitor of CPAR; and (R, S)-AMPA (100 μM), an activator of AMPA receptor. All chemicals were dissolved in DMEM medium. The concentration of propofol 6.7, 22.4, 69.6 μM corresponded to its commonly used infusion concentration in a rat model, which was 20, 40, and 60 mg/kg/h, respectively. All the treatments were applied for 6 h to mimic the clinical use of propofol in glioma patients.

2.3. MTT assay

The effect of propofol on cell viability was measured with an MTT assay. C6 glioma cells were seeded in 96-well plates at a cell density of 5000 cells/well and treated with corresponding drugs for 6 h. Then drugs were removed and culture was maintained for an additional 18 h. Subsequently, 5 mg/mL MTT was added to wells and incubated. After 4 h, MTT solution was removed and the formazan product was solubilized with 150 μL dimethyl sulfoxide, and absorbance at 570 nm was read with an ELISA reader (Elx 800, Bio-TEK, Winooski, VT).

2.4. Invasion assay

The effect of propofol on cell invasion was assayed with Transwell chambers (EMD Millipore, Billerica, MA) containing polycarbonate membranes with 8-μm pores. All chambers were precoated with Matrigel (BD Biosciences, Bedford, OH). C6 glioma cells were treated with drugs for 6 h, collected, and resuspended in media with 1% FBS. Approximately 30,000 cells were loaded in each upper chamber and medium containing 20% FBS was added to the lower chamber as a chemoattractant. After incubation for 18 h, cells in upper chambers were removed with cotton swabs, and cells at the chamber bottom (invaded cells) were stained with DAPI. Invading cells were photographed under a fluorescent microscope (NIKON ECLIPSE-80i, Nikon, Japan) and counted using Image-Pro Plus software. Average cell numbers from five random fields was defined as the number of invading cells of each chamber.

2.5. Migration assay

The effect of propofol on cell migration in C6 glioma cells was assessed using a scratch or wound healing assay. C6 glioma cells were seeded in 6-well plates at a cell density of 50,000 cells/well and incubated to reach 90% confluence. Subsequently, a straight scratch wound was made with a sterile plastic 200-μL micropipette tip in each well. Cells were treated as previously described and then cultured in media containing 1% FBS for 18 h. Images of each well were obtained with a microscope when the wound was made (T_0) and 24 h later (T_1). Wound closure was analyzed by comparing areas of each scratch field using Image Pro Plus.

2.6. Glu release assay

An Enzychrom Glu assay kit (EGLT-100, BioAssay System) was used for measuring Glu in solution according to the manufacturer's directions. Product color intensity measured at 550 nm is proportionate to Glu. In brief, C6 glioma cells were seeded in 96-well plates at a cell density of 5000 cells/well and treated with corresponding drugs. The supernatant of C6 glioma cells was transferred into another clean 96-well plate, and 80 μL of working reagent was added to each reaction well. The optical density was read at time “zero” (optical density $[OD]_0$) and again at OD_{30} after a 30-min incubation at room temperature. Glu standard calibration was measured at the beginning of the assay. Finally, a standard curve was created to convert ΔΔOD sample values to Glu concentration values.

2.7. Measurement of intracellular glutathione

Total intracellular glutathione (GSH) reflects the interplay of reduced GSH and oxidized glutathione (glutathione disulfide, GSSG). GSH and GSSG were measured using an enzymatic method according to the