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Original Article

Propofol decreases p53 levels to prevent cell death

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Abstract: Propofol can be protective against various insults in many systems. However, the exact mechanisms of propofol protection are still unknown. In the present study, we showed that propofol decreased p53 level through upregulating miR-6983-5p. This might be a possible mechanism of propofol promoting cell survival. In addition, propofol prevented neurons against staurosporine, etoposid, glutamate and serum deprivation treatments. Our study provides a possible mechanism that propofol regulating p53 level through miRNA to prevent cell death.

Keywords: Propofol, microRNA, anesthetics, cell death, p53

Introduction

Propofol (2, 6-disopropylphenol) is a widely used intravenous short-acting anesthetics [1]. Despite its anesthetic effects, propofol is also reported to be protective in cerebral ischemia or ischemia-reperfusion, Parkinson's disease, intracerebral hemorrhage, cerebral resuscitation, ischemia of spinal cords and Alzheimer's disease (AD) [1, 2], while the exact mechanisms of propofol protection are still unclear. MicroRNAs (miRNAs) are a group of short non-coding RNAs [3]. There are over one thousand miRNAs identified in mammals [4]. MiRNAs precursors are first processed into a single-stranded miRNA that interacts with the 3'UTR or 5'UTR area of the complementary mRNA sequences [5], resulting in translation repression or target degradation [6, 7], through which miRNAs regulate the protein level of target genes. A single miRNA can have up to several hundred target genes, and thus, may regulate multiple pathways at the same time [8, 9]. Many miRNAs have demonstrated implications in anesthesia [10-14]. We have previsously showed that various miRNAs are altered with propofol treatment in cultured primary mouse hippocampal neurons. Among 1908 mouse microRNAs examined, there are 40 microRNAs upregulated and 54 microRNAs downregulated with the cutoff as 2 fold change [15].

In the present study, we report that in mouse hippocampal neurons, miR-6983-5p, a microRNA that targets p53 mRNA 3'UTR, is highly upregulated with propofol treatment. Furthermore, we showed that propofol is protective against neuronal cell death induced by various insults, possibly through elevated level of miR-6983-5p. Our results point to microRNA regulation as a critical determinant of propofol protection mechanim.

Experimental procedures

Cell culture and treatments

Primary neurons were cultured from wild type C57 mouse hippocampus, following the regulations of Peking University Animal Care and Use Committee as previously described [16]. In brief, fresh fetal mouse hippocampal tissues were dissociated with 0.25% trypsin (Invitrogen, Carlsbad, CA), which was then inactivated by 10% decomplemented fetal bovine serum (FBS, HyClone, Logan, UT). The mixture was triturated through pipette to make a homogenous mixture. After filtering the mixture through 70 μm sterilized filters, the flow-through was centrifuged. The pellet was then washed once by phosphate buffer saline (PBS) and once by Dulbecco minimum essential medium (DMEM) in Earle's balanced salt solu-

tion containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, 1x antibiotic Pen-Strep (all from Invitrogen, Carlsbad, CA) with 5% FBS. Cells were then plated on poly-L-lysine (Sigma, St. Louis, MO) coated plates or glass coverslips at the density of 3×10^6 cells/ml. Neurons were incubated at 37°C in DMEM without phenol red with 5% FBS and with 5% circulating CO_2. Cytarabine was added to culture media 24 hours after plating at 10 μM to inhibit cell growth. Medium was changed every 48 hours. Cells were treated for experiments at 7 days in culture. Propofol was added to the medium at 100 nM for 6 hours.

Real-time PCR

Real-time PCR was done following the precedure described before [17]. MiR-6983-5p in the tissue, serum and cerebrospinal fluid (CSF) was measured by TaqMan MicroRNA assay kit for Mmu-miR-6983-5p according to the manufacturer's instruction (Life Technologies Corp.). In brief, cells were harvested and total RNA was isolated with TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China). Total RNA (2 μg) were reversely transcribed using TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). Real-time PCRs were done by using TransStart Green q PCR SuperMix UDG (Beijing TransGen Biotech Co., Ltd., Beijing, China). Real-time PCR quantifications were run in triplicate for each sample and the average were determined. In order to use the comparative Ct method for relative quantification, the amplification efficiency of target and housekeeping gene must be approximately equal. Quantification was done using the comparative Ct method, expression levels for the target gene was normalized to the GAPDH of each sample [$2^{-\Delta Ct}=2^{-(Ct(target\ gene)-Ct(GAPDH))}$]. Amplification was done for 45 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 30 s, 95°C for 1 min, 59°C for 30 s and 95°C for 30 s.

Sources of reagents and sequences of miRNAs and 3'UTR

Mouse miR-6983-5p (5'-UUGGAAGGGCAUACUGAUUCGG-3'), scramble microRNA control (5'-GTGTAACACGTCTATACGCCCA-3'), mimic miR-6983-5p (5'-UUGGAAGGGCGTCTATACGCCCA-3'), miR-6983-5p inhibitor (5'-AACCUUCCCGUAUGACUAAGCC-3') and mutant miR-6983-5p (5'-UACCUUCCGCAUACUGAUUCGG-3'), complementary sequence (5'-ACCUUCC-3') were all purchased from Qiagen. Several altered mouse p53 mRNA 3'UTR sequences used were as following: mutant p53 mRNA 3'UTR: 5'-GGAAGT-3'; MAP2-3'UTR: 5'-CATATTCATTCTTTACAAACCATAG-3'. All WT and modified 3'UTR sequences were cloned into pLenti-Luc-UTR or pLenti-EGFP-UTR vector backbone (Abm). MicroRNAs were transfected into SH-Sy5y cells with HiPerFect Transfection Agent (Qiagen).

Propofol (Sigma), staurosporine (STS, Sigma), etoposide (Etop, Sigma) and glutamate (Sigma) were stored in 100x stock and diluted with culture medium before use. C57 mice at 1 month old were treated with propofol (200 mg/kg) by intraparietal injection (i.p.). Cultured cells were treated with 2% propofol for 6 hours before assays.

Western blots and measurement of neuronal cell death

Western blots were done following the protocol described previously [16]. Anti-p53 (Abcam) and actin (Sigma) antibodies were diluted at 1:1000 for Western blots as primary antibodies. The relative density was calculated by the total absolute density of p53/actin control. Cell death was measured with terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining performed using the *in situ* cell death detection kit I as described by the manufacturer (Roche) [16].

Luciferase reporter assay

The Dual-Luciferase Reporter Assay System kit (E1910; Promega) was used to investigate the effect of miR-6983-5p on p53 expression. The constructs with the firefly (*Photnus pyralis*) luciferase gene downstream of the p53 3'UTR were co-transfected with the wild-type, mutant, scramble, mimic or inhibitor to miR-6983-5p into SH-Sy5y cells. Firefly luminescence was detected as indicated by the manufacturer. To control for transfection efficiency, the Renilla reniformis luciferase construct was co-transfected into the cells. The activity was corrected for the protein concentration of each sample and expressed as [(firefly/renilla luciferase) *10,000]. The luminescence was expressed as relative light units [18].

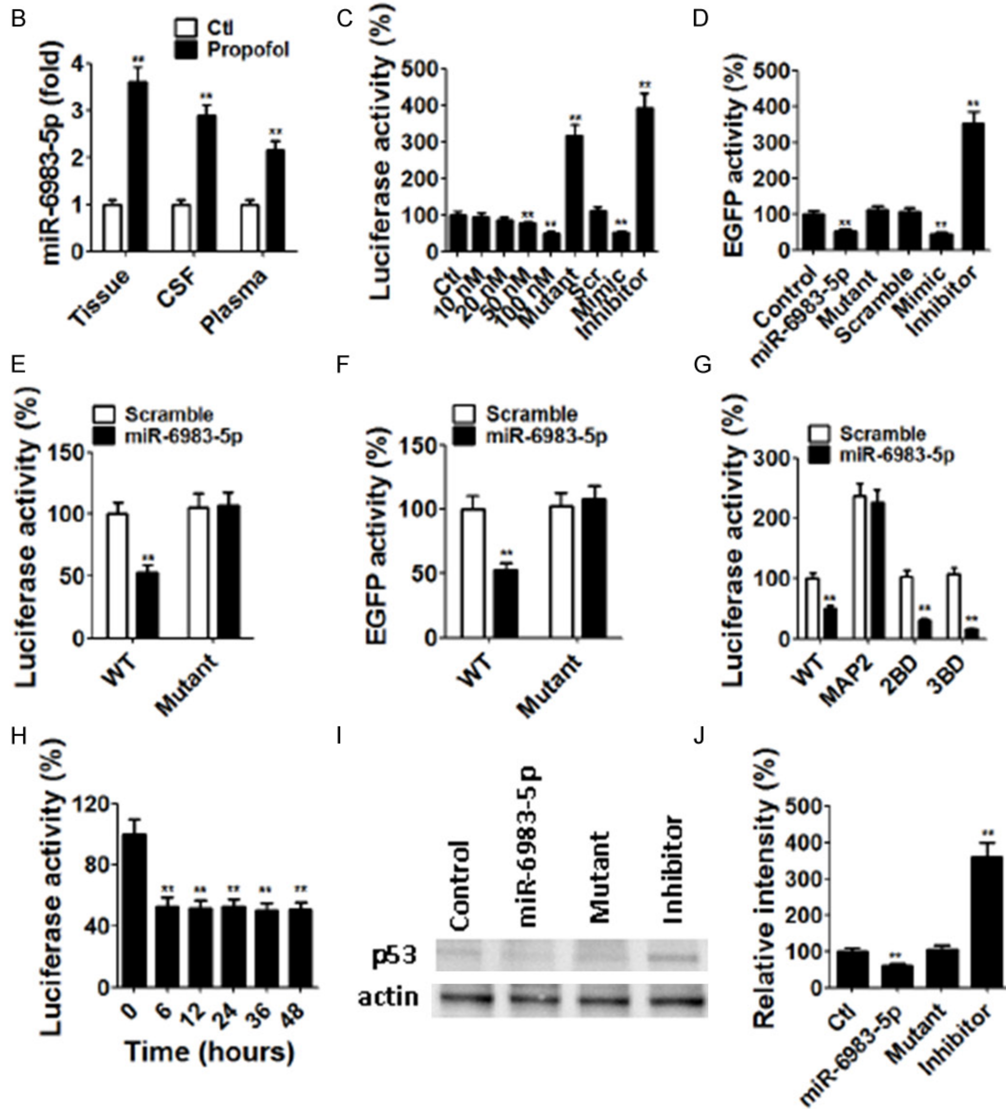

Figure 1. MiR-6983-5p regulated p53 mRNA 3'UTR. (A) Sequence alignment of mouse miR-6983-5p and p53 mRNA 3'UTR region. The red highlighted the complemtary regions. (B) MiR-6983-5p was increased in the hippocampal tissues, CSF and plasma of mice treated with propofol. (C) Dose response of miR-6983-5p on luciferase reporter assay in SH-Sy5y cells. (D) Dose response of miR-6983-5p on EGFP reporter assay in SH-Sy5y cells. (E) MiR-6983-5p reduced the luciferase activity with WT p53 mRNA 3'UTR, but not with mutant p53 mRNA 3'UTR. (F) MiR-6983-5p

reduced the EGFP reporter activity with WT p53 mRNA 3'UTR, but not with mutant p53 mRNA 3'UTR. (G) MiR-6983-5p did not alter the expression with unrelated MAP2 mRNA 3'UTR. With p53 mRNA 3'UTR constructed with 2 times or 3 times of the putative binding domain (2BD, 3BD) of each binding site, miR-6983-5p decreased luciferase activity remarkably in a dose dependent manner. (H) Time course of miR-6983-5p inhibition on p53 mRNA 3'UTR. (I) Western blot of p53 showed that miR-6983-5p (lane 2) reduced whereas inhibitor to miR-6983-5p (lane 4) increased the p53 level. Mutant miR-6983-5p (lane 3) did not alter p53 level. (J) Statistics of (I). Data represent Mean ± SE (n=3 for each group). **: P<0.01 compared with control.

Statistical evaluation

Statistical significance was assessed by one-way analysis of variances (ANOVA) followed by the Sheffé's test. A *p* value of less than 0.05 indicated statistical significance.

Results

p53 mRNA 3'UTR was a Target of miR-6983-5p

According to the miRNA target prediction tool website (http://www.microrna.org/microrna/home.do), one complementary sequences for miR-6983-5p is present in the 3'UTR of p53 mRNA in mouse (**Figure 1A**, highlighted with red). In the hippocampal tissues from 1 month old mice treated with propofol, miR-6983-5p was increased to about 4 folds (**Figure 1B**). Previous studies have showed that miRNAs can be secreted into the CSF and plasma [19]. Therefore, we observed that miR-6983-5p levels were upregulated in the CSF and plasma after propofol treatment (**Figure 1B**).

In SH-Sy5y cells, luciferase reporter assays were performed with the expression of firefly regulated by p53 mRNA 3'UTR complementary region to miR-6983-5p, and renilla as an internal control for transfection efficiency. We found that miR-6983-5p induced a dose-dependent inhibition of luciferase activity, whereas mutant miR-6983-5p significantly increased the luciferase activity, and scramble control miRNA had no effect (**Figure 1C**). The mimic of miR-6983-5p markedly inhibited the luciferase activity, while the inhibitor of miR-6983-5p increased the luciferase activity (**Figure 1C**). In SH-Sy5y cells, EGFP reporter assay confirmed the similar inhibitory effect of miR-6983-5p to p53 mRNA 3'UTR (**Figure 1D**).

We then mutated the p53 mRNA 3'UTR complementary sequence and performed luciferase assay and EGFP reporter assay with either scramble control miRNA or miR-6983-5p. Our results indicated that miR-6983-5p only suppressed the luciferase activity or EGFP fluoresence with WT p53 mRNA 3'UTR, but not with mutant 3'UTR (**Figure 1E** and **1F**), suggesting the specificity of the interaction between miR-6983-5p and p53 mRNA 3'UTR. To further validate that p53 mRNA 3'UTR was one of the targets for miR-6983-5p, we made mutant p53 mRNA 3'UTR with 2 repeats of the putative complementary binding domain (BD) sequence (2BD) and 3 repeats of the BD sequence (3BD) for each putative binding site. Luciferase reporter system showed that miR-6983-5p did not suppress the luciferase activity regulated by unrelated MAP2 mRNA 3'UTR (**Figure 1G**). With p53 mRNA 3'UTR, luciferase activity was greatly reduced in a BD dose-dependent manner (**Figure 1G**). Time course study showed that miR-6983-5p inhibited luciferase acitivity from 12 hours after treatment and kept roughly the same inhibition levels after 12 hours (**Figure 1H**). Western blotting of total p53 with miR-6983-5p, inhibitor to miR-6983-5p, and mutant miR-6983-5p indicated that miR-6983-5p indeed decreased p53 protein level (**Figure 1I** and **1J**), whereas inhibitor to miR-6983-5p increased p53 (**Figure 1I** and **1J**).

Propofol was protective against cell death

It is well-known that p53 is involved in regulation of cell death, especially apoptosis [20]. We then examined the vulnerability to various insults in cultured neurons with or without propofol treatment. Our data showed that STS [21, 22], Etop [22], glutamate [23] and serum deprivation [21, 22], induced more severe cell death in untreated neurons than in propofol treated neurons (**Figure 2A**). Exogenous administration of propofol at 2% for 6 hours in the culture medium decreased the vulnerability of neurons to all above insults (**Figure 2B**), suggesting that propofol may play an important role in neuronal vulnerability and viability.

Discussion

In the present study, we find that miR-6983-5p is upregulated in propofol treated neurons.

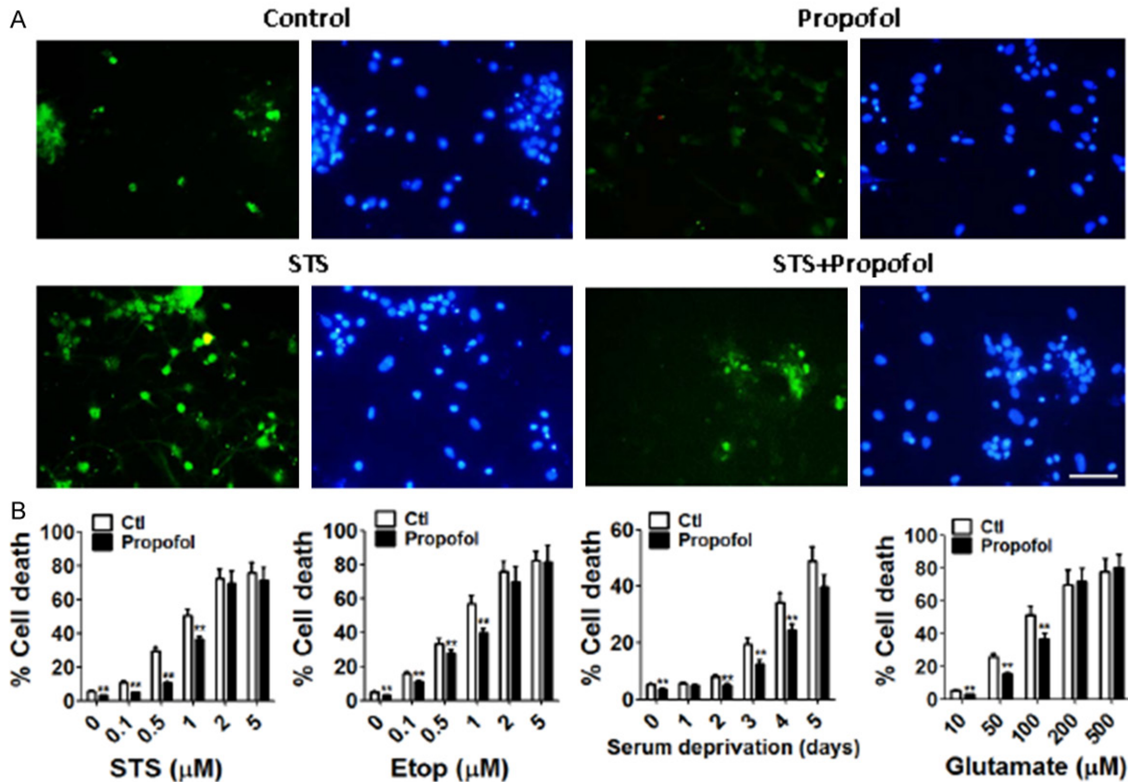

Figure 2. Propofol was protective against cell death. A. Representative images of cultured neurons treated with propofol or vehicle. Blue: Hoechst staining indicating nucli; Green: TUNEL staining indicating apoptotic cells. Scale bar: 50 μm. B. In cultured neurons, neuronal toxicity was induced by STS, Etop, glutamate or serum deprivation. The neurons were treated with propofol at 2% in the culture medium. Cell death was measured 6 hours after treatment. Data represent Mean ± SE (n=3 for each group). **: P<0.01 compared with control.

Furthermore, miR-6983-5p targets to the 3'UTR of p53 mRNA and decreases p53 level in neurons. Propofol is protective against cell death induced by STS, Etop, glutamate and serum deprivation. The mechanism of propofol protection is not fully understood yet. One possible explanation is that propofol can increase miR-6983-5p level, which targets and decrease the level of p53. Since the activation of p53 promotes cell death and apoptosis, decreasing p53 level could prevent cell death. Our current data are consistent with the previous observation that propofol may attenuate cell death through inhibition of p53 [24].

Several studies have reported the pretective role of propofol through various possible mechanisms [1]. For examples, activation of $GABA_A$ receptor has been showed to be associated with propofol protection against cell death induced by brain ischemia and acute mechanical injure [25, 26]. Propofol is structurally similar to some antioxidant, such as α-tocopherol [27]. Therefore, propofol could have antioxidative role against various insults. Propofol can inhibit the activation of glutamate receptors to reduce glutamete neurotoxcity [28, 29]. Propofol increases level of anti-apoptotic protein Bcl-2 and decreases level of pro-apoptotic protein Bax [30-32]. Other machanisms suggested to be related to propofol protective role include anti-inflammation, induction of heme oxygenase-1 expression, inhibition of auqaporin 4 overexpression [1]. Our study provides a possible mechanism that propofol regulating p53 level through miRNA to prevent cell death.

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Disclosure of conflict of interest

None.

Authors' contribution

C.P., L.S., and Z.C. performed all the experiments and analyzed the data. Y.W. conceptualized the study, performed analyses and drafted the manuscript.

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