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Original Article

Propofol inhibits neuronal apotosis by decreasing p53 levels via upregulating miR-592-5p

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Abstract: Propofol is one of a commonly used intravenous anesthetics. Many studies have shown that propofol can protect the cells from apoptosis by regulating different miRNA. In this study, we found that propofol decreased p53 level via upregulating miR-592-5p. The technique used were real time PCR, western blots and luciferase reporter assay. Moreover, we also found that propofol prevented neurons against actinomycin D, extracellular amyloid β, TNF-α and H_2O_2 treatments. This study shown that miR-592-5p could be regulated by propofol on preventing cell death.

Keywords: Propofol, p53, apoptosis, microRNA, anesthetics, miR-592-5p

Introduction

MicroRNAs (miRNAs) are shortnon-coding version of RNAs [1]. Precursors of miRNAs forms a single-stranded miRNA first. And that single-stranded miRNA then interacts with the complementary mRNA sequences on either 3'UTR or 5'UTR area [2]. After series of translation repression or target degradation,as a result, miRNAs regulate the protein level of target genes [2]. Propofol, 2, 6-disopropylphenol, is a commonly used intravenous anesthetic agent [3]. Many miRNAs have been examed to have implications in general anesthesia [4-8]. Different anesthetic agents can also lead different effects on mRNA expression [9]. We have previsously showed that various miRNAs are altered with propofol treatment in cultured primary mouse hippocampal neurons [10]. MiR-592-5p, the mRNAin mouse hippocampal neurons that increased after propofol treatment. MiR-592-5p was found to relate to aging and aging related disease in mice in previous studies [11].

In the this study, we foundthat miR-592-5p which targets p53 mRNA 3'UTR, is remarkbly upregulated with propofol treatment. Moreover, we showed that propofolis protective against neuronal cell death induced by various drugs, such as actinomycin D (ActD), extracellular amyloid β (Aβ), TNF-α and H_2O_2. Our results providea new cellular protective mechanism by microRNA regulation induced by propofol.

Experimental procedures

Cell culture and treatments

The source of mouse that we used in this experiement wassupported and regulated by Peking University Animal Care and Use Committee [12]. We took thehippocampusof wild type C57 mouse which was borned under 24 hoursto culture the primary neuron.The fresh fetal mouse hippocampuses were taken from the brain in Dulbecco minimum essential medium (DMEM) at 2-3°C. Then We transfered the DMEM out and dissociatedthe fresh fetal mouse hippocampal tissueswith 0.25% trypsin (Invitrogen, Carlsbad, CA). Cells were placed in 35 ml capsuls in 37°C incubator with 5% circulating CO_2 for 20 minutes. We put coverslip in the 24-well platesand added 65 μM poly-$_D$-lysine on the cover slips to maximize the tension of cover slips. The plates were also placed in the same incubator for 1.5-2 hours. To inactivate the trypsin, DMEM+fetal bovine serum (FBS) was added

to the cells, which was mixed by 5% decomplemented FBS (HyClone, Logan, UT) and DMEM. We mixed the cell with pipette for 10-12 times. After precipitation appeared, we transfered the clear upper cell layer to new culture mediums with obtaining 1.5 ml DMEM in each capsul and pipetted the cells to mix well. After settled down for 2 minutes, the clear upper cell layer was transfered to tubes. After centrifuging it, we collected the precipitation and added DMEM+FBS to resuspened the cells. For culture media, we washed out the poly-$_D$-lysine from the prepared 24-well plates and 35 ml dishes with deionized water 3 times. Then we plated the cells on poly-$_L$-lysine (Sigma, St. Louis, MO) glass coverslips or coated plates at the density of 3×10^6 cells/ml. We incubated the neurons at 37°C in DMEM+FBS with 5% circulating CO_2. After 4-6 hours, CNB (neurobasal+B27) was added to culture media to in with 200 μM on 24-well plates and 500 mM on 35 ml dishes. We added 10 μM Cytarabine 24 hours after plating to inhibit cell growth. We changed medium every 48 hours. Cells were treated at 7 days in culture. We added 100 nM propofol to the medium for 6 hours. The cells were then lysed.

Real-time PCR

The precudure of Real-time PCR was done similar as it was described before [13]. In this experiment, we made a mixture of forward primer, reverse primer, de-iodized water and supermix instead of adding them seperately. Based on the instruction (Life Technologies Corp.), TaqMan MicroRNA assay kit for mmu-miR-592-5p measured the miR-592-5p in the cerebrospinal fluid, serum and plasma. Working as the forward primer, TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China) collected the total RNA from the cell. TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China) was the reverse primer that used to reversely transcribe total RNA which was collected in 2 μg. TransStart Green q PCR SuperMix UDG (Beijing TransGen Biotech Co., Ltd., Beijing, China) was used for real-time PCRs. For each sample, Real-time PCR quantifications were 3 times replicated, and the average were determined. The house-keeping gene and amplification efficiency of target were kept in approximately equal, so that comparative Ct method for relative quantification can be used. The comparative Ct method was used for the quantificaiton. Expression levels for the target gene was normalized to the GAPDH of each sample [$2^{-\Delta Ct}=2^{-(Ct(target\ gene)-Ct(GAPDH))}$]. Amplification ran 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-592-5p (5'-AUUGUGUCAAUAUGCGAUGAUGU-3'), scramble microRNA control (5'-GTGTAACACGTCTATACGCCCA-3'), mimic miR-592-5p (5'-UUGUGUCGGUGTGCUGACGCCCA-3'), miR-592-5p inhibitor (5'-AACACAGCCGUAUGACUAAGCC-3') and mutant miR-592-5p (5'-UACCUUCCGCAUACUGAUUCGG-3'), complementary sequence (5'-AACACAG-3') were all purchased from Qiagen. Several altered mouse p53mRNA 3'UTR sequences used were as following: mutant p53 mRNA 3'UTR: 5'-TCTGTGTT-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).

In this experiment, we diluted the propofol (Sigma), staurosporine (STS, Sigma), etoposide (Etop, Sigma) and glutamate (Sigma) before use, which were stored in 100x stock. Propofol was intraparietally injected (i.p.) to 1 month old C57 mice at the amount of 200 mg/kg. Before assays, 2% propofol was used to treat cultured cells for 6 hours.

Western blots and measurement of neuronal cell death

The procedure of western blots was as same as it was described in previous study [12]. For primary antibodies in western blots, we diluted Anti-p53 (Abcam) and actin (Sigma) antibodies at 1:1000. We calculated the relative density from the total absolute density of p53/actin control, Based on the manufacturer (Roche), we measured cell death with terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining performed using the *in situ* cell death detection kit I [12].

Luciferase reporter assay

To determine the effect of miR-592-5p on p53 expression, The Dual-Luciferase Reporter

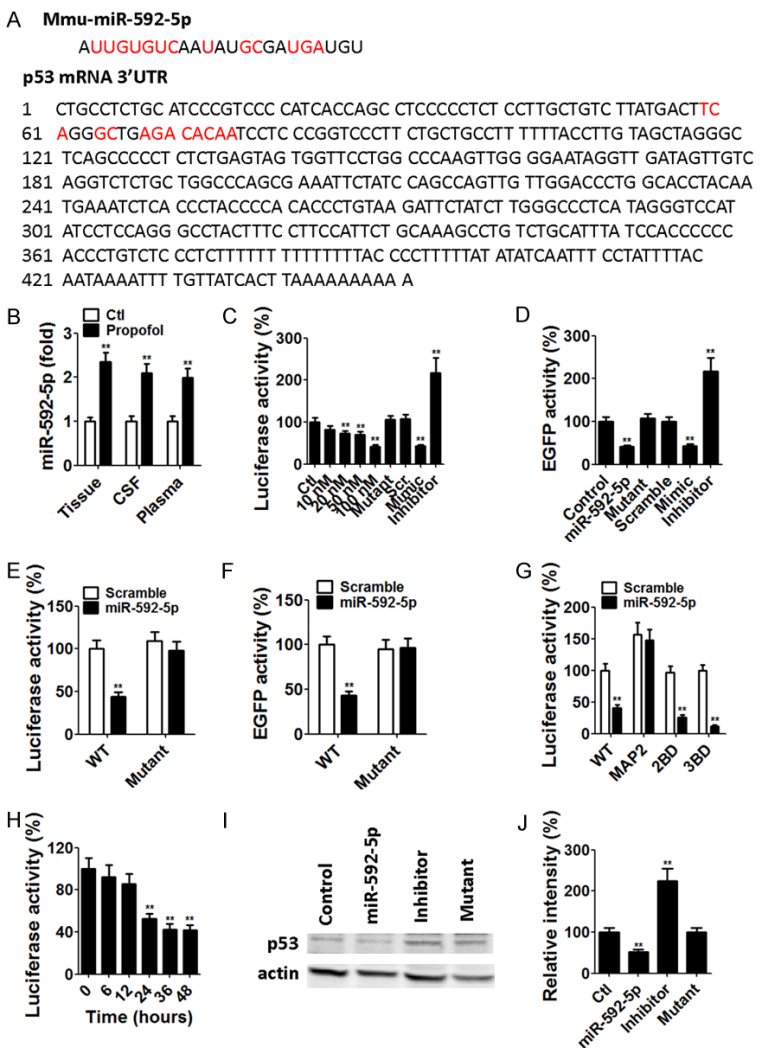

Figure 1. MiR-592-5p regulated p53 mRNA 3'UTR. A. The highlighted red was the matched sequence alignment of mouse p53 mRNA 3'UTR region and miR-592-5p. B. The amount (fold) of miR-592-5p significantly increased (P<0.01) in the hippocampal tissues, CSF and plasma of mice before and aftertreating with propofol. C. Dose response of miR-592-5p on luciferase reporter assay in SH-Sy5y cells. D. Dose response of miR-592-5p on EGFP reporter assay in SH-Sy5y cells. E. The luciferase activity of miR-592-5p significantly decreased (P<0.01) with WT p53 mRNA 3'UTR, while it slightly decreasedwith mutant p53 mRNA 3'UTR. F. The EGFP reporter activity of miR-592-5psignificantly decreased with WT p53 mRNA 3'UTR. None changing with mutant p53 mRNA 3'UTR. G. The luciferase activity of miR-592-5p had no change with unrlated MAP2 mRNA 3'UTR. The luciferase activity of miR-592-5p significantly decreased (P<0.01) with WT p53 mRNA 3'UTR, as well as p53mRNA 3'UTR constructed with 2 times or 3 times of theputative binding domain (2BD, 3BD) of each binding site. H. Inhibition of miR-592-5p on p53mRNA 3'UTR was decreasing by time, and it was siginificantly decreased after 12 hours. I. Western blot showed that p53 value inmimic miR-592-5p (lane 2) decresed, while p53 value increased in inhibitor miR-592-5p (lane 4). P53 value did not change in mutant miR-592-5p (lane 3). J. Statistics of I. Data represent Mean ± SE (n=3 for each group). **: changing significance at P<0.01 comparing with control.

Assay System kit (E1910; Promega) was used in this experiment. The constructs with the firefly (*Photnuspyralis*) luciferase gene downstream of the p53 3'UTR were co-transfected with the wild-type, mutant, scramble, mimic or inhibitor to miR-592-5pinto SH-Sy5y cells. Firefly luminescence was detected as indicated by the manufacturer. The*Renilla reniformis* luciferase construct was co-transfected into the cells in order to control for transfection efficiency. The activity was corrected for the protein concentration of each sample and expressed as [(firefly/renilla luciferase) *10,000]. The luminescence expression was shown as relative light unit [14].

Statistical evaluation

One-way analysis of variances (ANOVA) and Sheffé's test were used to analyze the Statistical significance. The statistic was significant at P<0.05.

Results

p53 mRNA 3'UTR wasaTarget of miR-592-5p

By using the miRNA target prediction tool website (http://www.microrna.org/microrna/home.do), we found the matching complementary sequences for miR-592-5p and 3'UTR of p53 mRNA in mouse (**Figure 1A**, highlighted with red). In the hippocampal tissues of 1 month old mice treated with propofol, miR-592-5p was increased to about 4 folds (**Figure 1B**). According toprevious study, miRNAs can diffuse into the plasmaand CSF [15]. We comparedthemiR-592-5p levels with or without propofol treatment in the plasma and CSF of mice (**Figure 1B**).

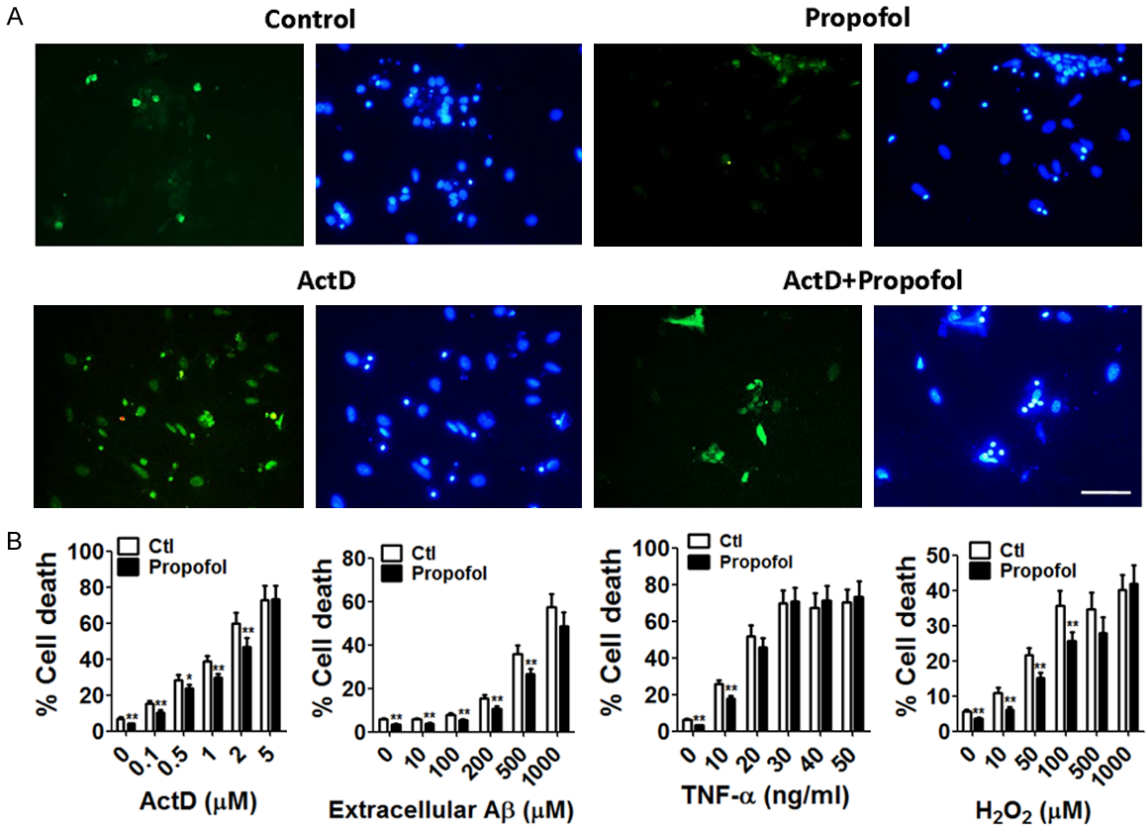

Figure 2. Propofol was protective against cell death. A. Representative images of cultured neurons treated with propofol or vehicle. Blue: Hoechst staining indicating nucli; Green: TUNEL staining indicating apoptotic cells. Scale bar: 50 μm. B. In cultured neurons, ActD, extracellular Aβ, TNF-α or H_2O_2 decreased neuronal toxicity. With TNF-α treatment, the change of neuronal toxicity was not significant after 20 ng/ml. Propofol was used to treat neurons at 2% in the culture medium. Cell death was measured 6 hours after treatment. Data represent Mean + SE (n=3 for each group). **: changing significance at P<0.01 comparing with control. *: changing significance at P<0.05 comparing with control.

Regulating by p53 mRNA 3'UTR complementary region to miR-592-5p in SH-Sy5y cells, luciferase reporter assays were done with the expression of firefly. The renilla worked as an internal controlfor transfection efficiency. MiR-592-5p was found to inhibit luciferase activity dose-independently, while the mutant miR-592-5p and scramble control miRNA had no effect (**Figure 1C**). The mimic of miR-592-5p had a significant inhibition ofthe luciferase activity, while the inhibitor ofmiR-592-5p increased the luciferase activity (**Figure 1C**). Moreover, EGFP reporter assay also had the similar effect on inhibitioin of miR-592-5p to p53 mRNA 3'UTR In SH-Sy5y cells (**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-592-5p. Our results indicated that miR-592-5p only suppressed the luciferase activity or EGFP fluorescence with WT p53 mRNA 3'UTR, but not with mutant 3'UTR (**Figure 1E** and **1F**), suggesting the specificity of the interaction between miR-592-5p and p53 mRNA 3'UTR. To further validate that p53 mRNA 3'UTR was one of the targets for miR-592-5p, 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-592-5p did not suppress the luciferase activity regulated by unrelated MAP2 mRNA 3'UTR (**Figure 1G**). Luciferase activity was greatly reduced in a BD dose-dependent manner with p53 mRNA 3'UTR (**Figure 1G**). Time course study showed that miR-592-5p 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 treatments of miR-592-5p, inhibitor to miR-592-5p, and mutant miR-592-5p indicated that miR-592-5p indeed decreased p53 protein level (**Figure 1I** and **1J**), whereas inhibitor to miR-592-5p increased p53 level (**Figure 1I** and **1J**).

Propofol was protective against cell death

As indicated in the previous studies, p53 has an impacton cell apoptosis [16]. Therefore, the cell viability to different drugs in cultured neurons with or without propofol treatment was examed in this experiment. Our data showed that ActD, extracellular Aβ, TNF-α and H_2O_2 produced more severe cell death in neurons without treatment than neurons with propofol treatment (**Figure 2A**). Exogenous administration of propofolat 2% for 6 hours in the culture medium reduced the viability of neurons in mentioned4 drugs (**Figure 2B**), showing that propofol may remarkably influnce the neuronal viability.

Discussion

In this study, we found that with the treatment of propofol, miR-592-5p targeted to the 3'UTR of p53 mRNA and decreased p53 level in neurons. Under the insults of ActD, extracellular Aβ, TNF-α and H_2O_2, propofol still inhibited cell apotosis. Although the machenisms of the propofol protecction were not yet unknown. One possible explanation could be that 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 [17].

Propofol plays a protective role via various possible mechanisms [3]. Study showed that propofol can activated $GABA_A$ receptor to against cell death which induced by acute mechanical injure and brain ischemia [18, 19]. Moreover, propofol could have antioxidative role against various insults because propofol is structurally similar to some antioxidant, such as α-tocopherol [20]. Propofol also can decrease the development of halothane-induced injury by inhibiting the NF-κB signal transduction pathway [21]. Other machanisms suggested to be related to propofol protective role include reduction of glutamete neurotoxcity,inhibition of auqaporin 4 over-expression,induction of heme oxygenase-1 expression [3, 22, 23]. Our study contributed one possible mechanism of the protectiveness of propofol, which is regulating p53 level through miRNA to prevent cell death.

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Disclosure of conflict of interest

None.

Authors' contribution

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

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