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Original Article

Propofol increases Bcl-2 Levels to protect cell from death

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Abstract: Bcl-2 is an important gene of apoptosis research, during the past decade, there has been an increasing interest in the relationship between Bcl-2 and the apoptosis, but only a few studies talk about the relationship between Bcl-2 and propofol, which is a wild use narcotic that can protect cells against various injuries. In the present study, we showed that propofol could down-regulate miR-297a-5p to increased Bcl-2 level. We think this might be a possible mechanism of propofol protecting cell from death. Furthermore, we use an apoptotic drug staurosporine to further improve the propofol's cell protecting function by showing that propofol prevented neurons against staurosporine. Our study supplies a possible mechanism that propofol could regulating Bcl-2 level through miRNA to protect cells.

Keywords: Propofol, microRNA, anesthetics, cell death, Bcl-2

Introduction

Bcl-2 family proteins are very important in the regulation of apoptosis, tumorigenesis and cellular responses to anti-cancer therapy, which has been studied intensively for the past decade owing to their importance in the regulation of apoptosis [1, 2]. At the same time, propofol (2, 6-disopropylphenol) is a widely used intravenous short-acting anesthetics [3], which has been also reported in protecting against cerebral ischemia or ischemia-reperfusion, intracerebral hemorrhage, cerebral resuscitation, ischemia of spinal cords, Parkinson's disease (PD) and Alzheimer's disease (AD) [3, 4], but we still don't know the exact mechanisms of propofol's protection. MicroRNAs (miRNAs) are a group of short non-coding RNAs [5]. There are more than one thousand miRNAs identified in mammals [6]. 3'UTR or 5'UTR area interact with a single-stranded miRNA which are the miRNA precursors [7], leading to translation repression or target degradation [8, 9], by miRNAs which regulate the protein level of the target genes. A single miRNA can conjugate with several hundred target genes, therefore, it may regulate multiple pathways at the same time [10, 11]. Many miRNAs have demonstrated implications in anesthesia [12-16]. We have a previous study showing that various miRNAs transform propofol treatment into cultured primary mouse hippocampal neurons. After examining 1908 mice microRNAs, we found that there are 40 microRNAs upregulated and 54 microRNAs downregulated with the cutoff as 2 fold change [17].

According to the present study, we reportedthat in the hippocampal neurons of mice, microRNA named miR-297a-5p, which targets Bcl-2 mRNA 3'UTR, is highly down-regulated with propofol treatment. In addition, we also showed that propofolprevents neuronal fromcelldeath, possibly through reduction in the level of miR-297a-5p. Our results indicate that microRNA regulation is a critical determining factor of propofol protection mechanism.

Experimental procedures

Cell culture and treatments

We culture the primary neurons by using wild type C57 mouse hippocampus, and follow the regulations of Peking University Animal Care

and Use Committee as previously described [18]. In brief, we dissociated the fresh fetal mouse hippocampal tissues into 0.25% trypsin (Invitrogen, Carlsbad, CA) by 20 minutes, then used Dulbecco's Modified Eagle Media (DMEM) mixed by 10% fetal bovine serum (FBS, HyClone, Logan, UT) to terminate. Then the mixture was put in a stationary state for a few minutes, and transferred into another sterilized centrifuge tube and then finally was centrifuged. The pellet was recoiling by 10% 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 and added 2 ml neurobasal medium along with B27 (Gibco, 17504-044), GlutaMax (Gibco, 35050-061), and Pen-Strep (Gibco, 15140-22) for every well after 30 minutes. Neurons were incubated in 5% circulating CO_2 at 37°C. Half of the medium was changed every 48 hours. Neurons were treated for experiments at the 7th day in culture. Propofol was added to the medium at 100 nM for 6 hours.

Real-time PCR

We do the real-time PCR as as per the following procedure described previously [19]. We measured miR-297a-5p in the tissue, serum and cerebrospinal fluid (CSF) by TaqMan MicroRNA assay kit according to the manufacturer's instruction (Life Technologies Corp.). In brief, we collected cells andinsolated total RNA with TRIGene reagent (GenStar BioSolutions Co., Ltd., Beijing, China). Then reverse transcription was done to be total RNA (2 mg) by using TransScript II First-Strand cDNA Synthesis SuperMix (Beijing TransGen Biotech Co., Ltd., Beijing, China). We only used TransStart Green qPCR SuperMix UDG (Beijing TransGen Biotech Co., Ltd., Beijing, China) to do the real-time PCRs. We ran the sample as triplicate and determine the average for real-time PCR quantifications. In order to use the Ct method to compare the relative quantification, the amplification efficiency of target and housekeeping gene must be approximately equal. After using the comparative Ct method to finish quantification, we normalized the expression levels for the target gene by 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-297a-5p (5'-AUGUAUGUGUGCAUGUGCAUGU-3'), scramble microRNA control (5'-GTGTAACACGTCTATACGCCCA-3'), mimic miR-297a5p (5'-UUGGAAGGGCGTCTAUGCAUGU-3'), miR-297a-5p inhibitor (5'-AACCUUCCCGUAUGACGUACA-3') and mutant miR-297a-5p (5'-AUGUAUGUGUGCAUGACGUACA-3'), complementary sequence (5'-ACATACA-3') were all purchased from Qiagen. Several altered mouse Bcl-2 mRNA 3'UTR sequences used were as following: mutant Bcl-2 mRNA 3'UTR: 5'-TGTATGT-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).

We used the 100× stored propofol (Sigma) and staurosporine (STS, Sigma) and diluted with the culture medium before useing it. One month old C57 mice 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

We do western blots by following the protocol described previously [18]. In brief, we diluted anti-Bcl-2 (Abcam) and actin (Sigma) antibodies at 1:1000 for Western blots as primary antibodies. Because actin is the house keeping gene, the relative density was calculated by the total absolute density of Bcl-2/actin control. We measured the cell death with terminal deoxynucleotidyl transferase-biotin dUTP nick-end labeling (TUNEL) staining performed using the *in situ* cell death detection kit I which described by the manufacturer (Roche) [18].

Luciferase reporter assay

We used the Dual-Luciferase Reporter Assay System kit (E1910, Promega) to investigate the effect of miR-297a-5p on Bcl-2 expression. In the meantime, we used co-transfected with the wild-type, mutant, scramble, mimic and inhibitor to miR-297a-5p into SH-Sy5y cells as the constructs of the firefly (Photnus pyralis) luciferase gene downstream of the Bcl-2 3'UTR.

Figure 1. Sequence alignment of mouse miR-297a-5p and Bcl-2 mRNA 3'UTR region. The red highlighted parts shows the complementary regions.

As indicated by the manufacturer, firefly luminescence was detected. In order to control the 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 [20].

Statistical evaluation

We used one-way analysis of variances (ANOVA) by the Sheffé's test which has statistical significance. A *p* value of less than 0.05 indicated statistical significance.

Results

Bcl-2 mRNA 3'UTR wasatarget of miR-297a-5p

According to the miRNA target prediction tool website (http://www.microrna.org/microrna/home.do), one complementary sequence for miR-297a-5p is present in the 3'UTR of Bcl-2 mRNA in mice (**Figure 1**, highlighted with red). After treating it with propofol in the hippocampal tissues which camefrom 1 month old mice, miR-297a-5p was decreased (**Figure 2A**). Previous studies have shown that miRNAs can be secreted into the CSF and plasma [21]. Therefore, we observed that miR-297a-5p levelsweredown-regulatedin the CSF and plasmaafter propofol treatment (**Figure 2A**).

In SH-Sy5y cells, luciferase reporter assays were performed with the expression of firefly regulated by miR-297a-5p which is the complementary region of Bcl-2 mRNA 3'UTR, and used renilla as an internal control for transfection efficiency. We found that miR-297a-5p induced a dose-dependent inhibition of luciferase activity, whereas mutant miR-297a-5p and scramble control miRNA had no effect (**Figure 2B**). The mimic of miR-297a-5p obviously inhibited the luciferase activity, although the inhibitor of miR-297a-5p increased the luciferase activity (**Figure 2B**). In SH-Sy5y cells, EGFP reporter assay confirmed the similar inhibitory effect of miR-297a-5p to Bcl-2 mRNA 3'UTR (**Figure 2C**).

We then mutated the Bcl-2 mRNA 3'UTR complementary sequence and performed luciferase assay and EGFP reporter assay with both scramble control miRNA and miR-297a-5p. Our results showed that miR-297a-5p only suppressed the luciferase activity or EGFP fluorescence with WT Bcl-2 mRNA 3'UTR, but did not significantly inhibit with mutant 3'UTR (**Figure 2D** and **2E**), which suggested the specificity of the interaction between miR-297a-5p and Bcl-2 mRNA 3'UTR. Further more, in order tofurther confirm that the Bcl-2 mRNA 3'UTR was one of the targets for miR-297a-5p, we mutanted Bcl-2 mRNA 3'UTR with the putative complementary binding domain sequence (BD), 2 repeats of the BD sequence (2BD) and 3 repeats of the BD

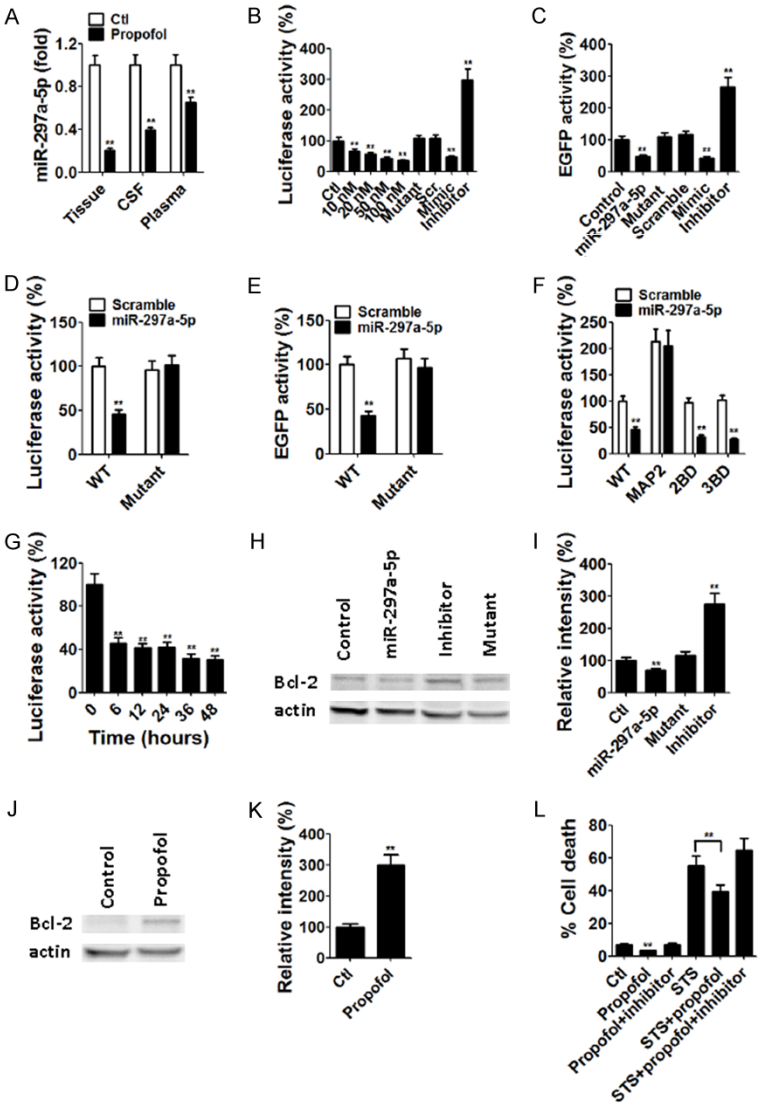

Figure 2. Propofol was protective against cell death. A. After treating with propofol, miR-297a-5pwas decreased in the hippocampal tissues, CSF and plasma in the mice. B. The dose response of miR-297a-5p on luciferase reporter assay in SH-Sy5y cells. C. The dose response of miR-297a-5p on EGFP reporter assay in SH-Sy5y cells. D. MiR-297a-5p reduced the luciferase activity with WT Bcl-2 mRNA 3'UTR, but not with mutant Bcl-2 mRNA 3'UTR. E. MiR-297a-5p reduced the EGFP reporter activity with WT Bcl-2 mRNA 3'UTR, but not significantlywith mutant Bcl-2 mRNA 3'UTR. F. MiR-297a-5p did not alter the expression with unrelated MAP2 mRNA 3'UTR. Within Bcl-2mRNA 3'UTR constructed with 2 times or 3 times of the putative binding domain (2BD, 3BD) of each binding site, miR-297a-5p decreased luciferase activity remarkably in a dose dependent manner. G. Time course of miR-297a-5p inhibition on Bcl-2mRNA 3'UTR. H. Western blot of Bcl-2 showed that mimic to miR-297a-5p (lane 2) reduced whereas inhibitor to miR-297a-5p (lane 4) increased the Bcl-2 level. Mutant miR-297a-5p (lane 3) did not alter Bcl-2 level. I. Statistics of H. Data represent Mean ± SE (n=3 for each group). **: P<0.01 compared with control. J. Western blot of Bcl-2 showed that propofol increased the Bcl-2 level. K. Statistics of J. Data represent Mean ± SE (n=3 for each group). **: P<0.01 compared with control. L. In cultured neurons, we compare the cell death rate results ofseveral different treatments such as adding propofol at 2%, propofol at 2% and Bcl-2 inhibitor, STS only, STS and propofol at 2%, and STS, propofol at 2% and Bcl-2 inhihitor in the culture medium, we measured cell death 6 hours after treatment.Data represent Mean ± SE (n=3 for each group). **: P<0.01 compared with control.

sequence (3BD) for each putative binding site. Luciferase reporter system showed that miR-297a-5p did not inhibit the luciferase activity regulated by unrelated MAP2 mRNA 3'UTR (**Figure 2F**). luciferase activity was greatly reduced in a BD dose-dependent manner where in Bcl-2 mRNA 3'UTR (**Figure 2F**). Time course study showed that luciferase acitivity was inhibited by miR-297a-5p from 12 hours after treatment and kept at almost the same inhibition levels after 12 hours (**Figure 2G**). Western blotting of total Bcl-2 with miR-297a-5p, inhibitor to miR-297a-5p, and mutant miR-297a-5p indicated that miR-297a-5p indeed decreased Bcl-2 protein level (**Figure 2H** and **2I**), whereas inhibitor to miR-297a-5p increased Bcl-2 (**Figure 2H** and **2I**).

Propofolprevent cell death by up-regulation the bcl-2 level

It is well-known that Bcl-2 family proteins areinvolved in regulation of cell death, especially apoptosis [1, 2]. We then examined the vulnerability to various injury in cultured neurons with or without propofol treatment. Our data showed that STS could induce severe cell death in untreated neurons than in propofol treated neurons [22, 23]. After that, we did a western where western blot of Bcl-2 showed that propofol increased the Bcl-2 level (**Figure 2J** and **2K**). In cultured neurons, neuronal toxicity was induced by STS, we compared the cell death rate cell death rate results of five different treatments such as (i) adding propofol at 2%, (ii) addingpropofol at 2% andBcl-2 inhibitor, (iii) adding STS only, (iv) adding STS and propofol at 2%, and (v) adding

STS. Propofol at 2% and Bcl-2 inhihitor in the culture medium remarkably decreased cell death at 6 hours after treatment (**Figure 2L**).

Discussion

As we know, the earliest metazoan that was analysed withregards to the Bcl-2 gene was the sponge [24], and so far Bcl-2 gene orthologues have been identified in all metazoan animals [25]. During the past few years, people found some viral gene products the signature helical fold of Bcl-2, where it functions in apoptosis regulation [26-28]. Moreover, propofol as an anesthetic has been widely used in short-acting anesthesia andprotects cells against various insults by increasing level of Bcl-2, which is an anti-apoptotic protein, and decreasingthe level of pro-apoptotic protein Bax. Our study has developed another possible mechanism, whichis that propofol regulating Bcl-2 level through miRNA prevents cell death.

In studies with those mechanisms, we found that neurons with propofol treatment could cause miR-297a-5p to be down-regulated. Besides that, miR-297a-5p targets 3'UTR of Bcl-2 mRNA and the decrease of miR-297a-5p increases Bcl-2 level in neurons. Propofol could protect against cell death induced by STS. However the mechanism of propofol protection has not been made fully clear yet. One possible explanation is that propofol can decrease miR-297a-5p level, which targets 3'UTR of Bcl-2 mRNAand increases the level of Bcl-2. Because the activation of Bcl-2 reduces cell death and apoptosis, increasing Bcl-2 level could prevent cell death. Our current data showed consistancy with the previous observation which isthat propofol may reduce cell death through increase of Bcl-2 [29]. In conclusion, our study indicates that propofol could prevent cell death by up-regulation the Bcl-2 level through decreasing the miR-297a-5p level which target the 3'UTR of Bcl-2 mRNA.

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Disclosure of conflict of interest

None.

Authors' contribution

L.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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