Propofol increases μ -opioid receptor expression in SH-SY5Y human neuroblastoma cells

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Received March 15, 2012; Accepted August 17, 2012

DOI: 10.3892/mmr.2012.1073

Abstract. The aim of the present study was to explore the effect of propofol, a intravenous sedative-hypnotic agent used widely in inducing and maintaining anesthesia, on μ -opioid receptor (MOR) expression in a human neuronal cell line. SH-SY5Y human neuroblastoma cells were treated with various concentrations of propofol (1, 5, 10 or 20 μ M) for different lengths of time (6, 12 or 24 h). Real-time quantitative RT-PCR showed that at a concentration range of 1-10 μ M, propofol increased MOR mRNA levels in a statistically significant dose- and time-dependent manner within 12 h of treatment. Western blot analyses demonstrated that propofol treatment for 12 h dosedependently increased the MOR protein levels. In the 12-h SH-SY5Y-treated cells, propofol dose-dependently increased MOR density (B_{max}) in the cell membranes. In addition, in the presence of the transcription inhibitor actinomycin D (1 mg/ml), propofol (10 μ M) had no significant effect on the MOR mRNA levels over time. The results suggested that propofol dose- and time-dependently enhances MOR expression in SH-SY5Y human neuroblastoma cells at the transcriptional level, leading to an increased density of ligand-binding MORs in the cell membranes. This study demonstrated for the first time a link between propofol and the opioid system, thereby providing new insights into propofol mechanism of action and potential for abuse.

Introduction

Propofol (2,6-diisopropylphenol) is a widely used intravenous sedative-hypnotic agent for the induction and maintenance of anesthesia and the sedation of critically ill patients (1). The advantages of this agent over others used for similar applications include a lower incidence of side effects (2) and improved quality of anesthesia (3). Although its clinical properties are well described, information concerning its potential for

Key words: propofol, µ-opioid receptor, transcription, abuse potential

abuse has been slow to emerge. However, a growing body of literature has documented propofol abuse in humans and abuse-like behavior in animal models (4). Propofol directly activates the γ -aminobutyric acid (GABA) receptors and inhibits the N-methyl-D-aspartate (NMDA) receptor in the nervous system (5). Previous studies also showed that the μ -opioid receptor (MOR) is functionally linked to GABA (6,7) and NMDA receptors in neurons (8). The findings suggest a correlation between propofol and the MOR, however, this association has yet to be studied.

Opioid systems are critical in the modulation of pain behavior and antinociception. Opioid peptides and their receptors are expressed throughout the nociceptive neural circuitry and critical regions of the central nervous system (CNS) and are involved in the regulation of reward-seeking behavior and emotions (9). The opioid receptors μ , δ and κ have been cloned. The most commonly used opioids for pain management act on the MOR, which is expressed primarily in the CNS and contributes significantly to the regulation of opioid-induced analgesia, tolerance and dependence (10). In the present study, we explored for the first time the effect of propofol on MOR expression in a human neuronal cell line, aiming to reveal potentially new mechanisms underlying the effects of propofol as well as its potential for abuse.

Materials and methods

Reagents. Cell culture media and fetal calf serum were purchased from Invitrogen (Carlsbad, CA, USA). Actinomycin D, propofol and all other reagents were obtained from Sigma (St. Louis, MO, USA). The selective MOR ligand [³H]DAMGO was purchased from Perkin-Elmer (Waltham, MA, USA). TRIzol reagent for RNA isolation and the SYBR-Green I kit were purchased from Invitrogen and Roche Diagnostics (Indianapolis, IN, USA), respectively. Anti-MOR antibody (sc-7489) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

Cell culture and treatment. The SH-SY5Y human neuroblastoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium supplemented with 10% fetal bovine

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serum as previously described (11). The cells were treated with various concentrations of propofol (1, 5, 10 or 20 μ M) for different time periods (6, 12 or 24 h). Propofol was dissolved in DMSO. Cells treated with DMSO were used as the control. The final concentration of DMSO in all samples was <0.3% (v/v). For actinomycin D treatment, the cells were pretreated with actinomycin D (1 mg/ml) for 30 min and then cultured for 12 or 24 h in medium containing actinomycin D (1 mg/ml) with or without propofol (10 μ M).

Real-time quantitative RT-PCR. RNA was prepared using the TRIzol reagent followed by purification using a Turbo DNA-free kit (Ambion, Austin, TX, USA). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using the LightCycler thermal cycler system (Roche Diagnostics) and the SYBR-Green I kit, following the manufacturer's instructions. The results were normalized against those of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same sample. The primers used were: for human MOR, forward, 5'-CTGGGTCAACTTGTCCCACT-3' and reverse, 5'-TGGACTAGAGGGCCAATGATC-3'; for human GAPDH, forward, 5'-GTCAGTGGTGGACCTGACCT-3' and reverse, 5'-TGCTGTAGCCAAATTCGTTG-3'. The mRNA levels of the treated cells are shown as fold or percentage changes from those of the untreated control cells (designated as 1 or 100%). Each experiment was repeated three times in triplicate. Results were shown as the mean \pm SD.

Western blot analysis. Immunoblotting was performed as previously described (12). Briefly, cells were dissolved in 250 μ l of 2X SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue and 5% 2-mercaptoethanol), and incubated at 95°C for 10 min. Equal amounts of proteins from each sample were separated by 8% SDS-PAGE and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h with a 1:1,000 dilution of anti-MOR antibody (sc-7489), washed and visualized using secondary antibodies conjugated with horseradish peroxidase (1:5,000, 1 h). Peroxidase was visualized with an ECL kit from GE Healthcare. The proteins were quantified prior to being loaded onto the gel and equal loading of extracts was verified by Ponceau staining.

 μ -opioid receptor binding assay. SH-SY5Y cell membranes were prepared by homogenizing cells in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1 mM dithiothreitol and 1 mM benzamidine, with a Polytron homogenizer. Following centrifugation (1,000 x g for 10 min at 4°C), supernatants were centrifuged (18,000 x g for 30 min at 4°C) and the pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂. The protein concentrations were determined by bicinchoninic acid assay. For th esaturation binding experiments, cell membranes (100 μ g/assay) were incubated in 100 mM Tris-HCl, pH 7.4, containing 0.3% bovine serum albumin with increasing concentrations of [³H] DAMGO (0.1-5 nM). Non-specific binding was determined in the presence of DAMGO (10 μ M). Following 90 min of incubation at 25°C, the bound ligand was isolated by rapid filtration

Table I. Relative μ -opioid receptor mRNA levels in SH-SY5Y cells under propofol treatment.

	Time		
Propofol (µM)	6 h	12 h	24 h
1	1.08±0.02	1.12±0.03	1.10±0.02
5	1.27 ± 0.04^{a}	$1.49 \pm 0.06^{a,c}$	1.53±0.06 ^{a,c}
10	$1.81 \pm 0.06^{a,b}$	1.98±0.09 ^{a-c}	2.03±0.09 ^{a-c}
20	$2.56 \pm 0.06^{a,b}$	2.86±0.09 ^{a-c}	2.92±0.09 ^{a-c}

The μ -opioid receptor mRNA levels of the treated cells are shown as fold changes from those of the untreated control cells (designated as 1). Comparisons of means among multiple groups were performed by one-way ANOVA followed by post hoc pairwise comparisons using the least significant difference method. ^aP<0.05 compared with propofol treatment at 1 μ M; ^bP<0.05 compared with propofol treatment at 5 μ M; ^cP<0.05 compared with 6 h of propofol treatment at each concentration.

on Whatman GF/B filters (Schleicher and Schuell, Riviera Beach, FL, USA). The filters were washed with 20 ml ice-cold 50 mM Tris-HCl buffer, pH 7.4, and left in scintillation fluid for 8 h prior to counting. Data were fitted by non-linear least-square regression and the Ligand program (13) was used to calculate receptor density (B_{max}), Hill slopes and ligand affinity (Kd). Data are expressed as fmol of [³H]DAMGO bound and normalized to cell protein content. Each experiment was repeated three times in triplicate. Results are expressed as the mean \pm SD.

Statistical analysis. Statistical analyses were performed with SPSS for Windows 10.0. Data values were shown as the mean \pm SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by post hoc pairwise comparisons using the least significant difference method. The significance level of this study was set at two-sided α =0.05.

Results

SH-SY5Y cells were treated with various concentrations of propofol (1, 5, 10 or 20 μ M) for different time periods (6, 12 or 24 h) and the endogenous MOR transcripts were evaluated using real-time quantitative RT-PCR. The MOR mRNA levels of the treated cells are shown as fold changes compared to those of the untreated control cells (designated as 1). At a concentration range of 1-10 μ M, propofol increased the MOR mRNA levels in a statistically significant dose- and time-dependent manner within 12 h of treatment (Table I).

Western blot analyses revealed that propofol treatment for 12 h dose-dependently increased the MOR protein levels in the SH-SY5Y cells (Fig. 1). In cells treated for 12 h, propofol dose-dependently increased the MOR density (B_{max}) in the cell membranes (Fig. 2). The results suggest that propofol significantly increases the density of ligand-binding MORs in the SH-SY5Y cell membranes by increasing the MOR mRNA levels.



Figure 1. Western blot analysis of μ -opioid receptor (MOR) expression in SH-SY5Y cells treated with propofol. (A) SH-SY5Y cells were treated with different concentrations of propofol (1, 5, 10 or 20 μ M) for 12 h. Cell lysates were subjected to western blot analyses of MOR expression. Lysates from untreated SH-SY5Y cells were used as the control. β -actin blotting was used as a loading control. Representative results are presented. (B) MOR and β -actin blots were measured by densitometry. The densities of the MOR blot were normalized against those of β -actin to obtain relative densities. Data are expressed as the mean \pm SD. Comparisons of means among multiple groups were performed by one-way ANOVA followed by post hoc pairwise comparisons using the least significant difference method. *P<0.05 compared with untreated control cells; *P<0.05 compared with propofol treatment at 1 μ M; *P<0.05 compared with propofol treatment at 5 μ M.



Figure 2. Saturation binding assay of μ -opioid receptor (MOR) density in the cell membranes of SH-SY5Y cells treated with propofol. SH-SY5Y cells were treated with different concentrations of propofol (1, 5, 10 or 20 μ M) for 12 h. Saturation binding assays were conducted on the cell membranes using [³H]DAMGO (0.05-5 nM). A single-site receptor binding model provided the best fit for data analysis. B_{max} values were estimated from non-linear regression analysis. B_{max} values of untreated SH-SY5Y cells were used as the control. Each experiment was repeated three times in triplicate. Data are expressed as the mean ± SD. Comparisons of means among multiple groups were performed by one-way ANOVA followed by post hoc pairwise comparisons using the least significant difference method. ^aP<0.05 compared with untreated control cells; ^bP<0.05 compared with propofol treatment at 1 μ M; ^cP<0.05 compared with propofol treatment at 5 μ M.

To evaluate the effects of propofol on MOR mRNA stability, the SH-SY5Y cells were treated with the transcription inhibitor actinomycin D (14). By blocking transcription, we were able to determine whether propofol affects the stability of MOR mRNA. The cells were pretreated with actinomycin D (1 mg/ml) for 30 min and then cultured for 6 or 12 h in medium



Figure 3. μ -opioid receptor (MOR) mRNA levels in SH-SY5Y cells treated with actinomycin D with or without propofol. SH-SY5Y cells were pretreated with actinomycin D (1 mg/ml) for 30 min and then cultured for 6 or 12 h in medium containing actinomycin D (1 mg/ml) with or without propofol (10 μ M). The MOR mRNA levels of the treated cells are shown as percentage changes from those of the untreated control cells (designated as 100%). Each experiment was repeated three times in triplicate. Data are expressed as the mean \pm SD. Comparisons of means among multiple groups were performed with one-way ANOVA followed by post hoc pairwise comparisons using the least significant difference method. 10 <0.05 compared with 6-h A+P. A, actinomycin D; P, propofol.

containing actinomycin D (1 mg/ml) with or without propofol (10 μ M). Real-time quantitative RT-PCR assays showed that the MOR mRNA levels significantly decreased with time after actinomycin D treatment. In the presence of actinomycin D, propofol had no significant effect on the MOR mRNA levels over time (Fig. 3). The results suggest that propofol increases MOR expression at the transcriptional level, rather than enhancing the stability of MOR mRNA at the post-transcriptional level.

Discussion

In the present study, we have demonstrated that propofol is able to significantly increase MOR expression in SH-SY5Y human neuroblastoma cells at the transcriptional level, leading to an increased density of ligand-binding MORs in the cell membranes. This study provides the first evidence suggesting a functional link between propofol and the opioid system.

The actions of propofol and opioids are functionally linked with GABA and NMDA receptors in neurons (5-8). In this study, we used SH-SY5Y cells since they are human neuronal cells that endogenously express MORs as well as GABA and NMDA receptors (14-16), making them an excellent tool for an *in vitro* study concerning propofol's effects on MOR expression and the underlying molecular mechanisms. Based on our findings, it would be of note to identify signaling pathways involved in the propofol-induced transcriptional regulation of the MOR gene in future studies.

Propofol dose- and time-dependently increases MOR expression (Table I) in neuronal cells, which results in an enhanced density of ligand-binding MORs in the cell membranes (Fig. 2). Thus, it is reasonable to suggest that long-term or frequent propofol sedation may potentiate the effects of endogenous μ -opioid peptides (e.g., endorphins and endomorphins) or μ -opioid analgesics (e.g., morphine and fentanyl), including side effects such as sedation, respiratory depression, euphoria and dependence. Significant experimental evidence suggests the endogenous opioid system (opioid peptides and receptors) with the development of dependence on a variety of drugs of abuse (17). Stimulation of the activity of distinct components of the endogenous opioid system by opioids or by other drugs of abuse, may mediate some of their reinforcing effects (17). Recent studies suggest that MOR and μ -opioid peptides are involved in the addictive processes induced by cannabinoids, nicotine and alcohol (18-20). Our findings suggest that by increasing MOR expression in neuronal cells, propofol has the potential to enhance the activity of the endogenous μ -opioid system and thus has the potential for abuse. A growing body of literature has documented propofol abuse in humans (4). The possibility of dependence and withdrawal is significant in determining propofol's potential for abuse. Previous studies have suggested that propofol dependence and withdrawal is a phenomenon that is primarily observed in prolonged propofol use (21-25). Evidence suggests that chronic second-hand exposure to the aerosolized intravenous anesthetics propofol and fentanyl may cause sensitization and subsequent opiate addiction among anesthesiologists and surgeons (26). The molecular mechanism underlying propofol abuse is unknown. Based on our findings, we propose that propofol abuse following long-term or chronic propofol exposure may be partially attributable to propofol-induced MOR expression and that the combined use of propofol and opioid analgesics may enhance the development of opioid dependence and addiction.

In addition to being a widely used sedative-hypnotic agent, propofol is also known to be a potent, dose-dependent depressant of respiration (27). The mechanism by which propofol modulates the central respiratory network is not fully understood. According to our findings, the sedative and respiratory depression effects of propofol may be partially attributable to propofol-induced MOR expression during long-term or chronic use, which may enhance the activity of the endogenous μ -opioid system. Long-term or chronic use of propofol may also exacerbate the depression of respiration by μ -opioid analgesics. However, further studies are required to demonstrate the interaction between propofol and the opioid system at the systems biology level.

In conclusion, propofol dose- and time-dependently enhances MOR expression in SH-SY5Y human neuroblastoma cells at the transcriptional level, leading to an increased density of ligand-binding MORs in the cell membranes. This study demonstrates for the first time a link between propofol and the opioid system, thereby providing new insights into the propofol mechanism of action and potential for abuse.

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