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Propofol up-regulates Mas receptor expression in dorsal root ganglion neurons

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Mas is a functional binding site for angiotensin (Ang)-(1-7), a critical component of the renin-angiotensin system that is involved in processing nociceptive information. A recent study reported the localization of Mas in rat dorsal root ganglia (DRG) and demonstrated that Ang-(1-7) produced a dose-dependent peripheral antinociceptive effect in rats through the Mas receptor by an opioid-independent mechanism. In the present study, we for the first time examined the effect of propofol on Mas expression in cultured DRG neurons. We treated rat DRG neurons with propofol at different concentrations (0.1, 0.5, 1, 5 or 10 μM) for different length of time (0.5, 1, 2, 4 or 6 h) with or without transcription inhibitor actinomycin D or different kinase inhibitors. Propofol increased the *Mas receptor* mRNA level in a statistically significant dose- and time-dependent manner within 4 h, which led to dose-dependent up-regulation of the Mas receptor protein level as well as Ang-(1-7) binding on the cell membrane. Actinomycin D (1 mg/ml) and p38 mitogen-activated protein kinase inhibitor PD169316 (25 μM) completely abolished the effect of propofol on Mas receptor expression in DRG neurons. In conclusion, we demonstrate that propofol markedly up-regulates Mas receptor expression at the transcription level in DRG neurons by a p38 MAPK-dependent mechanism. This study provides new insights into the mechanisms of action of propofol in peripheral antinociception, and suggests a new regulatory mechanism on the Ang-(1-7)/Mas axis in the peripheral nervous system.

1. Introduction

The renin-angiotensin system (RAS) is involved in processing of nociceptive information (Pelegrini-da-Silva et al. 2005; Sakagawa et al. 2000). Antinociception following intracerebroventricular injection of renin substrate, angiotensin (Ang) II, and Ang III has been demonstrated in several rodent pain models (Pelegrini-da-Silva et al. 2005). Treatment of genetically hypertensive rats with the angiotensin-converting enzyme inhibitor captopril during the developmental phase of hypertension has been reported to prevent analgesia (Irvine et al. 1995). Additional studies have demonstrated that exogenous administration of Ang II results in analgesia, a response sensitive to blockade by both opioids and angiotensin II receptor type 1 (AT1) antagonists (Haulica et al. 1986; Toma et al. 1986). However, injection of Ang II into the caudal ventrolateral medulla was recently shown to induce hyperalgesia by AT1 receptor activation (Marques-Lopes et al. 2010).

In addition to the angiotensin-converting enzyme (ACE)/Ang II/AT1 axis, the RAS possesses another regulatory axis composed by ACE2, Ang-(1-7), and Mas (Ferreira et al. 2012). Ang-(1-7) is now recognized as a critical component of the RAS. It can be generated directly from Ang II by ACE2 (Baronas et al. 2002; Der Sarkissian et al. 2006; Raizada and Ferreira 2007), and directly from Ang I by neutral endopeptidase and prolylendopeptidase (Rice et al. 2004; Stanziola et al. 1999). It is now well established that the G protein-coupled receptor Mas is a functional binding site for Ang-(1-7) (Ferreira and Santos 2005; Gomes et al. 2012). In the central nervous system (CNS), Ang-(1-7) acts as an important neuromodulator, particularly in areas associated with tonic and reflex control of arterial pressure, in the hypothalamus and in the dorsomedial and ventrolateral medulla (Santos et al. 2000). At these sites, the cardiovascular effects induced by Ang-(1-7) are blocked by a selective Mas antagonist (Santos et al. 2000; Stanziola et al. 1999), suggesting that the actions of Ang-(1-7) may be mediated by an interaction with Mas in the CNS. A recent study for the first time reported the localization of Mas in rat dorsal root ganglia (DRG) and demonstrated that Ang-(1-7) produced a dose-dependent peripheral antinociceptive effect in rats through the Mas receptor by an opioid-independent mechanism (Costa et al. 2012). Thus, as an essential component of RAS, the Ang-(1-7)/Mas axis also plays an important role in antinociception.

Propofol is one of the most popular intravenous agents for induction of general anesthesia. Advantages of this agent over others of similar applications include lower incidence of side effects (Tramer et al. 1997), rapid recovery (Trapani et al. 2000), and better quality of anesthesia (Schaer 1988). We previously demonstrated that propofol increased ACE2 expression in human pulmonary artery endothelial cells (Cao et al. 2012), which suggests a potential link between propofol and the Ang-(1-7)/Mas axis. In the present study, we for the first time exmained the effect of propofol on Mas expression in cultured rat DRG neurons.

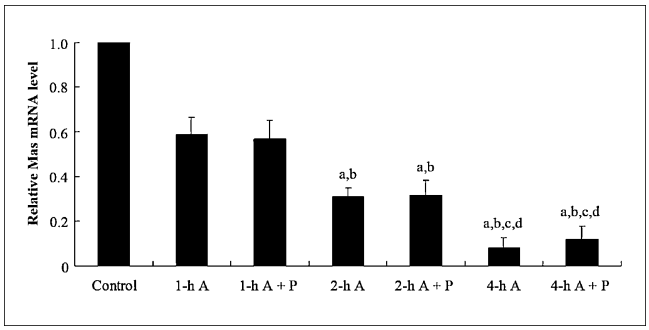

Fig. 1: Mas receptor mRNA level in rat dorsal root ganglion (DRG) neurons treated with actinomycin D with or without propofol Rat DRG neurons were pre-treated with actinomycin D (1 mg/ml) for 30 min, and then cultured for 1, 2 or 4 h in medium containing actinomycin D (1 mg/ml) with or without propofol (10 μM). The Mas receptor mRNA level of treated cells was shown as fold changes to that of untreated control cells (designated as 1). A, actinomycin D; P, propofol; h, hour. [a]$P < 0.05$ compared with 1-h A; [b]$P < 0.05$ compared with 1-h A + E; [c]$P < 0.05$ compared with 2-h A; [d]$P < 0.05$ compared with 2-h A + P.

2. Investigations and results

Rat DRG neurons were treated with propofol in different concentrations (0.1, 0.5, 1, 5 or 10 μM) for different length of time (0.5, 1, 2, 4 or 6 h). The *Mas receptor* mRNA levels were examined using real-time quantitative RT-PCR. The *Mas receptor* mRNA level of treated cells was shown as fold changes to that of untreated control cells (designated as 1). As shown in Table 1, propofol in the concentration range of 0.5 μM to 5 μM increased the *Mas receptor* mRNA level in a statistically significant dose- and time-dependent manner within 4 h of treatment. Propofol at 0.1 μM had no significant effect on the *Mas receptor* mRNA level over time. Treatment for 0.5 h with propofol at 0.1 μM–10 μM also showed no significant effect on the *Mas receptor* mRNA level.

To evaluate the effects of propofol on *Mas receptor* mRNA stability, rat DRG neurons were pre-treated with transcription inhibitor actinomycin D (1 mg/ml) for 30 min, and then cultured for 1, 2 or 4 h in medium containing actinomycin D (1 mg/ml) with or without propofol (10 μM). Real-time quantitative RT-PCR assays showed that the *Mas receptor* mRNA level significantly decreased with time after actinomycin D treatment (Fig. 1). In the presence of actinomycin D, propofol had no significant effect on the *Mas receptor* mRNA level over time (Fig. 1). The results suggest that propofol increases Mas receptor expression at the transcriptional level rather than increase *Mas receptor* mRNA stability at the post-transcriptional level.

To determine the signaling pathways involved in the promoting effect of propofol on *Mas receptor* gene transcription, we examined the *Mas receptor* mRNA levels in rat DRG neurons treated with propofol (10 μM) with or without different kinase inhibitors for 4 hours. As shown in Table 2, inhibition of phosphatidylinositol-3 kinase (PI3K) (LY294002; 50 μM), protein kinase C (Go6983; 250 nM), and mitogen-activated protein kinase (PD098059; 25 μM) had no significant effect on the *Mas receptor* mRNA level. In contrast, inhibition of p38 MAPK by selective blocker PD169316 completely reversed the promoting effect of propofol on *Mas receptor* gene transcription.

Western blot analyses showed that propofol treatment for 4 h dose-dependently increased the Mas receptor protein level in rat DRG neurons, which was reversed by PD169316 (Fig. 2). In line with the results, rat DRG neurons treated with propofol (10 μM) for 4 h showed a dose-dependent increase in Ang-(1-7) binding on the cell membrane, which was reversed by PD169316 (Fig. 3). Taken together, the results suggest that propofol can significantly increase the density of ligand-binding Mas receptor on the cell membrane of rat DRG neurons by up-regulating *Mas receptor* gene transcription by a p38 MAPK-dependent mechanism.

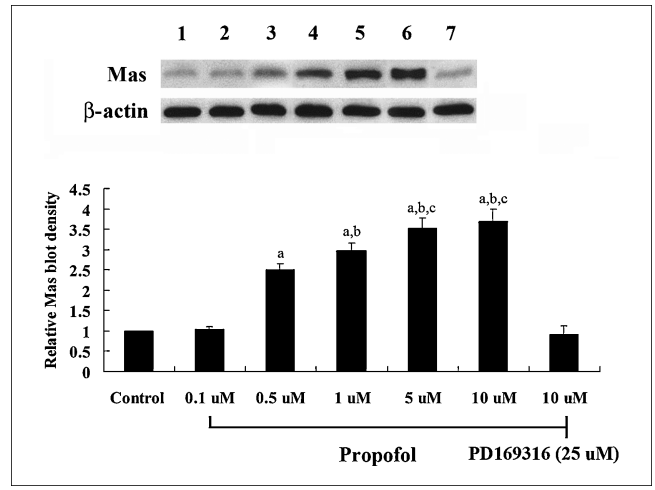

Fig. 2: Western blot analysis of Mas receptor expression in rat dorsal root ganglion (DRG) neurons. *A*, Rat DRG neurons were treated with propofol (0.1, 0.5, 1, 5, 10 μM) with or without PD169316 (25 μM) for 4 h. Cell lysates were subject to western blot analyses for Mas receptor expression. Lysates from untreated rat DRG neurons were used as a control (*lane 1*). *Lane 2*, propofol (0.1 μM); *lane 3*, propofol (0.5 μM); *lane 4*, propofol (1 μM); *lane 5*, propofol (5 μM); *lane 6*, propofol (10 μM); *lane 7*, propofol (10 μM) + PD169316 (25 μM). β-Actin blotting was used as a loading control. *B*, Mas receptor and β-Actin blots were measured by densitometry. The density of the Mas blot was normalized against that of β-actin to obtain a relative density, which was expressed as fold changes to the relative Mas density of untreated control cells (designated as 1). [a]$P < 0.05$ compared with untreated control cells; [b]$P < 0.05$ compared with propofol treatment at 0.5 μM; [c]$P < 0.05$ compared with propofol treatment at 1 μM.

3. Discussion

Ang-(1-7) is a biologically active member of the RAS (Silva et al. 2007). The physiological role of Ang-(1-7) was firmly established by two recent discoveries: (1) Identification of the ability of ACE2, an enzyme that generates Ang-(1-7) from Ang I or Ang II (Silva et al. 2007); (2) Characterization of the G proteincoupled receptor Mas as a receptor that is associated with several actions of Ang-(1-7) (Medeiros et al. 1997). A recent study demonstrated the localization of Mas in rat DRG and a

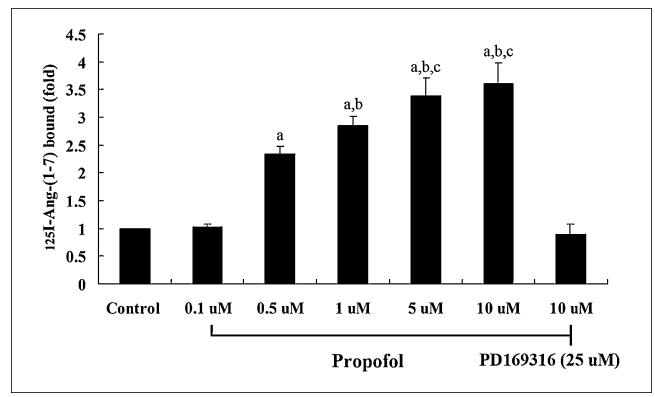

Fig. 3: Saturation binding assay of Mas receptor on the cell membrane of rat dorsal root ganglion (DRG) neurons. Rat DRG neurons were treated with propofol (0.1, 0.5, 1, 5, 10 μM) with or without PD169316 (25 μM) for 4 h. Saturation binding assays were conducted using increasing concentrations of ^{125}I-Ang-(1-7) (1-22 nM) on cell membranes. A single-site receptor binding model provided the best fit for data analysis. The disintegrations per minute (dpm) data was normalized against cell number (per 10,000 cells) and shown as fold changes to that of untreated control cells (designated as 1). [a]$P < 0.05$ compared with untreated control cells; [b]$P < 0.05$ compared with propofol treatment at 0.5 μM; [c]$P < 0.05$ compared with propofol treatment at 1 μM.

Table 1: Relative Mas receptor mRNA levels in rat dorsal root ganglion (DRG) neurons under propofol treatment

		Time (h)				
	0.5	1	2	4	6	
Propofol (μM)	0.1	1.01 ± 0.05	1.03 ± 0.05	1.04 ± 0.04	1.02 ± 0.05	1.07 ± 0.05
	0.5	1.04 ± 0.03	$1.21 \pm 0.08^{a,d}$	$1.80 \pm 0.11^{a,d,e}$	$2.67 \pm 0.16^{a,d,e,f}$	$2.81 \pm 0.18^{a,d,e,f}$
	1	1.05 ± 0.05	$1.85 \pm 0.13^{a,b,d}$	$2.68 \pm 0.15^{a,b,d,e}$	$3.05 \pm 0.21^{a,b,d,e,f}$	$3.25 \pm 0.23^{a,b,d,e,f}$
	5	1.08 ± 0.06	$2.76 \pm 0.17^{a,b,c,d}$	$3.01 \pm 0.13^{a,b,c,d,e}$	$3.59 \pm 0.25^{a,b,c,d,e,f}$	$3.77 \pm 0.28^{a,b,c,d,e,f}$
	10	1.06 ± 0.07	$2.84 \pm 0.22^{a,b,c,d}$	$3.14 \pm 0.25^{a,b,c,d,e}$	$3.79 \pm 0.25^{a,b,c,d,e,f}$	$3.93 \pm 0.26^{a,b,c,d,e,f}$

The Mas receptor mRNA level of treated rat DRG neurons was shown as fold changes to that of untreated control cells (designated as 1). [a] $P < 0.05$ compared with propofol treatment at 0.1 μM; [b] $P < 0.05$ compared with propofol treatment at 0.5 μM; [c] $P < 0.05$ compared with propofol treatment at 1 μM; [d] $P < 0.05$ compared with 0.5 h of propofol treatment at each concentration; [e] $P < 0.05$ compared with 1 h of propofol treatment at each concentration; [f] $P < 0.05$ compared with 2 h of propofol treatment at each concentration.

marked peripheral antinociceptive effect of Ang-(1-7) on rats through the Mas receptor in an opioid-independent pathway (Costa et al. 2012), suggesting an important role of the Ang-(1-7)/Mas axis in peripheral antinociception. Propofol is one of the most widely used intravenous anesthetics in clinic and thought to exert its pharmacological actions at both spinal and supraspinal level of CNS (Sun et al. 2005). It has been reported that besides its central actions, propofol also has peripheral antinociceptive effects (Sun et al. 2005). In the present study, we provide the first evidence suggesting a regulatory effect of propofol on the Ang-(1-7)/Mas axis in the peripheral nervous system, by showing that propofol up-regulates Mas expression in DRG neurons. We employed DRG neurons as a cell model in this study, because these cells are responsible for conducting information from the periphery to CNS.

We previously showed that propofol in the concentration range of 10 μM to 40 μM increased the ACE2 mRNA level in human pulmonary artery endothelial cells in a dose- and time-dependent manner within 24 h of treatment (Cao et al. 2012). In the present study, the ACE2 expression level was very low to none in DRG neurons and showed no significant change to propofol treatment over time. Propofol in the concentration range of 0.5 μM to 5 μM increased the *Mas receptor* mRNA level in a statistically significant dose- and time-dependent manner within 4 h of treatment. A selective p38 MAPK inhibitor, but not other kinase inhibitors, completely reversed the promoting effect of propofol, indicating that propofol up-regulates Mas expression by a p38 MAPK-dependent mechanism. Further studies are needed to elaborate the mechanisms of how propofol regulates Mas expression through p38 MAPK signaling.

Propofol has been proposed to have several mechanisms of action (Kotani et al. 2008). It activates γ-aminobutyric acid ($GABA_A$) receptors, inhibits the *N*-methyl-D-aspartate (NMDA) receptor, and modulates calcium influx through slow calcium-ion channels (Kotani et al. 2008). Recent research has also suggested that the endocannabinoid system may contribute significantly to propofol's anesthetic action and to its unique properties (Fowler 2004). In the present study, propofol at 10 μM increased Mas expression and Ang-(1-7) binding to the DRG neuron cell membrane by over 3.5 folds within 4 h. This suggests that propofol has a strong enhancing effect on the Ang-(1-7)/Mas axis, which may be a new mechanism of action of propofol. In addition, by demonstrating a significant peripheral antinociceptive effect of the Ang-(1-7)/Mas axis in an opioid-independent pathway, Costa et al. (2012) proposed that development of drugs activating the Mas receptor may be a useful tool in clinical treatment of pain. According to our results in the present study, propofol may be a good candidate.

In conclusion, we demonstrate that propofol markedly up-regulates Mas receptor expression at the transcription level in DRG neurons by a p38 MAPK-dependent mechanism. This study provides new insights into the mechanisms of action of propofol in peripheral antinociception, and suggests a new regulatory mechanism on the Ang-(1-7)/Mas axis in the peripheral nervous system.

4. Experimental

4.1. Reagents

Propofol, Ang-(1-7), actinomycin D, and kinase inhibitors LY294002, Go6983, PD098059, and PD169316 were purchased from Sigma (St. Louis, MO, USA). ^{125}I-Sodmm iodide (carrier free, 100 mCl/mL) was purchased from Amersham Biosciences (Piscataway, NJ, USA). TRIzol reagent for RNA isolation, and the SYBR Green Master Mix were purchased from Invitrogen (Carlsbad, Ca, USA) and PE Applied Biosystems (Foster City, CA, USA), respectively. Anti-MAS1 (M-13) (sc-54848) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Cell culture and treatment

Rat DRG neurons (R-DRG-505) and primary neuron medium (CC-3256) were purchased from Lonza Inc. (Houston, TX, USA). The cells were treated with propofol at different concentrations (0.1, 0.5, 1, 5 or 10 μM) for different length of time (0.5, 1, 2, 4 or 6 h). Propofol, actinomycin D and all kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO 0.05%). For kinase inhibitor treatment, Rat DRG neurons were pretreated with the kinase inhibitor for 30 min, and then incubated with both the kinase inhibitor and propofol (10 μM) for 4 h. Rat DRG neurons treated with propofol (10 μM) + DMSO (0.05%) was used as a control in the experiments. For actinomycin D treatment, the cells were pre-treated

Table 2: Relative Mas receptor mRNA levels in rat dorsal root ganglion (DRG) neurons in the presence of propofol with or without kinase inhibitors

Treatment	Relative ACE2 mRNA level
Control	3.79 ± 0.25^{a}
+LY294002 (50 μM)	3.55 ± 0.30^{a}
+Go6983 (250 nM)	3.62 ± 0.26^{a}
+PD098059 (25 μM)	3.65 ± 0.33^{a}
+PD169316 (25 μM)	0.95 ± 0.12

Rat DRG neurons were pre-treated with the kinase inhibitor for 30 min, and then incubated with both the kinase inhibitor and propofol (10 μM) for 4 h. Propofol and all kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO 0.05%). Rat DRG neurons treated with propofol (10 μM)+DMSO (0.05%) was used as the control. The Mas receptor mRNA level of treated cells was shown as fold changes to that of untreated control cells (designated as 1). [a] $P < 0.05$ compared with propofol (10 μM)+PD169316 (25 μM) treatment.

with actinomycin D (1 mg/ml) for 30 min, and then cultured for 1, 2 or 4 h in medium containing actinomycin D (1 mg/ml) with or without propofol (10 μM).

4.3. Real-time quantitative RT-PCR

RNA were prepared using TRIzol reagent followed by purification with TURBO DNA-free System (Ambion, Austin, TX, USA). The cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed on an Abi-Prism 7700 Sequence Detection System (Applied Biosystems), using the fluorescent dye SYBR Green Master Mix (PE Applied Biosystems) as described by the manufacturer. The results were normalized against that of *β-actin* in the same sample. The primers used are as follows: for rat *Mas*, 5′-CCTGCATACTGGGAAGACCA -3′ (forward) and 5′-TCCCTTCCTGTTTCTCATGG -3′ (reverse); for rat *β-actin*, 5′-TCCTCCTGAGCGCAAGTACTC -3′ (forward) and 5′-GTGGACAGTAGTGAGGCCAGGT -3′ (reverse). The mRNA level of treated cells was shown as fold changes to that of untreated control cells (designated as 1). Each experiment was repeated for three times in triplicates. Results are expressed as mean ± SD.

4.4. Western blot analysis

Rat DRG neurons were lysed in 250 μ l of 2 × SDS loading buffer (62.5 mM TrisHCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromphenol blue, 5% 2-mercaptoethanol), and incubated at 95 °C for 10 min. Equal amount of proteins (100 μg) for each sample were separated by 8-15% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore, Billerica, MA, USA). Membranes were incubated for 1 h with a 1:1000 dilution of primary antibody, and then washed and revealed using secondary antibodies with horseradish peroxidase conjugate (1:5000, 1 h). Peroxidase was revealed with an GE Healthcare ECL kit. Proteins were quantified before being loaded onto the gel, and equal loading of extracts was verified by Ponceau coloration.

4.5. [^{125}I]Ang-(1-7) binding assay

Rat DRG neurons in 12-well plates were rinsed twice with DMEM and equilibrated on ice with incubation buffer (DMEM containing 0.2% BSA and a protease inhibitors cocktail, pH 7.4) for 30 min. Subsequently, the plates were incubated at 4 °C for 60 min with incubation buffer containing 0.5 nmol/L ^{125}I-Ang-(1-7) (labeled as previously described (Gironacci et al. 2011)). Incubation was stopped by rinsing the cells three times with ice-cold PBS. Cells were solubilized by incubation with 0.1 mol/L NaOH for 60 min and the radioactivity was measured. Nonspecific binding was determined in the presence of 10 μmol/L unlabeled Ang-(1-7), which was no higher than 15%. Specific binding was calculated by the subtraction of nonspecific binding from total binding. The disintegrations per minute (dpm) data was normalized against cell number (per 20000 cells) and shown as fold changes to that of untreated control cells (designated as 1). Each experiment was repeated for three times in triplicates. Results are expressed as mean ± SD.

4.6. Statistical analysis

Statistical analyses were performed with SPSS for Windows 10.0 (SPSS Inc., Chicago, IL, USA). Data values were expressed as means ± 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 a two-tailed α=0.05.

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