

# Ketamine suppresses the substance P-induced production of IL-6 and IL-8 by human U373MG glioblastoma/astrocytoma cells

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**Abstract.** The neuropeptide substance P (SP) is an important mediator of neurogenic inflammation within the central and peripheral nervous systems. SP has been shown to induce the expression of pro-inflammatory cytokines implicated in the pathogenesis of several disorders of the human brain via the neurokinin-1 receptor (NK-1R). Ketamine, an intravenous anesthetic agent, functions as a competitive antagonist of the excitatory neurotransmission *N*-methyl-D-aspartate (NMDA) receptor, and also antagonizes the NK-1R by interfering with the binding of SP. In the present study, we investigated the anti-inflammatory effects of ketamine on the SP-induced activation of a human astrocytoma cell line, U373MG, which expresses high levels of NK-1R. The results from our experiments indicated that ketamine suppressed the production of interleukin (IL)-6 and IL-8 by the U373MG cells. Furthermore, ketamine inhibited the SP-induced activation of extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B). Taken together, these observations suggest that ketamine may suppress the SP-induced activation (IL-6 and IL-8 production) of U373MG cells by inhibiting the phosphorylation of signaling molecules (namely ERK1/2, p38 MAPK and NF- $\kappa$ B), thereby exerting anti-inflammatory effects. Thus, ketamine may modulate SP-induced inflammatory responses by NK-1R-expressing cells through the suppression of signaling molecules (such as ERK1/2, p38 MAPK and NF- $\kappa$ B).

## Introduction

The neuropeptide substance P (SP) is an important mediator of neurogenic inflammation within the central and peripheral nervous systems. SP has been implicated in pain, and also plays important roles in cancer (e.g., tumor cell proliferation, anti-apoptotic effects on tumor cells, angiogenesis, tumor cell invasion and metastasis) (1-8). SP is released from primary afferent nociceptors and sympathetic post-ganglionic neurons, and activates neighboring receptors, thereby triggering and spreading activation (6-11). Moreover, SP has been shown to induce the expression of pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8 (12,13), which are involved in the pathogenesis of several disorders of the human brain (such as multiple sclerosis, dementia complex, and Alzheimer's disease) (14), although it is currently a matter of debate as to whether SP plays a pathogenic or a protective role in these disorders. Previous research has indicated that the activation of an SP receptor [neurokinin-1 receptor (NK-1R)] elicits signals affecting the gene expression of some inflammatory cytokines (13). In addition, it has been reported that nanomolar concentrations of SP potently trigger the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), an important transcriptional activator, which regulates the production of various cytokines and other proinflammatory mediators (13).

The mitogen-activated protein kinase (MAPK) family of protein Ser/Thr kinases consists of at least three major subfamilies: i) the p42/44 MAPKs, which are also known as the extracellular signal-regulated kinases (ERKs)1/2; ii) the c-Jun NH<sub>2</sub>-terminal kinase/stress-activated protein kinases (JNK/SAPKs), including p46 JNK-1 and p54 JNK2; and iii) the p38 MAPK subfamily. MAPKs are activated under conditions of stress in response to a number of extracellular stimuli, including oxidative stress. Among these, the phosphorylation of p38 MAPK is induced in the dorsal horn of the spinal cord and in the dorsal root ganglia following peripheral nerve injury or inflammation (15-17), and the phosphorylation of ERK1/2 is induced by inflammatory stimuli in the trigeminal nucleus and the dorsal horn (16,18,19).

The release of glutamate and SP from the primary afferents, activates *N*-methyl-D-aspartate (NMDA) receptors

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**Key words:** ketamine, substance P, extracellular signal-regulated kinase 1/2, p38 mitogen-activated protein kinase, nuclear factor- $\kappa$ B, neurokinin-1 receptor, *N*-methyl-D-aspartate receptor, interleukin-6, interleukin-8, U373MG cell, anti-inflammatory effect

and NK-1Rs, respectively, thereby resulting in the increase of intracellular calcium concentrations in the dorsal horn neurons, the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (20,21) and the production of prostaglandins by constitutively expressed spinal cyclooxygenase-2 (COX-2) (22). Ketamine, an intravenous anesthetic agent, functions as a competitive antagonist of the excitatory neurotransmission NMDA receptor (23) and also antagonizes the NK-1R by interfering with the binding of SP (24). Notably, the anti-inflammatory effect of ketamine has also been demonstrated in various animal models, where it was observed that ketamine markedly suppressed the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6 following the stimulation of macrophages and peripheral leucocytes by lipopolysaccharide (LPS) (25). Moreover, it has been reported that ketamine inhibits the systemic production of inflammatory molecules by inhibiting the NF- $\kappa$ B signaling pathway (26). Based on these findings, we hypothesized that ketamine may also act on NK-1R (SP) and exert anti-inflammatory effects by modulating the MAPK and NF- $\kappa$ B signaling pathways.

Thus, in the present study, we examined the anti-inflammatory effects of ketamine on the SP-induced activation of the human astrocytoma cell line, U373MG, which expresses high levels of NK-1R.

## Materials and methods

**Materials.** The U373MG cell line (Uppsala; ECACC 08061901) was purchased from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, UK). SP was obtained from Sigma-Aldrich (St. Louis, MO, USA); ketamine was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan); minimum essential medium (MEM), non-essential amino acids (NEAAs), sodium pyruvate and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). RIPA buffer with protease inhibitor cocktail, sample buffer solution with reducing reagent (6X) for SDS-PAGE, running buffer solution (10X) for SDS-PAGE, Blocking One and Protein Ladder One Multi-color (Broad Range) for SDS-PAGE were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). A BCA protein assay reagent kit and enhanced chemiluminescence reagent, SuperSignal West Dura were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Mini-PROTEAN<sup>®</sup> TGX<sup>™</sup> precast gel and Trans-Blot<sup>®</sup> Turbo<sup>™</sup> Mini PVDF transfer packs were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA), and a Cytometric Bead Array (Human Inflammatory Cytokine kit) was obtained from BD Biosciences (San Jose, CA, USA).

**Antibodies.** Anti-phospho-ERK1/2 MAPK (Thr202/Tyr204) rabbit antibody (#9101), anti-ERK1/2 MAPK rabbit antibody (#9102), anti-phospho-NF- $\kappa$ B p65 (Ser536) rabbit monoclonal antibody (mAb; #3033), and anti-NF- $\kappa$ B p65 rabbit mAb (#8242) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); anti-phospho p38 MAPK rabbit antibody (Thr180/Tyr182; V121A) was obtained from Promega Corp. (Madison, WI, USA); anti-p38 MAPK (p38/SAPK2 $\alpha$ ; 612168) mouse mAb was purchased from BD Biosciences. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (API32P) and

HRP-conjugated goat anti-mouse IgG/IgM (AP308P) were obtained from Chemicon International (Temecula, CA, USA).

**Cell culture.** The U373MG cells were cultured in MEM supplemented with 1% (v/v) penicillin/streptomycin, NEAA, 1 mM sodium pyruvate and 10% heat-inactivated FBS. The cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**Western blot analysis.** The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with ketamine (0.1 and 1 mM) for 60 min, and then stimulated with SP (100 nM) for 10 min. Thereafter, the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and lysed in 0.1 ml of RIPA buffer (50 mmol/l Tris-HCl pH 7.6, 150 mmol/l NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, protease inhibitor cocktail and 0.1% SDS). The protein concentrations of cell lysates were measured using the Bradford assay (Thermo Fisher Scientific). The lysates were mixed with SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromophenol blue, and 5% 2-mercaptoethanol), and applied to SDS-PAGE 10% gels (Mini-PROTEAN TGX precast gel; 15  $\mu$ g protein/lane). Thereafter, the separated proteins were electroblotted onto polyvinylidene fluoride membranes (Trans-Blot Turbo Mini PVDF Transfer packs). Following incubation with Blocking One (Nacalai Tesque, Inc.), the blots were probed with a 1,000-fold dilution of rabbit anti-phospho ERK1/2, anti-phospho NF- $\kappa$ B or anti-phospho p38 MAPK antibody, and further probed with a 2,000-fold dilution of HRP-conjugated goat anti-rabbit IgG/IgM. The signals were detected by SuperSignal West Pico/Dura Chemiluminescent substrate (Thermo Fisher Scientific), and quantified using an LAS-3000 luminescent image analyzer and MultiGauge software (both from Fujifilm, Tokyo, Japan). Thereafter, the antibody was stripped using WB Stripping Solution Strong (Nacalai Tesque, Inc.) at room temperature for 15 min. The blots were probed with a 1,000-fold dilution of rabbit anti-ERK1/2, anti-NF- $\kappa$ B or anti-p38 MAPK antibody, and further probed with a 2,000-fold dilution of HRP-conjugated goat anti-rabbit IgG/IgM or HRP-conjugated goat anti-mouse IgG/IgM.

**Quantification of IL-6 and IL-8 levels.** The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, which was followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. The cells were then incubated with ketamine (1 mM) for 60 min, and stimulated with SP (100 nM) for 24 h. The culture supernatants were recovered, centrifuged at  $12,000 \times g$  for 10 min, and the levels of IL-6 and IL-8 in the medium were measured using a Cytometric Bead Array Human Inflammatory Cytokine kit (BD Biosciences), according to the manufacturer's instructions.

**Statistical analysis.** Data are expressed as the means  $\pm$  SD, and analyzed for significant differences using a one-way ANOVA followed by a multiple comparison test using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA,

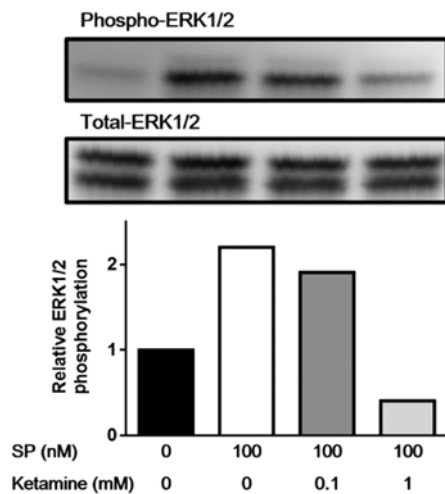


Figure 1. Dose-dependent inhibition of substance P (SP)-induced phosphorylation of extracellular signal-regulated kinase (ERK)1/2 by ketamine. The U373MG astrocytoma cells were plated into 12-well culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with or without ketamine (0.1 mM and 1 mM) for 60 min, and then incubated with or without SP (100 nM) for 10 min. The phosphorylation of ERK1/2 was detected by probing with anti-phospho-ERK1/2 MAPK (Thr202/Tyr204) antibody and HRP-conjugated goat anti-rabbit IgG. In order to confirm that equal amounts of the proteins were analyzed in each sample, the blots were stripped, and total ERK1/2 were detected by reprobing with anti-ERK1/2 MAPK antibody and HRP-conjugated goat anti-rabbit IgG. A representative image is shown, and the data are expressed relative to the cells incubated without SP and ketamine.

USA). A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

### Suppression of SP-induced ERK1/2 activation by ketamine.

Firstly, we examined the dose-dependent effect of ketamine on the phosphorylation of ERK1/2. As shown in Fig. 1, SP stimulation (100 nM) markedly induced the phosphorylation of ERK1/2 in the U373MG cells, and ketamine suppressed the SP-induced phosphorylation of ERK1/2 in a dose-dependent manner; the phosphorylation of ERK1/2 was only minimally inhibited by 0.1 mM ketamine, whereas the concentration of 1 mM ketamine caused marked inhibition. Thus, the concentration of 1 mM ketamine was used to repeatedly evaluate the effect of ketamine on the SP-induced phosphorylation of ERK1/2. As shown in Fig. 2, 1 mM ketamine significantly suppressed the SP-induced phosphorylation of ERK1/2 in the U373MG cells ( $P < 0.05$ ).

### Suppression of the SP-induced activation of p38 MAPK and NF- $\kappa$ B by ketamine.

We then evaluated the effects of ketamine on the phosphorylation of p38 MAPK and NF- $\kappa$ B. As shown in Fig. 3, stimulation of the U373MG cells with SP (100 nM) markedly induced the phosphorylation of p38 MAPK, and ketamine (1 mM) significantly suppressed the SP-induced phosphorylation of p38 MAPK ( $P < 0.05$ ). Similarly, stimulation of the U373MG cells with SP (100 nM) markedly induced the phosphorylation of NF- $\kappa$ B, and ketamine (1 mM) significantly suppressed the SP-induced phosphorylation of NF- $\kappa$ B (Fig. 4;  $P < 0.05$ ).

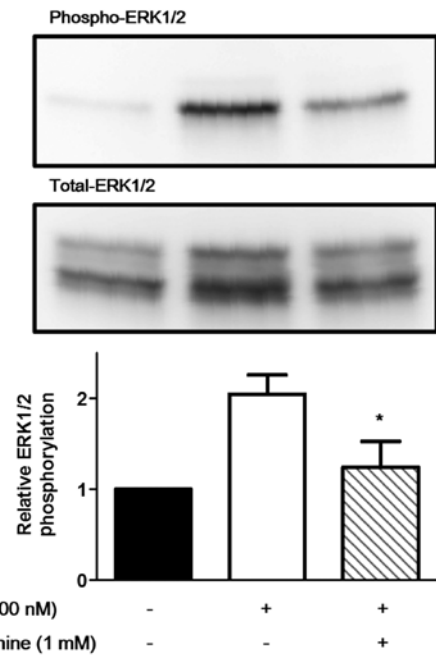


Figure 2. Inhibitory effect of ketamine on the substance P (SP)-induced phosphorylation of extracellular signal-regulated kinase (ERK)1/2. The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with or without ketamine (1 mM) for 60 min, and then incubated with or without SP (100 nM) for 10 min. The phosphorylation of ERK1/2 was detected by probing with anti-phospho-ERK1/2 MAPK (Thr202/Tyr204) antibody and HRP-conjugated goat anti-rabbit IgG. In order to confirm that equal amounts of the proteins were analyzed in each sample, the blots were stripped, and total ERK1/2 were detected by reprobing with anti-ERK1/2 MAPK antibody and HRP-conjugated goat anti-rabbit IgG. A representative image is shown. Data are the means  $\pm$  SD of 3 separate experiments, and expressed relative to the cells incubated without SP and ketamine. Data are compared between the SP-stimulated cells incubated with and without ketamine, \* $P < 0.05$ .

*Effects of ketamine on the SP-induced production of IL-6 and IL-8 by U373MG cells.* Finally, we evaluated the effects of ketamine on the production of IL-6 and IL-8 by U373MG cells. As shown in Fig. 5, the levels of IL-6 and IL-8 in the cell culture supernatants were markedly increased following stimulation with SP (100 nM). Notably, ketamine (1 mM) significantly suppressed the production of both IL-6 and IL-8 ( $P < 0.05$ ).

## Discussion

To the best of our knowledge, this is the first study to demonstrate the effects of ketamine on SP-induced inflammatory responses in U373MG glioblastoma/astrocytoma cells. In the present study, we revealed that ketamine suppressed the production of IL-6 and IL-8 in U373MG cells. Furthermore, ketamine inhibited the SP-induced activation of ERK1/2, p38 MAPK and NF- $\kappa$ B. Thus, ketamine may suppress the SP-induced activation (IL-6 and IL-8 production) of U373MG cells by inhibiting the phosphorylation of signaling molecules (namely ERK1/2, p38 MAPK and NF- $\kappa$ B), thereby exerting anti-inflammatory effects.

U373MG astrocytoma cells express a functional high-affinity SP receptor, NK-1R (27) and are capable of producing IL-6 in response to stimulation with SP (12). Thus,

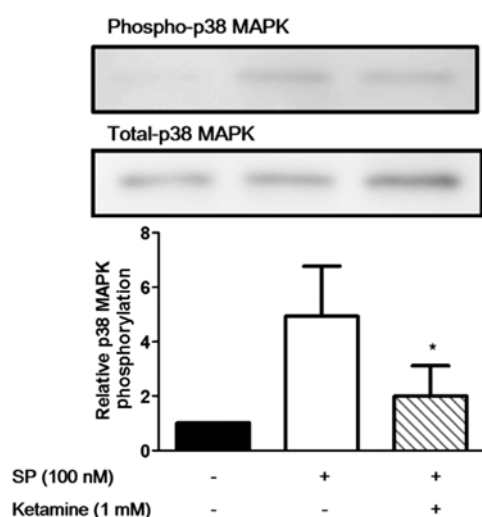


Figure 3. Inhibitory effect of ketamine on substance P (SP)-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK). The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with or without ketamine (1 mM) for 60 min, and then incubated with or without SP (100 nM) for 10 min. The phosphorylation of p38 MAPK was detected by probing with anti-phospho p38 MAPK antibody and HRP-conjugated goat anti-rabbit IgG. In order to confirm that equal amounts of the proteins were analyzed in each sample, the blots were stripped, and total p38 MAPK were detected by reprobing with anti-p38 MAPK antibody and HRP-conjugated goat anti-mouse IgG. A representative image is shown. Data are the means  $\pm$  SD of 3 separate experiments, and expressed relative to the cells incubated without SP and ketamine. Data are compared between the SP-stimulated cells incubated with and without ketamine. \* $P < 0.05$ .

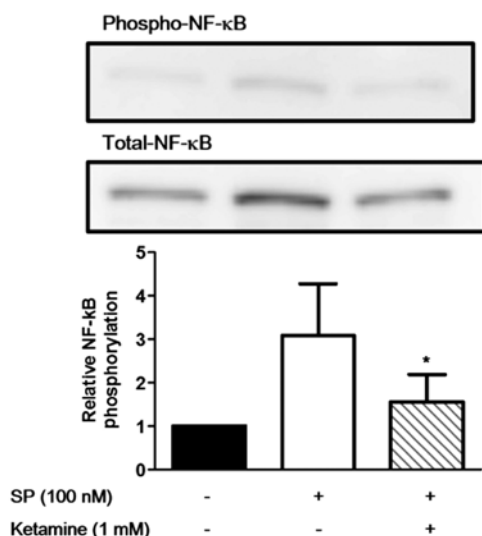


Figure 4. Inhibitory effect of ketamine on substance P (SP)-induced phosphorylation of nuclear factor-κB (NF-κB). The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with or without ketamine (1 mM) for 60 min, and then incubated with or without SP (100 nM) for 10 min. The phosphorylation of NF-κB was detected by probing with anti-phospho NF-κB p65 (Ser536) rabbit mAb and HRP-conjugated goat anti-rabbit IgG. In order to confirm that equal amounts of the proteins were analyzed in each sample, the blots were stripped, and total NF-κB were detected by reprobing with anti-NF-κB p65 rabbit mAb and HRP-conjugated goat anti-rabbit IgG. A representative image is shown. Data are the means  $\pm$  SD of 3 separate experiments, and expressed relative to the cells incubated without SP and ketamine. Data are compared between the SP-stimulated cells incubated with and without ketamine. \* $P < 0.05$ .

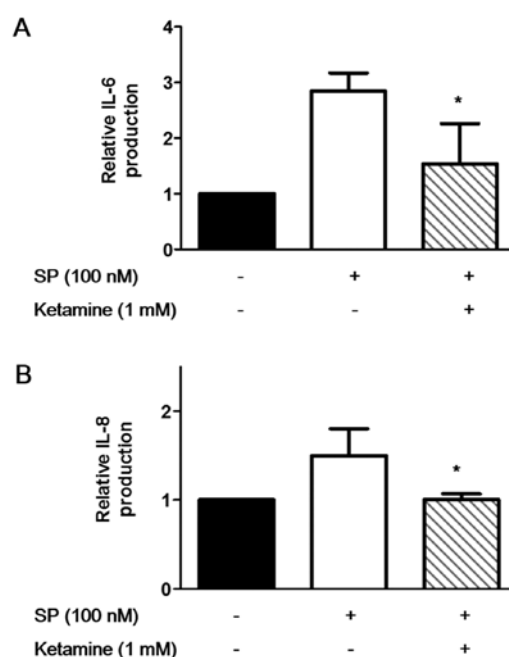


Figure 5. Inhibitory effects of ketamine on the production of interleukin (IL)-6 and IL-8 induced by substance P (SP). The U373MG cells were plated into 12-well tissue culture plates at a density of  $2 \times 10^5$  cells/well and incubated in MEM supplemented with 10% FBS for 12 h, followed by incubation in MEM supplemented with 0.5% FBS for 12 h at 37°C. Subsequently, the cells were incubated with or without ketamine (1 mM) for 60 min, and then incubated with or without SP (100 nM). After 24 h, the culture supernatants were harvested, and the levels of (A) IL-6 and (B) IL-8 were measured using a cytometric bead array. Data are the means  $\pm$  SD of 3 separate experiments, and expressed relative to the cells incubated without SP and ketamine. Data are compared between the SP-stimulated cells incubated with and without ketamine. \* $P < 0.05$ .

this cell line is widely used as an *in vitro* model in order to analyze the functions of the NK-1R. Previously, it has been demonstrated that the plasma levels of ketamine reached up to 25,800 ng/ml (108.4  $\mu$ M) in surgical patients 1 min after an intravenous injection of ketamine at a dose of 2.0-2.2 mg/kg (28). In the present study, we revealed that the phosphorylation of ERK1/2 was only minimally inhibited by 0.1 mM ketamine, whereas the dose of 1 mM ketamine caused a marked inhibition. Thus, it is possible that clinically relevant (100 mM) or higher concentrations of ketamine modulate the inflammatory responses of SP-stimulated NK-1R-expressing cells *in vivo* by suppressing the activation of signaling molecules (such as ERK1/2, p38 MAPK and NF-κB).

Neuroinflammation is involved in several diseases affecting the central nervous system (CNS) in humans, such as Parkinson's disease (PD) (29,30). As regards the pathogenesis of PD, neuroinflammation is a common feature, which is primarily induced by the long-term activation of glial cells (astrocytes and microglia) in the brain (31). It has also been demonstrated that activated glial cells produce pro-inflammatory cytokines, which play roles in the initiation and progression of neuroinflammation (29). Thus, anti-inflammatory drugs may be expected to reduce the risk and delay the pathogenic process of neuroinflammation by suppressing the activation of glial cells (32). Ketamine has been identified as a non-selective NMDA receptor antagonist. The pharmacological actions of ketamine include the

regulation of inflammatory and immune responses in neural tissues, which leads to decreases in the levels of proinflammatory cytokines including IL-6 and IL-8. Previously, ketamine has been reported to exert an anti-inflammatory effect on LPS-stimulated macrophages *in vitro* and *in vivo* by suppressing the MAPK pathways (33,34). The glial cells of the CNS are involved in regulating the immune response under conditions of neuropathic pain (35) and ketamine has been shown to reduce the LPS-induced production of TNF- $\alpha$  and prostaglandin E2 by astrocytes (36). Moreover, ketamine exerts an inhibitory effect on the activation of LPS-stimulated astrocytes by suppressing NF- $\kappa$ B activation through the reduction of Toll-like receptor 4 expression (26). As regards the effect of ketamine on the NK-1R, Okamoto *et al* demonstrated that ketamine inhibited NK-1R-mediated signaling by interfering with the binding of SP using NK-1R-expressing *Xenopus* oocytes using a whole-cell voltage clamp; however, the binding site of ketamine in the NK-1R is probably different from that of SP (24). Thus, the precise mechanism through which ketamine modulates SP-induced signaling remains unknown.

SP stimulates a number of intracellular signaling molecules, including members of the MAPK family (ERK1/2 and p38 MAPK) via the action of NK-1R. In fact, SP has been demonstrated to enhance the production of inflammatory chemokines by murine macrophages through ERK/p38 MAPK-mediated NF- $\kappa$ B activation (37). Moreover, it has been previously demonstrated that SP induces the expression of IL-6 through the activation of p38 MAPK, ERK1/2 and NF- $\kappa$ B in the U373MG astrocytoma cell line, which was also used in the present study (14,38). Taken together, these observations suggest that SP induces the production of cytokines/chemokines by NK-1R-expressing cells through the NK-1R, a principal receptor for SP, followed by the activation of signaling molecules (ERK1/2, p38 MAPK and NF- $\kappa$ B). Of note, ketamine may inhibit NK-1R-mediated signaling by interfering with the binding of SP to the receptor (24). Thus, ketamine may suppress the SP-induced activation of signaling molecules (ERK1/2, p38 MAPK and NF- $\kappa$ B), thereby suppressing the production of cytokines/chemokines by exerting an inhibitory effect on the NK-1R (interference of SP binding with the receptor).

In conclusion, our results demonstrate that ketamine inhibits the SP-induced phosphorylation of MAPK (ERK1/2 and p38 MAPK) and NF- $\kappa$ B, and suppresses the production of the proinflammatory cytokines IL-6 and IL-8. Moreover, the anti-inflammatory effect of ketamine is potentially mediated through the inhibition of signaling molecules (MAPK and NF- $\kappa$ B). Thus, ketamine may modulate SP-induced inflammatory responses by NK-1R-expressing cells through the suppression of signaling molecules (such as ERK1/2, p38 MAPK and NF- $\kappa$ B); however, a detailed examination of the anti-inflammatory effects of ketamine on glial cells (such as astrocytes and microglia cells) is warranted in the future.

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