Anti-inflammatory actions of gabapentin and pregabalin on the substance P-induced mitogen-activated protein kinase activation in U373 MG human glioblastoma astrocytoma cells

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Received February 16, 2017; Accepted August 3, 2017

DOI: 10.3892/mmr.2017.7368

Abstract. Gabapentin (GBP) and pregabalin (PGB) exert antinociceptive effects on chronic nociceptive responses with neuropathic or inflammatory conditions. Furthermore, it is considered that GBP and PGB exhibit anti-inflammatory effects by modulating the substance P (SP)-mediated neurokinin-1 receptor (NK1R; a SP receptor) response. Thus, in the present study, the effects of GBP and PGB on SP-induced activation were investigated in the human glioblastoma astrocytoma U373 MG cell line, which expresses high levels of functional high-affinity NK1R, and produces interleukin (IL)-6 and IL-8 in response to SP. The results indicated that GBP and PGB suppressed the SP-induced production of IL-6, and IL-8 in U373 MG cells. Furthermore, GBP and PGB inhibited the SP-induced phosphorylation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB, and the nuclear translocation of NF-κB in U373 MG cells. Together, these observations suggest that GBP and PGB likely prevent SP-induced IL-6 and IL-8 production in U373 MG cells via the inhibition of signaling molecules, including p38 MAPK and NF-κB, thereby exhibiting antineuroinflammatory effects.

Introduction

A neuropeptide substance P (SP) is an important mediator of neurogenic inflammation in the central and peripheral nervous systems. SP is implicated in pain, and also plays an important role in tumor cell proliferation, anti-apoptotic effects on tumor cells, angiogenesis, tumor cell invasion and metastasis (1-3). SP is released from primary afferent nociceptors and sympathetic postganglionic neurons, and activates neighboring receptors, thereby triggering spreading activation (4,5). Moreover, SP has been shown to induce the expression of proinflammatory cytokines and chemokines, such as interleukin (IL)-6 and IL-8 (6,7), which are involved in the pathogenesis of several human brain disorders (such as multiple sclerosis, dementia complex, and Alzheimer's disease) (8), although it is currently a matter of debate whether SP really plays a pathogenic role in these disorder. Previous report has shown that the activation of a SP receptor [neurokinin-1 receptor (NK1R)] elicits signals affecting inflammatory cytokine gene expression (7). In addition, it has been reported that SP potently triggers the activation of nuclear factor (NF)-κB, an important transcriptional activator, which regulates the production of various cytokines and other proinflammatory mediators (7).

Mitogen-activated protein kinases (MAPK), a family of protein Ser/Thr kinases, consist of at least three major subfamilies: i) the p42/44 MAPKs, which are also called extracellular signal regulated kinases (ERK-1 and ERK-2); ii) the c-Jun NH2-terminal kinase/stress-activated protein kinases (JNK/SAPK) including p46 JNK1 and p54 JNK2; and iii) the p38 MAPK subfamily. MAPKs are activated under stress conditions in response to a variety of extracellular stimuli, including oxidative stress. Among these MAPKs, phosphorylation of p38 MAPK is induced in dorsal horn of the spinal cord and dorsal root ganglia following peripheral nerve injury or inflammation (9-11).

Gabapentin (GBP) and pregabalin (PGB) are structural analogues of γ-amino butyric acid (GABA) with lipophilic characteristics, and were developed as potential anticonvulsants (12). However, many studies have shown that these substances do not act as GABA agonists and indeed have little demonstrable effect on any aspect of GABA transmission (13,14). Furthermore, a recent study has clarified the current understanding of gabapentinoid pharmacology that GBP and PGB do not inhibit any conventional subtype of voltage-gated calcium channel (VGCC), but rather selectively block calcium channels that contain the α2δ-1 subunit, with pharmacodynamics and cellular-specificities, depending on the structural and biochemical states of the α2δ-1 protein (15).
It is believed that blockade of VGCC containing the α2δ-1 subunit is the predominant pharmacological mechanism of both GBP and PGB (16). GBP binds potently to the α2δ-1 subunit and modulates calcium influx at nerve terminals, thereby, reducing the release of several neurotransmitters, including glutamate, noradrenaline, serotonin and SP (17-19). Interestingly, GBP could be a therapeutic agent, when given systemically, for treatment of neuropathic or postsurgical pain (20-24), and also reduces the experimental pain in humans after sensitization of the skin with capsaicin and heat (25). Furthermore, GBP and its derivative PGB reduce nociceptive behaviors of animal models with neuropathic pain or inflammation such as nerve ligation, injection of immune antigens, herpes infection, arthritis, diabetes, postoperative pain and thermal injury (26-30). In contrast, neither GBP nor PGB alters acute nociceptive responses (31,32). Thus, it is believed that the antinociceptive action of these substances depends on the chronic nociceptive responses with neuropathic or inflammatory conditions.

It has been demonstrated that the release of peptidergic neurotransmitters (including SP) from sensory neurons is increased during inflammation or in neuropathic pain models (33). In addition, SP is axonally transported to peripheral nerve endings, where it is released in response to traumatic stimuli and induces various biological effects (33). Importantly, GBP and PGB modulate the release of SP under the conditions corresponding to significant inflammation-induced sensitization of the spinal cord, which involves the action of SP on NK1 (SP) receptor (NK1R) (34). Based on the findings, we hypothesized that GBP and PGB may exert antinflammatory action by modulating the SP-mediated NK1R response. In this study, therefore, we investigated the antinflammatory effects of GBP and PGB on SP-induced activation of a human glioblastoma astrocytoma cell line U373 MG cell, which expresses the high levels of NK1R.

Materials and methods

Materials. U373 MG cell line (Uppsala; ECACC08061901) was purchased from European Collection of Cell Cultures (ECACC; Salisbury, UK). SP was obtained from Sigma-Aldrich Co., LLC., (St. Louis, MO, USA); GBP from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); PGB from Tokyo Research Chemicals (Tokyo, ON, Canada); Minimum essential medium (MEM), non-essential amino acids (NEAA), sodium pyruvate and fetal bovine serum (FBS) from Gibco BRL Life Technologies (Grand Island, NY, USA). Phosphate-buffered saline (PBS), RIPA buffer containing protease inhibitor cocktail, sample buffer solution containing reducing reagent (6X) for SDS-PAGE, running buffer solution (10X) for SDS-PAGE, Blocking One, WB Stripping Solution Strong, and Protein Ladder One Multi-color (Broad Range) for SDS-PAGE from Nacalai Tesque, Inc., (Kyoto, Japan). BCA protein assay reagent kit One Multi-color (Broad Range) for SDS-PAGE from Nacalai, WB Stripping Solution Strong, and Protein Ladder running buffer solution (10X) for SDS-PAGE, Blocking One, containing reducing reagent (6X) for SDS-PAGE, containing protease inhibitor cocktail, sample buffer solution containing reducing reagent from Cell Signaling Technology, Inc. (Danvers, MA, USA), anti-phospho p38 MAPK rabbit antibody (Thr180/Tyr182; V121A) from Promega Corporation (Madison, WI, USA), anti-p38 MAPK (p38/SAPK2a) mouse mAb (no. 612168) from BD Biosciences (San Jose, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (AP132P), HRP-conjugated goat anti-mouse IgG/IgM (AP308P) from Chemicon International (Temecula, CA, USA).

Preparation of whole cell lysate and western blot analysis. U373 MG cells were plated into 12-well tissue culture plates at a density of 1x10⁴ cells/well and incubated in MEM with 10% FBS for 12 h, followed by incubation in MEM with 0.5% FBS for 12 h at 37˚C. Subsequently, the cells were incubated with GBP or PGB (1 mM) for 60 min, and then stimulated with SP (100 nM) for 10 or 15 min. Thereafter, the cells were washed three times with ice-cold 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, 0.1% SDS and Protease Inhibitor Cocktail). Protein concentrations of cell lysates were measured with BCA protein assay reagent (Thermo Scientific). The lysates were mixed with SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.05% bromphenol blue, and 5% 2-mercaptoethanol), and applied to SDS-PAGE in 10% gels (Mini-PROTEAN® TGX™ Precast Gel; 10-20 μg protein/lane). Thereafter, separated proteins were electroblotted onto polyvinylidene fluoride membranes (Trans-Blot® Turbo™ Mini PVDF Transfer Packs). After incubation with Blocking One (Nacalai Tesque, Inc.), blots were probed with a 1,000-fold dilution of rabbit anti-phospho p38 MAPK antibody, anti-phospho ERK1/2 antibody or anti-phospho NF-κB antibody, and further probed with a 10,000-fold dilution of HRP-conjugated goat anti-rabbit IgG. Signals were detected with SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.), and quantified using LAS-3000 luminescence image analyzer (Fujifilm, Tokyo, Japan) and MultiGauge software (Fujifilm). Thereafter, the antibody was stripped using WB Stripping Solution Strong (Nacalai Tesque, Inc.) at room temperature for 15 min. Blots were probed with a 1,000-fold dilution of mouse anti-p38 MAPK antibody, rabbit anti-ERK1/2 antibody or anti-NF-κB antibody, and further probed with a 10,000-fold dilution of HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG/IgM. Signals were detected and analyzed, as described above.

Preparation of nuclear extract and western blot analysis. U373 MG cells were plated into 12-well tissue culture plates at a density of 1x10⁴ cells/well and incubated in MEM with 10% FBS for 12 h, followed by incubation in MEM with 0.5% FBS for 12 h at 37˚C. The cells were maintained at 37˚C in a 5% CO₂ humidified atmosphere.

Antibodies. Anti-phospho-ERK1/2 MAPK (Thr202/Tyr204) rabbit antibody (no. 9101), anti-ERK1/2 MAPK rabbit antibody (no. 9102), anti-phospho-NF-κB p65 (Ser536) rabbit monoclonal antibody (mAb; no. 3033), anti-NF-κB p65 rabbit mAb (no. 8242), and histone H3 (D1H2) XP rabbit mAb (no. 4499) from Cell Signaling Technology, Inc. (Danvers, MA, USA), anti-phospho p38 MAPK rabbit antibody (Thr180/Tyr182; V121A) from Promega Corporation (Madison, WI, USA), anti-p38 MAPK (p38/SAPK2a) mouse mAb (no. 612168) from BD Biosciences (San Jose, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (AP132P), HRP-conjugated goat anti-mouse IgG/IgM (AP308P) from Chemicon International (Temecula, CA, USA).

Cell culture. U373 MG cells were cultured in MEM supplemented with 1% (v/v) penicillin/streptomycin, and NEAA, 1 mM sodium pyruvate and 10% heat-inactivated FBS. The cells were maintained at 37˚C in a 5% CO₂ humidified atmosphere.
a density of 2x10^5 cells/well and incubated in MEM with 10% FBS for 12 h, followed by incubation in MEM with 0.5% FBS for 12 h at 37˚C. Subsequently, the cells were incubated with or without GBP (1 mM) or PGB (1 mM) for 60 min, and then incubated with or without SP (100 nM) for 10 min. (A) 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 amount of proteins were analyzed in each samples, the blots were stripped, and total p38 MAPK were detected by reprobing with anti-p38 MAPK antibody and HRP-conjugated goat anti-mouse IgG/ IgM. (B) The phosphorylation of ERK1/2 were detected by probing with anti-phospho-ERK1/2 (Thr202/Tyr204) antibody and HRP-conjugated goat anti-rabbit IgG. Total ERK1/2 were detected by reprobing with anti-ERK1/2 antibody and HRP-conjugated goat anti-rabbit IgG. A representative image is shown. Data are the mean ± standard deviation of 3 separate experiments, and expressed as relative to the cells incubated without SP, GBP, and PGB. Data are compared between the SP-stimulated cells incubated without and with GBP or PGB. *P<0.05.

SP (100 nM) for 15 min. Thereafter, the cells were washed three times with ice-cold PBS. Cells were detached by trypsin treatment, and washed in ice-cold PBS containing phosphatase inhibitors, and then centrifuged at 300 g for 5 min. Nuclear fractions were prepared using NE-PER nuclear and cytoplasmic extraction reagents by suspending the cell pellet in a hypotonic buffer, and centrifugation at 14,000 x g for 30 min. After collection of the supernatant (cytoplasmic fraction), the pellet (nuclear fraction) was lysed and solubilized in lysis buffer containing proteasome inhibitors. Protein concentrations were determined using with BCA protein assay reagent. The nuclear extract was mixed with SDS-PAGE sample buffer), and applied to SDS-PAGE in 10% gels, followed by western blot analysis using a 1,000-fold dilution of rabbit anti-NF-kB antibody and a 10,000-fold dilution of HRP-conjugated goat anti-rabbit IgG, and a 1,000-fold dilution of rabbit histone H3 and a 10,000-fold dilution of HRP-conjugated goat anti-rabbit IgG, as described in the above section.

Quantification of IL-6 and IL-8. U373 MG cells were plated into 12-well tissue culture plates at a density of 1x10^5 cells/well and incubated in MEM with 10% FBS for 12 h, followed by incubation in MEM with 0.5% FBS for 12 h at 37˚C. Then, the cells were incubated with GBP or PGB (1 mM) for 60 min, and stimulated with SP (100 nM) for 24 h. Culture media were recovered, centrifuged for 10 min at 12,000 x g, and the levels of IL-6 in the supernatants were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Moreover, the levels of IL-8 in the media were measured by ELISA kit, according to the manufacturer's instructions (R&D systems, Inc., Minneapolis, MN, USA).

Statistical analysis. Data are expressed as mean ± standard deviation, and analyzed for significant difference by a one-way ANOVA with multiple comparison test using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Suppression of SP-induced activation of p38 MAPK and ERK1/2 by GBP and PGB. First, we evaluated the effect of GBP and PGB on the phosphorylation of p38 MAPK. As shown in Fig. 1A, SP stimulation (100 nM) clearly induced the phosphorylation of p38 MAPK in U373 MG cells. Interestingly, GBP (1 mM) and PGB (1 mM) substantially suppressed the SP-induced phosphorylation of p38 MAPK, although only the suppression by PGB was statistically significant (P<0.05).

Next, we evaluated the effect of GBP and PGB on the phosphorylation of ERK1/2. As shown in Fig. 1B, SP stimulation (100 nM) markedly induced the phosphorylation of ERK1/2 in U373 MG cells; however, GBP (1 mM) and PGB (1 mM) did not essentially suppress the SP-induced phosphorylation of ERK1/2.

Suppression of SP-induced activation of NF-kB and nuclear translocation of p65 by GBP and PGB. Furthermore, we
evaluated the effect of GBP and PGB on the phosphorylation of NF-κB. SP stimulation (100 nM) substantially induced the phosphorylation of NF-κB in U373 MG cells. Importantly, GBP and PGB significantly abolished the SP-induced phosphorylation of NF-κB (Fig. 2A; P<0.05).

To further characterize the effect of GBP and PGB on the SP-induced activation of NF-κB, we evaluated the nuclear translocation of NF-κB. Thus, U373 cells were pretreated with GBP (1 mM) and PGB (1 mM) followed by stimulation with SP (100 nM), and the nuclear levels of p65 were analyzed. Nuclear p65 level was increased after SP stimulation compared with unstimulated cells; however, SP-induced p65 level was significantly reduced by GBP and PGB (Fig. 2B; P<0.05).

**Suppression of SP-induced IL-6 and IL-8 production by GBP and PGB.** Finally, we evaluated the effect of GBP and PGB on the IL-6 and IL-8 production by U373 MG cells. As shown in Fig. 3, both IL-6 and IL-8 were produced by resting U373 MG cells without SP. Importantly, the production of IL-6 and IL-8 was substantially increased by SP stimulation (100 nM). Noticeably, GBP and PGB (1 mM) significantly suppressed both the IL-6 and IL-8 production (P<0.05).

**Discussion**

To our knowledge, this is the first study to demonstrate the effects of GBP and PGB on the SP-induced inflammatory responses in glioblastoma astrocytoma U373 MG cells. U373 MG glioblastoma astrocytoma cells express a functional high-affinity NK1R (a SP receptor) (35) and are able to produce IL-6 and IL-8 in response to SP (6). In this study, we revealed that GBP and PGB suppressed the SP-induced production of IL-6 and IL-8 in U373 MG cells. Furthermore, GBP and PGB inhibited the SP-induced phosphorylation of p38 MAPK and NF-κB, and nuclear translocation of NF-κB in U373 MG cells. Thus, GBP and PGB likely prevent the SP-induced IL-6 and IL-8 production in U373 MG cells by inhibiting signaling molecules including p38 MAPK and NF-κB, thereby exhibiting anti-inflammatory action.

According to pharmacokinetic study (36,37), the maximum plasma concentration of GBP after administration of a single 400 mg GBP tablet to 12 volunteers was 3.33±1.19 µg/ml (approximately 20 µM). Moreover, it has been shown that 25 µM GBP and PGB reduced the SP-induced NF-κB activation in glioblastoma cell line in vitro (38). In our preliminary experiments, we observed that 100 µM GBP weakly but 1 mM GBP clearly suppressed the SP-induced phosphorylation of p38 MAPK and NF-κB, and production of IL-6 and IL-8. Thus, we examined the effect of GBP and PGB at a concentration of 1 mM on the SP-induced phosphorylation of signaling molecules, and production of cytokines.

The concentration of GBP used in this study (1 mM) was 50-fold higher than that of the maximum plasma concentration; however, we observed that GBP at 100 µM weakly suppressed the SP-induced phosphorylation of p38 MAPK and NF-κB, and production of IL-6 and IL-8 (data not shown). Thus, we speculate that GBP could exert the anti-inflammatory action in vivo, based on our findings.

GBP and PGB are anticonvulsants originally developed as spasmylocytic agents for the management of generalized or partial epileptic seizures resistant to conventional therapies (12). However, subsequent single center and multicenter, randomized

![Figure 2](image-url)
double-blind trials demonstrated that GBP is also effective for the management of pain of inflammatory and neuropathic origin, such as post herpetic neuralgia and painful diabetic neuropathy (39,40). Although PGB has not been extensively investigated as GBP, recent double-blind trials showed that PGB is also effective in the management of postoperative pain and diabetic neuropathy (39). In animal models of nociception, GBP reduces the mechanical or thermal hypersensitivity associated with nerve injury (31,41), incisional injury (26), inflammatory injury (27,41,42), and formalin-induced injury (42-44). PGB similarly reduces the mechanical or thermal hypersensitivity associated with injuries, described above (27,42,45). In contrast, both compounds do not exhibit substantive effect on acute pain in uninjured animal models.

An interesting action of SP is the induction and modulation of proinflammatory cytokine secretion by glial cells (7). The mechanism of SP-induced signal transduction is incompletely understood. However, it has been suggested that the activation of NF-κB is involved in SP-induced cytokine expression (7). Importantly, it is demonstrated that GBP and PGB decrease SP-induced NF-κB activation in human neuroblastoma and rat glioma cells, and that these drugs also inhibit NF-κB activation in rat spinal dorsal root ganglia cells pre-treated with SP (38). Interestingly, it has been reported that GBP and PGB attenuate the release of neuropeptides (such as SP) from inflamed spinal cord (34). Importantly, the present study demonstrated that GBP and PGB prevent the SP-induced IL-6 and IL-8 production in U373 MG cells by inhibiting p38 MAPK and NF-κB activation. Based on these findings, it could be speculated that gabapentinoids may exhibit anti-inflammatory action by suppressing not only the SP-induced cytokine production via the inhibition of p38 MAPK and NF-κB activation, but also the secretion of inflammatory neuropeptides (such as SP) at the inflamed neural tissue. In fact, it has been demonstrated that PGB binds potently to the α2β-1 subunit of calcium channels and modulates calcium influx at nerve terminals, thereby reducing the release of several neurotransmitters, including SP (46). However, it has been also reported that PGB and GBP suppress the degradation of IκB, thereby inhibiting nuclear localization of NF-κB (p65) in SH-SY5Y glioblastoma cells (45). Thus, gabapentinoid-mediated modulation of nuclear localization of NF-κB is also considered as a molecular mechanism for the actions of GBP and PGB.

The α2β-1 subunit of VGCC is involved in propagation of excitatory signals mediated by glutamate, calcitonin gene-related protein (CGRP), and SP (34). Its upregulation under pathological conditions is associated with hyperexcitatory states such as seizures and neuropathic pain. The causal link between α2β-1 subunit expression and hyperalgesia has been demonstrated in several experimental animal models of spinal nerve injury (47,48). Notably, PGB binds potently to the α2β-1 subunit and suppresses calcium influx at nerve terminals, thereby modulating hyperexcitatory states of neuronal synapse.

SP stimulates a number of intracellular signaling molecules including the members of MAPK family (ERK1/2 and p38 MAPK) via the action of NK1R. Moreover, SP induce the IL-6 and IL-8 production via the activation of p38 MAPK, ERK1/2 and NF-κB by U373 MG (49). In this study, GBP and PGB did not reduce the SP-induced ERK1/2 phosphorylation (Fig. 1B) but reduced p38 MAPK phosphorylation in U373 MG cells (Fig. 1A). Thus, our results suggest that GBP and PGB reduce the SP-induced production of IL-6 and IL-8 by the NK1R (a principal receptor for SP)-expressing U373 MG cells via the inhibition of signaling molecules p38 MAPK but not ERK1/2. It is possible that GBP and PGB may reduce the expression of NK1R, thereby suppressing the SP-induced production of IL-6 and IL-8. However, GBP and PGB did not reduce the SP-induced ERK1/2 phosphorylation, as mentioned above. Thus, GBP and PGB unlikely modulate the expression of NK1R.

In a neuropathic pain model, it is well documented that microglial activation, accompanying with p38 MAPK phosphorylation in the spinal cord, plays an important role in the development of neuropathic pain (50-52). Both peripheral inflammation and nerve injury induce p38 MAPK activation in spinal microglia, and p38 MAPK inhibitor SB203580 suppresses the inflammation-induced thermal hyperalgesia and spinal nerve ligation-induced mechanical allodynia (53). Moreover, intrathecal injection of minocycline, which inhibits spinal microglia activation by blocking p38 MAPK, exerts antinociceptive effect in both inflammation and neuropathic
pain models (52). These observations indicate that p38 MAPK activation plays important role in development of neuroinflammation. In addition, IL-6 is speculated to be involved in the development of neuropathic pain, because IL-6 mRNA was significantly elevated in both the dorsal and ventral horns in a neuropathic pain model of spinal nerve cryoneurolysis and spinal nerve tight ligation (54,55). IL-8 also contributes to the pathophysiology of inflammatory pain, because the expression of IL-8 was critically upregulated in the ipsilateral spinal cord dorsal horn after chronic constriction injury (56,57). In this study, GBP and PGB reduced IL-6 and IL-8 production possibly by suppressing p38 MAPK in U373 MG cell, and the results explain the pharmacological action of GBP and PGB on neuroinflammation (neuropathic pain).

In conclusion, we demonstrated that GBP and PGB suppressed the production of IL-6 and IL-8 in U373 MG cells. Furthermore, GBP and PGB inhibited the SP-induced activation of p38 MAPK, and NF-kB in U373 MG cells. Thus, GBP and PGB likely prevent the SP-induced IL-6 and IL-8 production by U373 MG cells via the inhibition of signaling molecules including p38 MAPK and NF-kB, thereby exhibiting anti-neuroinflammatory action.

Acknowledgements

This study was supported by Grant-in-Aid for Scientific Research (C) (15K10564).

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