Induction of brain-derived neurotrophic factor in enteric glial cells stimulated by interleukin-1β via a c-Jun N-terminal kinase pathway

Masanobu Fukumoto,* Toshihisa Takeuchi, Eiko Koubayashi, Satoshi Harada, Kazuhiro Ota, Yuichi Kojima and Kazuhide Higuchi

Internal Medicine (II), Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan

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Brain-derived neurotrophic factor (BDNF) is produced by enteric glial cells, enteric nervous system, visceral hypersensitivity, mitogen-activated protein kinase

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Irritable bowel syndrome (IBS), a common chronic functional disorder of the gastrointestinal tract, is characterized by abdominal pain and altered bowel habits and often results in reduced quality of life. Although the pathogenesis of IBS has not been fully elucidated, several mechanisms have been implicated: enhanced signaling perception in the brain, sensitization of dorsal horn neurons, and hypersensitivity of primary visceral afferent neurons of the intestinal organs. The genetic variants of IBS have been reported to be associated with these factors. For example, TPH1 polymorphism affects abdominal symptoms and QOL. Brain-derived neurotrophic factor (BDNF) is produced by neuronal cells or glial cells in the central nervous system (CNS) and plays important roles in neurotropic and neuroprotective functions. In one study, BDNF levels were elevated in dorsal root ganglion neurons during neuropathic and inflammatory processes, subsequently leading to the sensation of pain. In addition, BDNF levels are notably increased in the intestinal mucosa of patients with IBS as compared with controls, and the levels correlate with the severity and frequency of abdominal pain. Thus, BDNF is likely to be an important factor in the pathophysiology of IBS, although how it correlates with visceral pain in IBS patients has not been fully elucidated.

The enteric nervous system (ENS) is a huge neural network consisting of enteric neurons and enteric glial cells (EGCs) in the submucosal and myenteric plexuses. As well as surrounding neuron, EGCs play significant roles in the regulation of intestinal epithelial barrier functions, gastrointestinal motility, and neuromediator expression. Furthermore, it is known that EGCs have similar properties to astrocytes in physiological and chemical functions. A previous report shows that BDNF expression stimulated by interleukin-1β (IL-1β) is increased in the hippocampus, where astrocytes are abundantly present.

Recently, it was reported that IL-1β was increased in the hippocampus of a rodent model of stress and depression. A meta-analysis study also showed that serum IL-1β levels are elevated in patients with major depressive disorder. Various inflammatory cytokines are present in the peripheral blood mononuclear cell supernatants of IBS patients (diarrhea type), with serum IL-1β being present at the highest levels. IL-1β mRNA expression is also significantly increased in the rectal mucosa of post-infectious IBS patients. In addition, IBS patients more often complain that their abdominal symptoms are hypersensitive to stress compared with healthy controls. Considering all these findings, in the present study we aimed to determine whether BDNF is produced in EGCs after stimulation with IL-1β as a cellular mechanism in IBS pathogenesis.

Materials and Methods

CRL-2690™ (EFG/PK060399egfr) cell culture. Cells from the rat EGC cell line CRL-2690™ were purchased from ATCC (Manassas, VA). The cells were cultured in high glucose Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum in a humidified incubator (5% CO2, 37°C). Cells were seeded in 10 cm2 dishes (5.0 × 104 cells) for 4 days. The medium was replaced with a serum-free medium on day 5, then the cells were used in subsequent experiments.

Reagents. Recombinant rat IL-1β (≥97%) was purchased from R&D Systems (Minneapolis, MN), SP600125 (≥98%) and SB203580 (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO).

Real-time RT-PCR. First, EGCs were washed with phosphate-buffered saline (PBS), pH 7.4, and total RNA was extracted using
ISOGEN II (Nippon Gene, Tokyo, Japan) on ice precipitated with ethanol according to the manufacturer’s protocol. After dissolving in water, total RNA was reverse-transcribed to cDNA with a High Capacity RNA-to-cDNA kit (Life Technologies, Carlsbad, CA) in a ProFlex™ PCR System (Life Technologies). The primer and probe were used for TaqMan® Gene Expression Assays (Life Technologies) for BDNF (Rn02531967_s1) and for GADPH (Rn01775763_g1) as an endogenous control. TaqMan® Gene Expression Assays (1 μl/well), TaqMan® Fast Advanced Master Mix (10 μl/well) (Life Technologies), and cDNA (1 μl/well) were diluted with RNA-free water (7 μl/well), then the BDNF mRNA level was determined using a StepOnePlus™ Real-Time PCR System (Life Technologies) and 96-well optical plates. Each sample was run in duplicate and PCR was performed under the following reaction conditions: 1) 37 °C for 60 min, 2) 95 °C for 5 min, 3) maintained at 4 °C.

Western blotting. EGCs were washed with PBS and lysed in buffer containing 50 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), and 1% NP-40 with protease inhibitor (Complete mini, Roche Applied Science, Indianapolis, IN). To study the phosphorylation of mitogen-activated protein kinase (MAPK), we added a phosphatase inhibitor (PhosSTOP, Roche Applied Science). Protein concentration was quantified using a BCA Protein Assay kit (Life Technologies). Cell lystate was denatured with NuPAGE™ LDS Sample buffer (Life Technologies) and NuPAGE™ Sample reducing Agent (Life Technologies) for BDNF (Rn02531967_s1) and for GADPH (Rn01775763_g1) according to the manufacturer’s protocol.

The primary antibodies used were as follows: BDNF (#ab108319, Abcam plc, Cambridge, UK; 1:1,000), total extracellular signal-regulated kinase (ERK) (#4695, Cell Signaling Technology, Danvers, MA; 1:1,000), phosphorylated-ERK (#4370, Cell Signaling Technology; 1:1,000), total c-Jun N-terminal kinase (JNK) (#29252, Cell Signaling Technology; 1:1,000), phosphorylated-JNK (#4668, Cell Signaling Technology; 1:1,000), total p38 mitogen-activated protein kinase (MAPK) (#8690, Cell Signaling Technology; 1:1,000), phosphorylated p38 MAPK (#4511, Cell Signaling Technology; 1:1,000), β-actin (#A1978, Sigma-Aldrich, 1:1,000). The secondary antibodies were as follows: anti-rabbit IgG-horseradish peroxidase (HRP) (#ab205718, Abcam plc; 1:1,000), anti-mouse IgG-HRP (#sc-2005, Santa Cruz, Dallas, TX; 1:10,000). Immunoreactive signals were quantified using a Fusion imaging system (Vilber Lourmat, Marne-la-Vallée, France).

ELISA. Concentrations of total BDNF and proBDNF in EGC culture supernatants were determined using a BDNF ELISA kit (#ERBDNF, Life Technologies) and proBDNF ELISA kit (#BEK-2237, Biosensis, Thebarton, South Australia) according to the manufacturer’s protocol. Before the assay, supernatants were centrifuged at 10,000 × g for 5 min to remove debris.

Total BDNF. First, we added 100 μl supernatant samples and standards (0, 12.5, 30.7, 76.8, 192, 480, 1,200, and 3,000 pg/ml) to pre-coated microplate wells and incubated for 120 min at room temperature. After washing the plate 5 times, we added 100 μl antibody reagent and incubated for 30 min at room temperature. Then, 100 μl streptavidin-HRP conjugate was added to each well and incubated for 30 min at room temperature. Next, we added 100 μl TMB substrate solution. The absorbance was read at 450 nm on a plate reader and the concentration of proBDNF was calculated from a standard curve and expressed as pg/ml. The detection limit of this proBDNF assay kit was approximately 6 pg/ml.

Statistical analysis. Data are expressed as means ± SE. We statistically analyzed BDNF mRNA, BDNF protein, p-ERK1/2, p-JNK, and p-p38 MAPK protein expression among multiple groups using analysis of variance with Bonferroni correction. For analysis of differences in BDNF protein concentration between two groups, we used a t test. The significance level was set at p <0.05. All statistical analyses were carried out using JMP® pro ver. 13.0 (SAS Institute, Cary, NC).
Results

**BDNF expression in EGCs stimulated with IL-1β.** First, we confirmed that BDNF mRNA expression was significantly increased by IL-1β (ranges: 12.5–75 ng/ml) at 24 h (Fig. 1). In the time course study (from 6 h to 48 h), BDNF mRNA expression was significantly increased compared to the controls, with a 2.8-fold increase at 24 h, and a 4.1-fold increase at 48 h after IL-1β (50 ng/ml) stimulation (Fig. 2).

The protein expressions of mature BDNF (13.5 kDa) and proBDNF (a precursor of mature BDNF) (35 kDa) were increased for each concentration of IL-1β (3.125–75 ng/ml) (Fig. 3A and B). In addition, IL-1β (50 ng/ml) significantly increased both mature BDNF and proBDNF protein expression at 48 h with a 1.7-fold and a 2.7-fold increase, respectively, compared to the controls (Fig. 4A and B). Mature BDNF and proBDNF protein expression was not increased by 50 ng/ml IL-1β stimulation at 6 h or 24 h (Fig. 4A and B).

**BDNF protein contents in culture medium of EGCs stimulated with IL-1β.** While harvesting EGCs, we collected the culture medium and measured its BDNF contents after stimulation with IL-1β for 48 h using a BDNF ELISA kit, which can measure both mature BDNF and proBDNF levels. There was no significant difference in the total BDNF contents between controls (99.9 ± 1.02 pg/ml) and the IL-1β-stimulated group (99.6 ± 0.77 pg/ml) (Fig. 5A).

However, proBDNF protein levels were significantly lower in the IL-1β-stimulated group than in controls, according to measurement with another ELISA kit that specifically detects proBDNF protein (19.5 ± 4.60 pg/ml, in control group; 3.72 ± 3.25 pg/ml in IL-1β-stimulated group; p = 0.03) (Fig. 5B). Mature BDNF was more predominant in the culture medium in the IL-1β-stimulated group (about 96%) compared to controls (about 80%).

**Activation of MAPK in EGCs after stimulation with IL-1β.** For signal transduction analyses in EGCs after stimulation with IL-1β, we evaluated MAPK pathways. We found that IL-1β (50 ng/ml) did not activate ERK1/2 (44 kDa and 42 kDa) at 5, 15,
or 60 min (Fig. 6A). In contrast, phosphorylated-JNK was increased 4.8-fold (54 kDa) and 5.2-fold (46 kDa) 5 min after IL-1β stimulation compared to controls. At 15 min, it was increased 9.5-fold (both 54 kDa and 46 kDa), and then it gradually decreased to control levels over the next 60 min (Fig. 6B). Additionally, phosphorylated p38 MAPK was observed to be increased about 2-fold 5 and 15 min after IL-1β stimulation (Fig. 6C).

**Effects of inhibitors of respective MAPK pathways on IL-1β-induced BDNF protein expression in EGCs.** Prior treatment (30 min before) with SP600125 (30 μM), a JNK inhibitor, significantly cancelled the IL-1β-induced increase in both mature BDNF and proBDNF protein expression (Fig. 7A and B). We confirmed that the inhibitors (SP600125 and SB203580) had no effects on BDNF protein expression in EGCs (data not shown).

**Discussion**

The present study shows that BDNF is produced in EGCs via a JNK pathway and that mature BDNF protein may be predominantly secreted in EGC culture medium, at least under IL-1β-stimulated conditions. BDNF acts as a TrkB receptor and increases intercellular chloride levels in the dorsal horn neurons via downregulation of the potassium-chloride transporter. This change in transmembrane anion gradient induces GABAergic excitation in dorsal horn neurons, which may result in neuropathic pain. In colonic tissues, it has been reported that BDNF is produced by epithelial cells and is induced by fecal supernatants from diarrhea-dominant IBS patients. It is also suggested that BDNF may cause GABAergic excitation in enteric neurons, leading to abdominal symptoms. However, the detailed cellular mechanisms of BDNF production in the ENS are not fully understood, although there is evidence that inflammatory cells such as lymphocytes, macrophages, and mast cells infiltrate into the colonic tissues.

In the present study, BDNF was produced in EGCs by IL-1β stimulation. The findings that signal transduction of JNK and p38-MAPK was activated in EGCs after IL-1β stimulation may support the theory that BDNF is produced in EGCs. In other words, BDNF produced by EGCs as a component of the ENS may be associated with visceral sensations including pain through the neuronal networks in the ENS. Indeed, there are some studies about BDNF as a biomarker in the peripheral blood of patients of painful conditions, including fibromyalgia, knee osteoarthritis, and chronic low back pain. Therefore, BDNF produced by EGCs possibly affects the enteric neurons, which are implicated in producing symptoms of IBS such as abdominal pain and discomfort.

We also showed that the main inducer of BDNF production in EGCs may be a JNK pathway, and this pathway was particularly associated with BDNF production (or release). The function of mature BDNF is known to be neurite outgrowth, because it is reported that mediators isolated from IBS samples induced enteric neurite elongation in rat enteric neurons and human SH-SYSY cells. The results of the present report suggest that BDNF is produced in EGCs via a JNK pathway; however, it is also reported that the p38 MAPK pathway is important for BDNF expression in the rat hippocampus or microglia. Further examination of the association between intracellular signals other than MAPK and BDNF production and its processing mechanisms is needed. BDNF also plays an important role in the regulation of synaptic plasticity in parts of the CNS, such as the hippocampus and spinal cords.

Therefore, we consider that morphological studies using a co-culture system of EGCs and neurons or studies on the effects of neuronal features after stimulation of EGC culture medium will be important in the future to elucidate the pathology of IBS. To fully elucidate the pathogenesis of IBS, especially microinflammatory IBS, it will be necessary to evaluate the influence of other inflammatory cytokines such as tumor necrosis factor, IL-8, and IL-6 as well as IL-1β in future studies.

In summary, BDNF was induced by IL-1β in EGCs via JNK signaling, which may affect enteric neurons under stress. These findings may help to reveal the mechanism underlying the pathophysiology of IBS.

**Author Contributions**

MF performed all experiment and analysed the data and wrote the manuscript. EK provided technical support. TT, SH, KO, and YK revised the manuscript critically for important intellectual content. KH supervised this study and finally approved the manuscript.

**Abbreviations**

BDNF: brain-derived neurotrophic factor
CNS: central nervous system
EGC: enteric glial cell
ELISA: enzyme-linked immunosorbent assay
ENS: enteric nervous system
Fig. 6. Phosphorylated MAPK expression in EGCs in a time-course analysis after stimulation with IL-1β. EGCs were stimulated by IL-1β (50 ng/ml) for 5, 10, and 60 min (n = 4). BDNF protein levels were normalized to those of β-actin. (A) ERK expression was detected at 44 kDa and 42 kDa. Each value represents the mean ± SEM. (B) JNK expression was detected at 54 kDa and 46 kDa. Each value represents the mean ± SEM. *p<0.05 vs the control group, **p<0.01 vs control group.
Fig. 7. Effect of pretreatment with SP600125 or SB203580 on IL-1β-induced BDNF protein expression. EGCs were cultured in the presence of SP600125 (30 µM) or SB203580 (10 µM) for 30 min before stimulation with IL-1β (50 ng/ml) for 48 h (n = 4). BDNF protein levels were normalized to those of β-actin. (A) Mature BDNF was detected at 13.5 kDa (upper panel). Each column represents the mean ± SEM. *p < 0.01 vs the control group. (B) ProBDNF was detected at 35 kDa (upper panel). Each column represents the mean ± SEM. *p < 0.01 vs the control group.

ERK extracellular signal-regulated kinase
HRP horseradish peroxidase
IBS irritable bowel syndrome
IL-1β interleukin-1β
JNK c-Jun N-terminal kinase
MAPK mitogen-activated protein kinase
PBS phosphate-buffered saline

Conflict of Interest

No potential conflicts of interest were disclosed.

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