Rehmannia glutinosa induces glial cell line-derived neurotrophic factor gene expression in astroglial cells via cPKC and ERK1/2 pathways independently

Hai Yu[1], Kentaro Oh-Hashi[1], Tatsuhide Tanaka[1], Aihua Sai[2], Makoto Inoue[3], Yoko Hirata[1], Kazutoshi Kiuchi[1 *]

[1.] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan, [2.] Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan, [3.] School of Pharmary, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464-8650, Japan

[*] Corresponding author: Kazutoshi Kiuchi, Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.

E-mail: kiuchi@biomol.gifu-u.co.jp

Abstract

Among four herbs of traditional Chinese medicines used in the therapy of dementia, *Rehmannia glutinosa* (RG) was found to induce the gene expression of glial cell line-derived neurotrophic factor (GDNF) in C6 glioblastoma cells and primary cultured astrocytes. The RG-induced GDNF mRNA up-regulation in C6 glioblastoma cells was completely attenuated by the presence of a pan-specific protein kinase C (PKC) inhibitor (Ro-31-8220) and a MAPK-ERK kinase 1 (MEK1) inhibitor (U0126). A conventional PKC inhibitor (Gö6976) also significantly decreased GDNF gene induction. On the other hand, RG treatment was found to stimulate phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1/2), which preceded GDNF mRNA induction in C6 glioblastoma cells. However, none of the PKC inhibitors significantly changed RG-stimulated ERK1/2 phosphorylation. Therefore, RG-stimulated GDNF gene expression could be independently up-regulated through cPKC and ERK 1/2 pathways in C6 glioblastoma cells.

Keywords: *Rehmannia glutinosa* (RG); glial cell line-derived neurotrophic factor (GDNF); extracellular signal-regulated kinase (ERK); protein kinase C (PKC); astrocyte

Introduction

Although there are a lot of therapeutic theories regarding dementia in the field of traditional Chinese medicines (TCMs), the molecular mechanism and efficiencies of this traditional medicine for therapy have not been fully investigated. *Angelica*, *Astragali*, *Safflower*, and *Rehmennia glutinnosa* (RG), are herbs frequently used in the therapy of dementia. Among them, Shu Di-huang (processed RG) has recently been reported to improve the function of learning and memory in monosodium glutamate treated rats showing a chronic impairment of CA1 synaptic plasticity, and its restoring might include the up-regulation of hippocampal *c-fos*, nerve growth factor (NGF), *N*-methyl-D-aspartate receptor 1 (NMDAR1) and γ-aminobutyric acid receptor [1, 2].

Glial cell line-derived neurotrophic factor (GDNF) was purified and characterized in 1993 as a growth factor promoting survival of the embryonic dopaminergic neurons of the midbrain, i.e. those neurons that degenerate in Parkinson's disease (PD) [3]. In addition to dopaminergic neurons, GDNF promotes the survival of different neuronal populations, such as motoneurons in spinal cord [4], noradrenergic neurons in locus coeruleus [5, 6], Purkinje neurons in cerebellum [7], pyramidal neurons in hippocampus [8] and cholinergic neurons in the basal nucleus of Meynert [9]. As far as hippocampal neurons are concerned, exogenous GDNF decreased the neuronal loss in the CA2 and

CA3 region after traumatic brain injury [10], and the damaged level of pyramidal neurons in the CA3 and CA4 areas after kainate-induced excitotoxicity [11]. Recently, GDNF was reported to reduce the number of apoptotic cells and the activation of microglia evoked by aggregated Aβ(1-42) in rabbit hippocampus [12]. Therefore, a TCM capable of stimulating GDNF gene expression will become a prospective agent for treatment preventing progress of Alzheimer's disease.

In this study, we screened GDNF-inducing TCMs using C6 glioblastoma cells and found that RG effectively stimulated the expression of GDNF mRNA in primary cultured astrocytes as well as C6 glioblastoma cells. Subsequently, we investigated the related mechanism of GDNF gene expression.

Materials and methods

Reagents

Angelica, *Astragali*, *Safflower* and RG were purchased from Tsumura (Tokyo, Japan). Gö6976 (a conventional protein kinase C (cPKC) inhibitor), Ro-31-8220 (an ATP-competitive pan-specific PKC inhibitor), rottlerin (a novel PKCδ (nPKCδ)

inhibitor), U0126 (a mitogen-activated protein (MAP) kinase kinase (MEK1) inhibitor), SP600125 (a c-jun N-terminal kinase (JNK) Inhibitor) and SB202190 (a p38 kinase inhibitor) were purchased from Calbiochem (San Diego, CA, USA).

Cell culture and treatment

C6 glioblastoma cells were grown in Ham's F10 medium (Invitrogen) containing 3% horse serum and 7% fetal bovine serum, in a 37°C incubator, with 5% CO_2. Astrocytes were prepared from the cortex of neonatal SD rat pups [13]. In brief, rat cerebrum cortex was digested with 0.125% trypsin for 15 min. After incubation, 0.004% DNase, and 0.03% trypsin inhibitor were added to the dissociated tissue. The resultant cell suspension was diluted with DMEM supplemented with 10% fetal bovine serum and seeded into poly-L-lysine-coated dishes. Astrocytes on the dishes were cultured to confluence in a 5% CO_2 incubator at 37°C. The loosely adherent oligodendrocytes and microglial cells from the astrocyte-monolayer sheet were removed by appropriate shaking.

Preparation of crude herb extract

RG (30 g) was extracted with 600 ml boiling distilled water under reflux for 1 h. After filtration to remove insoluble debris, the extract was dried using a Freeze Drier (Labconco, USA) and was stored at -20°C until use. During use, every 100 mg of dry herb was resuspended in 1 ml of sterilized distilled water and was sterilized with a filter of 0.22 μm.

Cell proliferation assay

Cell proliferation was determined by use of a cell proliferation (WST-1) kit (Cell Counting Kit, Wako). Briefly, C6 glioblastoma cells (1000 cells/ well) were seeded overnight in 96-well plates. After 24 h of incubation, cell viability was documented for 1, 2 and 3 days of RG treatment at different concentrations (0.1 ~ 1 $mg \cdot ml^{-1}$). At the end of the experiments, cells were incubated with 10 μl of WST-1 reagent for 2 h. The absorbance of treated samples against a blank control was measured at 450 nm using a Multiskan MS-UV microplate reader (Labsystems). At least three independent experiments were performed for each study, and representative data is presented.

Hoechst 33258 staining

C6 glioblastoma cells in a twelve-well plate were treated with 1 $mg \cdot ml^{-1}$ of RG for 3 days, or with a potent non-specific protein kinase inhibitor, staurosporine (0.1 μM for 18 h) (Sigma), as a positive control for falling into apoptosis. The cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. Then, the cells were stained with 1 mM Hoechst 33258, and nuclei with apoptotic morphology were counted by fluorescence microscopy. Each treatment was repeated three times.

Measurement of GDNF mRNA levels by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from C6 glioblastoma cells and primary-cultured astrocytes. Briefly, cells grown in 60 mm dishes were lysed in Trizol (Invitrogen) to collect total RNA. cDNA was synthesized from total RNA with random 9mers using reverse transcriptase (Invitrogen). The reaction mixture contained 2.5 mM $MgCl_2$, 0.5 mM dNTP and 1 mM dithiothreitol. The reverse transcription product was added to a PCR reaction mixture (EX Taq PCR kit, Takara) containing 100 μM dNTP mix, 0.2 μM of each primer and 1.25 U Taq DNA polymerase in buffer supplied by the enzyme manufacturer. The following primer pairs were used. GDNF: sense 5'-GGTCACCAGATAAACAAGCGG-3' and antisense

5’-GGGTCAGATACATCCACACCGTTTAGCGGAA-3’; β-actin: sense 5'-TGTATGCCTCTGGTCGTACC-3' and antisense 5'-CAACGTCACACTTCATGATGG-3'. PCR was performed in a thermocycler (Takara). The PCR conditions for GDNF and β-actin were: 96°C for 5 min for predenaturation, followed by 19 or 28 cycles of denaturation at 96°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. The PCR products were separated through a 1.5% agarose gel and were stained with ethidium bromide. Images were captured using a Gel Print 2000i/VGA (Bio Image), and the intensity ratios between the GDNF and β-actin bands were determined using Intelligent Quantifier (Bio Image) computer software.

Western blotting

The cells were collected in phosphate-buffered saline (PBS) and lysed in lysis buffer [20mM Tris-HCl (pH 8.0), 1% tritonX-100, 10% glycerol and 2mM EDTA] containing a protease inhibitor mixture [10 $\mu g \cdot ml^{-1}$ aprotinin, 100 μM phenylmethylsulfonylfluoride (PMSF), 10 $\mu g \cdot ml^{-1}$ leupeptin and 1 mM NaF]. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 40 μg of protein were boiled in sodium dodecyl sulfate (SDS) sample buffer at 100°C for 3 min, electrophoresed on 10% SDS polyacrylamide gels,

transferred to polyvinyldifluoropyrimidone (PVDF) membranes (Amersham Biosciences, Buckinghamshire, England), and identified by enhanced chemiluminescence using an antibody against phosphorylated ERK (Cell Signaling). After detection of the phospho-proteins, the antibodies were stripped from the membrane by incubation with 62.5 mM Tris–HCl (pH 6.8), 2% SDS and 100 μM 2-mercaptoethanol at 55°C for 30 min. Then, expression levels of ERK protein on the membranes were measured by re-probing with rabbit antibodies against ERK1/2 (Cell Signaling).

Data analysis

Data are presented as mean ± S.E. of the mean of (SEM) the number of each experiment. The statistical analyses were performed by Fisher's PLSD test following one-way analysis of variance using StatView software.

Results

In the case of *Astragali* and *Safflower*, C6 glioblastoma cells fell to apoptosis 1 week after treatment at the concentration of 0.1 $mg \cdot ml^{-1}$. Therefore, these two herbs were excluded from the experiment due to their cytotoxicity. Although *Angelica* did not show

any cytotoxicity at the concentration of 1 $mg \cdot ml^{-1}$, it was unable to induce expression of the GDNF gene. On the other hand, RG efficiently stimulated GDNF gene expression at the concentration of 1 $mg \cdot ml^{-1}$. In order to evaluate the cytotoxicity of RG toward C6 glioblastoma cells, their proliferation after being treated with various concentrations (0.1 – 1 $mg \cdot ml^{-1}$) of RG for 3 days was monitored by the WST-1 assay. Up to two days after treatment, 0.25 $mg \cdot ml^{-1}$ of RG increased the growth rate of the cells while 1 $mg \cdot ml^{-1}$ of RG rather reduced it. However, RG in each concentration attenuated the proliferation of C6 glioblastoma cells 3 days after treatment (Fig. 1A). A high concentration of RG (1 $mg \cdot ml^{-1}$) significantly diminished the growth rate of C6 glioblastoma cells by about a half compared with that of control. To confirm whether this reduction was due to cell death or attenuation in proliferation, morphological changes of C6 glioblastoma cells were observed for 3 days after treatment with 0.25 and 1 $mg \cdot ml^{-1}$ of RG. The morphological change of C6 glioblastoma cells did not occur in each case up to 3 days after treatment (Fig. 1B). To determine apoptotic morphology, the cells were stained with Hoechst 33258. The percentage of apoptotic cells after 3-day treatment with 1 $mg \cdot ml^{-1}$ of RG was 7 ± 2 %, whereas almost a half of the cells after 18-h treatment with 0.1 μM staurosporine showed apoptotic morphology (Fig. 1C). The decrease in cell growth rate seems to have no relation to cell death due to cytotoxicity of RG. Therefore, RG concentrations between 0.1 and 1 $mg \cdot ml^{-1}$ were used in the following experiments.

Figure 2A shows that RG increased the expression level of GDNF mRNA in a dose-dependent manner in C6 glioblastoma cells, and 1 $mg \cdot ml^{-1}$ of RG significantly increase GDNF gene expression 24 h after RG treatment. The time course of GDNF gene expression stimulated by 1 $mg \cdot ml^{-1}$ of RG in C6 glioblastoma cells was examined. The expression level reached maximum at 24 h after treatment and declined down to the control level by 48 h after treatment (Fig. 2B). RG-stimulated GDNF gene expression was confirmed using primary-cultured astrocytes from neonatal rat cortex (Fig. 2C).

Since 4α-phorbol 12-myristate 13-acetate (PMA), a potent PKC activator, was reported to induce the GDNF gene in C6 glioblastoma cells [14], inhibitors for intracellular PKC signaling pathways were used to clarify whether RG-stimulated GDNF gene expression involved the PKC pathways. We found that the expression level of GDNF mRNA in the presence of 1 $mg \cdot ml^{-1}$ of RG was significantly reduced by treatment with Gö6976 (cPKC inhibitor) and completely reduced with Ro-31-8220 (pan-specific PKC inhibitor), but not rottlerin (nPKCδ inhibitor) treatment (Fig. 3). When C6 glioblastoma cells were treated with the above inhibitors at the same concentration without RG, the expression level of GDNF mRNA was slightly reduced but the change showed no significant difference (data not shown). These data suggest that PKCs including the conventional type are probably responsible for RG-stimulated GDNF gene expression.

Since serotonin and antidepressants (amitriptyline, clomipramine, mianserin, fluoxetine and paroxetine) were reported to mediate GDNF release through the MEK/ERK pathway in C6 glioblastoma cells [15, 16], we examined whether the MEK/ERK pathway was involved in GDNF mRNA induction enhanced by RG treatment. We found that U0126 (MEK1 inhibitor) decreased RG-stimulated GDNF gene expression while SP600125 (JNK inhibitor) and SB202190 (p38 inhibitor) did not (Fig. 4). Therefore, it is likely that the ERK1/2 phosphorylation is responsible for GDNF mRNA induction.

In order to confirm RG-activation of the MEK1-ERK1/2 pathway, the time course of ERK1/2 phosphorylation by RG treatment was analyzed using Western blotting. When C6 glioblastoma cells were treated with 1 $mg \cdot ml^{-1}$ of RG, the phosphorylation of ERK1/2 peaked at 3 h, and returned to the basal level at 12 h (Fig. 5A). The phosphorylation of JNK and p38 by RG was not observed in the same conditions (data not shown). As the RG-stimulated GDNF gene expression was completely inhibited by Ro-31-8220, the relationships between the PKC pathways and MEK1-ERK1/2 activation in C6 glioblastoma cells after the RG stimulation were investigated. The inhibitors against PKC isomers, Gö6976, rottelerin and Ro-31-8220 were applied to the culture medium prior to RG treatment. Figure 5B shows that none of the PKC inhibitors significantly affected the phosphorylation level of ERK1/2 in C6 glioblastoma cells treated by 1 $mg \cdot ml^{-1}$ of RG.

These results suggested that the cPKC and ERK1/2 pathways independently activated RG-stimulated GDNF gene expression.

Discussion

In the present study, we found that RG, one of TCMs used as a therapeutic agent for brain diseases, stimulated GDNF gene expression in C6 glioblastoma cells and primary cultured astrocytes. RG also activated both the cPKC and ERK1/2 pathways preceding GDNF induction. There are reports that intracellular signalling pathways including PKC isoforms, such as PKCδ and PKCμ, are upstream of the MEK1-ERK1/2 cascade [17, 18], however, none of them were found to influence the ERK1/2 pathway in this experiment. Only the cPKC pathway seems to independently participate in RG-stimulated GDNF gene expression against the ERK1/2 pathway. Nonetheless, there might be a signalling pathway up-stream of the MEK1-ERK1/2 cascade since ERK1/2 phosphorylation began to increase 1 h after RG treatment. This response is late compared with that triggered by PMA or IL-1β in astrocytes [19, 20]. On the other hand, the ERK1/2 pathway is known to participate in cell proliferation, differentiation and cell survival [21, 22]. A high concentration of RG (1 $mg \cdot ml^{-1}$) extended the duration of

ERK1/2 activation up to 6 h, and the proliferation of C6 glioblastoma cells was slightly suppressed up to 2 days and diminished 3 days after treatment compared with that of control. Marshall reported that small changes in ligand concentration could lead to sustained versus transient ERKs activation, and in some cell types, the former invariably leads to differentiation while the latter to proliferation [21]. Therefore, sustained activation of ERK1/2 by 1 $mg \cdot ml^{-1}$ of RG might induce differentiation rather than proliferation in C6 glioblastoma cells. In the case of low concentration (0.25 $mg \cdot ml^{-1}$), RG rather facilitated the proliferation of C6 glioblastoma cells (Fig. 1) while this concentration of RG failed to induce the GDNF gene expression (Fig. 2A). As RG was found to stimulate the cell-growth and GDNF mRNA induction at different concentrations, each phenomenon probably occurs through a different signalling cascade, respectively. In the case of antidepressant, amitriptyline induced the expression of GDNF mRNA in a dose dependent manner in C6 glioblastoma cells, and the increase in GDNF gene expression continued up to 48 h. GDNF augmentation by chronic amitriptyline treatment was inhibited by U0126, however, the phosphorylation profile of ERKs was not analyzed by Western blotting [17]. Further study is required to estimate relationship between the prolonged induction of GDNF gene and the duration of ERK1/2 activation.

Many of TCMs showing detoxification effects such as anti-aging and anti-Parkinson's disease properties have been studied [23-25], but investigations to

clarify mechanisms boosting the immune system's ability and promoting the circulation of blood are few. Anti-inflammatory therapies are known to not only decrease the expression levels of interleukin-1β (IL-1β) [26] and tumor necrosis factor-α (TNF-α) [27], but also to offer some protection against the development of Alzheimer's disease [28]. RG is reported to show anti-inflammatory properties such as inhibiting secretion of IL-1β□□□ TNF-α from primary-cultured astrocytes [29]. Pro-inflammatory agents such as IL-1β, TNF-α and lipopolysaccharide (LPS), which are known to activate nuclear factor-κB (NF-κB) [30-32], are reported to elevate GDNF release about 1.7 fold in C6 glioblastoma cells [33]. We have previously shown that the putative NF-κB binding site in intron 1 of the mouse GDNF gene effectively bound protein in the nuclear fraction extracted from the mouse astroglial cell-line TGA-3 treated with IL-1β [34]. We examined the effect of a specific NF-κB inhibitor, BAY11-7082, on GDNF gene expression stimulated with 1 $mg \cdot ml^{-1}$ of RG, however, prolonged cultivation with BAY11-7082 showed cytotoxicity and we could not estimate its effect (data not shown). Further study is needed to elucidate whether the NF-κB pathway is involved in RG-stimulated GDNF gene expression.

Recently, an anti-dementia drug, FK960, was reported to stimulate GDNF production through the ERK pathway in cultured astrocytes [35]. In addition, GDNF was reported to attenuate neuronal ER stress and inhibit the activation of GADD153, the

transcription factor triggering apoptosis, and the ER specific caspase, caspase-12, triggered by aggregated Aβ(1-42) in rabbit hippocampus [12]. Therefore, for evaluating the usefulness of RG for the treatment of Alzheimer's disease, it is necessary to figure out the details of the intracellular signalling pathways involved in RG-stimulated GDNF mRNA induction in astrocytes.

Acknowledgements

This work was supported by grants from Japan Society for the Promotion of Science (JSPS).

References

[1] Cui Y, Hou SL, Yan ZH, Chang ZF. Effect of shu di-huang on the expression of c-fos and NGF in hippocampi and learning and memory of rats damaged thalamic arcuate nucleus. Zhongguo Zhong Yao Za Zhi 2003; 28: 362-365.

[2] Cui Y, Yan ZH, Hou SL, Chang ZF. Effect of shu di-huang on the transmitter and

receptor of amino acid in brain and learning and memory of dementia model. Zhongguo Zhong Yao Za Zhi 2003; 28: 862-866.

[3] Lin LF, Doherty DH, Lile JD, Bektesh S, Collins. GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 1993; 260: 1130-1132.

[4] Henderson CE, Phillips HS, Pollock RA, Davies AM, Lemeulle C Armanini M, Simmons L, Moffet B, Vandlen RA, Simpson LC. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 1994; 266: 1062-1064.

[5] Arenas E, Trupp M, Akerud P, Ibanez CF. GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron 1995; 15: 1465-1473.

[6] Quintero EM, Willis LM, Zaman V, Lee J, Boger HA, Tomac A, Hoffer BJ,Stromberg I, Granholm AC. Glial cell line-derived neurotrophic factor is essential for neuronal survival in the locus coeruleus-hippocampal noradrenergic pathway. Neuroscience 2004; 124: 137-146.

[7] Mount HT, Dean DO, Alberch J, Dreyfus CF, Black IB. Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. Proc Natl Acad Sci U S A 1995; 92: 9092-9096.

[8] Humpel C, Hoffer B, Stromberg I, Bektesh S, Collins F, Olson L. Neurons of the

hippocampal formation express glial cell line-derived neurotrophic factor messenger RNA in response to kainate-induced excitation. Neuroscience 1994; 59: 791-795.

[9] Weis C, Marksteiner J, Humpel C. Nerve growth factor and glial cell line-derived neurotrophic factor restore the cholinergic neuronal phenotype in organotypic brain slices of the basal nucleus of Meynert. Neuroscience 2001; 102: 129-138.

[10] Kim BT, Rao VL, Sailor KA, Bowen KK, Dempsey RJ. Protective effects of glial cell line-derived neurotrophic factor on hippocampal neurons after traumatic brain injury in rats. J Neurosurg 2001; 95: 674-679.

[11] Cheng H, Fu YS, Guo JW. Ability of GDNF to diminish free radical production leads to protection against kainate-induced excitotoxicity in hippocampus. Hippocampus 2004; 14: 77-86.

[12] Ghribi O, Herman MM, Pramoonjago P, Spaulding NK, Savory J. GDNF regulates the Aβ-induced endoplasmic reticulum stress response in rabbit hippocampus by inhibiting the activation of gadd 153 and the JNK and ERK kinases. Neurobiol Dis 2004; 16: 417-427.

[13] Perego C, Vanoni C, Bossi M, Massari S, Basudev H, Longhi R, Pietrini G. The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal cocultures. J Neurochem 2000; 75: 1076-1084.

[14] Matsushita N, Fujita Y, Tanaka M, Nagatsu T, Kiuchi K. Cloning and structural organization of the gene encoding the mouse glial cell line-derived neurotrophic factor, GDNF. Gene 1997; 203: 149-157.

[15] Hisaoka K, Nishida A, Takebayashi M, Koda T, Yamawaki S, Nakata Y. Serotonin increases glial cell line-derived neurotrophic factor release in rat C6 glioblastoma cells. Brain Res 2004; 1002: 167-170.

[16] Hisaoka K, Nishida A, Koda T, Miyata M, Zensho H, Morinobu S, Ohta M, Yamawaki S. Antidepressant drug treatments induce glial cell line-derived neurotrophic factor (GDNF) synthesis and release in rat C6 glioblastoma cells. J. Neurochem 2001; 79: 25-34.

[17] Ueda Y, Hirai S, Osada S, Suzuki A, Mizuno K, Ohno S. Protein kinase C δ activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. J Biol Chem 1996; 271: 23512-23519.

[18] Hausser A, Storz P, Hubner S, Braendlin I, Martinez-Moya M, Link G, Johannes FJ. Protein kinase C μ selectively activates the mitogen-activated protein kinase (MAPK) p42 pathway. FEBS Lett 2001; 492: 39-44.

[19] Abe K, Saito H. The p44/42 mitogen-activated protein kinase cascade is involved in the induction and maintenance of astrocyte stellation mediated by protein kinase C. Neurosci Res 2000; 36: 251-257.

[20] Molina-Holgado E, Ortiz S, Molina-Holgado F, Guaza C. Induction of COX-2 and PGE(2) biosynthesis by IL-1β is mediated by PKC and mitogen-activated protein kinases in murine astrocytes. Br J Pharmaco. 2000; 131: 152-159.

[21] Marshall CJ. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell. 1995; 80: 179-185

[22] Pouyssegur J, Lenormand P. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Eur J Biochem 2003; 270: 3291-3299.

[23] Hiramatsu M, Liu J, Edamatsu R, Kadowaki D, Hamada H, Ohyama H, Watanabe S, Mori A. Combined treatment of Japanese herbal medicine (TJ-960) with low dose of dipropylacetate increased the latent time for pentylenetetrazol induced convulsion and decreased brain lipid peroxidation in mice. Neuroscience 1992; 18: 71-76.

[24] Iwasaki K, Wang Q, Seki H, Satoh K, Takeda A, Arai H, Sasaki H. The effects of the traditional Chinese meicine, "Banxia Houpo Tang (Hange-Koboku To)" on the swallowing reflex in Parkinson's disease. Phytomedicine 2000; 17: 259-263.

[25] Wang Q, Iwasaki K, Suzuki T, Arai H, Ikarashi Y, Yabe T, Toriizuka K, Hanawa T, Yamada H, Sasaki H. Potentiation of brain acetylcholine neurons by Kami-Untan-to (KUT) in aged mice: Implications for a possible antidementia drug. Phytomedicine 2000; 17: 253-258.

[26] Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM. The curry

spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci. 2001; 21: 8370-8377.

[27] Dziedzic T, Wybranska I, Dembinska-Kiec A, Klimkowicz A, Slowik A, Pankiewicz J, Zdzienicka A, Szczudlik A. Dexamethasone inhibits TNF-alpha synthesis more effectively in Alzheimer's disease patients than in healthy individuals. Dement Geriatr Cogn Disord. 2003; 16: 283-286.

[28] Etminan M, Gill S, Samii A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: systematic review and meta-analysis of observational studies. BMJ 2003; 327: 128-133. Review.

[29] Kim HM, An CS, Jung KY, Choo YK, Park JK, Nam SY. Rehmannia glutinosa inhibits tumour necrosis factor-α and interleukin-1 secretion from mouse astrocytes. Pharmacol Res 1999; 40: 171-176.

[30] Moynagh PN, Williams DC, O'Neill LA. Interleukin-1 activates transcription factor NF kappa B in glial cells. Biochem J 1993; 294: 343-347.

[31] Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 1994; 269: 4705-4708.

[32] Diehl JA, Tong W, Sun G, Hannink M. Tumor necrosis factor-α-dependent activation of a RelA homodimer in astrocytes. Increased phosphorylation of RelA and MAD-3 precede activation of RelA. J Biol Chem 1995; 270: 2703-2707.

[33] Verity AN, Wyatt TL, Hajos B, Eglen RM, Baecker PA, Johnson RM. Regulation of glial cell line-derived neurotrophic factor release from rat C6 glioblastoma cells. J Neurochem 1998; 70: 531-539.

[34] Tanaka M, Ito S, Kiuchi K. Novel alternative promoters of mouse glial cell line-derived neurotrophic factorgene. Biochim Biophys Acta 2000; 1494: 63-74.

[35] Koyama Y, Egawa H, Osakada M, Baba A, Matsuda T. Increase by FK960, a novel cognitive enhancer, in glial cell line-derived neurotrophic factor production in cultured rat astrocytes. Biochem Pharmacol 2004; 68: 275-282.

Legends of figures

Fig. 1. Effect of RG treatment on proliferation of C6 glioblastoma cells. (A) The cells (10^3 per well) were incubated with indicated concentrations (0, 0.1, 0.25, 0.5, 1 $mg \cdot ml^{-1}$) of water-extracted fraction of RG for 3 days. The cell proliferation was monitored by the WST-1 assay as described in the Materials and methods. The mean values were expressed as times of the control before RG treatment. The experiments were repeated three times. (B) The cell morphology was observed by microscopy at 1 day (a, b and c), 2 days (d, e and f) and 3 days (g, h and i) after treatment. C6 glioblastoma cells without RG treatment as control (a, d and g), the cells treated with 0.25 $mg \cdot ml^{-1}$ of RG (b, e and h), and 1 $mg \cdot ml^{-1}$ of RG (c, f and i). Bar =10 μm. (C) The cell morphology was observed by fluorescence microscopy using a blue filter 3 days after treatment. C6 glioblastoma cells without RG treatment (a), the cells treated with 1 $mg \cdot ml^{-1}$ of RG (b), or 0.1 μM of staurosporine as a positive control falling into apoptosis (c).

Fig. 2. Effect of RG on the expression level of GDNF mRNA in rat C6 glioblastoma cells. (A) C6 glioblastoma cells were treated with the indicated concentrations of RG for 24 h. Expression levels of GDNF mRNA were normalized with those of β-actin mRNA. Data are obtained from the duplicate samples of 3 independent experiments and shown as

the means ± SEM of ratios of each value toward control (0 mg·ml^{-1} of RG). *p < 0.05 vs control. (B) The expression level of GDNF mRNA was measured at indicated times following addition of RG at the final concentration of 1 mg·ml^{-1}. Data are shown as the means ± SEM (n = 6) of ratios of each value toward control (0 h). *p < 0.05, **p < 0.01 vs control. (C) Primary-cultured astrocytes from neonatal rat brain were treated with 1 mg·ml^{-1} of RG for 24 h. Data are shown as the means ± SEM (n = 6) of ratios of each value toward control (0 h). *p < 0.05.

Fig. 3. Effects of PKC inhibitors on RG-stimulated GDNF gene expression in C6 glioblastoma cells. Each PKC inhibitor was added to the culture medium 30 min before treatment of RG (1 mg·ml^{-1}). C6 glioblastoma cells were further cultured for 24 h. (A) Typical results of expression pattern for GDNF and β-actin mRNAs by RT-PCR according to the Materials and methods are shown. (B) Expression levels of GDNF mRNA were normalized with those of β-actin mRNA and shown as ratios toward control. (1) Control (without treatment), (2) RG (1 mg·ml^{-1}), (3) RG + Gö6976 (1 μM), (4) RG + rottlerin (2 μM), (5) RG + Ro 31-8220 (2 μM). Data are shown as the means ± SEM (n = 6) of ratios of each value toward control (no treatment). *p < 0.05, **p < 0.01 vs control (1). $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 vs RG (2).

Fig. 4. Effects of various inhibitors for MAPK cascades on the expression level of GDNF gene stimulated by RG in rat C6 glioblastoma cells. Each MAPK inhibitor was added to the medium 30 min before treatment of RG (1 mg·ml^{-1}). C6 glioblastoma cells were further cultured for 24 h. (A) Typical results of expression pattern for GDNF and β-actin mRNAs by RT-PCR according to the Materials and methods are shown. (B) Expression levels of GDNF mRNA were normalized with those of β-actin mRNA and shown as ratios toward control. (1) Control (without treatment), (2) RG (1 mg·ml^{-1}), (3) RG + U0126 (10 μM), (4) RG + SP600125 (30 μM), (5) RG + SB202190 (10 μM). Data are shown as the means ± SEM (n = 6) of ratios of each value toward control (without treatment). *$\underline{p}$ < 0.05, **$\underline{p}$ < 0.01 vs control (1). $^{\#}\underline{p}$ < 0.01 vs RG (2).

Fig. 5. RG stimulates ERK1/2 phosphorylation in C6 glioblastoma cells. (A) The cells were cultured with 1 mg·ml^{-1} of RG for the indicated times. Phosphorylation levels of ERK1/2 were detected according to the Materials and methods. (B) Each PKC inhibitor was added to the medium 30 min before the addition of 1 mg·ml^{-1} RG. C6 glioblastoma cells were further cultured for 3 h. (1) control (without treatment), (2) RG (1 mg·ml^{-1}), (3) RG + Gö6976 (1 μM), (4) RG + rottlerin (2 μM), (5) RG + Ro 31-8220 (2 μM), (6) RG + U0126 (10 μM). Data are shown as the means ± SEM (n = 6) of ratios of each value toward control (without treatment). *$\underline{p}$ < 0.05 vs control (1).

Figure revised

Click here to download high resolution image

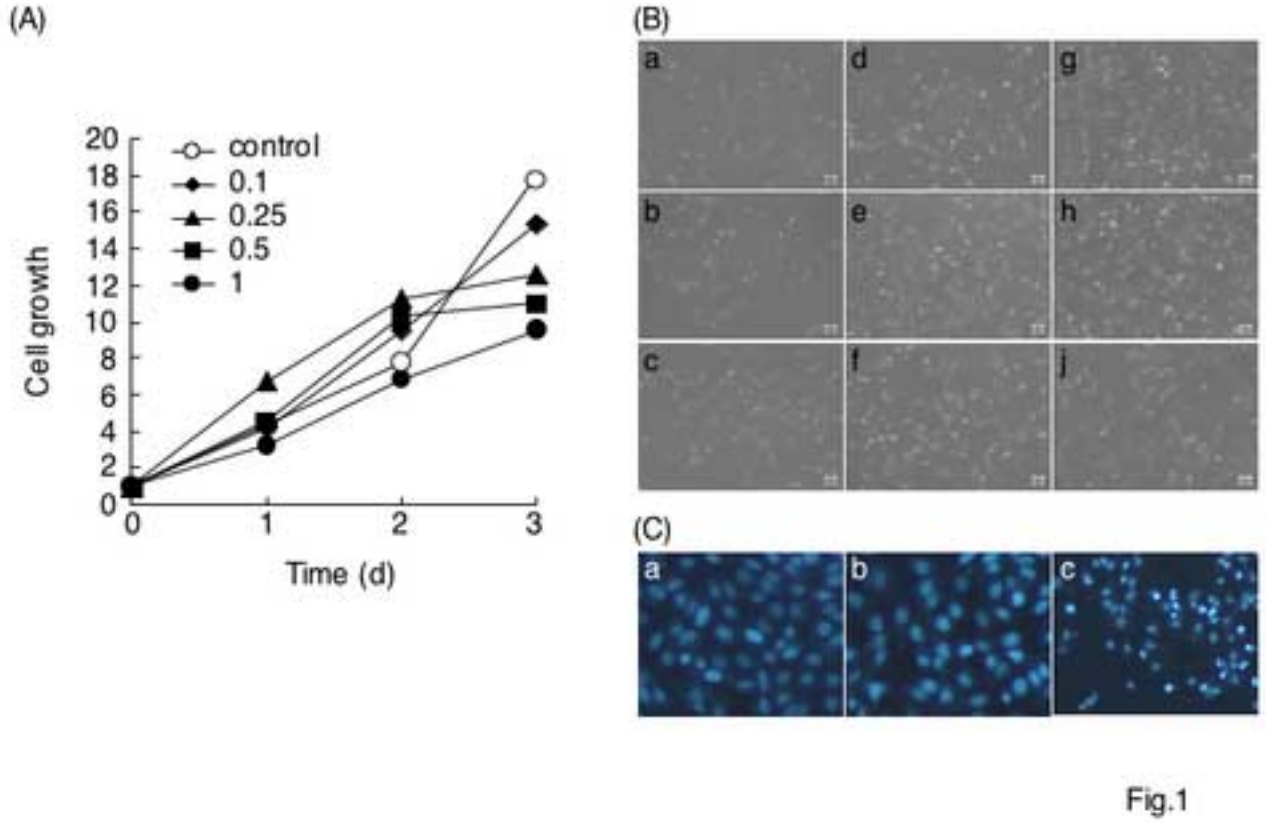

Fig.1

Figure revised

Click here to download high resolution image

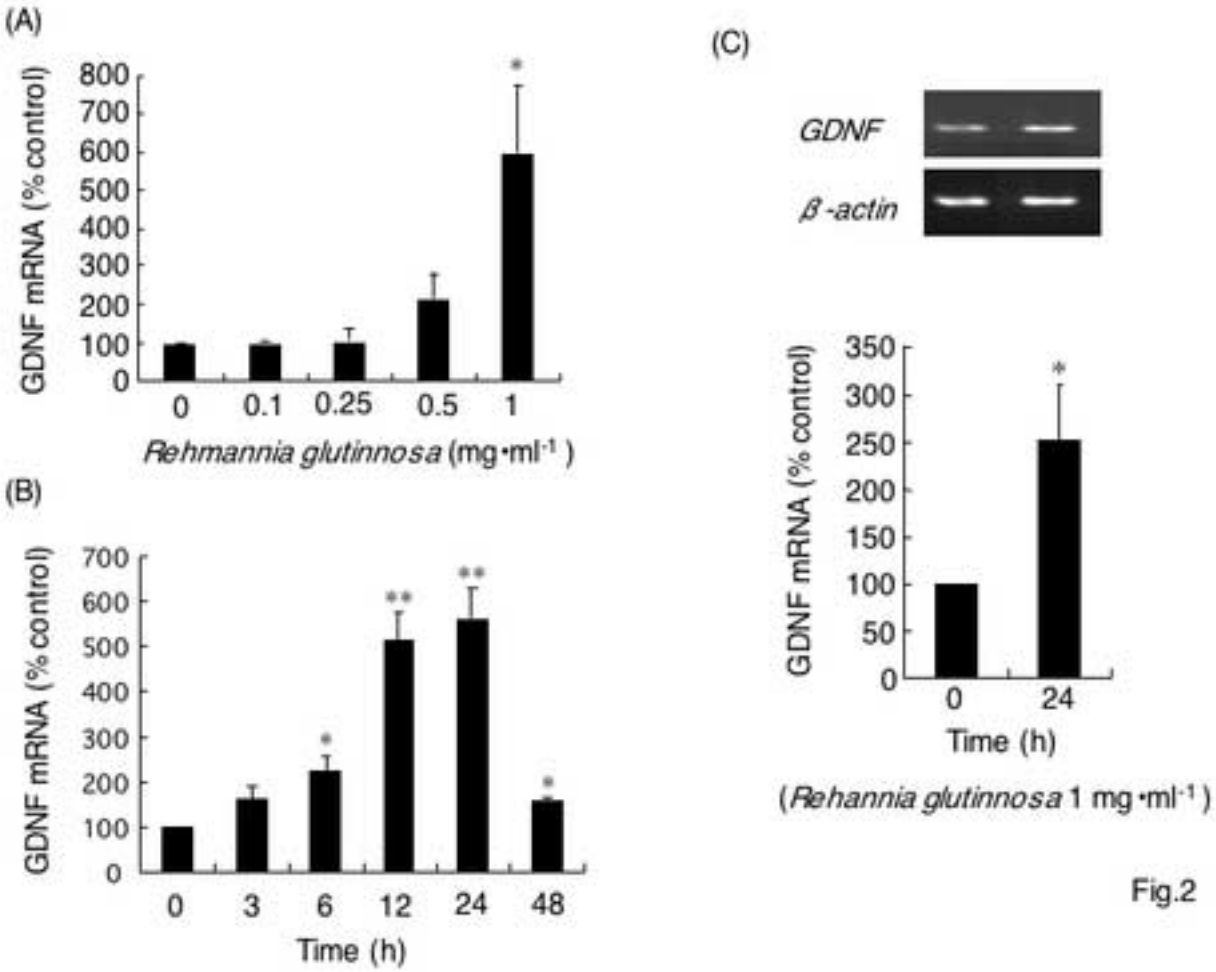

Fig.2

Figure(s)

Click here to download high resolution image

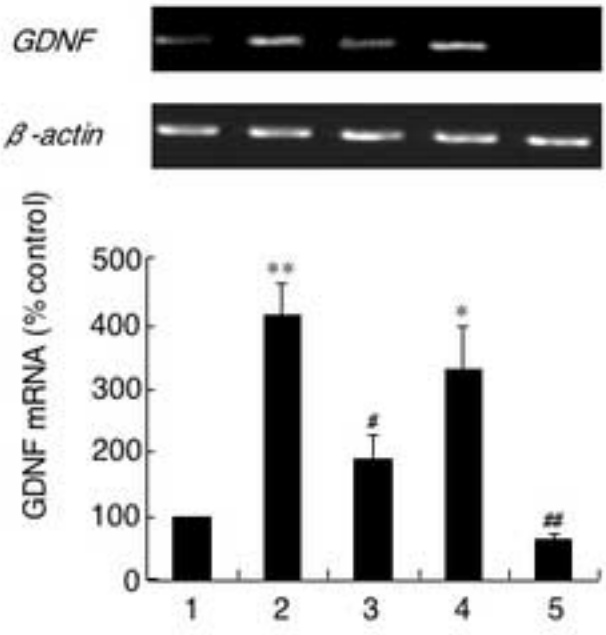

Fig.3

Figure(s)

Click here to download high resolution image

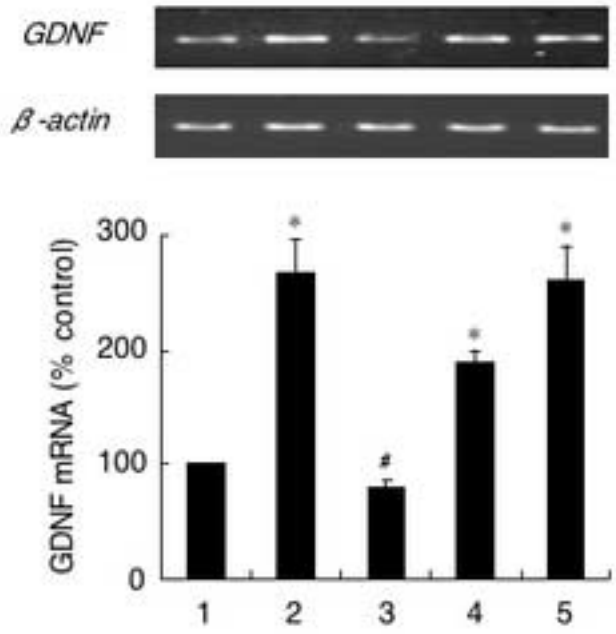

Fig.4

Figure(s)
Click here to download high resolution image

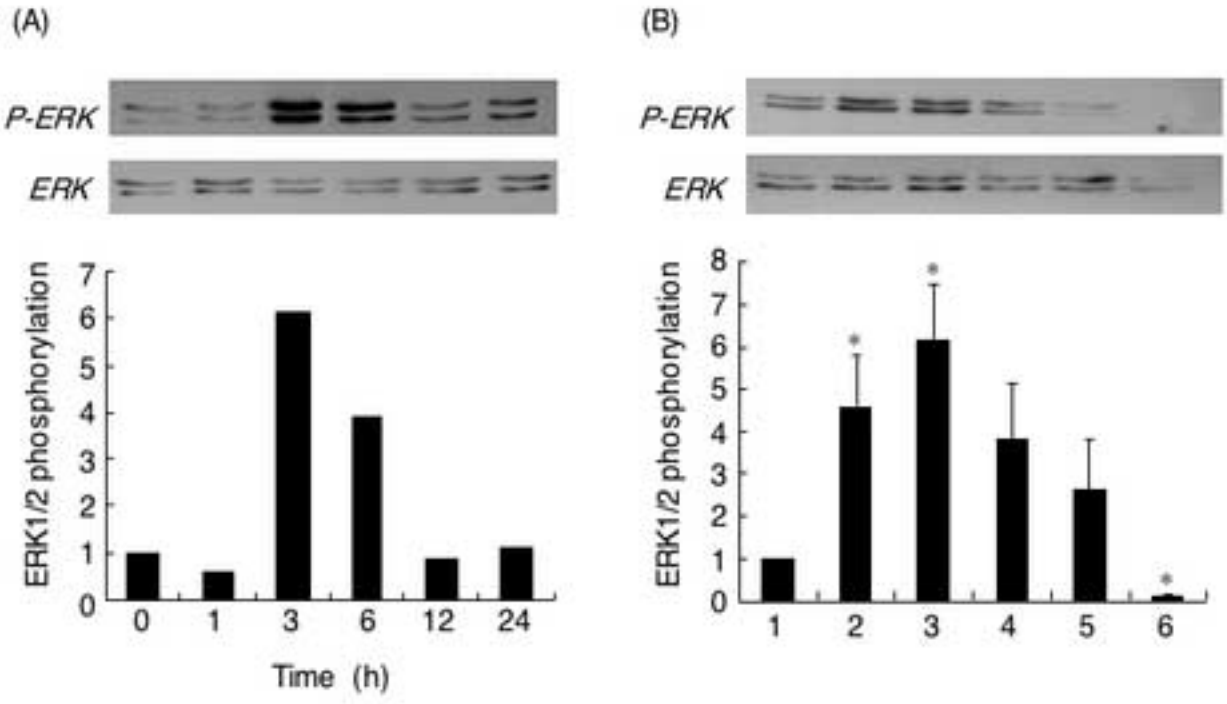

Fig.5