ER calcium discharge stimulates GDNF gene expression through MAPK-dependent and -independent pathways in rat C6 glioblastoma cells

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Abstracts

Glial cell line-derived neurotrophic factor (GDNF), a neurotrophic and differentiation factor, is expressed under several pathophysiological conditions but its regulatory signals have not yet been clarified. Here, we found that endoplasmic reticulum (ER) Ca^{2+} discharge by thapsigargin induced *GDNF* mRNA as well as *COX2* and *GRP78* expression in rat C6 glioblastoma cells. *GDNF* mRNA was immediately induced and peaked at 2 h by thapsigargin, and the alternative transcript consisting of exon 3 and exon 4 appeared to be most inducible. In spite of intracellular Ca^{2+} perturbation, Ca^{2+}-dependent PKC was not responsible for this induction. Instead, a PKCδ-specific inhibitor, rottlerin, suppressed the thapsigargin-induced *GDNF* mRNA expression. On the other hand, thapsigargin transiently enhanced phosphorylation status of mitogen-activated protein kinase (MAPK) pathway, including extracellular signal-regulated kinase (Erk), p38 MAPK and c-JUN amino-terminal kinase1 (JNK1) simultaneously; whereas specific inhibitors against MEK1 and JNK only reduced the thapsigargin-induced *GDNF* mRNA expression. In addition, a pan-PKC inhibitor (Ro-31-8220) attenuated the thapsigargin-enhanced phosphorylation levels of ERK1/2 and JNK1, whereas rottlerin did not. Thus, the present study demonstrated that the thapsigargin-stimulated ER Ca^{2+} discharge up-regulated *GDNF* gene expression through both MAPK –dependent and -independent pathways in C6 glioblastoma cells.

Key words: ER stress; GDNF; MAPK; PKC; glioblastoma

Glial cell line-derived neurotrophic factor (GDNF) was originally purified from rat B-49 conditioned medium and was characterized as a potent neurotrophic factor for cultured dopaminergic neurons from developing substantia - nigra [1]. GDNF is a distantly related member of the transforming growth factor-β (TGF-β) superfamily [2]; additional GDNF homologs have been also cloned [3 – 5]. Expression of GDNF is widespread in both the central and peripheral nervous systems, as well as outside the nervous system [6 – 9]. Targeted disruption of the mouse GDNF gene showed that GDNF plays a critical role in the development of both the kidney and enteric neurons during embryogenesis [10 – 12]. Therefore, GDNF has multifunctional properties to regulate development and differentiation of a variety of cell lineages and to act as a neurotrophic factor for specific kinds of neurons in the nervous system. GDNF is also up-regulated in astrocytes, microglial cells and macrophages in pathophysiological conditions, such as ischemic/hypoxic insults and spinal cord injury [13 – 16]. Recently, many investigators have indicated that GDNF is useful as a therapeutic agent against neurodegenerative disease [17]. Under pathological conditions, a number of pro-inflammatory stimuli are known to induce intracellular organelle dysfunctions such as energy crisis and endoplasmic reticulum (ER) stress; these dysfunctions are known to result in modified gene expression, morphological changes and cell death [18 – 20]. Recent findings suggest that ER stress is associated with several neurodegenerative disorders, such as ischemia [21] and Parkinson's disease [22]. Thapsigargin, which is reported to generate ER stress [23, 24], is a non-phorbol ester-type tumor-promoting agent that enhances discharge of intercellular Ca^{2+} store by specifically inhibiting the

ER Ca^{2+}–ATPase [25, 26]. This agent is known to up-regulate several inflammatory mediators, such as IL-6, TNF-α, prostaglandin E_2 and platelet-activating factor [27, 28]. However, little is known about the mechanism of *GDNF* gene induction-triggered by ER stress.

Here we show, in rat C6 glioblastoma cells, that thapsigargin primarily induces *GDNF* mRNA transcription from one of its three known alternative promoters; furthermore, our data indicate that this up-regulation might be effected through both MAPK-dependent and MAPK –independent pathways.

Rat C6 glioblastoma cells were maintained in F-10 Nutrient Mixture medium (Invitrogen) containing 7% fetal calf serum and 3% horse serum. For experiments, cells were grown to semi-confluence, and then the medium was exchanged with serum-free Eagle's minimum essential medium and cells were kept for 18 h. Cells were then treated with thapsigargin (0.5 μM) for the indicated times. The concentrations of other agents used were as follows; U0126 (10 μM), SB202190 (10 μM), SP600125 (30 μM), Gö6976 (0.2 μM), rottlerin (5 μM), Ro-31-8220 (1 μM). Cells were pretreated with each inhibitor for 30 min just before the addition of thapsigargin.

To estimate the mRNA level of each gene by reverse transcriptional-polymerase chain reaction (RT-PCR), total RNA was extracted from cells lysed with Trizol (Invitrogen), and converted to cDNA by reverse transcriptase using random ninemers to prime superscript III $RNase^-$ reverse transcriptase (RT) (Invitrogen) reactions as previously

described [29]. cDNA and specific primers were mixed and amplified with the PCR reaction mixture (EX Taq PCR kit, Takara). The RT-PCR primers used in this study are shown in Table 1. The typical reaction conditions were 0.5 min at 96°C, 0.5 min at 60°C, and 0.5 min at 72°C. The results represent 19 - 34 cycles of amplification, after which cDNAs were separated by electrophoresis on 2.0% agarose gels and visualized using ethidium bromide. Experiments were repeated and reproducibility was confirmed.

For Western blotting analysis, cells were lysed with 20 mM Tris buffer, pH 8.0, containing 137 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM sodium vanadate and 1% (v/v) Triton X-100. The protein concentration was determined by the method of Bradford [30]. Equal amounts of cell lysates were separated on 10% SDS-polyacrylamide electrophoresis gels, immunoblotted onto polyvinylidene difluoride membrane (Amersham) and identified by enhanced chemiluminescence (Amersham) using antibodies against phosphorylated or non-phosphorylated Erk, p38 MAPK and JNK1 (Cell Signaling).

The results are expressed means ± SEM of three independent experiments. Statistical analysis was carried out by one way-ANOVA followed by Fischer's PLSD test. The probability ($p < 0.05$) was considered to be statistically significant.

First, we investigated whether ER Ca^{2+} discharge by thapsigargin influences the expression level of *GDNF, COX2*, *iNOS* or *GRP78* mRNAs in C6 glioblastoma cells,

using RT-PCR. Within 1 h, thapsigargin up-regulated transcript levels of *GDNF* and *COX2*, which peaked at 2 h and declined to the basal level until 8 h after treatment (Fig. 1A). In contrast, the expression level of *GRP78*, one of the stress-inducible genes, gradually increased during the indicated time; *iNOS* levels also increased, but to a lesser extent than *GDNF* and *COX2* in C6 glioblastoma cells after thapsigargin treatment (Fig. 1A). The NFκ-B pathway was previously shown to mediate ER stress-induced COX2 expression in the mouse liver immortalized cell line, ML-1 [31], and we previously reported that the NFκ-B-binding site in intron 1 of the mouse *GDNF* gene could respond to cytokine [32]. However, Bay11-7082, a inhibitor of the NFκ-B pathway, hardly attenuated the thapsigargin-induced expression of *GDNF* mRNA or *COX2* in C6 glioblastoma cells (data not shown). Pahl and Baeuerle [24] reported that ER stress-mediated NF-κB induction has slower kinetics when compared with activation by external stimuli such as TNF-α and IL-1β. Therefore, the NFκ-B pathway seems not to be involved in the immediate induction of *GDNF* gene after thapsigargin treatment.

We previously characterized three alternative GDNF promoters in the 5' upstream regions of exons 1, 2, and 3 [32, 33]; we also reported that IL-1β, one of the pro-inflammatory factors, enhanced the levels of transcripts derived from each of the three promoters within 2 h in mouse glial cell line, TGA-3 [32]. We were therefore interested in determining which of the three promoters might be up-regulated by intracellular Ca^{2+} perturbation after thapsigargin treatment. As shown Fig. 1B, transcripts derived from each of the three alternative promoters was induced by thapsigargin at 1.5 h in C6 glioblastoma cells; yet the transcript consisting exons 3 and 4

(generated by promoter 3) seemed to be most abundant. We further analyzed the levels of thapsigargin-induced transcription by serial increases in PCR cycles; this analysis revealed that the promoter 3-derived transcript was the most inducible one (Fig. 1C). These results indicate that the promoter 3 plays an important role in up-regulating the *GDNF* gene expression in thapsigargin-induced ER stress. However, in our preliminary experiments (data not shown), no apparent response sequence to thapsigargin could be detected in the GDNF alternative promoters. Therefore, unidentified responsive elements might exist within intron 3 (between exon 3 and 4) or 3' UTR region of the GDNF gene.

We previously reported that PMA, which is known to activate two of the three PKC groups (conventional PKC and novel PKC isozymes) [34], up-regulated *GDNF* gene expression in C6 glioblastoma cells [35]. It was therefore of interest to determine which PKC signaling pathway is responsible for the immediate induction of *GDNF* mRNA by intracellular Ca^{2+} perturbation after thapsigargin treatment. In this study, three PKC inhibitors, Gö6976 (a cPKC-specific inhibitor) [36], rottlerin (a PKCδ-specific inhibitor) [37], and Ro-31-8220 (a pan-PKC inhibitor) [38] were utilized to address to this question. As shown in Fig. 2A, rottlerin and Ro-31-8220, but not Gö6976, suppressed thapsigargin-induced *GDNF* mRNA expression. Therefore, Ca^{2+}-dependent cPKCs were not responsible for the *GDNF* induction, despite the ER Ca^{2+} discharge by thapsigargin. Instead, two other Ca^{2+}-independent PKC groups might be responsible for the observed *GDNF* induction. In the case of PKCδ, tyrosine kinase Fyn, which was activated by thapsigargin within 0.5 min in rat liver epithelial

cells [39], was reported to phosphorylate and associate the tyrosine 187 of PKCδ in C6 glioblastoma cells and to play an important role in PKC signaling [40]. Further studies that employ a dominant negative form of each PKC isozyme may uncover a complex link between PKCs and the thapsigargin-induced *GDNF* gene expression. We next examined whether MAPK pathways, subdivided into Erk1/2, p38 MAPK or JNK, contribute to stimulate the thapsigargin-induced *GDNF* mRNA expression in C6 glioblastoma cells. Specific pharmacological inhibitors such as U0126 for MEK1-Erk1/2 [41], SB202190 for p38 MAPK [42], and SP600125 for JNK [43], were employed to elucidate the involvement of each kinase in various cellular events [29]. We found that inhibitors both to MEK1 and to JNK attenuate thapsigargin-induced *GDNF* mRNA expression in C6 glioblastoma cells (Fig. 2B). To investigate which MAPK cascades were activated after thapsigargin treatment, the phosphorylation levels of Erk1/2, p38 MAPK and JNK were analyzed. We found that all of these MAPKs were activated through the very acute phase, and each phosphorylation level peaked 5 min after thapsigargin treatment and then immediately declined to the basal level (Fig. 3A). No further activation of any of these kinases was observed over the 2 h following stimulation (data not shown). These results imply that both Erk1/2 and JNK engage in this transient up-regulation of *GDNF* mRNA in C6 glioblastoma cells. We further investigated the relationship between Ca^{2+}-independent PKC pathways and the MAPK cascade since PKCδ, a member of novel PKC isozymes, which is inhibited by rottlerin, is reported to be upstream of the MEK1-Erk1/2 cascade [44]. Moreover, PKCζ, one of atypical PKC isozymes, which is inhibited by Ro-31-8220, to directly activate Raf-1, an

upstream kinase of MEK1-Erk1/2 [45]. As shown in Figure 3B, Ro-31-8220 reduced Erk phosphorylation, which is consistent with the decrease in thapsigargin-induced *GDNF* mRNA expression in the presence of U0126 (Fig. 2B); whereas rottlerin showed no effect on Erk phosphorylation. Therefore, Ro-31-8220-sensitive pathways, including PKCζ, seem to up-regulate *GDNF* gene expression via the Erk1/2 cascade after thapsigargin treatment. In contrast, the thapsigargin-activated PKCδ pathway may induce *GDNF* mRNA in an Erk1/2-independent manner though we cannot exclude possibility that PKCδ has some effects on downstream of Erk1/2. Further, Ro-31-8220 almost blocked JNK phosphorylation in the present study despite this agent is reported to enhance the phosphorylation level of JNK by inhibiting MAPK phospatase-1 in Rat-1 fibroblasts [46]. Therefore, unidentified pathways affecting upstream kinases and/or phosphatases of JNK may be inhibited by Ro-31-8220 in C6 glioblastoma cells, which may in turn diminish thapsigargin-induced *GDNF* mRNA expression.

Although the mechanism by which diverse pathways, including PKCs and MAPKs, respond to ER stress remains elusive, we found that the *GDNF* gene expression was up-regulated by ER Ca^{2+} discharge after thapsigargin treatment and this immediate induction might be related to Ca^{2+}-independent PKC pathways, Erk1/2 and JNK cascades, but not to NF-κB pathway. The *GDNF* promoter 3 appears to play an important role in this up-regulation by intracellular Ca^{2+} perturbation. Further studies aimed at elucidating these complex relationships under the ER stress should provide insight into the molecular mechanisms underlying activation of *GDNF* gene expression and improving therapy for patients of neurodegenerative disorders.

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Figure Legends

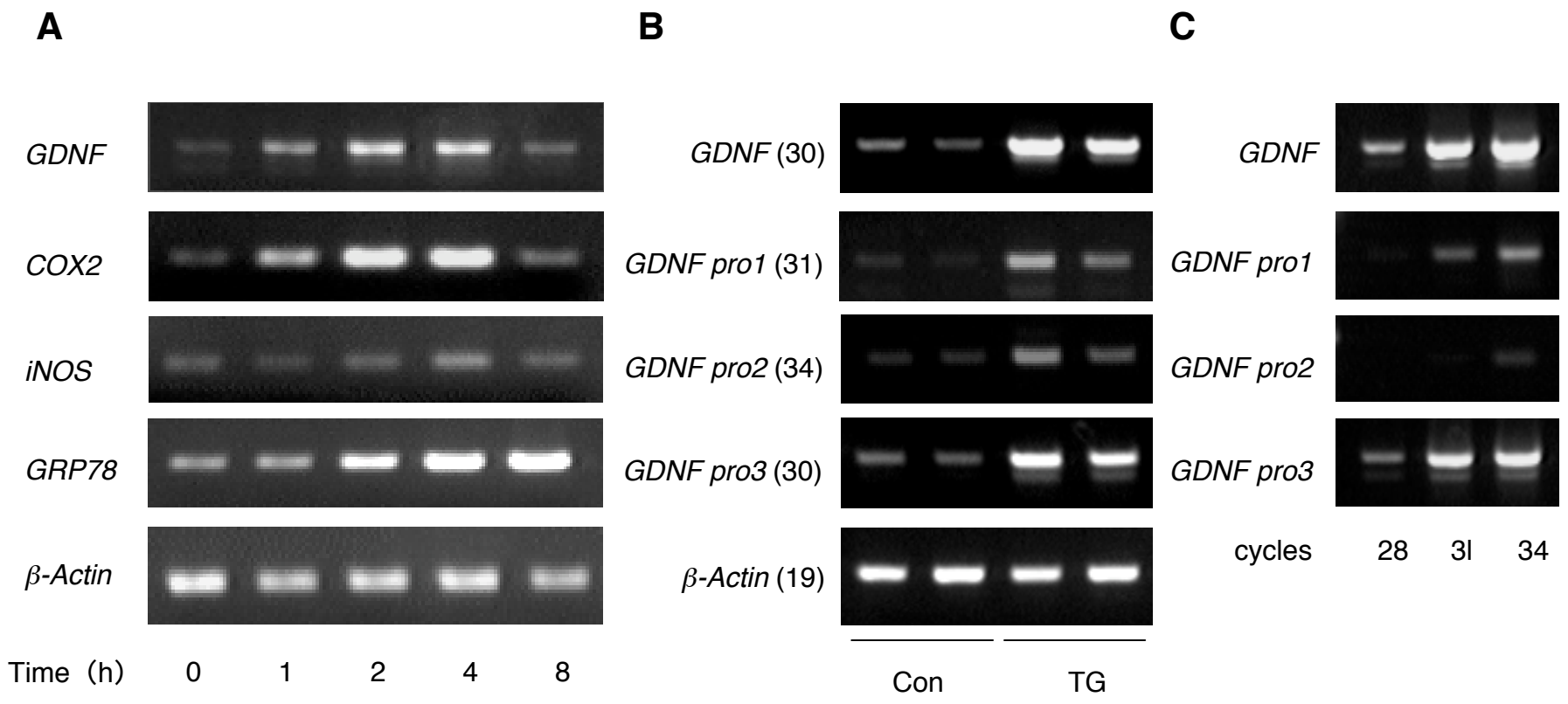
Fig. 1. Thapsigargin induced *GDNF* gene expression in C6 glioblastoma cells. (A) C6 glioblastoma cells were exposed to thapsigargin (TG) at 0.5 μM for the indicated times. Total RNA was isolated and mRNA expression levels of *GDNF*, *COX2*, *iNOS* and *GRP78* were analyzed by RT-PCR. (B) C6 glioblastoma cells were exposed to thapsigargin (TG) at 0.5 μM or vehicle for 1.5 h, and total RNA was isolated from the resultant cells. Each transcript derived from GDNF promoter 1, 2, and 3 (*GDNF pro1, 2,* and *3*) as well as that corresponding to *GDNF* coding region (*GDNF*) was analyzed by RT-PCR. Parentheses in the figure are PCR cycles used for detection of each transcript. (C) Expression levels of each *GDNF* transcript derived from promoters 1, 2, and 3 (*GDNF pro1, 2,* and *3*) as well as that corresponding to the coding region (*GDNF*) in thapsigargin-treated cells was estimated by serial increases in PCR cycle number.

Fig. 2. Effects of PKC and MAPK inhibitors on thapsigargin-induced *GDNF* mRNA expression in C6 glioblastoma cells. (A) After 0.5 h pretreatment with Gö6976 (0.2 μM, +Gö), rottlerin (5 μM, +Rot) or Ro-31-8220 (1 μM, +Ro) or vehicle, cells were treated with thapsigargin (TG) at 0.5 μM for 1.5 h. (B) After 0.5 h pretreatment with SP600125 (30 μM, +SP), SB202190 (10 μM, +SB) and U0126 (10 μM, +U0126) or vehicle, cells were treated with thapsigargin (TG) at 0.5 μM for 1.5 h. Total RNA was isolated from cells and each mRNA was analyzed by RT-PCR. The relative mRNA

level of *GDNF* was calculated by comparison of *β-Actin*-normalized values with the level of untreated control. Values represent means ± SEM of three independent experiments and are expressed as ratio of that of control. Data were analyzed by one way-ANOVA followed by Fischer's PLSD test to evaluate the effect of each kinase inhibitor on thapsigargin-induced *GDNF* mRNA expression; values marked with an asterisk are significantly different from the thapsigargin alone treatment ($p < 0.05$).

Fig. 3. Effects of PKC inhibitors on thapsigargin-induced MAPK phosphorylation in C6 glioblastoma cells. (A) The cells were incubated with thapsigargin (0.5 μM) for the indicated times. The resultant cell lysate was analyzed by Western blotting with antibodies against phosphorylated and non-phosphorylated Erk, p38 MAPK and JNK1. (B) After 0.5 h pretreatment with Gö6976 (0.2 μM, +Gö), rottlerin (5 μM, +Rot), Ro-31-8220 (1 μM, +Ro) or vehicle, cells were treated with thapsigargin (TG) at 0.5 μM for 5 min. Cell lysate was analyzed by Western blotting with antibodies against phosphorylated Erk and JNK1.

Figure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cycles 28 3l 34

Fig. 1

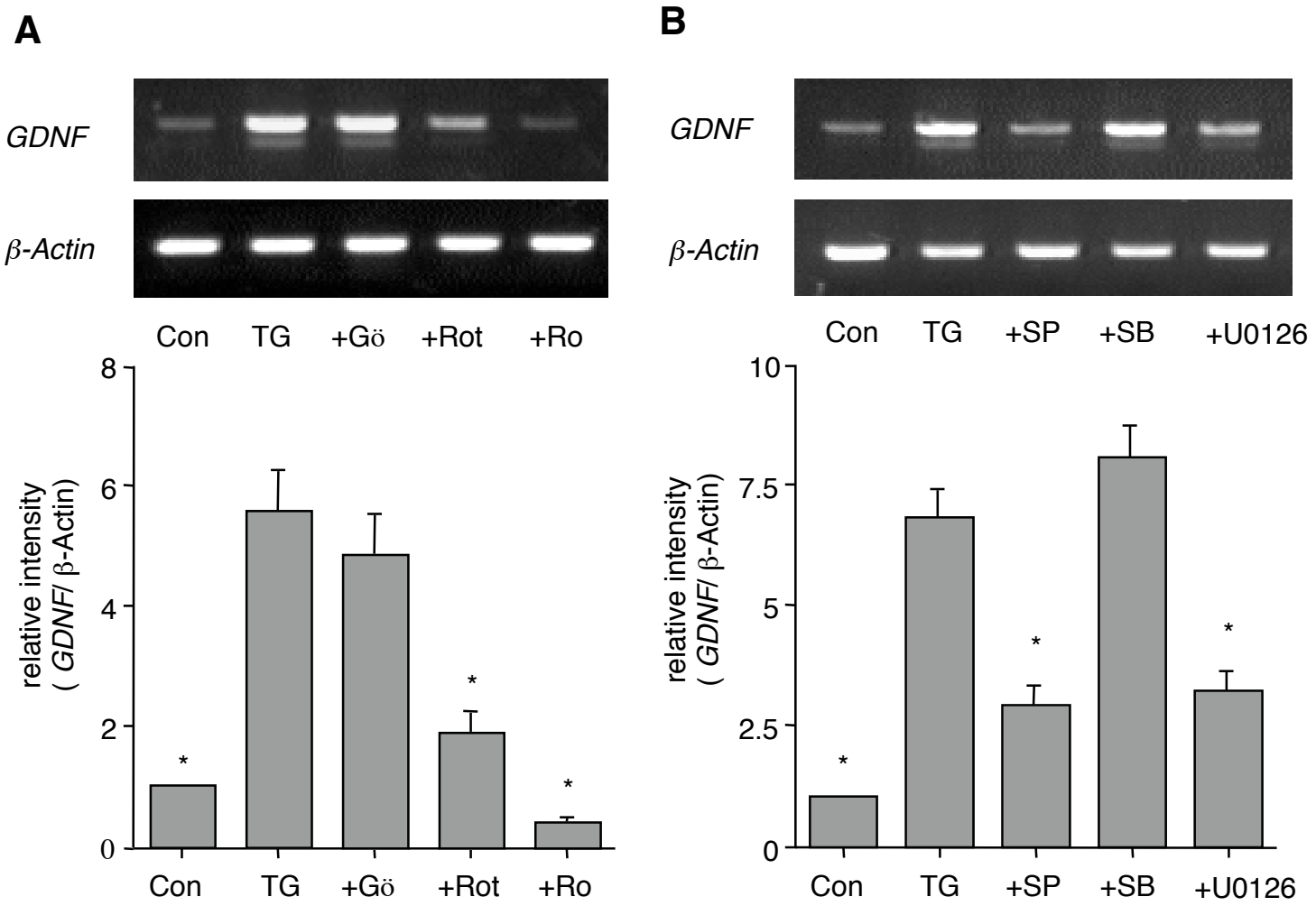
A
GDNF
β-Actin
Con
TG
+Gö
+Rot
+Ro
relative intensity
(GDNF/ β-Actin)
0
2
4
6
8
B
GDNF
β-Actin
Con
TG
+SP
+SB
+U0126
relative intensity
(GDNF/ β-Actin)
0
2.5
5
7.5
10

Fig. 2

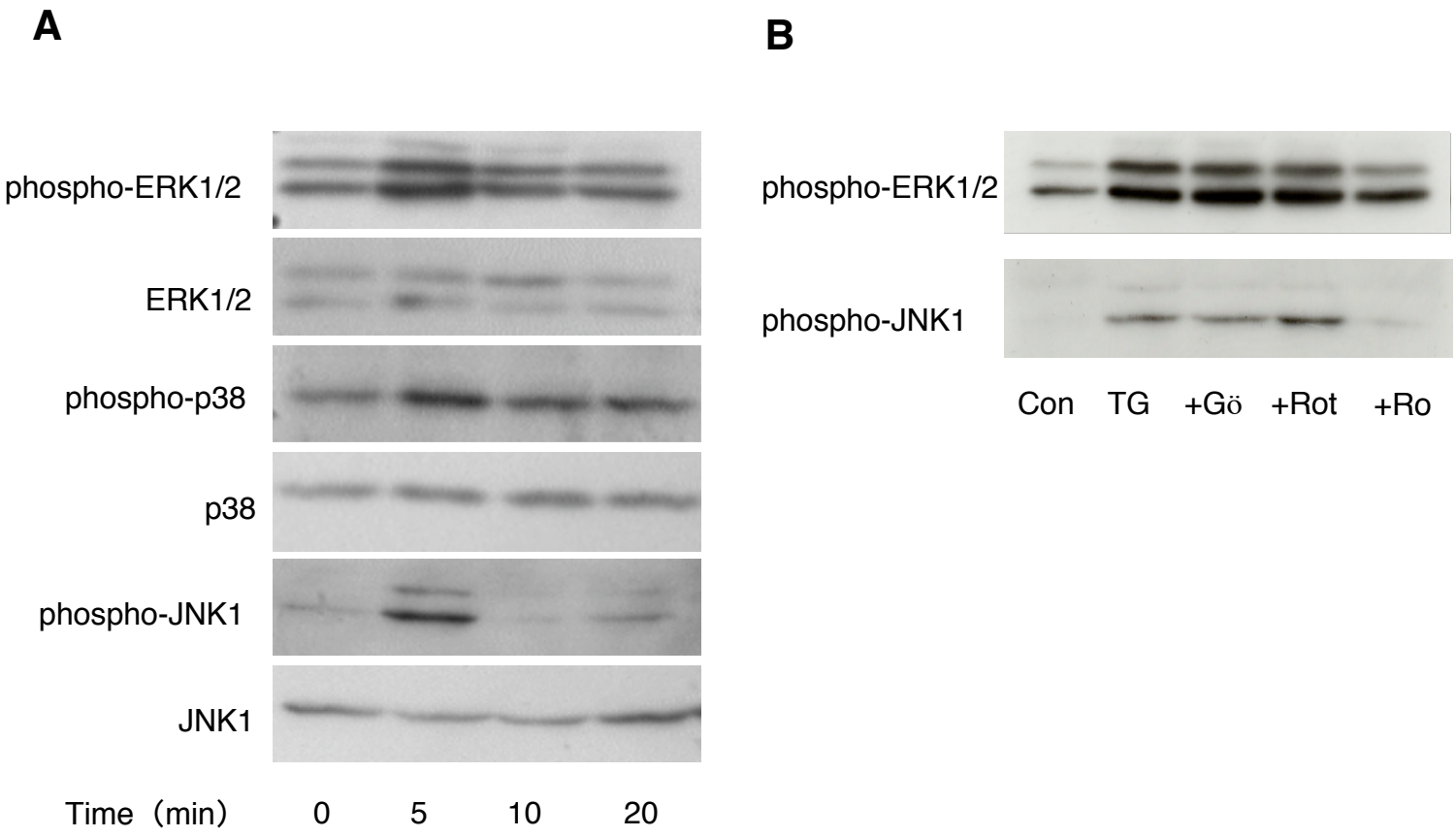
A
phospho-ERK1/2
ERK1/2
phospho-p38
p38
phospho-JNK1
JNK1
Time（min）
0
5
10
20
B
phospho-ERK1/2
phospho-JNK1
Con
TG
+Gö
+Rot
+Ro

Fig. 3

Table 1 PCR primer sequences used in this study

GDNF	sense;	5’- CGGGACTCTAAGATGAAGTTATGGGATGTCGTG -3’
	antisense;	5’- GGGTCAGATACATCCACACCGTTTAGCGGAATGC -3’
GDNF pro1	sense;	5’- TGGATTGCGTGCTCTTGCTC -3’
	antisense;	5’ -CATGACGTCATCAAACTGGTCAGGA -3’
GDNF pro2	sense;	5’ -GAACCCAACAGCTGCGGAGAAAA -3’
	antisense;	5’ -CATGACGTCATCAAACTGGTCAGGA -3’
GDNF pro3	sense;	5’ -TTCTCTTCCCCGCTGCCC -3’
	antisense;	5’ -CCACACCGTTTAGCGGAATGC -3’
COX2	sense;	5’- GAACAACATTCCCTTCCTTCG -3’
	antisense;	5’- GAAGTTCCTTATTTCCTTTCACACC -3’
iNOS	sense;	5'- TTGGTGTTTGGGTGCCGGC -3'
	antisense;	5'- CCATAGGAAAAGACTGCACCGAAG -3’
GRP78	sense;	5’- ACCAATGACCAAAACCGCCT -3’
	antisense;	5’- GAGTTTGCTGATAATTGGCTGAAC -3’
β-Actin	sense;	5’- TGTATGCCTCTGGTCGTACC -3’
	antisense;	5’- CAACGTCACACTTCATGATGG -3’