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Effect of JGK-263 as a new glycogen synthase kinase-3β inhibitor on extrinsic apoptosis pathway in motor neuronal cells

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ABSTRACT

Glycogen synthase kinase-3β (GSK-3β) has been identified as one of the important pathogenic mechanisms in motor neuronal death. GSK-3β inhibitor has been investigated as a modulator of apoptosis and has been shown to confer significant protective effects on cell death in neurodegenerative diseases. However, GSK-3β is known to have paradoxical effects on apoptosis subtypes, i.e., pro-apoptotic in mitochondrial-associated intrinsic apoptosis, but anti-apoptotic in death receptor-related extrinsic apoptosis. In this study, we evaluated the effect of a new GSK-3β inhibitor (JGK-263) on motor neuron cell survival and apoptosis, by using low to high doses of JGK-263 after 48 h of serum withdrawal, and monitoring changes in extrinsic apoptosis pathway components, including Fas, FasL, cleaved caspase-8, p38α, and the Fas–Daxx interaction. Cell survival peaked after treatment of serum-deprived cells with 50 μM JGK-263. The present study showed that treatment with JGK-263 reduced serum-deprivation-induced motor neuronal apoptosis by inactivating not only the intrinsic, but also the extrinsic apoptosis pathway. These results suggest that JGK-263 has a neuroprotective effect through effective modulation of the extrinsic apoptosis pathway in motor neuron degeneration.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive degeneration of both upper and lower motor neurons, motor weakness, muscular wasting, and respiratory failure, which results in high mortality [1–3]. The pathogenic mechanism of ALS is uncertain and the proposed theories suggest that it is the consequence of oxidative stress, glutamate toxicity, abnormal protein aggregation, calcium-mediated toxicity, genetic or immune defect, or apoptosis, which cause motor neuronal death [3].

Glycogen synthase kinase-3 (GSK-3) plays a significant role in cell survival, and the dysregulation of GSK-3 was suggested to be involved in the development of diverse human disease, such as mood disorder, schizophrenia, diabetics, cancer, and neurodegenerative diseases, including Alzheimer disease, Parkinson disease, and ALS [4–6]. In ALS, the increase of GSK-3 activity is investigated as a potential pathogenic mechanism associated with neuronal apoptosis. Proteomic screening methods, used to access the protein kinase, protein phosphatase, and phosphorylation states of phosphor-proteins in thoracic cord from sporadic ALS, showed elevated GSK-3β activity [7]. Immunohistochemical studies of the spinal cords of Japanese sporadic ALS patients showed significant up-regulation of GSK-3β expression [8]. Motor neurons transfected with G93A and A4 V mutant human Cu, Zn-superoxide dismutase (hSOD1) genes also showed increased GSK-3β activity; this was suggested to result from the inhibition of the phosphatidylinositol 3-kinase (PI3 K)/Akt pathways [9]. These consistent results support GSK-3 involvement in ALS pathology, so that a GSK-3β inhibitor may be an effective strategy in the treatment of ALS. Apoptotic signaling proceeds by either an intrinsic pathway or an extrinsic pathway.

The contradictory effects of GSK-3β on the intrinsic and extrinsic apoptosis pathways seem to be cell type- and context-depen-

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dent [10,11]. Motor neurons follow a more distinctive pattern and are classified as type III cells. In type III cells, Fas-triggered cell death requires not only the classical caspase-8 activation but also concomitant Daxx-p38-neuronal nitric oxide synthase (NOS) loop activation [12]. A recent study showed that the extrinsic apoptosis pathway plays an important role in motor neuron degeneration [12–14], but the effect of GSK-3β inhibition on type III cells, motor neurons, has not been reported. A recent series of data suggested that GSK-3β activity could also be an important factor in the extrinsic apoptotic pathway [10,15,16]. GSK-3 has two paradoxical effects on apoptosis. Previous studies mostly focused on the pro-apoptotic action of GSK-3β, and GSK-3β inhibitor suppressed the mitochondria-mediated intrinsic apoptosis pathway. Effects of GSK-3β inhibitor in the death receptor-mediated extrinsic apoptosis signal remained unknown.

A more effective approach would be to evaluate the effect of a GSK-3β inhibitor that suppresses cell death when acting through the intrinsic pathway and plays an anti-apoptotic role when the extrinsic pathway is active. We hypothesized that a new GSK-3β inhibitor, JGK-263, would affect the extrinsic apoptosis pathway. In addition, the study evaluated the activity and relevance of the extrinsic apoptotic pathway by treating motor neuron cells with JGK-263.

2. Materials and methods

2.1. Cell culture and JGK-263 treatment

Mouse motor neuron-like hybrid cells (NSC34) [17] were maintained in culture dish containing Dulbecco's modified Eagle medium (JBI, Korea) with 10% heat inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin–streptomycin (Gibco, Grand Island, NY, USA). Cells were kept in a humidified incubator at 37 °C under 5% CO_2. Cells were exposed to serum deprivation for 48 h with and without treatment with various concentrations of JGK-263 (0, 0.2, 1, 10, 50, 100 μM). Serum deprivation was used to induce the apoptosis in NSC-34 cells. JGK-263 (compound 17c) as a new GSK-3β inhibitor was supplied from Jeil Pharmaceutical Drug Discovery Laboratories (R&D Center; Jeil Pharmaceutical Co., Ltd.). JGK-263 showed good potency in enzyme and cell-based assays (IC_{50} = 111 nM, EC_{50} = 1.78 μM). Moreover, it has demonstrated desirable water solubility, PK profile, and moderate brain penetration [18].

2.2. Cell viability assay

NSC-34 cells were subcultured in 96-well plates at a density of 1.5×10^4 cells per well, and the next day, cells were exposed to serum deprivation for 48 h with and without treatment with various concentrations of JGK-263. After this treatment period, cell viability was evaluated by the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Tokyo, Japan).

2.3. Western blot analysis

Cell lysates were prepared from the proposed experimental conditions, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and blotted with anti-tau (Invitrogen, Camarillo, CA, USA), anti-phospho-tau (ser 396) (Invitrogen, Camarillo, CA, USA), anti-Fas (Santa Cruz Biotech, Delaware, CA, USA), anti-Fas ligand (Santa Cruz Biotech, Delaware, CA, USA), anti-cleaved caspase-8 (Novus Biologicals, Littleton, CO, USA), anti-p38α (Santa Cruz Biotech, Delaware, CA, USA), anti-Daxx (Cell signaling, Beverly, MA, USA), anti-cleaved caspase-3 (Cell signaling, Beverly, MA, USA), and anti-cytochrome *c* (Cell signaling, Beverly, MA, USA) antibody. For evaluation of cytosolic cytochrome *c* levels, cell suspensions were prepared according to the manufacturer's instruction (Mitochondria isolation kit for cultured cells; Thermo scientific, Rockford, IL, USA). Protein loading was controlled by probing for β-actin (Cell signaling, Beverly, MA, USA) antibody on the same membrane.

2.4. Immunoprecipitation

For immunoprecipitation, protein samples were incubated overnight at 4 °C with anti-Fas antibody. The complexes formed were immunoprecipitated using protein A-Sepharose. The Sepharose beads were boiled in SDS–PAGE sample buffer, and the samples were resolved by SDS–PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed using anti-FADD or anti-Daxx antibody.

2.5. Statistical analysis

The data are presented as the mean ± standard errors (SE) from more than three or four independent tests. Data were analyzed by Prism software (GraphPad Software, San Diego, CA, USA) using either Student's *t* test or Tukey's multiple comparison tests after one-way ANOVA (GraphPad Prism Software). Differences were considered statistically significant when $p < 0.05$.

3. Results

In vitro motor neuron apoptosis, induced by serum deprivation, revealed that both the intrinsic and the extrinsic apoptotic pathway were well activated [19]. For this reason, we selected 48 h of serum deprivation as the condition used in further studies. A CCK-8 assay was done after treatment of NSC-34 cells that had been serum-deprived for 48 h with JGK-263 treatment (0, 0.2, 1, 10, 50, 100 μM). The mean (% of the cell viability in serum-deprived condition) ± SE cell viability after treatment with each concentration of JGK-263 was determined. Treatment with increasing concentrations of JGK-263 upto 50 μM increased cell viability, compared to the control (45.92 ± 5.37% survival). Treatment with 50 μM JGK-263 resulted in the highest cell viability (85.23 ± 9.91%, $p < 0.001$). The protective effect peaked with 50 μM JGK-263, but above 100 μM (32.83 ± 5.19%) these protective effects were reduced (Fig. 1A).

GSK-3β activity was indirectly measured by measuring the immunoreactivity (IR) ratio of phosphorylated tau (ser396) to total tau after JGK-263 treatment. Western blot results for the phosphorylated tau (ser396) and total tau following each concentration shown as Fig. 1. As the dose of JGK-263 increased, the IR ratio of phosphorylated tau (ser396) to total tau decreased. We confirmed that JGK-263 effectively inhibited GSK-3β in NCS-34 cells, and this inhibitory action showed a dose-dependent pattern (Fig. 1B).

Next, we investigated late stage apoptosis by monitoring levels of cleaved caspase-3; a significant reduction in the cleaved caspase-3 signal was noted, in a dose-dependent manner. Significant reduction in cleaved caspase-3 signals was noted for groups treated with concentrations of JGK-263 above 10 μM, compared with the control group ($p < 0.001$; Fig. 1C).

To evaluate the effect of serum deprivation in NSC-34 cells and of treatment with JGK-263 on the external apoptotic pathway, levels of Fas, FasL, cleaved caspase-8, and p38α were investigated by Western blot analysis. We determined that JGK-263 treatment did not induce an increase in the levels of Fas/FasL activation, which constitutes the first component of the extrinsic apoptosis pathway, in serum-deprived cells (Fig. 2A and B).

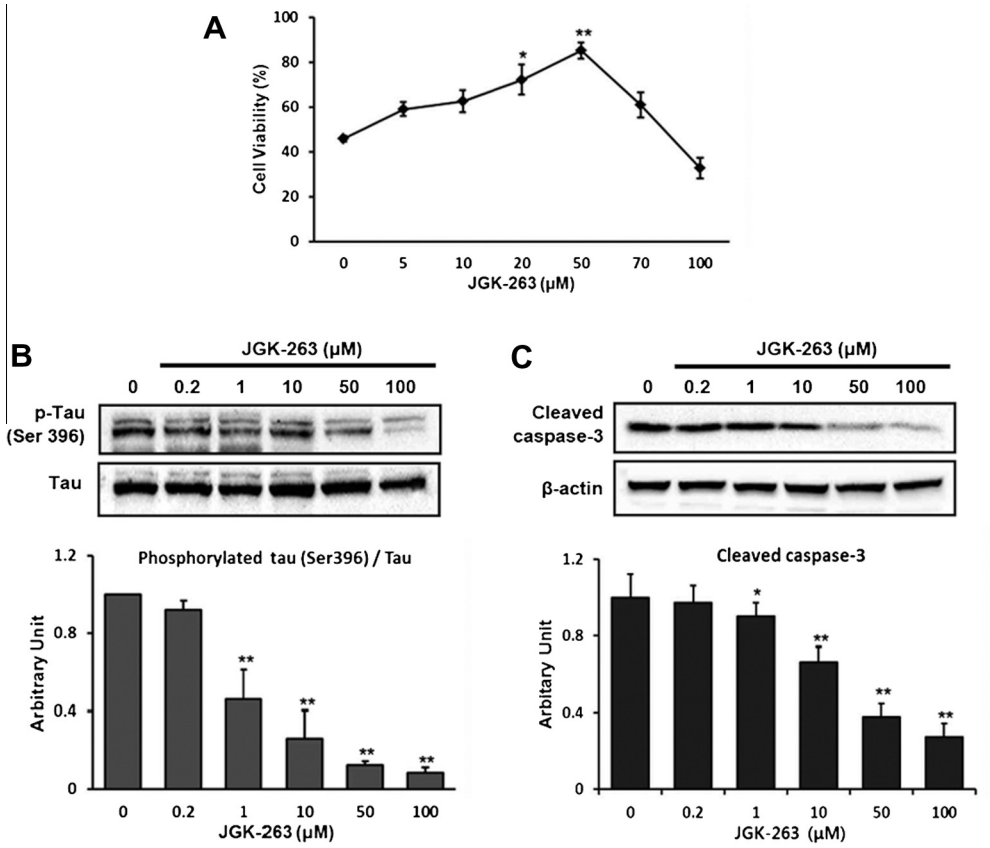

Fig. 1. Effect of JGK-263 on cell viability and late apoptosis in serum-deprived NCS-34 cells. (A) CCK-8 assay after JGK-263 treatment of 48 h serum-deprived NSC-34 cells. Cell viability after treatment of cells with each JGK-263 concentration were determined and are shown as mean (% of the cell viability in normal condition) ± SE. The protective effect peaked after treatment with 50 μM JGK-263; however, above 100 μM, these protective effects were reduced. (B) JGK-263 activity was indirectly measured by measuring the immunoreactivity ratio of phosphorylated tau (ser396) to total tau after JGK-263 treatment. NSC-34 cells were incubated in serum-deprived media with or without JGK-263 (0, 0.2, 1, 10, 50, 100 μM). Western blotting results for the phosphorylated tau (ser396) and total tau following treatment with each concentration is indicated. As the dose of JGK-263 increased, the immunoreactivity ratio of phosphorylated tau (ser396) to total tau decreased. Quantitative data of the immunoreactivity ratio is presented as arbitrary units. (C) NSC-34 cells in late apoptosis were indirectly assessed by evaluating the change in cleaved caspase-3 signaling by Western blotting. β-actin was used as the loading control. Quantitative data of cleaved caspase-3 immunoreactivity is expressed in arbitrary units, normalized to the loading control. Significant reductions in cleaved caspase-3 signals were noted. Data shown represent the mean ± S.E. of three independent experiments performed in triplicate. $^{*}p < 0.01$, $^{**}p < 0.001$ compared with cells treated with serum-deprivation only.

Furthermore, to identify changes in the upstream components of the extrinsic pathway, represented by Fas–FADD interaction, we performed immunoprecipitation. We immunoprecipitated NSC-34 cell lysates with Fas antibody, followed by Western blotting using a FADD antibody as probe; we confirmed that Fas–FADD interactions were significantly decreased in cells treated with JGK-263 (10–100 μM; Fig. 2C).

When cells were treated with JGK-263, cleaved caspase-8 decreased in a dose-dependent manner. Cleaved caspase-8 was significantly decreased after treatment with 50 μM (0.7 ± 0.06, $p < 0.01$) and 100 μM (0.67 ± 0.05, $p < 0.01$) JGK-263 (Fig. 2D).

Cytochrome c is one of the central mediators of the mitochondrial/intrinsic apoptotic pathway. As shown in Fig. 2E, treatment of NSC-34 cells with JGK-263 significantly decreased levels of cytochrome c in the cytosolic fraction in a dose-dependent manner.

Motor neurons are classified as type III cells. In type III cells, Fas-triggered cell death requires not only the classical caspase-8 activation, but also concomitant activation of the Daxx–p38–neuronal nitric oxide synthase (NOS) loop [12]. Some studies have reported that GSK-3β inhibitor treatment promotes death receptor-mediated apoptosis. Therefore, to identify changes upstream of the extrinsic pathway, represented by Fas–Daxx interaction, we performed immunoprecipitation. By means of immunoprecipitation of NSC-34 cell lysates with Fas antibody, followed by Western blotting using Daxx antibody as probe, we confirmed that Fas–Daxx interactions were significantly decreased in the JGK-263-treated cells compared with non-treated cells. Treatment with increasing concentrations of JGK-263 upto 10 μM decreased Fas–Daxx interaction significantly. Treatment with 50 or 100 μM JGK-263 resulted in an increase in Fas–Daxx interaction (Fig. 3A).

Thereafter, we determined that JGK-263 treatment induced a decrease in the level of p38α, Daxx downstream signal, under serum-deprivation. When cells were treated with JGK-263, p38α decreased in a dose-dependent manner. Levels of p38α were significantly decreased after treatment of cells with 1 μM (0.69 ± 0.07, $p < 0.01$) and 100 μM (0.64 ± 0.09, $p < 0.01$) JGK-263 (Fig. 3B). p38α, which is downstream of the motor neuron-specific extrinsic apoptosis pathway, was unchanged. Dose-dependent decrease of p38α was independent of extrinsic apoptosis pathway Fas–Daxx interaction in the JGK-263-treated cells.

These results clearly demonstrate that this effect was not influenced by factors in the downstream region of the extrinsic apoptosis pathway. These data also indicate a significant effect of JGK-263 in the Daxx–p38α extrinsic apoptosis pathway in motor neuron cells.

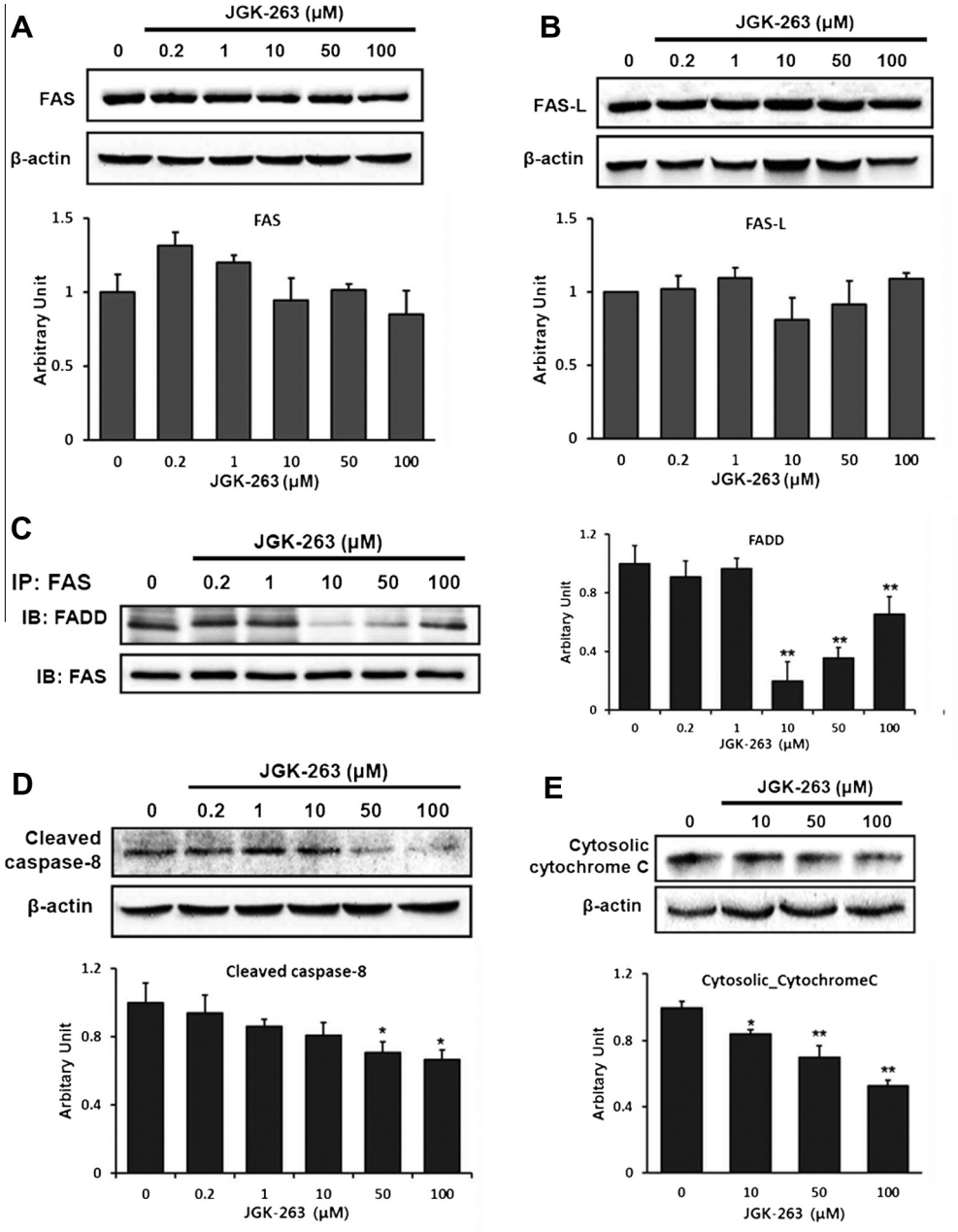

Fig. 2. Change in the classical FADD–caspase 8 extrinsic apoptosis pathway markers by JGK-263 treatment. (A, B) Fas, Fas ligand, the first step of the classical FADD–caspase 8 extrinsic pathway, did not show any significant changes in immunoreactivity in response to different JGK-263 concentrations. (C) By means of immunoprecipitation of NSC-34 cell lysates with Fas antibody, followed by Western blotting and probing with an anti-FADD antibody, interaction of Fas–FADD were found to be significantly decreased in the JGK-263 treated cells (10, 50 μM). Data shown represent the mean ± S.E. of 3 independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.001$ compared with cells treated with serum-deprivation only. (D) Immunoreactivity of cleaved caspase-8 decreased in dose-dependent manner. Data shown represent the mean ± S.E. of 3 independent experiments performed in triplicate. $^{*}p < 0.01$ compared with cells treated with serum-deprivation only. (E) Cytosolic cytochrome *c*, showed similar changes as seen in cleaved caspase-3. Significant reductions in cytosolic cytochrome c signals were noted to occur in a dose-dependent manner. Data shown represent the mean ± S.E. of three independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.001$ compared with cells treated with serum-deprivation only.

4. Discussion

The present study showed that treatment of motor neuron cells with JGK-263, a new GSK-3β inhibitor, reduced serum deprivation-induced apoptosis by inactivating the extrinsic apoptosis pathway. These results suggested that JGK-263 has a neuroprotective effect through modulating of the extrinsic apoptosis pathway in motor neuron degeneration.

Apoptosis can be processed in two different ways: one entails activation of the intrinsic apoptosis pathway, which brings the mitochondrial disruption, and the other is by initiating the death receptor-mediated extrinsic apoptosis pathway. Interestingly, JGK-263 is a unique compound that acts effectively in both apoptosis pathways. However, in a previous report, GSK-3 was shown to promote cell death caused by the mitochondrial intrinsic apoptotic pathway, but inhibits the death receptor-mediated extrinsic apoptotic pathway. Consequently, inhibitors of GSK3 provide protection from intrinsic apoptosis signaling, but potentiate extrinsic apoptosis signaling [14]. In most previous reports, investigations of GSK-3 inhibitor focused on the protective effect on intrinsic apoptosis, and suggested this as a possible therapeutic approach for many neurodegenerative diseases, such as ALS [8,20,21]. Only a few evaluated changes in extrinsic apoptosis signaling after artificial GSK inhibition, and none considered the effect of GSK-3β

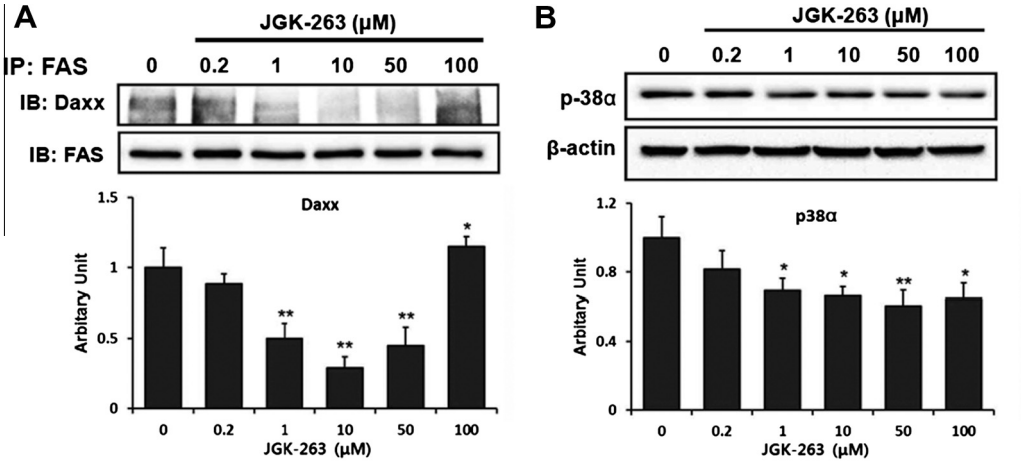

Fig. 3. Daxx–p38a extrinsic apoptosis pathway is inactivated by JGK-263 treatment. (A) By means of immunoprecipitation of NSC-34 cell lysates with an anti-Fas antibody, followed by Western blotting and probing with an anti-Daxx antibody, interaction of Fas–Daxx were significantly decreased in cells treated with JGK-263 from 1 μM to 50 μM. Data shown represent the mean ± S.E. of 3 independent experiments performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.001$ compared with cells treated with serum-deprivation only. (B) Significant reductions in p38α signals were noted to occur in a dose-dependent manner. Data shown represent the mean ± S.E. of three independent experiments performed in triplicate. $^{*}p < 0.01$, $^{**}p < 0.001$ compared with cells treated with serum-deprivation only. These data indicated that JGK-263 has some significant effect on the Daxx–p38α extrinsic apoptosis pathway in motor neuron cells.

inhibitor on the extrinsic apoptosis. Extrinsic apoptosis signaling is associated with death receptors, which are a subset of the tumor necrosis factor (TNF) family. Well-known death receptors and their specific ligands are TNF R1–TNF, Fas–Fas ligand, DR4/DR5–TRAIL [14]. Activated death receptors, such as Fas, react with the cytoplasmic protein FADD and pro-caspase-8 to form the death-inducing signaling complex (DISC).

In type III cells, such as motor neurons, Fas-triggered cell death requires not only the classical caspase-8 activation, but also requires that the Daxx–p38–neuronal nitric oxide synthase (NOS) loop be activated concomitantly [12]. A recent study showed that the extrinsic apoptosis pathway plays an important role in motor neuron degeneration [12–14], but the effect of GSK-3β inhibition on type III motor neurons has not been reported. Here, we described the alteration of extrinsic apoptosis components, including Fas, FasL, caspase-8, p38α, and Fas–FADD and Fas–Daxx interaction. In the absence of serum, treatment with different doses of JGK-263 did not influence the death receptor Fas and FasL. However, the amount of cleaved caspase-8 was decreased in a dose-dependent manner. The relative immunoreactive ratio of p38α, Fas–FADD, and Fas–Daxx interaction, determined by Western blotting and immunoprecipitation, were decreased after treatment with specific doses of JGK-263. These changes of extrinsic markers according to JGK-263 concentration may provide some explanation for the results of the viability assay. Contrary to most of the previous studies of GSK-3β inhibitors on the type I extrinsic pathway, above 10 μM, JGK-263 inhibits the Fas–FADD interaction, which is in the upstream regions of the extrinsic apoptosis pathway, even though the inhibitory effect of JGK-263 is lost at 100 μM. However, cleaved caspase-8, which is in the downstream region of the extrinsic apoptosis pathway, decreased in a dose-dependent manner. Our findings clearly indicate that this effect was not influenced by the downstream regions of the extrinsic apoptosis pathway.

Similarly, a dose-dependent decrease of p38α was independent of Fas–Daxx in the extrinsic apoptosis pathway in JGK-263-treated cells. This indicates that this effect was not influenced by the downstream regions of the extrinsic apoptosis pathway. These data indicate a significant effect of JGK-263 in the Daxx–p38α extrinsic apoptosis pathway in motor neuron cells.

In a previous report, GSK-3 promoted cell death caused by the mitochondrial intrinsic apoptotic pathway, but inhibits the death receptor-mediated extrinsic apoptotic pathway [14]. Therefore, it is important to inhibit GSK-3 selectively and the net effect of the GSK-3 inhibitors. Our results showed that JGK-263 inhibits the downstream section of the extrinsic apoptosis pathway. Additionally, as shown in Fig. 2E, treatment of NSC-34 cells with JGK-263 significantly decreased levels of cytochrome c in the cytosolic fraction. Cytochrome c is one of the central mediators of the mitochondrial/intrinsic apoptotic pathway. The findings of this study show that JGK-263 does not have a net extrinsic apoptosis pathway-related effect on motor neuron apoptosis.

The limitation of this study is that we did not reveal the effect of JGK-263 on the extrinsic apoptosis pathway. However, GSK-3 is involved in a number of central intracellular signaling pathways, including cellular proliferation, migration, inflammation, immune responses, glucose regulation, and apoptosis [22]. Due to its involvement in a great number of signaling pathways, JGK-263 may have positive therapeutic effects on motor neurons.

In this study, we investigated the working of extrinsic apoptosis pathway by using JGK-263 treatment in motor neuronal cells. JGK-263 may be effective in preventing motor neuron cell death, in contrast to previously reported results. The present study suggests that JGK-263 has a neuroprotective effect involving the inactivation of extrinsic apoptotic pathways in motor neuron cells, and may be a novel promising therapeutic agent.

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