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Original Research

Effects of C-Jun N-terminal kinase on Activin A/Smads signaling in PC12 cell suffered from oxygen-glucose deprivation

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Abstract: Activin A (Act A), a member of transforming growth factor-β (TGF-β) superfamily, is an early gene in response to cerebral ischemia. Growing evidences confirm the neuroprotective effect of Act A in ischemic injury through Act A/Smads signal activation. In this process, regulation networks are involved in modulating the outcomes of Smads signaling. Among these regulators, crosstalk between c-Jun N-terminal kinase (JNK) and Smads signaling has been found in the TGF-β induced epithelial-mesenchymal transition. However, in neural ischemia, the speculative regulation between JNK and Act A/Smads signaling pathways has not been clarified. To explore this issue, an Oxygen Glucose Deprivation (OGD) model was introduced to nerve-like PC12 cells. We found that JNK signal activation occurred at the early time of OGD injury (1 h). Act A administration suppressed JNK phosphorylation. In addition, JNK inhibition could elevate the strength of Smads signaling and attenuate neural apoptosis after OGD injury. Our results indicated a negative regulation effect of JNK on Smads signaling in ischemic injury. Taken together, JNK, as a critical site for neural apoptosis and negative regulator for Act A/Smads signaling, was presumed to be a molecular therapeutic target for ischemia.

Key words: JNK, Activin A, Smads, Oxygen–Glucose Deprivation (OGD).

Introduction

With the introduction of recombinant tissue-type plasminogen activator (rt-PA) and endovascular intervention, therapies for ischemic stroke have progressed greatly in recent years (1,2). However, these therapies are only applicable to a small percentage of stroke patients for a narrow therapeutic time window (3,4). Therefore responded endogenous neuroprotective process to ischemic injury is still attracting widespread interests in the fields of stroke therapy (5). Multiple cellular signaling pathways have been identified in this process, providing molecular targets for therapeutic strategies (6,7).

Growing evidences indicate that Activin A (Act A), a member of the transforming growth factor-β (TGF-β) superfamily, plays a neuroprotective role through Smads-dependent signal activation (8,9). Studies on Act A/Smads signaling have attracted much attention in cerebral ischemia, neuronal differentiation and brain development (10-12). In these processes, the elaborate network of regulators are involved in modulating the inputs, activity, and outcomes of Act A/Smads signaling (13). Some of these regulatory mechanisms have been uncovered, such as follicle-stimulating hormone and Smad anchor for receptor activation (14,15). However, multiple regulations and crosstalk mechanisms of Act A/Smads signaling are still not fully understood. C-Jun N-terminal kinase (JNK), as a member of mitogen activated protein kinase (MAPK) family, is one of these regulatory factors. Crosstalk between JNK and Smads signaling has been found in the TGF-β induced epithelial-mesenchymal transition and carcinogenesis (16,17). But the speculative regulation between JNK and Act A/Smads signaling has not been clarified in ischemic injury.

To address this issue, an Oxygen Glucose Deprivation (OGD) model was introduced to nerve-like PC12 cells in this study. Results showed that OGD injury could induce JNK signal activation, which was suppressed by Act A addition. Moreover, JNK inhibitor (SP600125) administration could promote Smads signal activation through enhanced Smad3 phosphorylation and Smad4 nuclear translocation. Meanwhile, the apoptosis and death rate of cells suffered from OGD injury reduced with SP600125 addition. These results indicated that JNK could negatively regulate Smads signal activation in ischemia. Therefore, JNK, as a negative regulator for Act A/Smads signaling and critical site for neural apoptosis, may serve as a novel molecular target for ischemic therapy.

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Materials and Methods

Cell culture, differentiation and treatment conditions

Rattus PC12 pheochromocytoma cells were purchased from Cell Bank of the Chinese Academy of Sciences and cultured as previous described (18). After attachment, they were treated with serum-free Dulbecco modified Eagle medium (DMEM) containing nerve growth factor (NGF 2.5S; Promega, Madison, WI, USA) (50 ng/ml) for 7 days to generate nerve-like PC12 cells (18), which was used as Control group in this study. Unless specific notification, all experiments were performed with nerve-like PC12 cells treated as above.

After differentiation, PC12 cells were incubated in DMEM containing 50 ng/ml Act A (Sigma, New York, NY, USA) for 24 h (12). Then they were exposed to OGD with the same concentration of Act A and named as Act A group. While cells without Act A exposure were named as Blank group. SP600125 (BioSource International, Camarillo, CA, USA), the chemical inhibitor of JNK, could inhibit JNK phosphorylation by competing with the ATP-binding site (19). It was added 30 min before OGD exposure at 10 μM with 0.2 % Dimethyl Sulphoxide (DMSO) and maintained at the same concentration during OGD (20). Then nerve-like PC12 cells with SP600125 administration were treated as Inhibitor group. Cells with the same amount of DMSO addition were taken as DMSO group. To establish the OGD model, cells were explored to glucose free DMEM containing 1 mM NaS_2O_4 in hypoxic conditions (37 °C, 5 % CO_2 and 95 % N_2) for 0, 1, 3 h, after carefully washed with glucose-free DMEM for three times (21).

Cell Survival Rate Assay

Cells were seeded in a 96-well plate in 1×10^4 per well and treated with OGD for 0, 1 and 3 h. Every subgroup had 3 parallel wells and the blank wells worked as control. At the end of OGD exposure, fresh DMEM culture containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 20 μl, 5 mg/mL in PBS, Sigma, St. Louis, MO, USA) was added into each well and incubated for 4 h. Then the media were removed before DMSO (100 μl) was added into each well. After carefully shaken the plate for 5 min, the optical absorbance (*A*) of each well was read using a Universal Microplate reader (Bio-TEK Instrument.inc.) spectrophotometer at 490 nm. The percentage of survival cells in each group was calculated as follows: (*A* of OGD X h experimental subgroup-*A* of blank control subgroup)/(*A* of OGD 0 h subgroup from the corresponding group-*A* of blank control subgroup) × 100 %.

Western blot analysis

For protein analysis, cells were lysed using total lysis buffer RIPA (Beyotime Institute of Biotechnology, P0013B, China) with 1 % (v/v) protease and phosphatase inhibitors. Protein concentrations in the supernatant were estimated by BCA protein assay (Pierce; Rockford, IL). After diluted with RIPA, samples were boiled at 100 °C for 5 minutes in sodium dodecyl sulfate (SDS) and then separated by SDS-PAGE. Bands were electroblotted onto Amersham Hybond-P polyvinylidene difluoride transfer membranes and further processed according to standard Western protocols. The following primary antibodies were used: mouse monoclonal to β-actin (Santa, SC-47778, 1:1000, 43KDa), mouse monoclonal to Act A (Abcam, ab89307, 1:500, 56 KDa), rabbit polyclonal to JNK1 (Abcam, ab10664, 1:1000, 48KDa), rabbit polyclonal to JNK1-phosphoT183+T183 (Abcam, ab18680, 1:1000, 48KDa), rabbit polyclonal to Smad3 (ThermoFisher scientific, PA5-17378, 1:1000, 52KDa), rabbit monoclonal to Smad3-phosphoS423+425 (ThermoFisher scientific, MA5-14936, 1:1000, 52KDa). Protein bands were visualized using enhanced chemical fluorescence (Amersham Pharmacia Biotech, Buckinghamshire, England), analyzed by ImageJ software. Each experiment was done at least three times independently.

Immunofluorescence

Cells seeded on coverslips were fixed with 4 % paraformaldehyde and permeabilized with 0.3 % Triton-100/PBS. Then they were blocked in 2 % bovine serum albumin and incubated in rabbit anti-Smad4 antibody (Abcam, ab40759, 1:100) at 4 °C overnight before treated with Cy3 marked goat anti-rabbit IgG (Boster, Wu Han, BA1032, China, 1:50) and DAPI (Roche, Mannheim, No. 10236276001, Germany, final concentration: 1μg/ml). At last they were dehydrated by gradient alcohol by turns and observed under a confocal laser scanning microscope (CLSM, Olympus Life Science Europa GmbH, Hamburg, Germany) at 554 nm (Cy3 marked red fluorescence detection) and 340 nm (DAPI). The fluorescence intensity was evaluated by biological fluorescence image analysis software (Olympus Fluoview Ver.2.1a). The immunofluorescence staining of microtubule-associated protein 2 (MAP2) in nerve-like PC12 cells was also carried out following the instruction above, with rabbit anti-MAP2 antibody (Abcam, ab32454, 1:200) and FITC marked goat anti-rabbit IgG (Boster, Wu Han, BA1105, China, 1:50). After staining, it was observed by a fluorescence microscope equipped with a photomicrograph system.

Cell Apoptosis Tested by Flow Cytometry

After treated with OGD injury, nerve-like PC12 cells were collected and washed with cold PBS (4 °C). Then cells were suspended with 500 μl Binding Buffer, and stained by 5 μl Annexin V-FITC and 5 μl Propidium Iodide (Kaiji Bio Co., Nanjing, China) for 10 min at room temperature avoiding light. At last they were analyzed using flow cytometry. All reactions were done in triplicate.

Statistical analysis

SPSS 10.0 (Chicago, IL, USA) was used for statistical analysis. Results were presented as mean values ± S.E. of (n) determinations, which were derived from three to five independent experiments. The significance of difference between means was assessed by Student's *t*-test (single comparisons) or by ANOVA (for multiple comparisons), with $P<0.05$ considered statistically significant.

Results

NGF exposure induced PC12 cell differentiation

To confirm the neural characteristics of PC12 cells

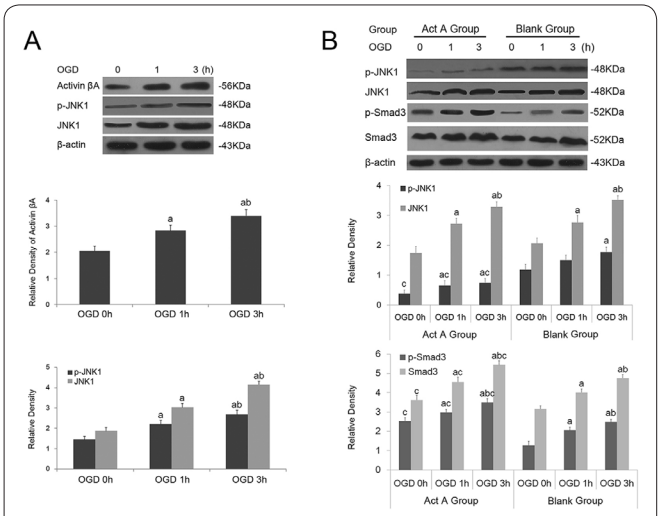

Figure 1. Act A administration reduced JNK signal activation. (A) Increased Act A and JNK1 expression in response to OGD injury. (B) Act A addition enhanced Smad3 expression and phosphorylation, but reduced the level of phosphorylated JNK1. Immunoblots for β-actin were performed as a loading control. Values were mean ± SEM. (n≥3/group, [a] $P<0.05$ versus OGD 0 h group, [b] $P<0.05$ versus OGD 1 h group in A; [a] $P<0.05$ versus the Blank or Act A group-OGD 0 h, [b] $P<0.05$ versus the Blank or Act A group-OGD 1 h, [c] $P<0.05$ versus the corresponding Blank group in B).

after NGF exposure, the morphological changes of cells were observed, as well as the fluorescence intensity of MAP2. It was apparent that with NGF treatment PC12 cells stopped proliferation and began to differentiate into nerve cells. Normal PC12 cells were hemi-suspending with a round or polygonal shape in small clumps. After NGF (50 ng/ml) exposure for 7 days, they stopped proliferation and began to adhere and extend branching varicose processes similar to neurons in primary cell culture as described in the previous study (18). Immunocytochemical analysis showed that the nerve-like PC12 cells were immunopositive for MAP2, which is a cytoskeletal marker, mainly locating in neurons. That means NGF-induced differentiated PC12 cells were capable to simulate nerve cells in *vitro*.

Ischemic injury induced JNK signal activation

To explore the regulation mechanism of JNK on Act A/Smads signaling in ischemia, the expressions of JNK1 and Act A were first explored in response to OGD injury. On the whole, Act A and JNK1 were both early activated in nerve-like PC12 cells subjected to OGD injury. As shown in Figure 1-A, the level of Act A protein increased with the extension of OGD injury. JNK phosphorylation also occurred at the early time of OGD (1 h). At OGD 3 h, the levels of total and phosphorylated JNK1 expression increased by 123% and 85%, compared to that at OGD 0 h.

Act A administration reduced JNK pathway in ischemia

Since OGD injury could elevate Act A expression and JNK1 phosphorylation, the regulation mechanism between JNK and Act A/Smads signal was further detected with Act A addition. As shown in Figure 1-B, in the Blank group, OGD injury induced Act A/Smads and JNK signal activation through increased Smad3 and JNK1 phosphorylation. With Act A administration, the levels of total and phosphorylated Smad3 were higher than that in the Blank group before and after OGD exposure. Although the levels of total JNK1 in the Act A group were almost the same as that in the Blank group, the expression of phosphorylated JNK1 reduced in response to Act A addition before and after OGD injury. These results suggested that Act A addition could suppress JNK1 phosphorylation in response to OGD injury.

JNK inhibition enhanced Smads signal activation

To explore the effect of JNK on Smads signaling, the activation of JNK was suppressed through JNK inhibitor SP600125 administration (Figure 2-A), which could inhibit JNK phosphorylation by competing with the ATP-binding site (19). In general, JNK inhibition could promote Smads signal activation through the enhanced fluorescence intensity of Smad4 in nuclei and the increased expression of phosphorylated Smad3. The strength of Smads signaling can be seen in Figure 2 through the distributions of Smad4 in nuclei and the level of phosphorylated Smad3 (22). Under a CLSM, red color represents Smad4 stained with Cy3 marked antibody, and the nucleus of cells appeared blue stained with DAPI. In the Inhibitor group-OGD 0 h, the fluorescence intensity of Smad4 in nuclei was stronger than that in the corresponding DMSO group. After OGD treatment, it gradually increased, which was significantly high in the Inhibitor group-OGD 3 h, compared to that in the

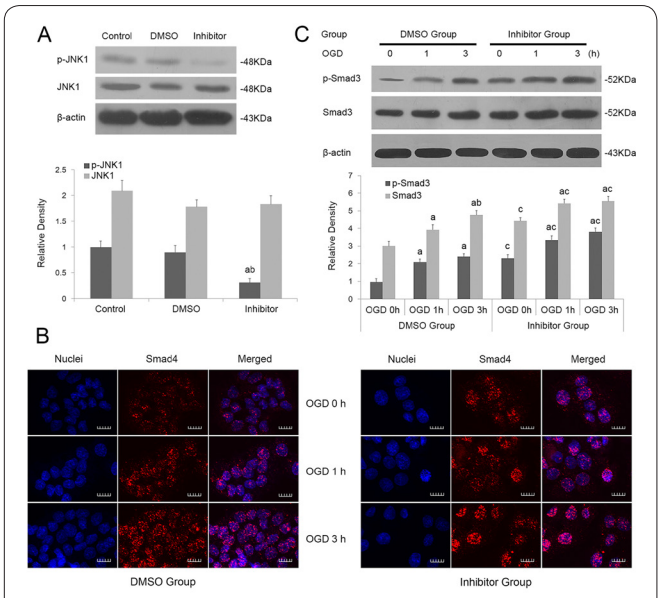

Figure 2. JNK inhibition promoted Smads signal activation. (A) JNK inhibitor (SP600125) administration suppressed JNK1 phosphorylation. (B) JNK inhibition enhanced Smad4 nuclear translocation in nerve-like PC12 cells. Cells stained with anti-Smad4 (red) and DAPI (nucleus, blue) were observed by a confocal laser scanning microscope. In the Inhibitor group, the fluorescence intensity of Smad4 in nuclei was much higher than that in the corresponding DMSO group before and after OGD injury. *Scale bars*=10 μm. (C) JNK inhibition promoted Smad3 expression and phosphorylation. Immunoblots for β-actin were performed as a loading control. Values were mean ± SEM. (n≥3/group, [a] $P<0.05$ versus the Control group, [b] $P<0.05$ versus the DMSO group in A; [a] $P<0.05$ versus the DMSO or Inhibitor group-OGD 0 h, [b] $P<0.05$ versus the DMSO or Inhibitor group-OGD 1 h, [c] $P<0.05$ versus the corresponding DMSO group in C).

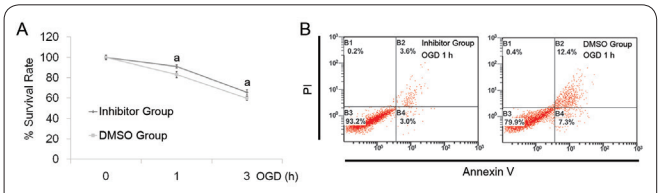

Figure 3. JNK inhibition promoted neural survival in response to OGD injury. (A) Alleviated neural death in response to SP600125 addition. Values were normalized to incorporation in the 0 h of OGD treatment in each group. Bars indicated values which were the mean ± SEM from three independent experiments. (B) JNK inhibition mitigated neural apoptosis after 1 h of OGD exposure. (n≥3/group, [a] $P<0.05$ versus DMSO group with the same time of OGD exposure in A).

DMSO group-OGD 3 h. Besides, the levels of total and phosphorylated Smad3 gradually increased with the extension of OGD injury. In the Inhibitor group, they were higher than that in the corresponding DMSO group before and after OGD exposure. These results indicated that JNK pathway could negatively regulate responded Smads signal activation after ischemia.

Inhibition of JNK signaling promoted neural survival

To identify the biological effect of JNK signaling on cerebral ischemia, MTT assay was performed to nerve-like PC12 cells pre-treated with JNK inhibitor SP600125. It was evident that JNK inhibition could attenuate cell death and apoptosis after OGD injury. As shown in Figure 3-A, the survival rates of cells decreased with the extension of OGD injury. In the Inhibitor group, they were slightly higher than that in the DMSO group. Since the disparities of survival rates in two groups were obvious at OGD 1 h compared to at OGD 3 h, the neural apoptosis at OGD 1 h was further detected by flow cytometry. Compared to the DMSO group, JNK inhibition mitigated the injury induced cellular apoptosis in the Inhibitor group (Figure 3-B).

Discussion

As a member of TGF-β superfamily, Act A has been found to play the neuroprotective role through Act A/Smads signaling after the injury-induced endogenous Act A elevation (9,12). In the canonical Act A/Smads signaling, extracellular Act A activates intracellular Smad2/3 through binding to its surface receptors (23). Then the phosphorylated Smad2/3 assembles heterodimer complexes with Co-Smad (Smad4), translocates to nuclei and takes part in the regulation of target genes through DNA binding (24). The Act A responses are not solely the result of the Smads cascade activation, but interactions and regulations of Smads signaling with a variety of other signal pathways (13). They control Act A signaling at multiple levels, including accessibility of Act A to the receptors, activation of receptors, localization and activation of Smads, and regulation of Smads-dependent transcription (11). However, the elaborate network of regulators has not been fully understood in neural ischemia.

JNK, as a member of MAPK family, is one of these regulators. The MAPK family consists of several subfamilies, among which p38 and extracellular signal-regulated kinase1/2 (ERK1/2) could also be activated by ischemia and geared towards apoptosis and cell survival respectively (25). It was reported that MAPK/ERK pathway involved in preconditioning induced brain protection in a global ischemia (26). While JNK was first found to promote neural apoptosis in ischemic stroke and neural degenerative disease (27,28). JNK1 is the major isoform responsible for the high level of JNK activity in the brain. It is phosphorylated and activated by external noxious stimuli through a kinase cascade (29,30). Activated JNK1 phosphorylates c-Jun and then increases the transcription activity of the activator protein-1 (AP-1) complex (31,32). After translocating to the nucleus, phosphorylated c-Jun further promotes neural cell death through binding to DNA and regulating gene transcription in *vivo* and *vitro* (33,34). These data illustrate the pro-apoptosis effect of JNK signaling. Recently, the effect of JNK pathway has been extended to crosstalk with Smads signaling. In rat hepatic stellate cells, JNK participated in TGF-β induced Smad2/3 phosphorylation after acute liver injury (35). While c-Jun, as the downstream of JNK pathway, was found to act as a Smad3 co-repressor and control TGF-β responses through a negative feedback loop in human hepatoma cell line HepG2 (36). These results suggested that active JNK pathway may modulate Act A induced Smads signal activation in ischemia, but the speculative regulation mechanism remained unclear.

To further explore this problem, an OGD model was introduced to nerve-like PC12 cells to create an environment of ischemia in *vitro*, which neural cells in penumbra suffered from after ischemic stroke (21). As an immediate-early response of Act A/Smads signaling, the level of Act A protein increased with the extension of OGD injury (Figure 1-A). JNK signal activation also occurred at OGD 1 h and enhanced with time. But the early activated JNK signaling was reduced by Act A administration (Figure 1-B). While the kinetics of Smads signaling was enhanced in Act A group. These results suggested that Act A administration could promote Smads signal activation, but reduce JNK pathway. Further, JNK, as a downstream agent of Act A, participated in the pathological process of cerebral ischemia.

Moreover, JNK inhibition could enhance Smads signal activation. SP600125, as a derivative of anthrone, is widely used in biochemical studies as an inhibitor of JNK (37,19). With SP600125 addition, the phosphorylation of JNK was suppressed in nerve-like PC12 cells (Figure 2-A). Since Smad4 was required for the functional formation of transcriptional complexes with phosphorylated Smad2 or Smad3 and participated in DNA binding after translocating into nuclei (22). The distributions of Smad4 in nuclei were observed to quantify the strength of Act A/Smads signaling, together with the level of total and phosphorylated Smad3. In the Inhibitor group, the fluorescence intensity of Smad4 in nuclei and the level of phosphorylated Smad3 were both higher than that in the corresponding DMSO group before and after OGD treatment. These results indicated that JNK pathway could negatively regulate the strength of Smads signaling at both resting and activation stages.

On the one hand, within certain time, the strength of JNK pathway increased with the extension of OGD injury (Figure 1). That early activated and dynamically

changed JNK pathway would restrict the cascade reaction of Smads signaling. On the other hand, the expression of Act A increased in response to OGD injury. Even though the enhanced Smads signal activation in response to increased Act A expression was in agreement with the previous study and has been well established as receptor-dependent Smads activation (9,12). The elevated Smads signal activation in this study could also be associated with the reduced kinetics of JNK pathway due to the increased Act A expression, considering the negative regulation effect of JNK on Smads signaling found in this study. Therefore, Act A, as an upstream of both JNK and Smads pathways, complicated the negative regulation mechanism of JNK on Smads signaling in ischemia. Meanwhile, JNK may accurately modulate Act A/Smads signaling in ischemia due to the uniform extracellular signal derived from Act A.

Since the role of MAPK cascades in regulating neural death and survival after ischemia depend on the types of cells, magnitude and timing of insults (38). The effect of JNK on PC12 cells was explored through JNK inhibitor (SP600125) addition. As shown in Figure 3, SP600125 administration reduced the death rate and apoptosis of cell in response to OGD injury. That means JNK inhibition could mitigate ischemic injury. Therefore, JNK was proposed to be a novel molecular target for Act A induced neuroprotection, considering its effects on cellular apoptosis and Smads signal regulation. However, the protective effect and underlying mechanisms of JNK inhibition on ischemic stroke remain to be further explored in neurovascular unit before application it in *vivo* ischemia.

Acknowledgments

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