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Full Length Research Paper

Effects of RNA interference targeting Smad7 on nerve cells ischemic injury induced in PC12 cells

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Ischemic cerebrovascular disease is a global health problem. According to the World Health Organization, ischemic stroke is actually the most common cause of death in the world. ActA/smads signaling pathways was shown to be required for the differentiation-associated physiological apoptosis of stroke. Although smad7 is an important regulator of ActA/smads signaling via a negative feedback circuit, its effects have not been well understood. This experiment primarily investigated the apoptosis in ischemic cerebral injury by targeting silence of smad7. In this study, we used nerve growth factor (NGF) and oxygen–glucose deprivation (OGD) to stimulate PC12 cells and convert them into neurons in order to establish an ischemia *in vitro* model. Combined with the small interfering technology of smad7, we also used flow cytometric (FCM) and 4,6-diamidino-2-phenylindole(DAPI) to identify apoptosis rate. The results show that OGD 16 h apoptosis rate was 25.53%, while OGD 16 h combined with sismad7 apoptosis rate was 16.76%. It was also observed that the apoptosis rate decreased in ischemic injury when sismad7 was targeted. This study therefore provides a reference for further study of ActA/smads signaling pathways on acute ischemic brain damage.

Key words: Oxygen–glucose deprivation (OGD), ischemia tolerance model, smad7, ActA/Smad pathway.

INTRODUCTION

Ischemic stroke occurs when the blood supply to the brain is obstructed. Accumulating evidence suggests that the cell death observed during the first few hours of cerebellar ischemia is a result of apoptosis as opposed to necrosis, which was considered the predominant form of cerebellar damage generated by ischemia. Moreover, effective methods of preventing and controlling ischemic cerebrovascular disease have been a topic of great interest. The ischemic damage of nerve cells leads to the disruption of a series of complex signaling pathways that produces an effect on corresponding biological functions and thus affects the function of the brain. A better understanding of the role of the signal transduction mechanisms that underlie brain ischemic injury could identify key targets for neuroprotective substances.

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Some reports suggest that smad7 can also have proinflammatory effects. Smad7 overexpression has been noted in mucosa and mucosal T cells from patients with inflammatory bowel disease, and blockade of smad7 restores TGF-β signaling and reduces proinflammatory cytokine generation in this context (Monteleone et al, 2001). Overexpression of smad7 in mouse T cells promotes airway hyperreactivity and enhances airway inflammation (Nakao et al, 2000), whereas inhibition of smad7 suppresses autoimmune encephalitis (Kleiter et al, 2007). Thus, it appears that the role of smad7 in the regulation of inflammation and immunity is complex, possibly dependent on cell lineage, context, and duration of inflammatory response, and needs to be more completely defined before over-expression of smad7 really becomes viable as a therapeutic goal.

For expounding Actin/Smads pathways about ischemic brain injury and signal transduction mechanism and looking for nerve protective substances that can be applied to clinical practice and key targets of Actin/Smads

pathways, a stable *in vitro* neuronal ischemia model had been established (Mei et al., 2011). Nerve growth factor (NGF) can induce the differentiation of PC12 cells into neuron-like cells. The sympathetic neuron-like cells are characterized by electrical excitability, expression of neuron-specific genes and neurite outgrowth (Dichter et al., 1977; Greenberg et al., 1985). PC12 cells were cultured in different oxygen conditions. The metabolic activity of PC12 cells was measured in the final 4 h prior to cellular characterization using an alamarBlue assay (Serotec) following the manufacturer's instructions (Hamid et al., 2004). A neuronal ischemia model was then obtained based on research at molecular level (Mei et al., 2011).

In this experiment, small interference targeting smad7 on *in vitro* neuronal ischemia model was used to observe the changes of cell apoptosis. This is to investigate the role of smad7 on ischemic cerebrovascular disease and signal transduction mechanism so as to look for nerve protective substances that can be applied clinically in the future and the key targets in ActA/Smads pathways. Protecting and promoting neurological recovery will bring the new hope for the treatment of ischemic cerebrovascular disease.

MATERIALS AND METHODS

Cell culture, differentiation and preparation of oxygen–glucose deprivation (OGD) model

PC12 cells were purchased from the Cell Bank of the Chinese Academy of Sciences. The cell line was maintained in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS, GIBCO), 100 IU/ml streptomycin, 100 IU/ml penicillin and 2 mmol/L L-glutamine. The cells were then treated with 100 ng/ml nerve growth factor (NGF 2.5S; Promega, Madison, WI). After 24 h, the cells were transfected with promoter constructs and cultured for an additional five days in a medium containing NGF (Mei et al., 2011). PC12 cells were treated with NGF for six days. Cells were then washed three times with DMEM, and the cells were cultured with DMEM in the presence of no sugar and 1 mmol/L NaS_2O_4 in hypoxic conditions (37°C, 5% CO_2 and 95% N_2) for 16 h (Mei et al., 2011).

Smad7 immunocytochemical analysis

The cells were fixed with 4% paraformaldehyde/phosphate buffered saline (PBS) and permeabilized with 0.1% Triton X-100 in PBS for 10 min. The cells were then incubated in 5% goat serum/PBS for 1 h at room temperature. Cells were washed again then incubated at 4°C overnight in the presence of anti-smad7 (1:1,000 dilution, Santa SC-9183). After washing twice with PBS, the cells were incubated with fluorescently labeled secondary FITC-rabbit anti-goat (Santa) for 1 h at room temperature. The results were observed by a fluorescence microscope equipped with a photo-micrograph system (Kumar et al., 2006).

Silencing of Smad7 in PC12 cells by siRNA

Sequences targeting smad7 gene were constructed using Dharmacon siDESIGN Center (Dharmacon Inc., Dallas, TX): siRNA (1435-1458 on NM 030858.1), sense 5′-AAC GAU CUG CGC UCG UCC GGC GUD TDT- 3′, antisense 3′-DTD TUU GCU AGA CGC GAG CAG GCC GCA-5′. These siRNA were used at 100 nm with concentrations standard transfection technique using oligo-fectamine (Invitrogen). As a control, we used 100 nm random siRNA.

Real-time polymerase chain reaction (RT-PCR)

Total RNA from PC12 cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc.) according to the manufacturer's instructions and was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies Inc.) at 42.8°C with random hexamer priming. An RNA control tube containing all RT reagents except the reverse transcriptase was included as a negative control to monitor genomic DNA contamination. The mRNA levels of smad7 were measured by RT-PCR using β-actin as the internal standard. The sequences of primers for smad7 and β-actin were as follows: Smad7, sense 5'-AAC CCC CAT CAC CTT AGT CG -3' and antisense 5'- TGC TCC GCA CTT TCT GTA CC -3'; β-actin, sense 5'- GAC CCC TTC ATT GAC CTC AAC -3' and antisense 5'-GAT GAC CTT GCC CAC AGC CTT -3'.

Western blot analysis

PC12 cells were treated with NGF for six days and OGD treatment was performed for 16 h after sismad7. They were washed twice with cold PBS and then 1×10^6 cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate) containing protease inhibitors (1% cocktail and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Total proteins were separated with 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked for with Tris-buffered saline with 0.1% Tween 20 (pH 7.6, TBST) for 1 h at room temperature and then immunoblotted with anti-smad7 (1:1,000 dilution, Santa SC-9183) at 4°C overnight. After washing twice with TBST, the membrane was incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Santa) for 1 h at room temperature and was washed three times with TBST. Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Biosciences, Piscataway, NJ), and the membranes were exposed to Lumi-Film Chemiluminescent Detection Film (Roche). Loading differences were normalized using a monoclonal ß-actin antibody. Quantitative analysis of Western blot data was performed by measuring the intensity of the band signals with the use of NIH Image analysis software.

Hoechst 33258/propidium iodide (PI) staining

Cell death was determined by propidium iodide (PI) and Hoechst 33258 double fluorescent staining. PC12 cells were cultured on cover slides. After injury by OGD for 16 h and followed by 24 h recovery, the cells were stained with PI (10 µg/ml) and Hoechst 33258 (10 µg/ml, Sigma, USA) and then fixed by 4% paraformal-dehyde. For each cover slide, 1000 to 1500 cells were examined under a fluorescence microscope (Figure 5) (Olympus BX51, Japan) and photographed with a digital camera (Olympus, Japan). The results were expressed as the percentages of apoptotic cells and necrotic cells, respectively.

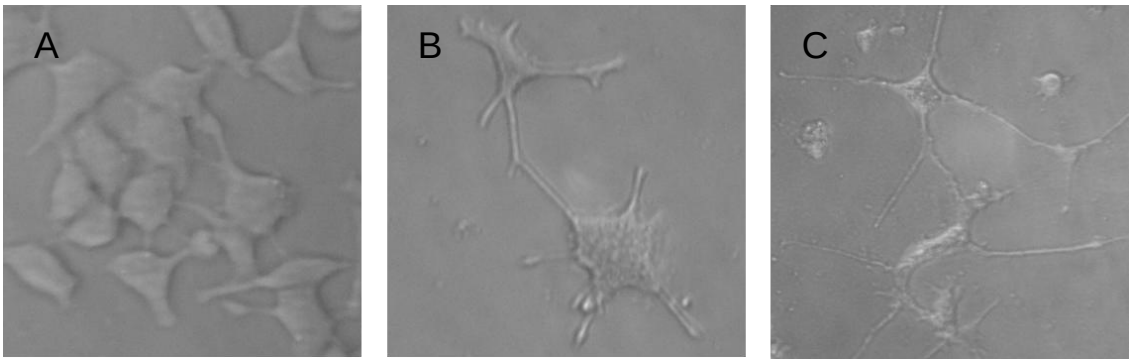

Figure 1. The morphological changes of PC12 cells (×200). Bars = 20 μM, Invert microscope, Olympus IX71, Japan.

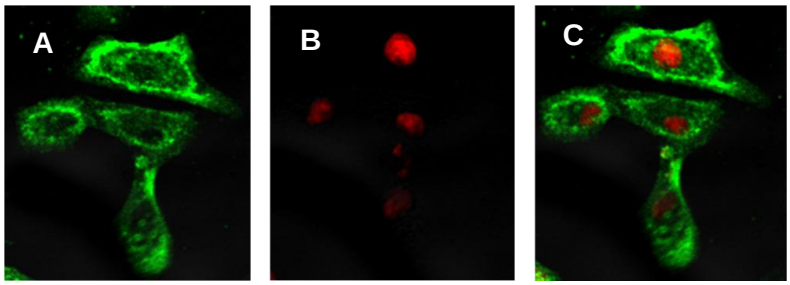

Figure 2. The morphological changes captured with fluorescence microscopy following immunofluorescence staining (×200). (A) anti-smad7 and FITC-labeled IgG staining under the blue excitation. (B) Nuclear counter staining of PI under purple excitation. (C) Merged image of the FITC and PI staining.

Flow cytometry analysis

To quantitatively assess the apoptosis, Annexin V-FITC and PI double-staining followed by flow cytometry was used. Cells (1×10^5) were harvested and stained with Annexin V-FITC and PI using a double staining kit (Kaiji Bio Co., Nanjing, China) according to the manufacturer's instructions. The cells were then immediately analyzed by flow cytometry. Signals from apoptotic cells were localized in the lower right quadrant of the resulting dot-plot graph.

Statistical analysis

SPSS software was used for statistical analyses, and values are presented as means ± SD. An ANOVA was used to compare the mean values. P-values less than 0.05 were considered to indicate statistically significant differences.

RESULTS

Morphological changes of PC12 cells

PC12 cells were pretreated with 100 ng/ml NGF for one, three or six days. The differentiated cells were photographed under a phase contrast microscope. PC12 cells were pretreated with NGF (100 ng/ml) for one day, and the differentiated cells were counted as described in Figure 1. The results show that treatment with NGF (100 ng/ml) stimulated neuron-like differentiation of PC12 cells as seen under the microscope. PC12 cells changed into neurons after one day of NGF treatment and later formed synapses. Synapses extended up the length of the cell after three days of treatment. The synaptic length increased six- to eight-fold after six days of treatment. Experiments using representative PC12 cells obtained from four cell depositories yielded essentially the same results. PC12 cells were exposed to the indicated concentrations of NGF for three days, and the differentiated cells were counted. From the results obtained, significant differences were detected.

Immunofluorescence analysis

The results (Figure 2) show that PC12 cells was cultured for three days and then assessed with smad7 immunofluorescence staining. Application of Pro Plus 6.0 software to add image fusion after confirm green fluorescent was used for PC12 cells transformation of neurons appearance cell. Smad7 immunofluorescence stain had a strongly positive expression, while PBS control had negative fluorescence.

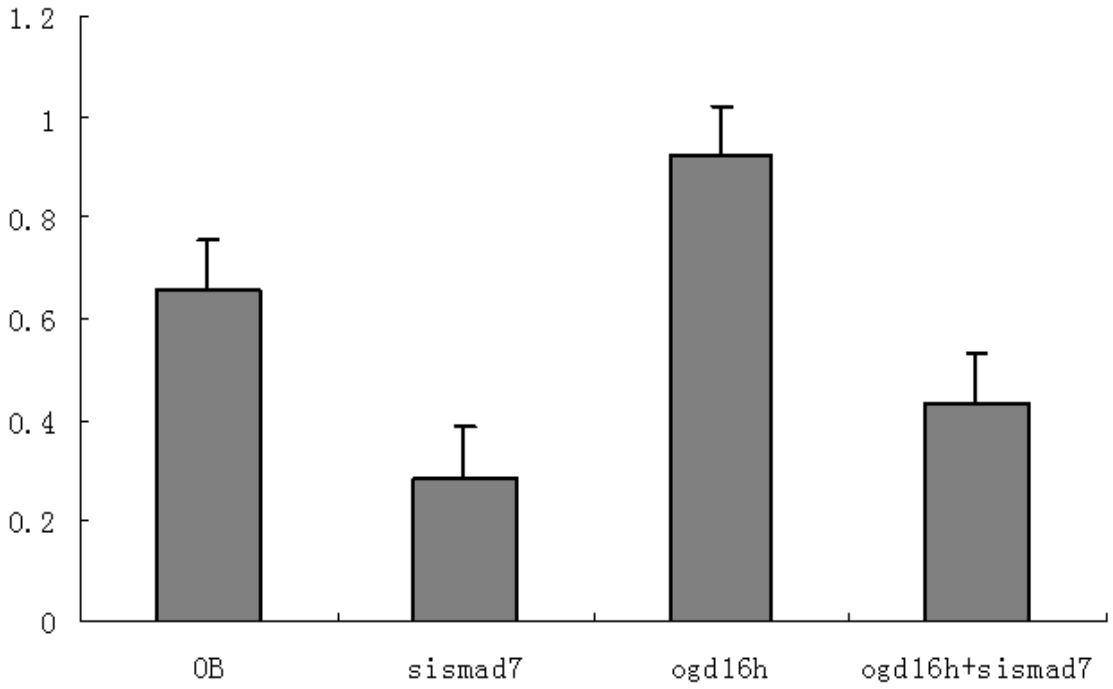

Figure 3. Smad7 mRNA changes, 1, control; 2, PC12 cells combined with sismad7; 3, OGD 16 h; 4, sismad7 combined with OGD 16 h; Smad7 mRNA of normal PC12 cells is more, OGD16h increase, OGD16h combined with sismad7 mRNA level is lower than former. The data are shown with mean ± SD from three independent experiments.

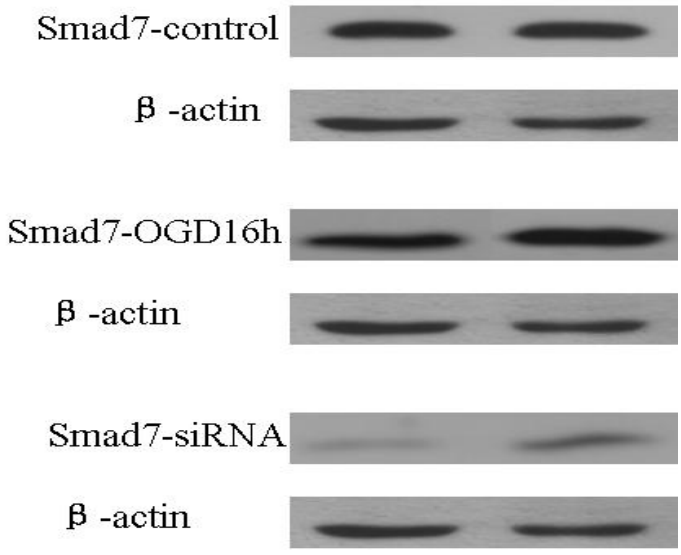

Figure 4. Expression of smad7 in PC12 cells. Equal cell lysates were separated on 10% SDS-PAGE. Smad7 protein was detected by immunoblotting with smad7 antibody.

RT-PCR

The results show that the mRNA levels of smad7 were different. Smad7 mRNA of normal PC12 cells was high, OGD16 h was the highest, while OGD16 h combined with sismad7 mRNA level was lower than former (Figure 3). The data are shown with mean ± SD from three indepen-dent experiments; *P <0.05.

Smad7 protein expression chances in PC12 cells

Western blot analysis of the effects of sismad7 treatment on smad7 expression after OGD 16 h (Figure 4) showed that OGD 16 h could decrease smad7 protein expression compared with the OGD 16 h combined sismad7. The expression of smad7 protein was significantly increased. Expression of smad7 protein in vehicle control or geniposide treated PC12 cells following normalization with a loading control (β-actin). The data are shown with mean ± SD from three independent experiments. *P <0.05.

Hoechst 33258/PI staining

The representative microphotographs show the PC12 cells as detected by PI staining after OGD-reperfusion induced injury. The representative microphotographs also showed the apoptotic cells as detected by Hoechst 33258 staining after OGD-reperfusion Induced. However, sismad7 prevented PC12 cells from OGD-reperfusion injury. The summarized data show percentage changes in the numbers of normal and apoptotic cells. Data are expressed as mean ± SD; n = 4 wells for each group;

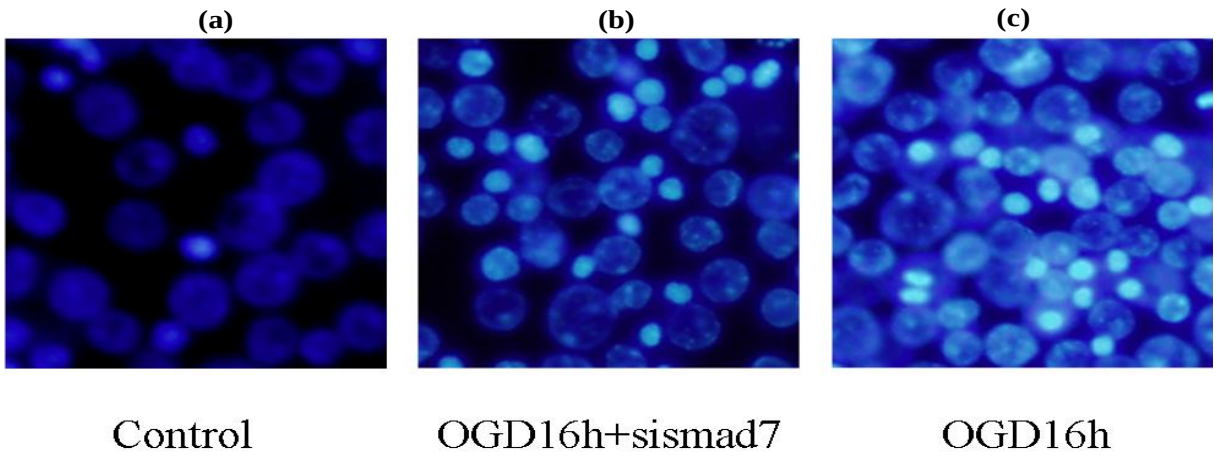

Figure 5. The cell death analyzed by double fluorescent staining with Hoechst 33258 and propidium iodide (PI). (a) Control, (b) sismad7 combined with OGD 16 h, (c) OGD 16 h.

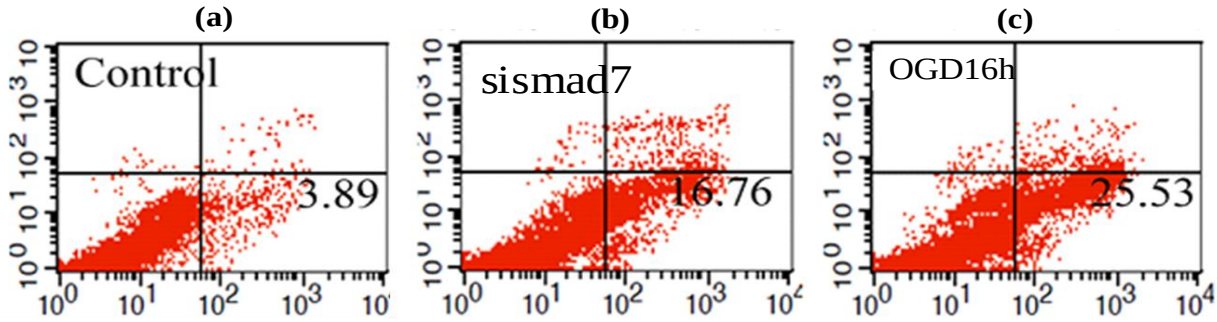

Figure 6. Flow cytometric analysis of OGD 16 h and combined with sismad7 induced apoptosis in PC12 cells. A: control, B: OGD 16 h combined with sismad7, C: OGD 16 h. Cells were treated with OGD or combined sismad7 and cultured for 16 h. The cells were then analyzed with flow cytometry following AnnexinV-FITC/PI staining.

*P<0.05 and **P<0.01, compared to control and other group.

OGD 16 h and combined with sismad7 induced apoptosis in PC12 cells

To assess the apoptosis of differentiated PC12 cells treated with OGD and sismad7, the cells were analyzed with Annexin V-FITC and PI double-staining flow cytometry. Signals from each group of cells were located in the lower right quadrant of the dot-plot graph as shown in Figure 6. Compared with the control (transfected but untreated cells), the proportions of apoptotic cells treated with OGD after 16 h, and combined sismad7 were 25.53 and 16.76%, respectively.

DISCUSSION

The I-Smad smad7, in the context of canonical TGF-β signaling, has been implicated in both inhibition (Azuma et al, 2005; Javelaud et al, 2005) and promotion (Liu et al, 2003; Halder et al, 2008) of tumorigenesis. In early tumorigenesis, it has been suggested that loss of smad7 is protective, yielding more R-Smad phosphorylation and subsequently more apoptosis and growth arrest of cancerous cells (Halder et al, 2005). In contrast, increased abundance of smad7 during late tumorigenesis can yield less R-Smad phosphorylation and consequently fewer downstream events, such as epithelial-mesenchymal transformation (Valcourt et al, 2005) and cell migration (Javelaud et al, 2005), thus providing a protective effect. Tang et al. (2008) shed new light on the role of smad7 in cancer and how its noncanonical function can promote increased cell-cell adhesion, which may potentially inhibit cancer metastasis. They demonstrated that smad7 directly interacted with β-catenin to inhibit its phosphorylation by the target kinases glycogen synthase kinase 3β (GSK-3β) and casein kinase Iα (CKIα), resulting in decreased β-catenin degradation (Tang et al, 2008). Unphosphorylated, stabilized β-

catenin complexes with E-cadherin, yields a strengthened cell-cell adhesion (Tang et al., 2008). Whether smad7 regulates the phosphorylation of other intracellular proteins remains unknown, but smad7 does interact with other proteins that can be phosphorylated, including, protein inhibitor of activated signal transducer (PIASy) and activator of transcription (STAT) (Imoto et al, 2003). Likewise, smad7 binds both the R-Smad transcriptional co-activator, p300, and its activating kinase, Akt, creating a possible scenario for smad7 to further regulate ActA/Smads signaling in a noncanonical manner. The extent to which smad7 is a general phosphorylation inhibitor is unknown; hence there is need for further studies.

The purpose for the study of the signal transduction pathways of ActA/Smads was to ascertain whether they have certain functions in specific diseases. In this study, ischemic brain injury; the function of signal transduction pathways of ActA/Smads are analyzed. Some researchers suggested that it exists as some nerve protective effects. However, little research has been done about the function on smad7 to cerebral ischemia. As a kind of signal protein, the biological function of smad7 is inhibiting ActA/Smads signaling pathways. Then the cells will have in a series of physiological and biochemical changes and produces the corresponding function. In this study, NGF stimulated PC12 cell combined with OGD was adopted to establish nerve cells ischemia model. Small interfering technology was applied to silence smad7 before nerve cells ischemia model in pc12 cells. Based on the results obtained, it was observed that the cell apoptosis rate was different. The results show that cell apoptosis rate was lower in targeted silence group of smad7 compared with that not silenced. It was therefore confirmed that smad7 is not protective in cerebral ischemia damage. On the other hand, ActA/Smads signal transduction pathways may pass antiapoptotic and generate neural protection. This mechanism involved remains to be further studied.

Conclusion

In this study, we examined the smad7-induced apoptosis of the signaling pathway of ActA/smads in ischemic stroke. Smad7 are highly sensitive to the apoptosis in cerebral ischemic injury. Our results, however, demonstrate that smad7 has no neuroprotective effects. Moreover, targeting silence of smad7 might be a valuable therapeutic approach for cerebral ischemia. Therefore, further investigation is required to fully understand the role of smad7 in post-ischemic injury, which will eventually lead to clinical interventions that will salvage brain cells that are at risk in ischemic cerebrovascular disease.

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