Suppression of c-jun influences nNOS expression in differentiated PC12 cells

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Abstract. In various animal models of central neuronal diseases, both c-jun and nNOS genes are expressed inside injured neurons; however, the mechanism of these two genes in neuronal diseases remains uncertain. Our previous studies have shown that c-jun expression always occurs prior to expression of nNOS in motoneuron injuries. We aimed to determine whether there is a correlation between c-jun and nNOS, and whether the crosstalk between these two genes regulated the pathological progression of injury-induced neuronal degeneration. In the present study, we used the neuron-like differentiated PC12 cells, which express c-jun and nNOS, to examine whether c-jun is the upstream molecule modulating nNOS expression. The c-jun small interfering RNAs (c-jun siRNA) were transfected into PC12 cells and cells were treated for 72 h *in vitro*. Western blotting and immunofluorescence were used to check the protein levels and the expression of c-jun and nNOS in differentiated PC12 cells. The results from the immunofluorescence experiments showed that the c-jun and nNOS proteins were co-expressed in the differentiated PC12 cells. The results from the western blotting experiments revealed that the protein levels of c-jun were significantly decreased by c-jun siRNA. Moreover, the nNOS protein levels were also downregulated in differentiated PC12 cells following c-jun siRNA treatment. The present study found that siRNA used against c-jun not only knocked down c-jun, but also downregulated the nNOS protein expression in differentiated PC12 cells. These results indicate that there is a functional relationship between c-jun and nNOS in differentiated PC12 cells.

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Introduction

c-Jun is a member of the AP-1 family of leucine zipper transcription factors, and it forms DNA-binding homodimers with other family members, such as jun-D, c-Fos and ATF-2. The transcription factor c-jun is a useful model used in the study of the complexity and specificity of signaling. This inducible transcription factor directs changes in gene expression in response to various extracellular stimuli (1). c-Jun is involved in cell death and survival. In particular, c-Jun has been termed 'a killer protein' for developing neurons and is regarded as essential for neuronal apoptosis via the transcriptional activation of c-jun target genes encoding pro-apoptotic proteins (2). However, c-jun also plays a protective role against programmed cell death (3). Thus, the biological role of c-Jun in regulating cell death or survival concerns the cell type and also depends on the nature of the death-inducing signal. PC12 cells are a model for dopaminergic cells with well-defined steps triggered by apoptosis stimuli (in response to metabolic, oxidative stress or apoptotic signals). Therefore, an advantage of the PC12 model over other neuronal cell lines is that it has a markedly different response to various stress stimuli. Previous studies on the function of c-Jun in PC12 cells suggested c-jun as an executor of death signals in neuronally differentiated PC12 cells, as well as in fibroblasts (4). The molecular basis for the role of c-jun in apoptosis is not fully understood. However, regulation of the c-jun gene in differentiated PC12 cells may be useful in the study of the molecular basis of the role of c-jun in apoptosis.

Nitric oxide (NO) performs a variety of physiological and pathological functions in the nervous system. NO in cells is produced by nitric oxide synthase (NOS), of which there are three isotypes: nNOS or bNOS (neuronal NOS), eNOS (endothelial NOS) and iNOS (inducible NOS). In the nervous system, NO is involved in learning and memory, neurotransmitter release, development and neuronal damage or degeneration (5). It has also been postulated that NOS levels are induced during the differentiation of PC12 cells by nerve growth factor (NGF), and nNOS and NO are required for PC12 cell differentiation (6). In NGF-treated PC12 cells, nNOS was induced at RNA and protein levels, resulting in increased NOS activity (7). Mounting evidence suggests that the crosstalk between NO/nNOS and the c-jun N-terminal kinase (JNK) signaling pathway was present in the different

neuronal cell lines and in various physiological and pathological processes (8). Subsequent to inhibition of proteasome activity, the increased nNOS protein levels correlated with activation (phosphorylation) of JNK. JNK activation was suppressed upon nNOS inhibition, thus establishing a pecking order in which nNOS upregulation and formation of reactive oxygen species are upstream of JNK phosphorylation (9). c-jun is also a substrate for a related group of JNKs (10). Therefore, a correlation has been suggested between c-jun and nNOS expression in differentiated PC12 cells *in vitro*.

In the present study, we first detected the expression of c-jun and nNOS in differentiated PC12 cells, and then a c-jun siRNA transfection strategy was used to study whether the nNOS levels in differentiated PC12 cells change following silencing of the c-jun gene.

Materials and methods

Cell culture and transfection. The differentiated PC12 cell line was purchased from the Shanghai Cellular Institute of China Scientific Academy (Shanghai, China). Differentiated PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin A and 100 U/ml streptomycin). Cells were grown to the subconfluent stage at 37˚C in a humidified atmosphere containing 5% CO_2. Prior to siRNA transfection manipulations, PC12 cells were plated ($2x10^5$ cells/dish) onto 35-mm tissue culture plates and grown in DMEM growth medium without antibiotics, since adding antibiotics to the medium during transfection reduces transfection efficiency. The PC12 cells were then randomly divided into two sets. One set of the PC12 cells was used to detect the expression of c-jun and nNOS by immunofluorescence double labeling, while the other set was treated with growth medium only (normal control), non-silencing siRNAs and c-jun siRNAs (Cell Signaling Technology, Inc., Beverly, MA, USA), respectively, to investigate the expression of c-jun and nNOS protein by western blot analysis. One day after plating, differentiated PC12 cells were transfected for 6 h with siRNA duplexes (100 nmol/l of c-jun siRNA or control non-silencing siRNA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, and the transfection medium was changed after 6 h. Cells were incubated at 37˚C in a CO_2 incubator for 72 h until they were ready to be assayed for gene knockdown.

Immunofluorescence double labeling for c-jun and nNOS in culture cells. For immunofluorescence double labeling, we followed the procedures described in our previous studies (11). The cultured cells in the 96-well plates were gently washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 30 min. After rinsing three times with PBS, the cells were treated with 0.3% Triton X-100 at room temperature for 20 min. The cells were then gently washed with PBS for 15 min, for 5 min each time. Subsequently, the cells were treated with 0.1% BSA and 0.3% Triton at room temperature for 30 min. The cells were then incubated with rabbit anti-c-jun (1:400; Cell Signaling Technology, Inc.) and mouse anti-nNOS (1:3,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. After washing three times with PBS, the cells were incubated with TRITC-conjugated anti-rabbit IgG (1:400; Sigma, St. Louis, MO, USA) and FITC-conjugated anti-mouse IgG (1:200; Sigma) antibodies at room temperature for 2 h in the dark. The cells were then washed extensively with PBS, and Hoechst 33258 was included in the last wash to stain the nuclei. Staining without primary antibodies was used as a negative control. The cells were examined and images were captured using fluorescence microscopy (Zeiss, New York, NY, USA).

Western blot analysis for c-jun and nNOS. To semi-quantify the c-jun and nNOS protein expressed in differentiated PC12 cells after treatment, western blot analysis was performed as previously described (11). Briefly, PC12 cells were grown to subconfluence in growth medium in 60-mm dishes and then scraped down gently by a cell slicker. The cell pellet was homogenized in 0.2 ml buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 μg/ml bacitracin, 20 μg/ml soybean trypsin inhibitor, 10 mM NACl and 0.25% Triton X-100) for 10 min on ice, followed by centrifugation at 15,000 rpm for 30 min. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. For western blotting, samples (20 μg protein/lane) were separated on 8% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE), with a pre-stained protein ladder (5 μl) as a molecular weight marker, and then transferred to polyvinylidene difluoride (PVDF; Pall, Port Washington, NY, USA) membranes.

To detect c-jun and nNOS, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 at room temperature for 2 h, and incubated overnight at 4˚C with c-jun polyclonal antibody (1:1,000; Cell Signaling Technology, Inc.), nNOS polyclonal antibody (1:500; Santa Cruz Biotechnology) or anti-β-actin (1:1,000; Boster, China). After washing and incubation for 2 h at room temperature with a secondary antibody conjugated with horseradish peroxidase (1:4,000), the membranes were washed five times in 0.1% Tween-20 in TBS. Immunoreactive bands were visualized by the supersignal west Pico Trial kit (Pierce, Rockford, IL, USA) and blots were exposed to X-ray film (Fujifilm, Japan). The intensity was quantified in both bands. Relative levels of protein in the different lanes were compared by analyzing scanned images using the NIH IMAGE program. The studies were performed a minimum of three times using independent cultures.

Statistical analysis. The statistical calculations and data handling were performed using SPSS version 16.0. Variables were expressed as medians or means ± standard deviation ($\bar{\chi} \pm$ SD), including the range. A one-way ANOVA was applied to detect differences among groups followed by Tukey-Kramer multiple comparison tests. Differences were considered significant at $p<0.05$.

Results

Coexpression of c-jun and nNOS in differentiated PC12 cells. Cells were cultured as described in the experimental procedures to examine whether there was a correlation between c-jun and

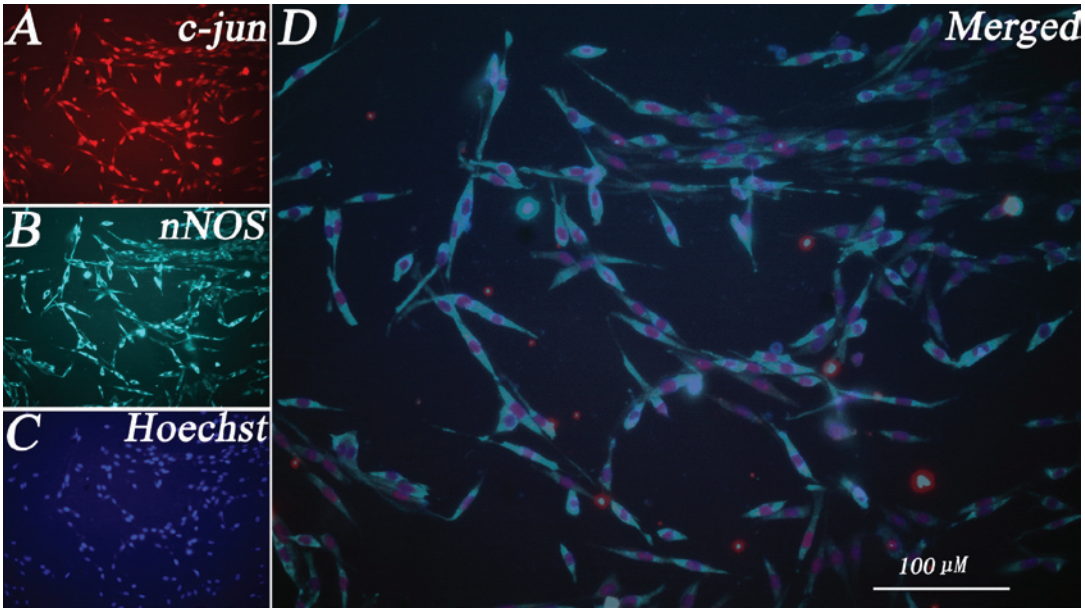

Figure 1. Coexpression of c-jun and nNOS by immunofluorescent double labeling in differentiated PC12 cells (magnification, x10). (A) c-Jun immunoreactivity (ir) was present in the nuclei. (B) nNOS-ir was present in the cytoplasm. (C) All the nuclei of the differentiated PC12 cells were labeled with Hoechst 33258. (D) The results showed that c-jun and nNOS were coexpressed in almost all the differentiated PC12 cells.

nNOS in differentiated PC12 cells. In the first series of experiments, the expression of c-jun and nNOS in differentiated PC12 cells was analyzed by immunofluorescent double labeling detection. Due to their sympathetic neuronal-like phenotype, differentiated PC12 cells are characterized by a large soma and two unbranched processes, with the length of the processes being more than twice the cell body (Fig. 1). Our result showed that c-jun was clearly expressed in differentiated PC12 cells, while c-jun immunoreactivity (ir) was present in the nuclei (Fig. 1A). nNOS was also expressed in differentiated PC12 cells, while nNOS-ir was present in the cytoplasm (Fig. 1B). All the nuclei of the differentiated PC12 cells were labeled with Hoechst 33258 (Fig. 1C). We then examined whether c-jun and nNOS expression were colocalized inside the same cells. Immunofluorescence double labeling further indicated that c-jun and nNOS were coexpressed in the same differentiated PC12 cells. The results indicated that almost all the differentiated PC12 cells coexpressed c-jun and nNOS (Fig. 1D).

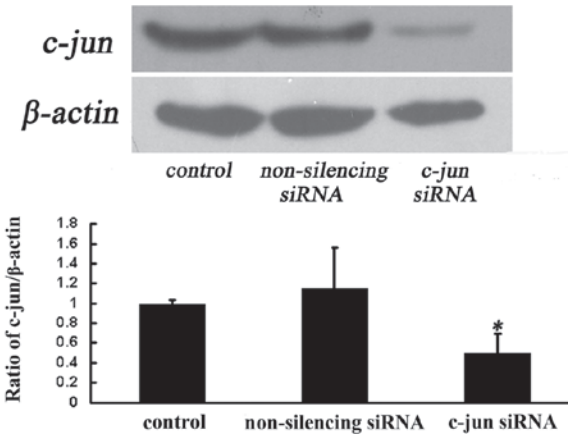

Figure 2. Effects of c-jun siRNA on the c-jun gene in cultured differentiated PC12 cells. The results showed that c-jun protein was downregulated after 72 h of transient transfection of c-jun siRNA in differentiated PC12 cells.

Transfection of siRNA knocks down the c-jun gene in differentiated PC12 cells. To investigate whether c-jun siRNA silences c-jun expression in differentiated PC12 cells, we evaluated the protein levels of c-jun in the cells after 72 h of c-jun-siRNA treatment by western blotting. The result indicated that PC12 cells transfected with c-jun siRNA exhibited significantly lower levels of c-jun protein (Fig. 2A). Statistical analysis showed that the c-jun protein in c-jun-siRNA-treated cells was decreased by ~50% as compared to that in non-silencing-siRNA-treated cells after 72 h of transfection (Fig. 2B). This finding suggested that the transient transfection of c-jun siRNA to cultured differentiated PC12 cells was able to selectively knock down the c-jun gene.

Suppression of c-jun expression affects nNOS expression in differentiated PC12 cells. Findings of a previous study showed that the impairment of proteasome activity and consequent increases in nNOS levels lead to nitrative stress that involves the coordinated response of the JNK/c-jun cytosolic signaling pathway (9). To investigate the impact on the nNOS gene resulting from the downregulation of c-jun expression by the siRNA transfection, nNOS protein expression was evaluated by western blotting. Cells were harvested 72 h after transfection for western blot analysis on nNOS protein. Our data indicated that such silencing of c-jun siRNA resulted in a significant decrease in the protein expression of nNOS after 72 h of transfection (Fig. 3). It is suggested that c-jun siRNA knocks down the level of c-jun protein efficiently and affects nNOS protein expression in differentiated PC12 cells.

Discussion

In the present study, we demonstrated that almost all the differentiated PC12 cells coexpressed the c-jun and nNOS

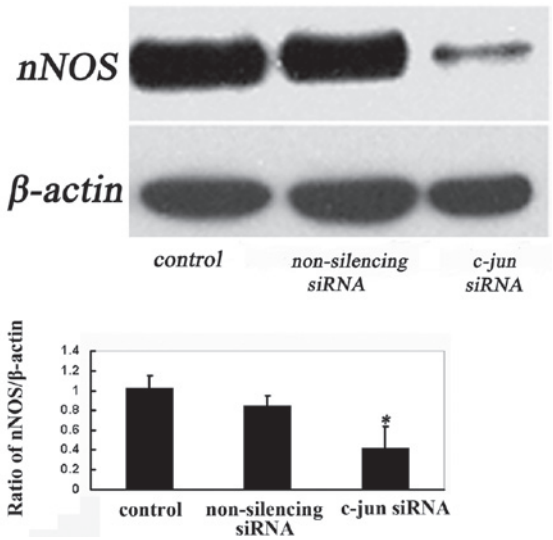

Figure 3. Suppression of c-jun expression affects nNOS expression in differentiated PC12 cells. Cells were harvested 72 h after transfection for western blot analysis on nNOS protein. c-jun siRNA resulted in a significant decrease in the protein expression of nNOS after 72 h of transfection.

genes. For the first time c-jun siRNA was shown to effectively inhibit the expression of c-jun *in vitro* in differentiated PC12 cells. We also found, for the first time, that suppression of c-jun affected nNOS expression in differentiated PC12 cells using the c-jun siRNA strategy.

In response to NGF treatment, PC12 cells adopt a sympathetic neuronal phenotype (12). c-Jun is involved in neuronal differentiation in response to NGF and in death upon NGF withdrawal. In previously published studies, in which neuronally differentiated PC12 cells or sympathetic neurons were used, apoptosis induced by NGF withdrawal was prevented by dominant-negative mutants or by microinjected antibodies specific for c-Jun (2). In addition, the constitutively active mutant of c-Jun was sufficient to trigger apoptosis in cerebellar granule neurons (13). Taken together, these results indicate that activation of c-Jun induces apoptosis in differentiated PC12 and other neuronal cells. However, the molecular basis for the role of c-jun in apoptosis is not fully understood. It may depend on the target gene activated by c-jun in the respective cell type, or the relevance or crosstalk of related proteins. In various animal models of central neuronal diseases, the expression of c-jun and nNOS genes is induced inside injured neurons.

c-Jun is known to be expressed in NOS immunoreactive neurons in the lateral geniculate nucleus of experimental glaucomatous rats (14). Moreover, NO-regulated activation of c-Jun N-terminal kinase 1/2 is associated with neuronal survival in hippocampal neurons in a rat model of ischemia (15). Compromised proteasome degradation elevates nNOS levels and induces programmed cell death in differentiated PC12 cells (9). In our previous study, there may have been a functional crosstalk between c-jun and nNOS in motoneurons, and this may have had an impact on motoneuron degeneration (16). Our study also showed that c-jun phosphorylation acts as the upstream molecule that inhibits the nNOS expression in injured spinal cords within the first 2 weeks after root-avulsion injury. Additionally, a functional relationship between c-jun and nNOS in motoneurons is possible (17).

In the above-mentioned studies, c-jun expression always occurred prior to expression of nNOS in motoneuron injuries. In the present study, we investigated whether there is a correlation between c-jun and nNOS and whether the crosstalk between these two genes regulated the pathological progress of injury-induced neuron degeneration. Using a c-jun siRNA strategy for the first time, we found that suppression of c-jun affects nNOS expression in differentiated PC12 cells. The results are in line with the previous studies. Firstly, PC12 cell differentiation is accompanied by the coexpression of c-jun and nNOS. Secondly, other studies indicated c-jun as an executor of death signals (4), and that elevation of the nNOS levels induces apoptotic cell death in neuronally differentiated PC12 cells (9). Thirdly, our data demonstrated that the suppression of c-jun affects nNOS expression in differentiated PC12 cells. The present findings suggest that we cannot exclude the nonspecific effects of c-jun siRNA on both the c-jun and nNOS genes, however, we believe that the c-jun siRNA purchased underwent specific verification after manufacture. Therefore, based on the present study, the levels of c-jun and other related genes should be investigated by regulating change of the nNOS gene. Thus, investigation of the relationship between the c-jun and nNOS genes is crucial.

The present study has shown that there is a coexpression of c-jun and nNOS in differentiated PC12 cells. siRNA transfection effectively knocks down the c-jun gene and suppression of c-jun affects nNOS expression in differentiated PC12 cells. More studies are required, however, to gain a better understanding of the relationship between c-jun and nNOS and the underlying molecular mechanism involved.

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