Korean J Physiol Pharmacol
Vol 11: 239-246, December, 2007

Involvement of Endoplasmic Reticulum Stress Response in the Neuronal Differentiation

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Expressions of endoplasmic reticulum stress response (ERSR) genes were examined during the neuronal differentiation of rat fetal cortical precursor cells (rCPC) and rat pheochromocytoma PC12 cells. When rCPC were differentiated into neuronal cells for 7 days, early stem cell marker, nestin, expression was decreased from day 4, and neuronal markers such as neurofilament-L, -M and Tuj1 were increased after day 4. In this condition, expressions of BIP, ATF6, and phosphorylated PERK as well as their down stream signaling molecules such as CHOP, ATF4, XBP1, GADD34, Nrf2 and $p58^{IPK}$ were significantly increased, suggesting the induction of ERSR during neuronal differentiation of rCPC. ERSR was also induced during the differentiation of PC12 cells for 9 days with NGF. Neurofilament-L transcript was time-dependently increased. Both mRNA and protein levels of Tuj1 were increased after the induction, and the significant increase in NeuN was observed at day 9. Similar to the expression patterns of neuronal markers, BIP/GRP78 and CHOP mRNAs were highly increased at day 9, and ATF4 mRNA was also increased from day 7. These results strongly suggest the induction and possible role of ERSR in neuronal differentiation process. Further study to identify targets responsible for neuronal induction will be necessary.

Key Words: Endoplasmic reticulum, Neuronal differentiation, Stress response, Neuronal precursor cell

INTRODUCTION

Cell replacement is crucial for recovery from many neurodegenerative disorders, because there are not yet neuroprotective therapies that can prevent cell loss. New neurons and oligodendrocytes which can be produced from various stem cells and neural precursors could help develope cell replacement therapies. Adult neurogenesis can occur in the mammalian central nervous system through manipulating endogenous neural progenitors. Neuronal precursors have the capacity to differentiate in response to neural-inducing signals, migrate and form proscribed functional circuits, thereby creating the central and peripheral nervous systems, respectively. However, in spite of the reports showing the presence of neural stem cells in the adult brain such as subgranular layer or subventricular zone of the central nervous system as well as the involvement of several molecules in adult neurogenesis (Grote and Hannan, 2007), little is known about the molecular mechanism for differentiation and integration of new neurons in the adult central nervous system.

The endoplasmic reticulum (ER) is an important organelle involved in proper folding and posttranslational modification of proteins, transport to the Golgi apparatus, and the biosynthesis of lipids. Many disturbances, including perturbed calcium homeostasis or cellular redox status, elevate the rates of secretory protein synthesis and, when the need for protein folding exceeds the capacity of ER, misfolded or unfolded proteins are accumulated in the ER. To alleviate such a stressful situation, eukaryotic cells activate a series of self-defense mechanisms collectively referred to as the ER stress response (ERSR) (Zhao and Ackerman, 2006). A specific signaling in response to ER stress is known as the unfolded protein response, which is an early event and initiated by three different membrane receptors, PERK, IRE1, and ATF6 (Yoshida, 2007). The unfolded protein response pathway regulates various aspects of cellular metabolism and even influences fate of cells resulting in apoptosis. ER stress-induced apoptosis has been reported in various human diseases and, more interestingly, ER stress has been suggested to be involved in the pathogenesis of human neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease and prion diseases. Although many studies have been focused on ER stress-induced apoptosis, ER stress-mediated cell differentiation has only recently been reported; for example, erythropoiesis, adipogenesis, chondrocyte and osteogenic differentiations, and eyes and bone developments (Cui et al, 2000; Gass et al,

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ABBREVIATIONS: AbFGF, basic fibroblast growth factor; CMF-HBSS, Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution; ER, endoplasmic reticulum; ERSR, ER stress response; NF-L, neurofilament-L; NF-M, neurofilament-M; rCPCs, rat fetal cortical precursor cells.

2002; Pereira et al, 2004; Yang et al, 2005; Zambelli et al, 2005). ER is abundant and well developed in neurons, and the expression of an ERSR protein was observed in the developing brain (Chan et al, 1996), however, ER stress response in neurogenesis has not yet been studied.

In this study, we investigated the involvement of ERSR in cellular differentiation process such as the transition of rat fetal cortical precursor cells and PC12 cells into neuron-like cells, and found for the first time that ERSR are specifically induced during the neuronal differentiation.

METHODS

Cell culture and neuronal differentiation of rat fetal cortical precursor cells (rCPCs)

Rat fetal cortical precursor cells were isolated and cultured as previously described (Chang et al, 2004). Briefly, timed-pregnant Sprague-Dawley rats were purchased from Dae Han Biolink (Korea). Fetal CNS tissues were dissected from fetal day 14 (E14; plug day = 0) rat cortex. Cells were isolated by mechanical dissociation in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS) and plated on 10 cm culture dishes (Corning, USA) precoated with ploy-L-ornithine (15ug/ml; Sigma, USA)/fibronectin (1ug/ml; Sigma) at the density of 2×10^6 cells. Cells were allowed to proliferate in the presence of 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, USA) in serum-free medium (N2; Johe et al, 1996). Cell clusters, grown on the adherent culture surface, were dissociated at the fourth day of *in vitro* expansion and replated onto 12-mm glass coverslips (Bellco, USA) or 6-cm culture plates precoated with poly-L-ornithine/fibronectin at the cell density of 4×10^4 and 5×10^5 cells, respectively. After additional bFGF-dependent expansion for 4 days, differentiation of the cortical precursors was induced in the absence of bFGF in N2 medium, and bFGF was withdrawn from the medium to promote differentiation for 7 days.

Culture and neuronal differentiation of rat PC12 cells

Rat pheochromocytoma PC12 cells were purchased from American Type Culture Collection, and maintained on 100-mm diameter culture dishes precoated with poly-L-lysine (10 μg/ml) at 37°C under an atmosphere of 5% CO_2 and 95% air. Cells were grown in RPMI1640 medium supplemented with 5% fetal bovine serum, 10% horse serum, 2 mM L-glutamine, penicillin, and streptomycin. For neuronal differentiation, cells were cultured in the medium with nerve growth factor (100 ng/ml, Sigma) for 9 days with the medium change every two days.

Total RNA isolation and real time PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen, USA), and RNA concentration was determined spectrophotometrically. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, USA). The reaction was carried out in a Perkin-Elmer 9700 PCR machine with the following parameters: 15 min at 25°C, 60 min at 37°C, 5 min at 99°C, and 5 min at 4°C. For real time PCR, PCR reactions containing SYBR Green QPCR Master Mix (2×) (Takara, Japan), ROX reference dye, DNase-free water, 0.5 μl of 5 pM each of primers and 100 ng of template cDNA were analyzed using M×3000P Multiplex Quantitative PCR instrument (Stratagene, USA) equipped with version 3.0 Mxpro Q-PCR software. The relative amount of mRNAs was calculated using the comparative Ct method with the *β-actin* mRNA as an internal control. The primers used for real time PCR analysis are listed in Table 1.

Northern blot analysis

Cells were washed with ice-cold PBS, and total RNA was extracted with Trizol RNA isolation Reagents (Invitrogen). Full-length cDNAs of *BIP* and *CHOP* was generated with RT-PCR using the total RNA isolated from PC12 cells. The primers used for amplification were as follows: 5'-AGTT-CACTGTGGTGGCGGCGGCG-3' as a forward primer and 5'-CCGCATCGCCAATCAGACGCTCCC-3' as a reverse primer of *BIP*; 5'-AACCTTCACTACTCTTGACCCTGC-3' as a forward primer and 5'-GTGCCACTTTCCTCTCATTCTCCT-3' as a reverse primer of *CHOP*. cDNAs thus obtained was confirmed by sequencing. RNA samples were electrophoresed on 1% agarose gel containing 2.2 mol/l of formaldehyde and transferred onto nylon membranes using a vertical minigel apparatus. The nylon membranes were hybridized with *BIP* and *CHOP* cDNAs and GAPDH cDNA probes, and washed

Table 1. Sequences of primers used for real time PCR

Name	Forward	Reverse
BIP	5'-atcaacccagatgaggctgtagca-3'	5'-agaccttgattgttacggtgggct-3'
CHOP	5'-tgaactgttggcatcacctcctgt-3'	5'-cctctcctttggtctaccctcagt-3'
Gadd34	5'-cctgaatggagtaaagca-3'	5'-ctccccaactttcttatc-3'
ATF4	5'-gtggccaagcacttcaaacctcat-3'	5'-catgtgtcatccaacgtggccaaa-3'
Tuj1	5'-agtaccctgaccgcatcatgaaca-3'	5'-tggtagcagacacaaggtggttga-3'
NF-L	5'-ctgctaagaagaaagattgagcc-3'	5'-ctgaactcataagcatggacc-3'
NF-M	5'-acatcaccgtagagcgcaaagact-3'	5'-actcgagctcaatgctcttggact-3'
ATF6	5'-ggatttgatgccttgggagtcagac-3'	5'-attttttttctttggagtcagtccat-3'
XBP1	5'-agcataggcctgtctgcttcacta-3'	5'-tggtaaagtccagcacttgggagt-3'
Nrf2	5'-agtcccagcaggacatggatttga-3'	5'-cttgtttgggaatgtgggcaacct-3'
P58IPK	5'-agagaaagcccagcggttactgaa-3'	5'-attgctaccacctccttgctgagt-3'
CNPase	5'-agagctgcagttccctttccttca-3'	5'-tgtcatcgagcacaagaaccctga-3'
GFAP	5'-atctgtgtcagaaggccacctcaa-3'	5'-tgttagttggagatgcagggtggt-3'
β-actin	5'-tgcccatctatgagggttacg-3'	5'-tagaagcatttgcggtgcacg-3'

twice in 2×SSC with 0.1% SDS and twice in 1×SSC with 0.1% SDS at 65°C for 30 min. Immunolabeled bands were detected using DAB.

Immunoblot analysis

Cells were resuspended in RIPA cell lysis buffer (20 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and proteinase inhibitor cocktail). After incubation on ice for 30 min, the homogenate was centrifuged at 13,200 ×g for 30 min at 4°C. The supernatant was isolated and the protein concentration was determined using Bradford method (Pierce, USA). Forty micrograms of total protein was separated by 9 or 12% SDS-PAGE, and transferred onto a nitrocellulose membrane (Schleicher & Schell, USA). The membranes were blocked with 3% BSA and incubated overnight at 4°C with the antisera against BIP (1:500 dilution, BD Biosciences, USA), phosphorylated PERK (1:200 dilution, Cell Signaling, USA), NeuN (1:500 dilution, Chemicon, USA), Tuj1 (1:500 dilution, Covance, USA), CHOP (1:200 dilution, Santa Cruz, USA), GAPDH (1:1,000 dilution, Sigma), and α-tubulin (1:1,000 dilution, Sigma). Antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1:1,000 dilution, Santa Cruz) and the immunoreactive bands were visualized with ECL method (Amersham Pharmacia, USA).

Immunocytochemistry

rCPC were cultured on 12 mm glass coverslips (Bellco) precoated with ploy-L-ornithine/fibronectin, and were differentiated as described above. At the indicated times, cells were washed with 0.01 M PBS and then fixed in 4% paraformaldehyde for 30 min on ice. After the fixation, the slides were washed with PBS/0.1% bovine serum albumin (PBS/BSA), blocked for 1 h with 10% normal donkey serum in PBS/BSA containing 0.3% Triton X-100 at room temperature, and then incubated overnight with mouse monoclonal anti-Tuj1 antibodies (1:500, Covance) at 4°C. The slides were washed three times with PBS/BSA, and incubated for 1 h with Alexa 594-conjugated anti-mouse IgG (1:1,000, Invitrogen) at room temperature, and mounted with Vectashield with DAPI (Vector Laboratories, USA). Fluorescence images were visualized through a Zeiss 510LSM META laser-scanning microscope (Carl Zeiss, Germany).

Statistical analysis

All values were expressed as mean ± SEM from at least 3 independent experiments. Data were analyzed using one-way ANOVA between subjects, and *post hoc* comparisons were made using the Tukey HSD test. Statistical analyses were performed using Graphpad Prism 4 for Windows (GraphPad Software Inc. USA). In all cases, statistical significance was set when p value was less than 0.05.

RESULTS

Neuronal differentiation of rat fetal cortical precursor cells

We isolated rat fetal cortical precursor cells (rCPC) from the cortex of E14 fetus of Sprague-Dawley rats, and induced

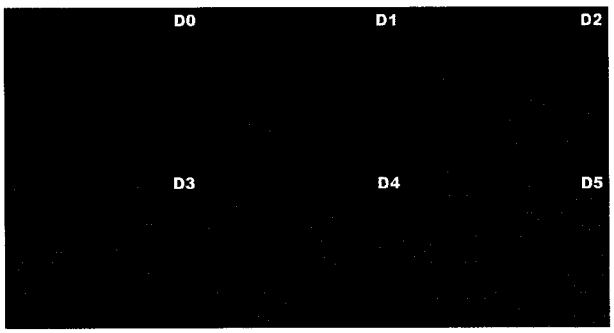

Fig. 1. Expression of Tuj1 during neuronal differentiation of rat fetal cortical precursor cells. Rat fetal cortical precursor cells were isolated and differentiated into neuronal cells as described in Methods, and the expression of the neuronal marker Tuj1 expression was examined during differentiation period using immumocytochemical staining method. Tuj1-positive neuronal cells which was labeled with red fluorescent (Alexa 594) are increased time-dependently by neuronal differentiation for 5 days. Cells stained with DAPI (blue fluorescent) indicate undiffentiated cells. Note similar number of DAPI-positive cell population through the differentiation period. Magnifications: 250 x.

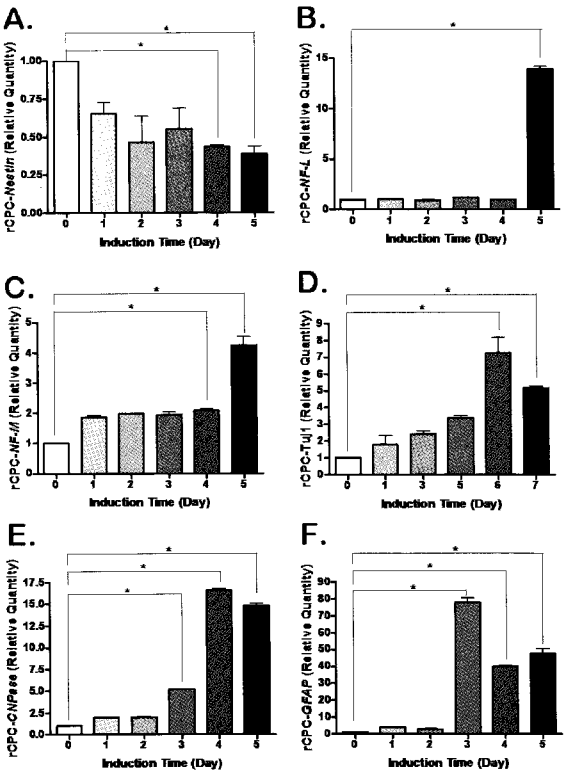

Fig. 2. Expression of neuronal and glial differentiation markers during neuronal differentiation of rat fetal cortical precursor cells. Rat fetal cortical precursor cells were isolated and differentiated into neuronal cells as described in Methods, and the expression of the early neuronal stem cell marker nestin (A), three neuronal markers; neurofilament-L (*NF-L*) (B) and neurofilament-M (*NF-M*) (C), Tuj1 (D), and markers for oligodendrocytes CNPase (E) and astrocytes GFAP (F) were examined using real-time PCR. The relative quantities were normalized to the expression of *β-actin*. Data are mean ± SEM. *$p<0.05$.

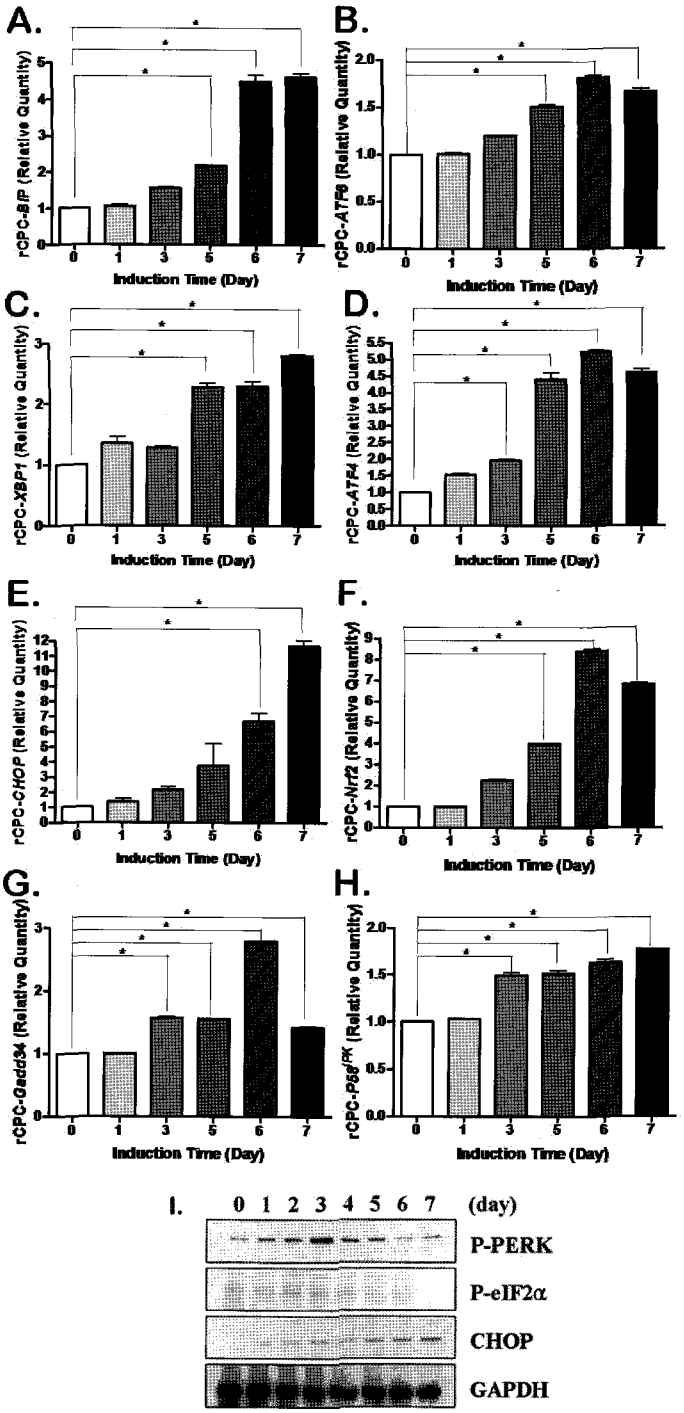

Fig. 3. Expression and activation of endoplasmic reticulum stress genes during neuronal differentiation of rat fetal cortical precursor cells. Rat fetal cortical precursor cells were isolated and differentiated into neuronal cells as described in Methods, and the expression of ER stress-related genes were examined using real time PCR (A~H) and immunoblot (I) analyses. Expression of an ER molecular chaperon BIP increased in a dose-dependent manner (A). Transcription of an ER stress sensor molecule ATF6 was increased rapidly from day 5 (B), and it was activated, evidenced by the increase of its target molecule XBP1 transcription (C). The expression of PERK signaling pathway of ER stress response was examined. Expressions of phosphorylated forms of PERK (P-PERK) and eIF2α (P-eIF2α) were examined by immunoblot analysis (I). P-PERK and P-eIF2α showed transient expression patterns which reached the peak level at day 3. Transcriptions of downstream targets of eIF2α were examined with real time PCR. *ATF4* (D) was remarkably up-regulated from day 3, and CHOP showed typical time-dependent increase at both mRNA (E) and protein (I) levels. Another target molecule of PERK, *Nrf2* (F) was increased eight-fold at day 6. Two negative regulators of PERK, *Gadd34* (G) and $p58^{IPK}$ (H) were up-regulated from day 3. The relative quantities from real time PCR were normalized to the expression of *β-actin* in real time PCR, and GAPDH was used for a loading control for immunoblot analysis. Data are mean ± SEM. *$p < 0.05$.

neuronal differentiation by the removal of bFGF from the media. Immunocytochemical staining of neuronal marker Tuj1 showed time-dependent increase in Tuj1-positive cells (Fig. 1). Although we did not calculate the ratio of Tuj1-positive cells to DAPI positive total cells, less than 10% of total cells were differentiated even at day 5. When we compared the DAPI-positive cell population, total cell number appeared to be stably maintained during neuronal differentiation process. To evaluate the neuronal differentiation at the molecular level, we examined the expression patterns of early neuronal stem cell marker, and neuronal and glial cell markers using quantitative real-time PCR analysis (Fig. 2). Transcriptions of nestin, an early neuronal stem cell marker, was time-dependently decreased (Fig. 2A). In contrast, expressions of two mature neuronal markers (*neurofilament-L* and *neurofilament-M*) (Fig. 2B, 2C) and an immature neuronal marker (*Tuj1*) (Fig. 2D) increased from 5 days after the induction. Expressions of astrocyte (*GFAP*) or oligodendrocyte (*CNPase*) markers were also increased in this situation. These results indicate that rCPC were successfully differentiated into both neuron- and glial-like cells *in vitro*.

Expression of ERSR genes during neuronal differentiation of rCPC

ER stress leads to the increase of an ER chaperon BIP, followed by the expression and activation of three ER stress sensor molecules (ATF6, IRE1, and PERK). Using real-time PCR and immunoblot analysis, we examined the expression of several ERSR genes during neuronal differentiation (Fig. 3). The expression of *BIP* was time-dependently increased from day 5 (Fig. 3A). *ATF6* expression was also increased from day 5 (Fig. 3B), and the time-dependent increase in the transcription of *XBP1*, a target molecule of ATF6, indicated the activation of ATF6 (Fig. 3C). PERK-mediated signaling pathway was activated during neuronal differentiation. On immunoblot analysis, PERK and its downstream target eIF2α were transiently activated by phosphorylation with the peak levels at 3 day (Fig. 3I). Transcriptions of downstream targets of eIF2α were examined. *ATF4/CREB2* was up-regulated from day 3 (Fig. 3D), and *CHOP/Gadd153* was time-dependently increased at both mRNA (Fig. 3E) and protein (Fig. 3I) levels. Expression of another target molecule of PERK, Nrf2 (Cullinan and Diehl, 2006; Cullinan et al, 2003) increased eight-fold at day 6 (Fig. 3F). GADD34 and $p58^{IPK}$ have been suggested as negative feedback regulators for PERK signaling: GADD34 inhibits stress-induced gene expression and promote recovery from translational inhibition by dephosphorylating eIF2α (Novoa et al, 2001), whereas $p58^{IPK}$ prevents dimerization and autophosphorylation of PERK (Tan et al, 1998), thus interfering with PERK's ability to phosphorylate eIF2α. To examine their role in the transient activation of PERK signaling pathway, the expression levels of *Gadd34* and $p58^{IPK}$ during neuronal differentiation were measured (Jiang and Wek, 2005). As we expected, delayed expressions of both were observed from day 3, suggesting their negative feedback role for P-PERK and P-eIF2α (Figs. 3G, 3H). These findings strongly suggest the induction of ERSR during neuronal differentiation of rCPC.

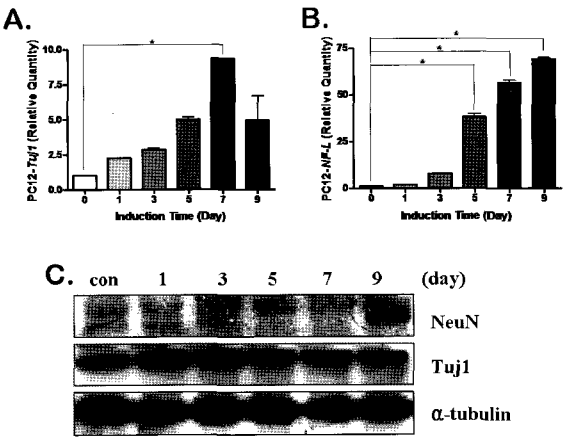

Fig. 4. Expression of neuronal and glial differentiation markers during neuronal differentiation of PC12 cells. Rat PC12 cells were cultured and differentiated into neuronal cells using nerve growth factor as described in Methods, and the expression of neuronal markers were examined using real time PCR (A, B) and immunoblot (C) analyses. Tuj1 mRNA (A) and protein (C) levels were rapidly increased after the induction of neuronal differentiation. Transcription of *neurofilament-L* (*NF-L*) was significantly and time-dependently increased (B), and the protein expression of another neuronal marker NeuN was observed at day 9 (C). The relative quantities were normalized to the expression of β-actin in real-time PCR, and α-tubulin was used for loading control in immunoblot analysis. Data are mean ± SEM. *$p<0.05$.

Induction of neuronal differentiation and ERSR in NGF-treated PC12 cells

Induction of ERSR during neuronal differentiation was also examined in PC12 cells which have long been used for *in vitro* neuronal differentiation studies. When we treated PC12 cells with NGF for 9 days, an immature neuronal marker *Tuj1* reached the peak at day 7 (Fig. 4A). *Neurofilament-L* levels showed typical time-dependent increase. Expression of another neuronal marker NeuN was observed clearly at day 9, revealed by immunoblot analysis (Fig. 4C). These findings show successful neuronal differentiation of PC12 cells. In this condition, we examined the expression of ERSR genes, using real time PCR and Northern blot analysis. As shown in Fig. 5, transcriptions of BIP, ATF4 and CHOP all began to increase from day 7 and reached significantly higher level at day 9.

DISCUSSION

Stress response is a conserved mechanism that protects cells to respond to a variety of environmental and metabolic conditions. In mammalian cells, ER stress is induced in many physiological and pathological conditions (Yoshida, 2007). Here, we presented strong evidences that ERSR may be an important factor for neuronal differentiation. In rCPC, expressions of neuronal markers began to increase from 5 or 6 days after the induction. BIP, a major chaperone protein of the ER lumen and a marker for ERSR, was increased time-dependently from day 5. Moreover, our data showing the activation of all three ER stress sensor

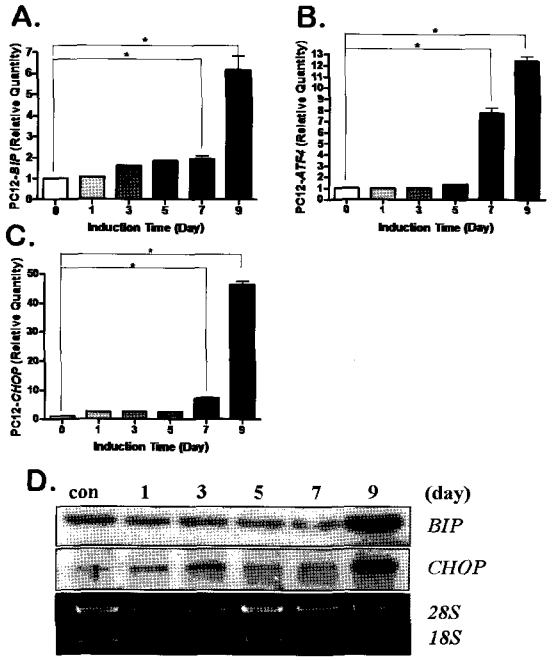

Fig. 5. Expression of endoplasmic reticulum stress genes during neuronal differentiation of PC12 cells. Rat PC12 cells were cultured and differentiated into neuronal cells using nerve growth factor as described in Methods, and the expression of ER stress-related genes were examined using real time PCR (A~C) and Northern blot (D) analyses. Expression of an ER molecular chaperon BIP began to increase from day 7 and reached very significantly high level at day 7 (A). *ATF4* (B) was remarkably up-regulated from day 7, and *CHOP* also increased from day 7 (C). Results form Northern blot analysis showed similar expression pattern of *BIP* and *CHOP* to those from real time PCR. The relative quantities from real time PCR were normalized to the expression of *β-actin* in real time PCR, and ribosomal RNAs were used for a loading control for northern blot analysis. Data are mean ± SEM. *$p<0.05$.

molecules as well as downstream signaling pathways definitely indicated the occurrence of ERSR by differentiation. Besides rCPCs, we also found the induction of ERSR in neuronally differentiated rat PC12 cells as well as human umbilical cord mensechymal stem cells. These findings indicate that the occurrence of ERSR may be a common mechanism during neuronal differentiation process. When temporal expressions were compared between ER stress-related genes and neuronal markers, ERSR appeared to be not a phenomenon secondary to the neuronal differentiation. However, only small portion of the rCPC differentiated into Tuj1-positive neuronal cells and moreover, the expressions of glial markers such as GFAP and CNPase were clearly increased during neuronal differentiation of rCPC. For direct evidence for the relationship between neuronal differentiation and ERSR, the coexpression of ERSR proteins and neuronal markers should be shown using immunocytochemical study.

Insufficient adaptive responses to ER stress result in the initiation of apoptosis, and functions of ER stress-related proteins have been studied primarily from the view of ER-mediated apoptosis. Therefore, it is necessary to examine the pro-apoptotic role of the ERSR which was induced in our experiments. The expressions of ER stress proteins, including even those which are well-known to be associated with apoptosis, were increased in this situation. CHOP is a central mediator for ER stress-induced apoptosis, and its expression during ER stress is up-regulated in proportion to the level of apoptotic cell death without delay (Eymin et al, 1997; Han et al, 2005). Reports showing specific activation of ATF6 during apoptosis in myoblasts (Nakanishi et al, 2005) and the delay of proteasome inhibitor-induced apoptosis by the loss of eIF2α phosphorylation (Jiang and Wek, 2005) also suggest their roles in ERSR-induced apoptosis. However, our data from DAPI staining (Fig. 1) and microscopic examination (data not shown) strongly indicate the stable maintenance of rCPC during neuronal differentiation, suggesting that ERSR was induced during the differentiation, but the potency was not enough to induce apoptosis. It is also possible that mechanisms to increase cell survival could counteract the pro-apoptotic signals. Among the ER stress proteins which enhance cell survival, the transcriptions of two cytoprotective transcription factors, *ATF4* and *XBP1*, were increased or activated by NIM. *Nrf2*, which has been known to enhance cell survival during ER stress (Cullinan et al, 2003; Harris et al, 2004), was also time-dependently increased. In addition to these findings demonstrating non-apoptotic role of ERSR during neuronal differentiation from rCPC, the potential roles of ER stress-related proteins in various cellular differentiation processes have been suggested by many authors. ERSR occurs during myoblast differentiation both *in vivo* and *in vitro* (Nakanishi et al, 2007; Nakanishi et al, 2005), CHOP regulates the differentiations of erythrocytes, osteocytes, chondrocytes and B cells (Cui et al, 2000; Pereira et al, 2004; Skalet et al, 2005; Yang et al, 2005), XBP1 induces osteogenic and plasma cell differentiations (Iwakoshi et al, 2003; Zambelli et al, 2005), and IRE1 increases lymphopiesis of B cells (Zhang et al, 2005). These findings strongly suggest that ERSR potentially plays a role in neuronal differentiation.

Our data showing the possible role of ERSR in neuronal differentiation raise an important question. How ERSR was induced by neuronal differentiation conditions? Currently, only a few reports show possible correlations between the components which have commonly been used for neuronal induction and ERSR. BDNF has been reported to suppress ER stress-induced upregulation of CHOP (Chen et al, 2007). In our study, BDNF induced ERSR during the neuronal differentiation of human umbilical cord mesenchymal stem cells. NGF which was used for neuronal differentiation of PC12 cells apparently induced ERSR. Related to this finding, the expression of neurotrophin receptor $p75^{NTR}$ is initiated very specifically upon the induction of neural differentiation of BMSCs (Yaghoobi and Mowla, 2006), and its homologous protein, NRADD, has been reported to mediate ER stress-induced apoptosis (Wang et al, 2003). Although it is not certain at this time, these findings show the possibility that many factors or conditions used for neuronal differentiation may induce ERSR. Furthermore, Lu *et al* recently demonstrated that chemical neuronal induction itself may be a kind of cellular stress (Lu et al, 2004). Moreover, considering the fact that many signals are shared by both pathways, it is also possible that signals leading to the differentiation into neuronal fate may be initiated or synergistically enhanced by ERSR.

Signaling molecules involved in ERSR have been reported to be required during development (Reimold et al, 2000; Urano et al, 2000), and a novel strategy that ER controls developmental pathways by a specific ER retention and release mechanisms has emerged (Yamamoto et al, 2005). Although it is premature to predict real players for neuronal differentiation with our current results, it is quite probable that one or more transcription factors may induce the expression of protein(s) responsible for differentiation. During ERSR, expressions or activities of many transcription factors are increased: ATF6, IRE1 and PERK are activated following dissociation from BIP; ATF6, a bZip transcription factor, increases a potent transcription factor XBP1 which is spliced into an active form by IRE1; PERK activates Nrf2, a Cap 'n' Collar family of bZip transcription factors, and phosphorylates eIF2α which in turn activates NFκB and enhances the expression of a transcription activator, ATF4; and transcriptions of two additional bZIP regulators, CHOP/GADD153 and ATF3, are increased by ATF4. Roles of these factors during neuronal development can indirectly be assessed from the studies using developing brain or knock-out mice. Data regarding developmental expression of ER stress proteins are very scant. Chan *et al* showed the expression of Nrf2, a substrate of PERK, in the developing brain (Chan et al, 1996). Although no obvious abnormalities in brain was found in the mice deficient of Nrf2, PERK, CHOP, ATF4 or ATF6 genes (Chan et al, 1996; Harding et al, 2001; Masuoka and Townes, 2002; Yamamoto et al, 2007; Zinszner et al, 1998), embryonic lethalities were observed in IRE1$\alpha^{-/-}$, XBP1$^{-/-}$ or eIF2^{S51A} knockin mice, suggesting critical role of these genes in the development of mice (Reimold et al, 2000; Scheuner et al, 2001; Urano et al, 2000). However, Iwawaki *et al* failed to detect ERSR in the brain of a transgenic mouse model at any embryonic stage or in the early and late postnatal stages (Iwawaki et al, 2004). To verify the role of ER stress proteins in *in vitro* neuronal differentiation or development of nervous system, further in-depth studies to examine their expressions during developmental period and to identify the candidate target molecules for these transcription factors are required.

In conclusion, we demonstrated for the first time the involvement and the active role of ERSR during neuronal differentiation, adding one more new function to the broad spectrum of ER's roles. These findings which suggest a novel mechanism of neuronal differentiation have many implications in in vitro differentiation, transplantation of neuronal precursor cells and mesenchymal stem cells, neural development and neurodegenerative diseases.

ACKNOWLEDGEMENT

This work was supported by a grant R13-2002-005-02002-0 from the MRC of the Korea Science & Engineering Foundation.

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