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Cross-talk between IGF-1 and estrogen receptors attenuates intracellular changes in ventral spinal cord 4.1 motoneuron cells due to interferon-gamma exposure

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Abstract

Insulin-like growth factor-1 (IGF-1) is a neuroprotective growth factor that promotes neuronal survival by inhibition of apoptosis. In order to examine whether IGF-1 exerts cytoprotective effects against extracellular inflammatory stimulation, ventral spinal cord 4.1 (VSC4.1) motoneuron cells were treated with interferon-gamma (IFN- γ). Our data demonstrated apoptotic changes, increased calpain:calpastatin and Bax:Bcl-2 ratios, and expression of apoptosis related proteases (caspase-3 and -12) in motoneurons rendered by IFN- γ in a dose-dependent manner. Post-treatment with IGF-1 attenuated these changes. In addition, IGF-1 treatment of motoneurons exposed to IFN- γ decreased expression of inflammatory markers (cyclooxygenase-2 and nuclear factor-kappa B:inhibitor of kappa B ratio). Furthermore, IGF-1 attenuated the loss of expression of IGF-1 receptors (IGF-1R α and IGF-1R β) and estrogen receptors (ER α and ER β) induced by IFN- γ . To determine whether the protective effects of IGF-1 are associated with ERs, ERs antagonist ICI and selective siRNA targeted against ER α and ER β were used in VSC4.1 motoneurons. Distinctive morphological changes were observed following siRNA knockdown of ER α and ER β . In particular, apoptotic cell death assessed by TUNEL assay was enhanced in both ER α and ER β -silenced VSC4.1 motoneurons following IFN- γ and IGF-1 exposure. These results suggest that IGF-1 protects motoneurons from inflammatory insult by a mechanism involving pivotal interactions with ER α and ER β .

Keywords

IGF-1; Calpain; Apoptosis; IGF-1 receptor; Estrogen receptor

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INTRODUCTION

Accumulating evidence indicates that microglia, endogenous CNS cells, potentially contribute to neuron injury and death in the pathology of many neurodegenerative diseases (Zhao *et al.* 2006, Xiao *et al.* 2007). In particular, microglia-mediated cytotoxicity is a common major participant in the pathogenesis of neurodegeneration (Huang *et al.* 2005, Smith *et al.* 2012). Microglia are innate immune cells of the CNS and are activated in various pathological states, including CNS injury, ischemia, and infection. Their activation results in initiation of immune reaction, phagocytosis, and production of many cytotoxic factors such as reactive oxygen and nitrogen species, excitotoxic glutamate, and histamine (Walter & Neumann 2009, Nakajima & Kohsaka 2001). The release of these factors from microglia activated by the pro-inflammatory cytokine IFN- γ has been reported (Meda *et al.* 1995). Cytokines, which are small signaling molecules (8–30 kDa) classified as proteins, possess potential actions to regulate local cellular activities including survival, growth, and differentiation (Smith *et al.* 2012). The expression of cytokine is known to increase in response to infection, injury and disease. Release of pro-inflammatory cytokines may also be toxic to neuron or other glial cells. In order to investigate the mechanisms involved in neurodegeneration relevant to motoneuron diseases, the hybrid cell line ventral spinal cord 4.1 (VSC 4.1) motoneuron cell was exposed to pro-inflammatory Th1 cytokine IFN- γ in the current study. This in vitro motoneuron cell model may be used for studying neurodegeneration involving multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and spinal cord injury (SCI). In addition, the effect of insulin like growth factor-1 (IGF-1) was examined for its efficacy to render cytoprotective potential in motoneuron.

IGF-1 is well known as a polypeptide growth factor with a variety of functions in both neuronal and non-neuronal cells (Zheng & Quirion 2006). In particular, IGF-1 has neurotrophic and neuroprotective roles essential for the survival of neurons in vivo and in vitro and also enhances survival of spinal motor neurons in disease of the spinal cord (Dobrowolny *et al.* 2005). Deficiency of IGF-1 contributes to pathology of degenerative cerebellar ataxias (Torres-Aleman *et al.* 1996) and AD (Mustafa *et al.* 1999), whereas normal levels of IGF-1 enhance functional recovery in transient ischemia (Selvamani & Sohrabji 2010). The role of IGF-1 in motoneuron disease and its mechanism of action are important from both pathogenic and therapeutic points of view (Narai *et al.* 2005). Notably, the formation of oligodendrocytes (oligodendrogenesis) and synthesis of myelin are regulated by several neurotrophic factors including IGF-1 (Skihar *et al.* 2009). Overexpression of IGF-1 increases myelin content in CNS (Ye *et al.* 1995), while deficiency of IGF-1 significantly reduces both myelination and the numbers of oligodendrocytes (Beck *et al.* 1995). In addition, the application of IGF-1 in EAE rats has identified its potential value in attenuating inflammatory changes in the CNS (Chesik *et al.* 2007). Since IGF-1 is not only a potent neurotrophic factor but also a survival factor for the oligodendrocyte lineage and since it possesses an effective myelinogenic capacity, it has been considered to be a strong candidate for the therapy of various neurodegenerative diseases including AD, Parkinson's disease (PD), ALS, MS, and SCI.

Recent studies have identified the principle underlying mechanism for the neuroprotective effects of IGF-1 in suppressing apoptosis in motor neurons (Tsai *et al.* 2011). IGF-1 blocks

Bim induction, cytochrome c release, and activation of intrinsic apoptotic signaling in cerebellar granule neurons (Linseman *et al.* 2002b). Also, IGF-1 contributes to reduce intracellular Ca^{2+} level, and thus modulates calpain expression. Calpain is an intracellular nonlysosomal, ubiquitously expressed, Ca^{2+} -regulated cysteine protease. Calpain exists in two forms: μ -calpain and m -calpain, requiring μM and mM Ca^{2+} concentrations for activation, respectively (Banik *et al.* 1992). Calpain activity is increased in post-mortem brain tissue from MS patients (Shields *et al.* 1999) and is co-localized with damaged axons (Diaz-Sanchez *et al.* 2006); therefore, calpain may be critically involved in the pathogenesis of CNS injuries. Calpain activity is regulated by an endogenous inhibitor, calpastatin (Higuchi *et al.* 2005). However, calpastatin is a large protein and is not an ideal candidate for drug therapy. Recent studies have shown that IGF-1 maintains calpastatin levels and attenuates apoptosis in photoreceptor cells (Arroba *et al.* 2009). In addition, IGF-1 is known to modify intracellular calcium levels (Sanchez *et al.* 2011). Thus, instead of calpastatin, IGF-1 may have therapeutic potential to regulate apoptosis induced by Ca^{2+} -dependent calpain activation. The biological actions of IGF-1 are mediated by a heterotetrameric tyrosine kinase membrane receptor, IGF-1 receptor (IGF-1R α and IGF-1R β) (Butler *et al.* 1998). It has been reported that IGF-1R and estrogen receptors (ER α and ER β) are co-expressed in many neurons and glial cells in the CNS (Quesada *et al.* 2007). Additionally, both factors interact to regulate neural function (Garcia-Segura *et al.* 2006). Several studies have shown that there is a cross-talk between IGF-1R and ER (Quesada & Micevych 2004, Gonzalez *et al.* 2008, Garcia-Segura *et al.* 2000). Although, the neuroprotective effects of IGF-1 have been previously described, the correlation between IGF-1 and ER, also their connection with regulation of calpain in motoneuron cells remains unknown. Here, we hypothesize that IGF-1 attenuates various intracellular changes in motoneuron cells induced by inflammatory stimulation. The present study demonstrated changes in morphology and protein expression in VSC4.1 motoneuron cells induced by IFN- γ exposure and the cellular protection afforded by IGF-1. In addition, the interaction between IGF-1 and ER was determined.

MATERIALS AND METHODS

Materials

The VSC4.1 motoneuron cell line was formed by fusion of dissociated embryonic rat ventral spinal cord neuron with mouse N18TG2 neuroblastoma cell (Crawford *et al.* 1992, Smith *et al.* 1994). This hybrid motoneuron-neuroblastoma cell line (VSC 4.1) was a gift from Dr. Stanley H. Appel (Methodist Neurological Institute, Houston, TX, USA). VSC4.1 cells were grown in monolayer to 70% confluency in Dulbecco's modified Earle's medium (DMEM)/Ham's F12 50/50 Mix with L-glutamine and 15 mM HEPES supplemented with penicillin (100 IU/mL) and streptomycin (100 $\mu\text{g/mL}$) (Cellgro, Mediatech, Manassas, VA, USA). Complete medium contained 2% Sato's components and 2% of heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA). Recombinant rat IFN- γ was obtained from R&D Systems (Minneapolis, MN, USA), and used the midpoint of ED₅₀ range as the midpoint in the titration for calculation of IFN- γ based on manufacturer's instructions. The dose 600 ng/mL of IFN- γ was equivalent to 300 units/mL. In addition, recombinant rat IGF-1 (Sigma-Aldrich, St. Louis, MO, USA), ER antagonist ICI 182780 (Tocris Cookson, Ellisville, MO,

USA), small interfering RNA (siRNA) for ER α and β (Flexi Tube siRNA, Cat. 1027417, Qiagen, USA) and Lipofectamine RNAiMAX (Invitrogen, NY, USA) were used based on manufacturer's instructions.

Cell culture and treatments

For VSC 4.1 motoneuron cell culture, 75-cm² flasks (Corning, NY, USA) were pre-coated with 0.01% poly-L-ornithine (Sigma Aldrich, St. Louis, MO, USA) in 0.6% boric acid solution (pH 8.4). VSC4.1 cells were cultured in complete medium at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and were refreshed every alternative day. In all, 60–70% confluence was attained in 3–4 days. Cells were redistributed at a density of 10⁶ cells in pre-coated 75-cm² flasks. Motoneuron cells were then either exposed to IFN- γ and/or IGF-1. All treatment procedures were conducted in low-serum medium (0.5% FBS). To analyze dose-dependent effects, IFN- γ was given at concentrations of 100, 200, 400, 600, or 1000 ng/mL followed by addition of different doses (50, 100, or 200 ng/mL) of recombinant rat IGF-1 at 15 min after optimal dose of IFN- γ (600 ng/mL) treatment to observe the IGF-1 effects for neuronal cell death. Treatment of motoneuron cells with IFN- γ and/or IGF-1 was carried out for 48 h. For cell viability assays, determination of morphological changes due to apoptosis, and immunofluorescent staining, VSC4.1 cells were collected and disseminated in pre-coated six-well plates at density of 3–5 $\times$ 10⁴ cells/well in which treatments were performed.

Silence of ER α and ER β expression

To inhibit ERs activation, cells were treated with ICI182780 (ICI; 10 μ M) for 24 h before exposure to IFN- γ and IGF-1. Particularly, siRNA oligonucleotide targeting ER α (Esr1, Cat.SI03080231) and ER β (Esr2, Cat.SI03100657) were also transfected in VSC4.1 motoneuron cells to knockdown expression of endogenous ER α and ER β . Briefly, VSC4.1 cells were seeded in pre-coated six-well plates with sterile glass cover slips or 75-cm² flasks. Various doses of ER α and ER β siRNA (10, 25, or 50 nM) were added with serum- and antibiotic-free medium. For transfection, Lipofectamine RNAiMAX was prepared by dilution in serum- and antibiotic-free medium (Invitrogen). RNAi and transfection solutions were mixed together and allowed to incubate at room temperature for 20 min before being added to cell culture media. This medium was replaced with complete medium at 24 h after transfection, and transfected cells were allowed to incubate for 24 h more before further treatment. To compare the morphological features of motoneuron cells after transfection for 48 h, cells grown on cover slips were viewed under bright field conditions on an inverted Olympus BH-2 microscope at 100 $\times$ and 200 $\times$ magnifications (Olympus, Melville, NY, USA). To determine the suppression of ER α and ER β protein levels by transfection with ER α and ER β siRNA, cells grown in 75-cm² flasks were collected and Western blot analysis performed as described below. Based on morphological changes of transfected cells and protein level of ERs, 50 nM was selected as an optimal dose to silence ER α and ER β expression. As previously described, VSC4.1 cells were seeded in six-well plates and 75-cm² flasks and transfected with 50 nM of ER siRNA for 48 h. Cells were then treated with 600 ng/mL IFN- γ followed by 200 ng/mL IGF-1 (15 min post-treatment).

Cell viability assay

To assess cell viability following IFN- γ and IGF-1 exposure, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was used. The assay shows the conversion of bright yellow MTT dye to dark blue formazan crystals by reduction in viable cells. When dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), MTT has a specific absorption maximum at 570 nm (Samantaray *et al.* 2011a). Following IFN- γ and IGF-1 treatments, medium was replaced with 0.5% serum medium containing MTT reagent (0.1 mg/ml) and incubated at 37°C for 1 h. Formazan crystals were precipitated by centrifugation at 1900 $\times$ g (Eppendorf Centrifuge 5804R, Germany) for 10 min, medium was removed, and crystals were dissolved in 1 mL/well of DMSO. Plates were read in an Emax Precision Microplate reader at 570 nm with reference wavelength set at 630 nm using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). Optical density was compared by setting the control at 100%

Wright staining for detection of apoptotic morphological features

VSC4.1 cells were cultured in six-well plates and treated with IFN- γ and/or IGF-1 for 48 h; cells were collected and gently spun for 5 min to obtain a pellet. These cells were resuspended in phosphate-buffered saline (PBS; Cellgro, Mediatech, Manassas, VA, USA) and sedimented onto microscopic slide glasses and fixed with 95% ethanol for 10 min. Finally, cells were stained using the Hema 3 hematoxylin/eosin stain set (Cat 122–911, Fisher Scientific, Kalamazoo, MI, USA): Hema 3 Fixative, Hema 3 Solution 1 (eosin), and Hema 3 Solution 2 (hematoxylin), according to the manufacturer's protocol. Next, cells were gently washed with distilled water and dried completely. Cells were viewed at 200 $\times$ magnification on an Olympus BH-2 microscope. Images were captured by a non-confocal camera to detect the characteristics of apoptotic features including cell shrinkage, cell membrane blebbing, chromatin condensation, and/or apoptotic body formation.

TUNEL assay

DNA fragmentation and cell death was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay. VSC4.1 motoneuron cells were grown on sterile glass cover slips and treated with IFN- γ (600 ng/mL), IGF-1 (200 ng/mL), ICI (10 μ M) or ER α and ER β siRNA (50 nM) as previously described. Cells were fixed with 4% methanol-free paraformaldehyde in PBS for 15 min followed by washing twice with PBS for 5 min. After washing, cells were immersed in 0.2% Triton X-100 in PBS for 5 min and washed twice in PBS for 5 min each. To prevent evaporation, cells were equilibrated for 10 min then incubated with fluorescein-conjugated 12-dUTP reagent (Apoptosis Detection System, Promega, Madison, WI, USA) at 37°C for 2 h. The labeling reaction was terminated by addition of 2 $\times$ NaCl/Na-citrate (SSC) (Promega) for 15 min at room temperature. Cells were washed with PBS, and cell nuclei were counterstained and finally mounted with antifade Vectashield™ (Vector Laboratories, Burlingame, CA, USA). Fragmentation of DNA was visualized by green fluorescence and fluorescent images were viewed and captured in Olympus BH-2 microscope at 200 $\times$ magnification. Experiments were perfect in triplicate.

Protein analysis by Western blotting

Methods used to detect changes in protein levels were described previously (Nozaki *et al.* 2011, Park *et al.* 2012). Collected VSC4.1 cell pellets were homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.4, 1 mM PMSF, and 5 mM EGTA) to extract protein. Protein content was determined using a standard Lowry protein assay, and each sample was diluted with the same volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5 mM β -mercaptoethanol, and 10% glycerol). Protein samples containing 12 μ g were separated on 4–20% linear gradient SDS-PAGE gels according to standard procedures. After electrophoresis, proteins were transferred onto PVDF membranes for immunoblot analysis. The blots were probed at 4°C overnight with primary IgG antibodies against calpastatin, Bax, Bcl-2, caspase-3, caspase-12, NF- κ B, I- κ B, Cox-2, IGF-1R α and IGF-1R β , ER α and ER β , Akt-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and m-calpain that was raised in our laboratory (Banik *et al.* 1983). Primary IgG antibody against β -actin (Sigma-Aldrich) was used to standardize protein loading. The appropriate horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary IgG antibody (MP Biomedicals, Solon, OH, USA) were applied to the blots at 37°C for 1 h. For subsequent detection of specific proteins, the enhanced chemi-luminescence (ECL) system was used (GE Health Care, Piscataway, NJ, USA). Blots were immediately processed to digital images using FluorChem FC2 system (AlphaInnotech, San Leandro, CA, USA). Protein bands were quantified using the Image J software program (<http://rsb.info.nih.gov/ij/>). The amount of protein was calculated as percentage relative to control.

Statistical analysis

Results were assessed using IBM SPSS Statistics Version 20 (IBM, Armonk, New York, USA) and compared using one-way analysis of variance (ANOVA) with Tukey post-hoc test at a 95% confidence interval. Data were presented as mean $\pm$ SD of separate experiments (n = 3). Significant difference compared with control was indicated by * p < 0.05 or ** p < 0.01; compared with IFN- γ treatment was indicated by # p < 0.05 or ## p < 0.01; and compared with IFN- γ +IGF-1 post-treatment was indicated by @ p < 0.05 or @@ p < 0.01.

RESULTS

IFN- γ reduces cell viability and induces apoptotic changes in a dose-dependent manner in motoneuron cells

To identify effects of the inflammatory cytokine IFN- γ on cell viability and apoptosis, VSC4.1 motoneuron cells were treated with rat recombinant IFN- γ at various doses (100, 200, 400, 600, or 1000 ng/mL) for 48 hours. MTT reduction was used as a cell viability assay and showed that motoneurons responded to IFN- γ in a dose-dependent manner (Fig. 1A). Two doses of IFN- γ , 600 and 1000 ng/mL, significantly reduced cell viability by 22.34% and 44.77%, respectively, compared to control (p < 0.05). Apoptotic features were detected by Wright staining under a light microscope (Fig. 1B). There were cell shrinkage, membrane blebbing, chromatin condensation, and formation of membrane-bound apoptotic bodies in VSC4.1 motoneuron cells treated with IFN- γ . To determine whether extracellular inflammatory stimulation affects the expression of calpain and apoptosis related proteins, Western blot was performed. Expression of calpain was elevated in a dose-dependent

manner, while calpastatin (endogenous inhibitor) protein level was reduced (Fig. 1C). A significant increase in the calpain:calpastatin ratio was seen in the cells treated with IFN- γ (600 and 1000 ng/mL) compared with control cells ($p < 0.05$). Pro-apoptotic Bax expression was increased resulting in a significant increase in Bax:Bcl-2 ratio compared with control cells ($p < 0.05$ at 400 ng/mL, and $p < 0.01$ at 600 and 1000 ng/mL) (Fig. 1D). Our results showed a significant induction of active 12 kDa caspase-3 fragments ($p < 0.01$ at 600 and 1000 ng/mL) in VSC4.1 motoneuron cells compared with control cells (Fig. 1E). Also, 50 kDa caspase-12 was up-regulated significantly at 600 and 1000 ng/mL ($p < 0.01$) (Figure 1F). Based on these results, we selected the 600 ng/mL IFN- γ dose for all subsequent studies.

IGF-1 attenuates apoptosis and calpain and apoptosis related protein expression in motoneuron cells following IFN- γ stimulation

To clarify the neuroprotective effects of IGF-1 against inflammatory stimulation, we treated VSC4.1 motoneuron cells with various doses of IGF-1 (50, 100, and 200 ng/ml) at 15 min following IFN- γ stimulation. Cell viability was reduced by 35.77% with 600 ng/mL of IFN- γ , whereas IGF-1 attenuated these changes in a dose-dependent manner (Fig. 2A). Post-treatment with IGF-1 showed significant cytoprotective effects at doses of 100 and 200 ng/mL ($p < 0.05$). Morphological features of apoptosis were determined by Wright staining (Fig. 2B). Post-treatment with IGF-1 in the presence of IFN- γ significantly attenuated the morphological changes and apoptotic cell death in VSC4.1 motoneuron cells. Whether treatment with IGF-1 attenuates the changes in protein expression induced by IFN- γ in VSC4.1 motoneuron cells, Western blot was also examined. Post-treatment of motoneuron cells with IGF-1 in the presence of IFN- γ significantly decreased calpain:calpastatin ratio compared with the cells treated only with IFN- γ ($p < 0.01$) (Fig. 2C). The pro-apoptotic protein Bax was diminished in the cells post-treated with IGF-1 (Fig. 2D) and the Bax:Bcl-2 ratio was significantly decreased after post-treatment with IGF-1 ($p < 0.01$). Active 12 kDa caspase-3 fragments ($p < 0.01$) were also decreased after treatment of cells with IGF-1 compared to those treated with IFN- γ alone (Fig. 2E). We also demonstrated a significant decrease in caspase-12 in cells treated with 200 ng/mL IGF-1 ($p < 0.01$) (Fig. 2F).

IGF-1 provides anti-inflammatory effect following IFN- γ exposure in motoneuron cells and its association with IGF-1 and estrogen receptor expression

To examine whether extracellular inflammatory stimulation induces secondary intracellular inflammatory changes in motoneuron cells, VSC4.1 motoneurons were treated with IFN- γ (600 ng/mL). IGF-1 (50, 100, or 200 ng/ml) was added to IFN- γ treated cells and the effects on inflammatory changes were examined. There was significantly increased expression of 72 kDa Cox-2 in the cells treated with IFN- γ compared to control ($p < 0.01$), and post-treatment with 200 ng/mL IGF-1 markedly attenuated this change (Fig. 3A). A significant increase in expression of 65 kDa NF- κ B and an elevated NF- κ B:I- κ B ratio were evident in cells treated with IFN- γ compared to control ($p < 0.01$). Post-treatment with IGF-1 (100 and 200 ng/mL) significantly attenuated this ratio by increasing I- κ B levels ($p < 0.01$) compared with the cells stimulated with IFN- γ alone (Fig. 3B). To determine the effects of IGF-1 on the expression of its receptors and ERs in motoneuron cells secondary to extracellular inflammatory stimulation, the expression of IGF-1R α , IGF-1R β , ER α , and ER β protein

levels were determined in VSC 4.1 cells treated with IFN- γ and IGF-1. A significant decrease was found in IGF-1R α protein levels ($p < 0.05$) but not IGF-1R β in the cells following IFN- γ exposure (Fig. 3C). However, post-treatment with IGF-1 significantly increased the expression of both IGF-1R α and IGF-1R β ($p < 0.01$). Moreover, ER α protein levels were decreased following 600 ng/mL IFN- γ ($p < 0.01$) while ER β protein levels were unaffected (Fig. 3D). Post-treatment with IGF-1 showed up-regulation of both ER α and ER β expression compared with the cells treated only IFN- γ ($p < 0.01$).

Antagonism of ERs induced apoptosis in VSC4.1 motoneuron cells

The previous results support that IGF-1 has neuroprotective effects against the inflammatory cytokine IFN- γ . In addition, these neuroprotective effects were correlated with increased expression of IGF-1R and ER at the protein level. To further determine whether ERs are involved in the cytoprotective mechanisms, ICI (10 μ M), which acts as an antagonist of ER α and ER β , was used in VSC4.1 cells for 24 h to suppress ER activation. Cells were then stimulated with IFN- γ and then treated with IGF-1 as described above. Western blot analysis showed that marked elevation in the Bax:Bcl-2 ratio ($p < 0.05$) through IFN- γ stimulation was significantly restored by IGF-1 ($p < 0.01$). Also, a more significant increase in the Bax:Bcl-2 ratio was found following antagonism of ERs compared to IFN- γ and IGF-1 exposure alone (Fig. 4A). Up-regulation of active caspase-3 (12 kDa) was shown in cells stimulated with IFN- γ ($p < 0.05$), and IGF-1 was capable to block activation of caspase-3, and protected motoneuron cells. However, ICI exposure increased cleavage of caspase-3 in motoneuron cells (Fig. 4B; $p < 0.05$). Therefore, these results indicate that ERs are involved in the neuroprotective effects of IGF-1 after IFN- γ exposure.

Morphological features of ER-silenced motoneuron cells by siRNA

Although ERs may contribute to protection of IGF-1, their involvement in cytoprotection of motoneuron cells exposed to pro-inflammatory cytokine remains unclear. Therefore, ER subtypes were knocked down by selective siRNA targeting either ER α or ER β to compare the inhibitory effects of ER α and ER β individually in motoneuron cells. VSC4.1 cells were prepared as described above and knock-down of ER α and ER β was induced with various doses of siRNA (10, 25, or 50 nM). After transfection for 48 h, phenotypes of ER-silenced motoneuron cells were observed under bright field conditions on an inverted microscope and captured at 100 $\times$ and 200 $\times$ magnifications. ER α -silenced cells exhibited marked morphological changes suggestive of apoptosis including shortening of neurite branches, rounded cell bodies and loss of inter-cellular connections when compared to control cells. ER β -silenced cells also displayed altered phenotypes such as longitudinal shape of cell body (Fig. 5A). To confirm the suppression of ERs expression in motoneuron cells, protein levels of ER α and ER β were examined by Western blot. As seen in Figure 5B and 5C, 50 nM of ERsiRNA reduced the expression of ER α and ER β protein levels significantly ($p < 0.01$), thus we choose the 50 nM dose for ER siRNA for all subsequent experiments.

Cross-talk between IGF-1 and ER for neuroprotection attenuates intracellular changes in motoneuron cells

Apoptosis in response to IFN- γ exposure was evaluated with TUNEL assay in motoneuron cells. As seen from representative microphotographs, IFN- γ (600 ng/mL) substantially increased TUNEL-positive cell numbers compared to control cells, and further treatment with IGF-1 (200 ng/mL) attenuated these changes. On the other hand, the cytoprotective effect of IGF-1 was lost in cells treated with the ERs antagonist ICI (10 μ M). Motoneuron cells with specific-knockdown of ER α and ER β separately showed apoptotic changes following IFN- γ exposure (Fig. 6A). Silencing of ER α in IGF-1-treated cells significantly increased TUNEL-positive motoneuron cells, and a similar effect was seen following ER β deletion. To determine whether Akt-1 expression, a marker of cell survival and protein synthesis, is affected by suppression of ERs in motoneuron cells, Akt-1 protein levels were examined by Western blot (Fig. 6B). Interestingly, the VSC4.1 cells treated with the ERs antagonist ICI before IFN- γ /IGF-1 exposure showed the lowest levels of Akt-1 ($p < 0.01$). Since ICI blocked both ER α and/or ER β , the inhibition of both ER α and ER β in motoneuron cells may affect Akt-1 expression regardless of exogenous IGF-1. To distinguish the involvement of ER α and ER β in maintenance of Akt-1 protein expression, we used siRNA to silence these receptors in VSC4.1 motoneurons. ER α -silenced motoneuron cells showed a significant reduction in Akt-1 protein expression compared to the cells treated IFN- γ and IGF-1 ($p < 0.01$). Akt-1 protein levels in ER β -silenced motoneuron were also decreased ($p < 0.05$). These results suggest that both ER α and ER β may contribute to the neuroprotection mediated by IGF-1 against the pro-inflammatory cytokine IFN- γ .

DISCUSSION

Recent findings have shown pro-inflammatory cytokine activation is commonly associated with neuronal degeneration. Mounting evidence suggests that cytokine involvement in various neurodegenerative disease including AD (Swardfager *et al.* 2010, Rogers & Lue 2001), PD (Gao *et al.* 2008, Sawada *et al.* 2006, Su *et al.* 2008), ALS (Ono *et al.* 2001, Sekizawa *et al.* 1998), and SCI (Thompson *et al.* 2013, Guerrero *et al.* 2012). Furthermore, production of pro-inflammatory Th1 cytokines, including IFN- γ and TNF- α , by CD4 $^{+}$ T cells is increased in MS (Lassmann & Ransohoff 2004). Several mechanisms of cytokine-mediated neuronal cell death have been described such as modulation of neuronal excitability and neurotransmitter release (Smith *et al.* 2012), direct interactions between cytokine and neurotransmitter receptors (Gardoni *et al.* 2011), and activated microglial production of nitric oxide (NO) and cytokines (Bal-Price *et al.* 2002). Finally, these subsequent effects may contribute to excitotoxic injury and apoptosis in neurons. One central finding of the current study was that pro-inflammatory cytokine IFN- γ showed cytotoxic effects, reducing cell viability of motoneuron and inducing various morphological apoptotic changes, upregulation of cysteine proteases calpain, caspase-3, caspase-12, and pro-apoptotic Bax, and down-regulation of anti-apoptotic Bcl-2. IFN- γ exposure has been reported to cause the elevation of intracellular Ca $^{2+}$ levels (Ye *et al.* 2012), and Ca $^{2+}$ -dependent cell death has been previously described in VC4.1 motoneuron cells (Smith *et al.* 1994). Thus, the increased Ca $^{2+}$ generated by exposure of motoneurons to IFN- γ will activate and upregulate calpain. Increased calpain will degrade its specific endogenous

inhibitor calpastatin, a suicide substrate of calpain, keeping the activated enzyme unregulated (Blomgren *et al.* 1999). In view of these findings, calpain and its specific endogenous inhibitor calpastatin have been suggested to be involved in the apoptosis mechanism (Arroba *et al.* 2009). Calpain has often been implicated in the cleavage of a number of Bcl-2 family members to induce the apoptotic process (Lopatniuk & Witkowski 2011). For example, active calpain cleaves Bax at its N-terminus (at Asp33) and generates a potent pro-apoptotic 18 kDa fragment, leading to apoptosis (Lopatniuk & Witkowski 2011, Gao & Dou 2000). Our results suggest that IFN- γ may induce the intrinsic apoptotic pathway through increases in pro-apoptotic Bax and Bak, and reduction of anti-apoptotic Bcl-2 and Bcl-xL levels. These proteins undergo oligomerization to form pores to effect mitochondrial membrane permeability changes (Nicotra & Parvez 2002). As a consequence, cytochrome c and apoptosis-inducing factor (AIF) are released into the cytosol leading to subsequent activation of caspase pathways. Caspases belong to the cysteine protease family and play crucial roles in regulating pathological cell death. Caspase-9 mediates apoptotic signals after mitochondrial damage (Li *et al.*, 1997), while caspase-12 mediates endoplasmic reticulum (ER) stress-specific apoptosis (Nakagawa *et al.*, 2000). Caspase-3 is activated by calpain and acts as a final executioner to induce apoptosis (Gonzalez *et al.* 2008). Thus, calpain plays a critical role that is upstream of caspases in the apoptotic cell death (Samantaray *et al.* 2011a).

Another finding of current study indicates that IGF-1 provides diverse cytoprotective effects including prevention of decrease of cell viability and attenuation of apoptotic and inflammatory changes. IGF-1 was found to mediate down-regulation of calpain, Bax, active caspase-3, caspase-12, Cox-2 and NF- κ B in motoneurons. Several previous studies have reported that IGF-1 inhibited the apoptotic changes in Purkinje cells (Crocì *et al.* 2011), motor neurons (Tsai *et al.* 2011), cerebellar granule neurons (Linseman *et al.* 2002a), and oligodendrocytes (Mason *et al.* 2000). Additionally, Pons *et al.* (2000) demonstrated that IGF-I mediates a site-specific dephosphorylation of I- κ B α (phospho-Ser32) and inhibits the nuclear translocation of NF- κ B (p65) induced by TNF- α exposure in astrocytes (Pons & Torres-Aleman 2000). These neuroprotective, anti-apoptotic, anti-inflammatory effects of IGF-1 are thought to be mediated through receptor-mediated signaling pathways. Binding of IGF-1 to its receptors (IGF-1R α and IGF-1R β) induces receptor autophosphorylation and activation of the intrinsic tyrosine kinase domain. Furthermore, the IGF-1 signaling induces production of other growth factors, including brain-derived neurotrophic factor (BDNF) (Ding *et al.* 2006), vascular endothelial growth factor (VEGF), (Lopez-Lopez *et al.* 2004), and the ovarian hormone estradiol in the CNS (Garcia-Segura *et al.* 2010, Mendez *et al.* 2006). Thus, the finding in the present study suggests that ER α and ER β were up-regulated following IGF-1 treatment, indicating an interaction between IGF-1 and ERs. A number of studies have suggested that the interaction between IGF-1 and estrogen promotes neuroprotection (Quesada & Micevych 2004, Gonzalez *et al.* 2008, Azcoitia *et al.* 1999, Garcia-Segura *et al.* 2010). Estrogen is known to regulate brain development, neural function, and neuroendocrine events, as well as to modulate synaptic plasticity, neurogenesis, and the response to injury in the nervous system (Mendez *et al.* 2006, Garcia-Segura *et al.* 2010, Gonzalez *et al.* 2008, Kahlert *et al.* 2000). Mediation of the protective effects of estrogen by its receptors (ER α and ER β), which belong to the steroid/thyroid

nuclear receptor superfamily, has been proposed (Sribnick *et al.* 2006, Kahlert *et al.* 2000). Binding of estrogen to its receptors induces ERs dimerization and translocation to the cell nucleus where ERs regulate the transcription of target genes either by binding to Estrogen Response Elements (ERE) within the promoter sequence or by binding to other transcription factors (TF's) (Mendez *et al.* 2006). There appear to be several differences in ER α and ER β signaling. Estrogen-induced MAPK activation is mediated by ER α , but not through ER β (Kahlert *et al.* 2000). In addition, ER α forms a part of macromolecular complex with IGF-1R and with several components of IGF-1R signaling cascade such as p85, insulin receptor substrate 1 (IRS1), GSK3 β , and β -catenin (Mendez *et al.* 2006, Garcia-Segura *et al.* 2010). The difference between the ER subtypes in terms of the ability to interact with IGF-1R may be in part due to different N-terminal activation function 1 (AF-1) domains since ER α possesses constitutive AF-1 activity while ER β does not (Kahlert *et al.* 2000). Although ERs related neuroprotection has been repeatedly demonstrated in different models, the involvement of non-classical ERs in IGF-1-mediated cytoprotection in motoneuron remains unclear. The anti-apoptotic effect of IGF-1 was diminished by the ER antagonist ICI 182,780, as reported in the present study. Increased pro-apoptotic Bax and active caspase-3 was reproduced by inhibition of ERs. In turn, the effects of estradiol were blocked by inhibiting the synthesis of IGF-1 via a specific IGF-1 antisense oligonucleotide (Duenas *et al.* 1996). Antagonism of ERs attenuated the anti-apoptotic effect of IGF-1, but did not demonstrate whether the specific ER subtype was responsible. Thus, to determine the contributions of both ERs to the IGF-1 effects, siRNA oligonucleotide targeting ER α and ER β were transfected separately to silence their expression in motoneuron cells. Interestingly, both ER α and ER β -silenced VSC4.1 cells showed salient morphological changes, and these altered phenotypes suggest an important role for ER subtypes in motoneuron survival under basal conditions. Although marked morphological changes were found in motoneuron cells treated with ER α and ER β siRNA, the motoneuron cells survived. It is thought that each ER may compensate when the other was inhibited by its siRNA. Estrogen and its receptors (ERs) possess cytoprotective effects via inhibition of apoptosis, particularly by modifying intracellular calcium levels through modulating L-type Ca²⁺ channels (Carrer *et al.* 2003, Mermelstein *et al.* 1996, Sribnick *et al.* 2006). In addition, ERs contribute to neuroprotection via diverse signal pathways, including IGF-1. Since estrogen and its receptors have central roles in neuroprotection and anti-inflammation (Sribnick *et al.* 2006, Samantaray *et al.* 2011b), the effects of siRNAs of ER would be a challenging topic to investigate the mechanism of cross-talk with IGF-1 in motoneuron cells. The synergistic actions of IGF-1 and ER are also supported by our findings. ERs antagonism by ICI resulted in numerous TUNEL-positive cells, also both ER α and ER β -knockdown increased numbers of TUNEL-positive cells. Based on these observations, suppression of ER signaling may decrease the anti-apoptotic and neuroprotective effects of IGF-1. Whether the phosphatidylinositol 3-kinase (PI3K)/ Akt pathway is affected by inhibition of ERs was also determined. IGF-1 triggers multiple signaling pathways including PI3K/Akt pathway to promote glycogen synthase kinase 3 (GSK3), mammalian target of rapamycin (mTOR), and other key regulatory proteins, facilitating protein synthesis in neuronal cells (Dudek *et al.* 1997, Zheng & Quirion 2006). Moreover, Campbell *et al.* (2001) and Varea *et al.* (2010) have shown that ER α activates the PI3K/Akt pathway (Campbell *et al.* 2001, Varea *et al.* 2010). Our results demonstrate that a reduction in Akt-1 was occurred in motoneuron cells

following antagonism of ERs by ICI or knockdown of specific ER subtypes. Interestingly, antagonism of ERs resulted in the lowest level of Akt-1, in particular ER α -silenced cells showed a greater decrease in Akt-1 protein levels than ER β knockdown. Thus, ER α may have a predominant role of protein synthesis and cell survival. The major difference between the two ER α and ER β s is the ability to interact with the IGF-1 pathway. Kahlert et al (2000) have reported that ER β was not capable to bind to the IGF-1R, thus no phosphorylation and activation of IGF-1R were found (Kahlert et al. 2000). On the other hand, ER α binds to the IGF-1R (heterodimer of IGF-1R and ER α) and activates the IGF-1R signaling cascade (Varea et al. 2010). Particularly, activation of ERK signaling via ER α located in the plasma membrane plays a central role in the neuritogenesis activating ERK signaling, MAPK pathway, and regulation of intracellular Ca²⁺ concentration (Varea et al. 2010). Furthermore, ER α also interacts with IGF-I receptor and with components of the PI3K-Akt signaling pathway, contributing to the glucose metabolism, synaptic plasticity, stabilization of microtubules, and protein synthesis. Therefore, the present data may support that binding of ER α to the IGF-1R is an influential mechanism for estrogen to activate the IGF-1R. However, these data do not rule out the possibility that both ER α and ER β may be required for the neuroprotection mediated by IGF-1 against the inflammatory cytokine IFN- γ .

Overall, the major findings obtained from current study indicate that IGF-1 is effective in preventing inflammatory and apoptotic changes in motoneuron cells against pro-inflammatory stimulation by IFN- γ . In addition, these results may be connected to the cross-talk between IGF-1 signaling and ERs (Fig. 7). Thus, IGF-1 may have potential therapeutic implications for preventing secondary changes in neuro-inflammatory or neuro-degenerative diseases, including MS, ALS, and SCI.

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Abbreviations

IFN-γ	interferon gamma
IGF-1	insulin like growth factor-1
VSC4.1	ventral spinal cord cells clone 4.1
NF-κB	nuclear factor-kappa B
I-κB	inhibitor of kappa B
Cox-2	cyclooxygenase-2
IGF-1R	insulin like growth factor-1 receptor
ER	estrogen receptor

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Figure 1. Effects of pro-inflammatory cytokine IFN-γ on cell viability and apoptotic cell death in motoneuron cells

IFN-γ was treated with various doses (100, 200, 400, 600, or 1000 ng/mL) for 48 hours, and dose-dependent induction of cell death and apoptotic changes were showed in VSC4.1 motoneuron cells. (A) MTT assay was used to assess cell viability after 48 h exposure to IFN-γ in VSC4.1 cells. IFN-γ stimulation diminished the cell viability in a dose-dependent manner. (B) Apoptotic cells were determined by Wright staining in VSC4.1 cells following 48 h exposure of IFN-γ. Representative microphotographs of control and cells stimulated with IFN-γ. Images were taken by light microscope after Wright staining at 200×

magnification. Arrows indicate damaged and apoptotic cells. Calpain and apoptosis related protein expressions were altered in a dose-dependent manner following IFN- γ stimulation. Representative Western blot to show levels and determination of percent changes in (C) 80 kDa calpain and 110 kDa calpastatin, (D) 23 kDa Bax and 26 kDa Bcl-2, (E) 32 kDa pro-caspase-3 and 20/12 kDa active caspase-3 and (F) 50 kDa caspase-12. ** $p < 0.01$, * $p < 0.05$, significantly different from the controls.

Figure 2. Cytoprotective effects of IGF-1 in VSC4.1 motoneuronal cells

Cells were treated with IFN- γ (600 ng/mL) for 48 h, then IGF-1 (50, 100, or 200 ng/mL) was added 15 min after the IFN- γ treatment. (A) MTT assay showed that IGF-1 prevented the decreased of cell viability following IFN- γ . (B) Attenuation of apoptotic changes by IGF-1 was founded in motoneuronal cells by Wright staining. The arrows indicate apoptotic cells (200 $\times$ magnification). IGF-1 reduced the expression of calpain and apoptosis related proteins in VSC4.1 motoneuron cells following IFN- γ treatment. Representative Western blot to show levels and determination of percent changes in (C) 80 kDa calpain and 110 kDa

calpastatin, (D) 23 kDa Bax and 26 kDa Bcl-2, (E) 32 kDa pro-caspase-3 and 20/12 kDa active caspase-3 and (F) 50 kDa caspase-12. ** $p < 0.01$, * $p < 0.05$, significantly different from the controls; ## $p < 0.01$, # $p < 0.05$, significantly different from the cells treated with IFN- γ (600 ng/mL).

Figure 3. Anti-inflammatory effect of IGF-1 is associated with IGF-1 receptor and estrogen receptors in motoneuronal cells

The expression of inflammation related transcription factors and Cox-2 were risen up in VSC4.1 motoneuron cells by IFN- γ stimulation. Attenuation of Cox-2 expression and NF- κ B:I- κ B ratio by IGF-1 was showed in motoneuron cells. Moreover, the expressions of IGF-1R (α and β) and ER (α and β) were altered in VSC4.1 motoneuron cells following IFN- γ stimulation. Treatment with IGF-1 significantly increased the expression of both IGF-1R (α and β) and ER (α and β) compared with IFN- γ treatment alone. Representative Western blot to show levels and determination of percent changes in (A) 72 kDa Cox-2, (B) 65 kDa NF- κ B and 49 kDa I- κ B, (C) 130 kDa IGF-1R α and 97 kDa IGF-1R β and (D) 66

kDa ER α and 56kDa ER β . ** $p < 0.01$, * $p < 0.05$, significantly different from the controls; ## $p < 0.01$, # $p < 0.05$, significantly different from the cells treated with IFN- γ (600 ng/mL).

Figure 4. Antagonizing of ER pathway reproduced the apoptotic changes in motoneuronal cells treated with IFN- γ and IGF-1

The expression of pro-apoptotic Bax protein and 20/12 kDa active caspase-3 were up-regulated, however anti-apoptotic Bcl-2 protein was down-regulated in cells treated with IFN- γ (600 ng/mL). IGF-1 (200 ng/mL) attenuated these changes, whereas ICI (10 μ M), antagonist of ERs reversed the anti-apoptotic effect of IGF-1. Representative Western blots to show levels and determination of percent changes in (A) 23 kDa Bax and 26 kDa Bcl-2, (B) 32 kDa pro-caspase-3 and 20/12 kDa active caspase-3. * p < 0.05, significantly different from the controls; ## p < 0.01, # p < 0.05, significantly different from the cells stimulated with IFN- γ (600 ng/mL); @ p < 0.05, significantly different from the cells treated with IFN- γ (600 ng/mL) and IGF-1 (200 ng/mL).

Figure 5. Characterization of silencing of ERα and ERβ in VSC4.1 motoneuron cells

To determine whether ERs are involved in cytoprotection against inflammatory insult, the expression of ERα and ERβ were deleted in motoneuron cell by ERsiRNA. (A) Typical features of motoneuron cells were observed in control cells; however, the cells inhibited ERs showed particular morphological changes. The shapes which are round cell body and less dendrite were caused by treatment of ERαsiRNA (50 nM), moreover 50 nM of ERβsiRNA resulted in longitudinal cell body. Images were viewed under invert microscope and captured at 200× magnification. The levels of inhibition of ER proteins by ERsiRNA

treatments were confirmed biochemically. The expressions of ER α and ER β proteins were reduced in a dose-dependent manner in motoneuron cells. Representative Western blots to show levels and determination of percent changes in (B) 66 kDa ER α and (C) 56kDa ER β . ** $p < 0.01$, * $p < 0.05$, significantly different from the controls.

Figure 6. Participation of ER in neuroprotective effect of IGF-1 on motoneuron cells stimulated pro-inflammatory cytokine

To determine whether the ERs were connected with cytoprotection of IGF-1, 10 μ M of ICI, which is antagonist of ER α and ER β , was added to cells. Also, to distinguish the involvement of ER α and ER β respectively, 50 nM of ER α and ER β siRNA were transfected to cells according to protocol from manufacture. Then inflammatory insult IFN- γ (600 ng/mL) and IGF-1 (200 ng/mL) were treated to cells for 48 h. (A) Representative microphotographs of TUNEL-positive cells were founded in cells after IFN- γ exposure, depicting motoneuron cell death. IGF-1 attenuated these TUNEL-positive changes.

However, TUNEL-positive cells prominently emerged in the cells blocked with ICI. In particular, the cells knocked out of ER α and ER β had the increases of TUNEL-positive cells compared with the cells treated with IFN- γ and IGF-1. These results indicated that ER α and ER β may associate to neurprotection in motoneuron cells. Images were viewed under fluorescent microscope and captured at 200 $\times$ magnification (green fluorescence). (B) Akt-1 expression was slightly diminished in cells after IFN- γ stimulation, and IGF-1 attenuated the expression. Interception of ERs by ICI significantly decreased the expression of Akt-1 protein, as well as suppression of ER α and ER β caused by ERsiRNA disrupted Akt-1 protein expression. Interestingly, knock down of ER α showed the decrease of Akt-1 protein level compared to ER β inhibition. Therefore, ER α may have a predominant involvement of protein synthesis and cell survival. Representative Western blot to show levels and determination of percent changes in 62 kDa Akt-1. @@ $p < 0.01$, @ $p < 0.05$, significantly different from the cells treated IFN- γ and IGF-1.

Figure 7. Schematic diagram of interaction between IGF-1 and ERs that have been verified in the motoneuron cell in vitro

IGF-1 may activate both MAPK/ERK and the PI3K/Akt pathways and this may affect various neuronal functions via heterodimer of IGF-1R and ERα. Activation of IGF-1R may affect ERs expression and, thus, regulate ER-mediated transcription. In turn, activation of ERs may influence IGF-1R expression and IGF-1R-mediated functional events (This scheme was based on Cardona-Gomez *et al.* 2002).