Biochemical and Biophysical Research Communications 429 (2012) 93–98

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Transcriptional regulation of specific protein 1 (SP1) by hypoxia-inducible factor 1 alpha (HIF-1α) leads to *PRNP* expression and neuroprotection from toxic prion peptide

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ARTICLE INFO

*Article history:*
Received 20 October 2012
Available online 3 November 2012

*Keywords:*
Specific protein 1
Prp(106–126)
HIF-1α
Prion disease

ABSTRACT

Our previous study demonstrated that hypoxia-inducible factor-1 (HIF-1)-mediated neuroprotective effects are related to cellular prion protein (PrPc) gene (*PRNP*) regulation under hypoxic conditions. However, the mechanism of HIF-1α-mediated *PRNP* gene regulation in prion-mediated neurodegenerative disorders is not clear. Transcription factor specific protein 1 (SP1) is necessary for *PRNP* transcription initiation, and SP1 gene expression is regulated through HIF-1α activation under hypoxic conditions. Thus, we hypothesized that HIF-1α-mediated neuroprotection is related to the SP1 transcription pathway as a result of *PRNP* gene regulation. Inhibition of SP1 expression blocked the HIF-1α-mediated protective effect against prion-mediated neurotoxicity. Also, knockdown of HIF-1α induced downregulation of SP1 expression and sensitivity to prion-mediated neurotoxicity, whereas upregulation of SP1 transcriptional activity lead to protection against prion-mediated neuron cell death and *PRNP* gene expression even in HIF-1α depleted cells. This report is the first study demonstrating that HIF-1α-mediated SP1 expression regulates PrPc transcription, and upregulation of SP1 induced by HIF-1α plays a key role in protection from prion-mediated neurotoxicity. These studies suggest that transcription factor SP1 may be involved in the pathogenesis of prion diseases and also may be a potential therapeutic option for neurodegeneration caused by the pathological prion protein, PrPsc.



## 1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcriptional activator that consists of two subunits, alpha and beta, among which HIF-1α is broadly expressed in mammalian tissues [1]. Recent studies have shown that HIF-1α has neuroprotective properties [2–4]. Wu et al. reported that accumulation of HIF-1α attenuated rotenone-induced apoptosis in a cell model of Parkinson's disease [4]. Also, our previous study suggested that prion protein fragment [PrP(106–126)]-mediated neurotoxicity was blocked by overexpression of HIF-1α [2]. These observations suggest that regulation of HIF-1α activation may have therapeutic benefits in neurodegenerative disorders, including prion disease.

Transmissible spongiform encephalopathies, or prion diseases, are a group of progressive neurodegenerative disorders characterized by accumulation of the misfolded prion protein PrPsc [5–8]. PrPsc, a protease-resistant prion protein, is converted from the normal prion protein PrPc, which is naturally expressed in neurons [9].

PrP(106–126), containing the amino acid residues 106–126 of human PrPc, is different from PrPsc but is found to have some similar characteristics of PrPsc, including neurotoxicity and the ability to form amyloid fibrils *in vivo* and *in vitro* [10–13]. These properties are useful for the *in vitro* examination of prion-mediated neurotoxicity.

Some reports have shown that modulation of PrPc expression protects against neurodegenerative diseases, including Alzheimer's disease and Huntington's disease [14,15]. Upregulation of PrPc inhibited β-secretase cleavage of the amyloid precursor protein and reduced amyloid beta (Aβ) formation [15]. On the other hand, Aβ levels in scrapie-infected mice were significantly increased [15]. Also, overexpression of PrPc in neuronal cells transfected with huntingtin fragments (Htt) decrease Htt-mediated neurotoxicity, whereas PrPsc-infected cells showed increased Htt-mediated neurotoxicity [14]. These findings indicate that upregulation of PrPc expression may be a key factor in the prevention of neurodegenerative disorders.

Our previous study showed that overexpression of PrPc caused by exposure to hypoxic conditions or upregulation of HIF-1α prevents prion peptide-mediated neurotoxicity [16,17]. However, the regulatory mechanism of hypoxia-inducible HIF-1α-mediated PrPc expression is not clear.

It is well known that specific protein 1 (SP1), belonging to the SP/KLF family of transcription factors, regulates gene expression

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http://dx.doi.org/10.1016/j.bbrc.2012.10.086

in the development of an organism [18–20]. Transcription factor SP1 contains zinc finger motifs that directly bind to DNA and enhance gene transcription [21]. Some reports have shown that SP1 transcription factors are key factors for manipulating prion gene (*PRNP*) expression [22,23]. Bellingham et al. suggested that *PRNP* expression is regulated through the interaction of transcription factor SP1 and metal transcription factor-1 [23]. Also, a recent study showed that HIF-1 binds to hypoxia response elements on the SP1 promoter to stimulate the SP1 gene transcription activity [24]. It is proposed that upregulation of HIF-1α expression protects against prion-mediated neurotoxicity through an increase in SP1-mediated *PRNP* expression. Indeed, some studies demonstrated that HIF-1α-mediated transcriptional activity is regulated by the SP1 transcription factor under hypoxic conditions [25,26]. For *PRNP* expression, however, the relationship between SP1 and HIF-1α is not yet identified.

Thus, this study focused on the influence of SP1 transcriptional activity on hypoxia-inducible HIF-1α-mediated *PRNP* expression and analyzed the influence of SP1-mediated *PRNP* expression during prion-mediated neurotoxicity. Our research showed that upregulation of HIF-1α expression increased SP1 protein levels. Also, depletion of SP1 in neuronal cells by siRNA led to prion peptide-mediated neuron cell death and decreased transcriptional activity of *PRNP*, in spite of HIF-1α activation. The present results demonstrated that regulation of hypoxia-inducible HIF-1α affects the transcriptional activity of SP1-mediated *PRNP* expression, which consequently regulates PrP(106–126)-induced neurotoxicity.

## 2. Materials and methods

### 2.1. Cell culture and reagents

The neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture collection (ATCC, Rockville, MD, USA). Mouse neuronal cell lines ZW 13–2 and Zpl 3–4 established from the hippocampus of ICR ($PRNP^{+/+}$) and Zürich I ($PRNP^{-/-}$) mice, respectively, were kindly provided by Professor Yong-Sun Kim (Hallym University, Chuncheon, Kangwon-do, South Korea). SH-SY5Y cells were cultured in minimum essential medium (MEM; Hyclone Laboratories, Logan, UT, USA), whereas ZW 13–2 and Zpl 3–4 cells were grown in DMEM (Hyclone Laboratories) that contained 10% fetal bovine serum (Invitrogen-Gibco, Grand Island, NY, USA) and gentamycin (0.1 mg/ml) in a humidified incubator maintained at 37 °C and 5% $CO_2$. Deferoxamine, doxorubicin and insulin-like growth factor-1 (IGF-1) were purchased from Sigma.

### 2.2. Construction of HIF-1α shRNA plasmid

The shRNA against the HIF-1α gene was a kind gift of Dr. Yong-Nyun Kim (National Cancer Research, Goyang, Gyeonggi-do, South Korea). The shRNA plasmid constructs for HIF-1α (shHIF-1α) were constructed in the pL-UGIP vector. The shRNA for HIF-1α was obtained using the oligonucleotide sequences 5′-CTGATGACCAG-CAACTTGA-3′ and 5′-TCAAGTTGCTGGTCATCAG-3′ as the forward and reverse primers, respectively. SH-SY5Y cells were transfected with shHIF-1α, and stable transfectants were selected in puromycin after 24 h recovery in standard growth medium. SH-SY5Y cells transfected with a mock vector were used as a control.

### 2.3. RNA interference

SH-SY5Y cells were transfected with SP1 small interfering RNA (siRNA; Stealth RNAi, *VHS40867*; Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 according to the manufacturer's instructions. After a 48 h culture, knockdown efficiency was typically measured at the protein level by immunoblot. Stealth RNAi Negative Control (Invitrogen, Carlsbad, CA, USA) was used as a control.

### 2.4. PrP(106–126) treatment

Synthetic PrP(106–126) (sequence: Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) was synthesized by Peptron (Seoul, Korea). The peptides were dissolved in sterile dimethyl sulfoxide at a 12.5 mM concentration and stored at −80 °C.

### 2.5. Annexin V assay

Apoptosis was assessed in detached cells using an Annexin V assay kit (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol. Annexin V levels were determined by measuring the fluorescence at 488 nm excitation and 525/530 nm emission wavelengths using a Guava easyCyte HT System (Millipore, Billerica, MA, USA).

### 2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL analysis was performed to measure the degree of cellular apoptosis using the *in situ* Apo-BrdU DNA Fragmentation Assay Kit (BioVision, San Francisco, CA, USA) following the manufacturer's instructions. Cells were washed with phosphate buffered saline, fixed with paraformaldehyde for 15 min, preincubated with 50 μL of DNA-labeling solution (10 μL TdT reaction buffer, 0.75 μL TdT enzyme, 8 μL Br-dUTP) for 1 h at 37 °C, and then incubated with 5 μL anti-BrdU-fluorescein isothiocynate antibody for 0.5 h at room temperature. Finally, cells were mounted with DakoCytomation fluorescent medium and visualized using fluorescence microscopy. Cells were counterstained with propidium iodide (PI) to show all cell nuclei.

### 2.7. Western Blot

After SH-SY5Y cells were lysed in buffer [25 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and protease inhibitor mixture], proteins were electrophoretically resolved by 10–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubation with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. The antibodies used for immunoblotting were anti-SP1 (Millipore, Billerica, MA, USA), anti-HIF-1α (BD Bioscience, San Diego, CA, USA), anti-PrPc (Millipore, Billerica, MA, USA), anti-murine PrP (provided by Professor Yong-Sun Kim), and anti-ß-actin (Sigma–Aldrich). Images were examined using a Fusion FX7 imaging system (Vilber Lourmat, Marne-la-Vallée, France).

### 2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from SH-SY5Y cells using the Hybrid-RTM kit (GeneAll Biotechnology, Seoul, Korea). cDNA was synthesized using the TaKaRa Prime Script™ 1st strand cDNA synthesis kit (Takara Bio Inc., Tokyo, Japan) following the manufacturer's instructions. The following primers were designed:

HIF-1α: forward 5□-CGC AAG TCC TCA AAG CAC AG-3□; reverse 5□-TGG TAG TGG TGG CAT TAG CA-3□;
*PRNP*: forward, 5□-GTG CAC GAC TGC GTC AAT-3□; reverse, 5□-CCT TCC TCA TCC CAC TAT CA-3□;

β-actin: forward, 5□-GCA AGC AGG AGT ATG ACG AG-3□; reverse, 5□-CAA ATA AAG CCA TGC CAA TC-3□.

All reactions with Thunderbire™ SYBR qPCR mix (TOYOBO, Osaka, Japan) were performed on the CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

### 2.9. Statistical evaluation

All data are expressed as mean ± standard deviations (SD) and compared using the Student's *t*-test, analysis of variance (ANOVA), and Duncan's test with SAS statistical software. Results were considered significant at $^{*}p < 0.05$ and $^{**}p < 0.01$.

## 3. Results

### 3.1. Neuroprotective effect of HIF-1α against prion-mediated neurotoxicity under hypoxic conditions is dependent on SP1 expression

Our previous study showed that prion peptide-induced neuron cell death was blocked through increased HIF-1α expression, and a recent study suggested that the protective effect of HIF-1α is regulated through the SP1 transcription pathway [16,27]. Thus, we evaluated the effect of SP1 and the relationship between SP1 and HIF-1α signaling pathways on PrP(106–126)-induced neuron cell death (Fig. 1). To determine whether SP1 operated by expression of HIF-1α to block PrP(106–126)-induced neuronal apoptosis, the SP1 gene in cells was knocked down (si-SP1 RNAi) using the SP1 RNAi oligomer and then exposed to PrP(106–126) with or without HIF-1α activation by hypoxia (Fig. 1). As shown in Fig. 1A, the PrP(106–126)-induced increase in the population of Annexin V positive cells was decreased by HIF-1α activation caused by hypoxic conditions, whereas knockdown of SP1 in PrP(106–126)-treated cells reduced the protective effect of HIF-1α activation. Consistent with these results, the TUNEL assay (Fig. 1C) showed that depletion of the SP1 gene inhibited the protective effect of HIF-1α activation on PrP(106–126)-induced apoptosis. We next assessed whether the regulation of HIF-1α activity affected the expression of SP1 mRNA and protein levels (Fig. 2). The levels of SP1 and HIF-1α mRNAs and proteins were detected using qRT-PCR and Western Blots. The SP1 mRNA levels were increased in cells exposed to hypoxic conditions, whereas the HIF-1α mRNA levels were not changed (Fig. 2B and D). On the other hand, treatment with the HIF-1α inhibitor doxorubicin decreased SP1 mRNA levels, although it did not change HIF-1α mRNA levels (Figs. 2D and 4D). Also, Western Blot assay identified a marked increased in SP1 and HIF-1α protein levels under hypoxic conditions in a time-dependent manner (Fig. 2A). However, doxorubicin decreased SP1 and HIF-1α protein levels in cells despite exposure to hypoxic conditions (Fig. 2C). These results provide convincing evidence that regulation of the SP1 gene plays a pivotal role in the protective effect of HIF-1α on prion peptide-induced neuron cell death, and SP1 gene expression may be upregulated through HIF-1α activation in low-oxygen conditions.

### 3.2. SP1 signaling pathway regulates PrPc expression and prevents prion-mediated neuronal apoptosis under hypoxic conditions

Some reports showed that *PRNP* expression is regulated through the SP1 transcription pathway [22,28]; therefore, we next examined whether an increase in SP1 activity affected HIF-1α-mediated *PRNP* expression. To determine whether SP1 transcriptional activity regulates *PRNP* expression by activation of HIF-1α, cells were treated with si-SP1 RNAi oligomers (Fig. 3). As shown in Fig. 3A, hypoxia-induced HIF-1α activation increased PrPc gene and protein expression, whereas knockdown of the SP1 gene using SP1

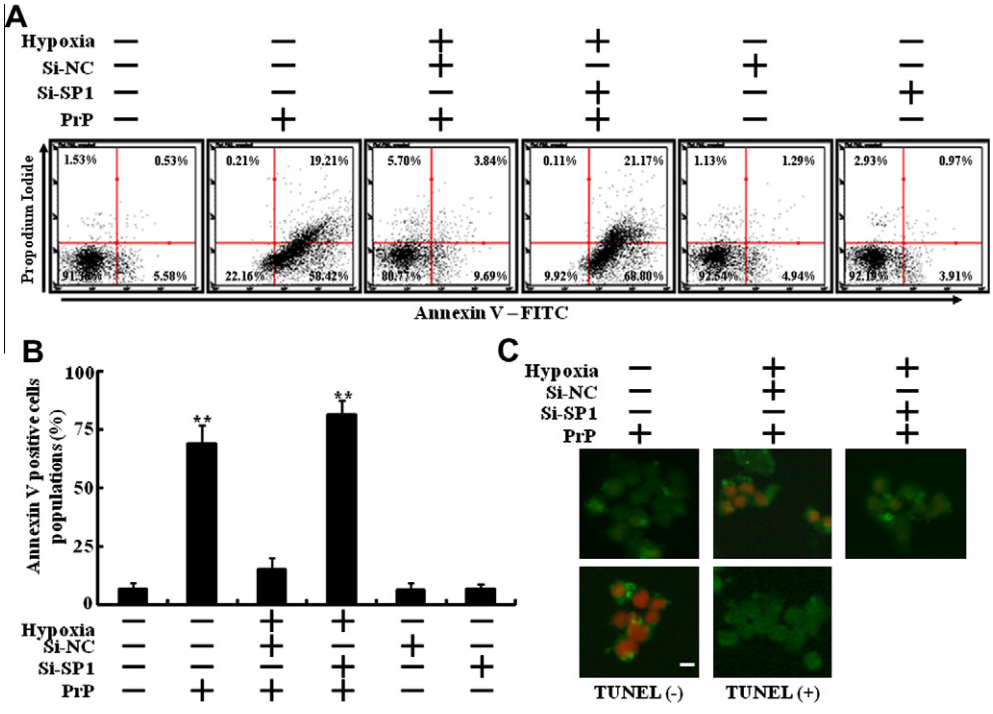


**Fig. 1.** Knockdown of SP1 blocks protection of hypoxia in PrP(106–126)-treated cells. (A) SP1 gene in SH-SY5Y neuronal cells was knocked down (si-SP1 RNAi) using the SP1 RNAi oligomer and then exposed to hypoxic conditions with or without 50 μM PrP(106–126) for 24 h. Cell viability was measured by Annexin V assay, (B) bar graph indicating the averages of Annexin V positive cells. $^{*}p < 0.05$, $^{**}p < 0.01$: significant differences between control and each treatment group and (C) representative immunofluorescence images of TUNEL-positive (green) SH-SY5Y cells exposure to hypoxic conditions after the knockdown of the SP1 gene (si-SP1 RNAi) using the SP1 RNAi oligomer and then treated with 50 μM of PrP(106–126) for 24 h. The cells were counterstained with PI (red) to show all cell nuclei. Magnification 400×, scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

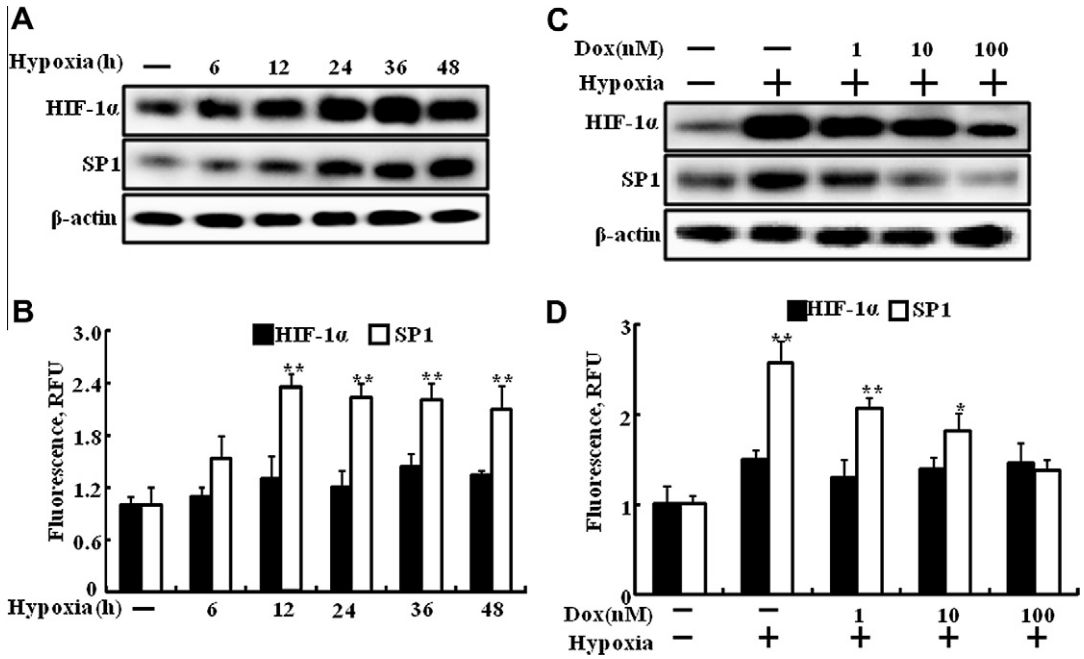


**Fig. 2.** Hypoxia increases HIF-1α and SP1 levels. SH-SY5Y cells exposed to hypoxic conditions in a time-dependent manner. (A) The hypoxia-exposed cells were assessed for HIF-1α and SP1 production by Western Blot analysis. Results were normalized with β-actin, (B) the cells were assessed for HIF-1α and SP1 gene production by qRT-PCR analysis. Results were normalized with β-actin, (C) SH-SY5Y cells were treated with doxorubicin in a dose-dependent manner. The cells were assessed for HIF-1α and SP1 production by Western Blot analysis. Results were normalized with β-actin and (D) doxorubicin-treated cells were assessed for HIF-1α and SP1 gene production by qRT-PCR analysis. Results were normalized with β-actin. $^{*}p < 0.05$, $^{**}p < 0.01$: significant differences between control and each treatment group.

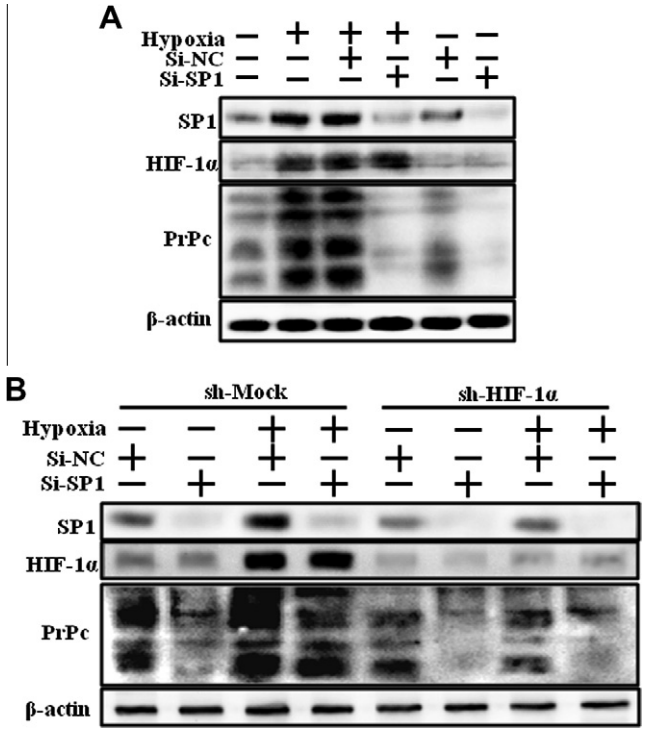


**Fig. 3.** HIF-1α promotes expression of PrPc by regulation of SP1. (A) SP1 gene of cells was knocked down (si-SP1 RNAi) using the SP1 RNAi oligomer and then exposed to hypoxic conditions. The cells were assessed for HIF-1α, PrPc and SP1 production by Western Blot analysis. Results were normalized with β-actin and (B) SP1 gene was knocked down in shHIF-1α- or mock-transfected SH-SY5Y cells using the SP1 RNAi oligomer and then exposed to hypoxic conditions. The cells were assessed for HIF-1α, PrPc and SP1 production by Western Blot analysis. Results were normalized with β-actin.

RNAi oligomers inhibited the HIF-1α-mediated PrPc gene and protein levels in SH-SY5Y cells. We next examined the influence of HIF-1α activation on SP1-mediated *PRNP* expression (Fig. 3). Western Blot analysis showed that shHIF-1α cells had decreased expression of SP1 and PrPc protein levels under hypoxic conditions (Fig. 3B). In contrast, hypoxia increased the HIF-1α, SP1, and PrPc mRNA and protein levels in mock-transfected cells (Fig. 3B). However, PrPc expression was inhibited through knockdown of SP1 genes in mock-transfected cells (Fig. 3B).

Next, we studied whether the upregulation of SP1 prevents PrP(106–126)-induced neuronal cell death. SP1 activator IGF-1 prevents PrP(106–126)-induced neurotoxicity (Fig. 4B) and increased SP1 protein levels (Fig. 4A) in mock- and shHIF-1α-transfected cells, respectively, independently of oxygen status. Collectively, these results suggest that upregulation of SP1 protein expression increases *PRNP* expression and prevents PrP(106–126)-induced neuronal cell death.

## 4. Discussion

The present study demonstrates that activation of HIF-1α inhibits prion peptide-induced neuronal apoptosis through SP1 upregulation and that PrPc expression induced by HIF-1α stabilization is regulated by SP1 transcriptional activity in neuronal cells under hypoxic conditions. Notably, hypoxia-inducible HIF-1α-mediated induction of SP1 lead to increased PrPc expression in neuron cells, which, in turn, may confer neuroprotection.

We previously reported that activation of HIF-1α prevents PrP(106–126)-induced apoptosis in neuronal cells and that inhibition is associated with HIF-1α-activated PrPc expression [2]. In the same study, PrPc overexpression was shown to prevent PrP(106–126)-induced neurotoxicity in HIF-1α-depleted neuronal cells [2]. Another study reported that regulation of PrPc protects neuronal cells from neurodegenerative disorders, including Alzheimer's

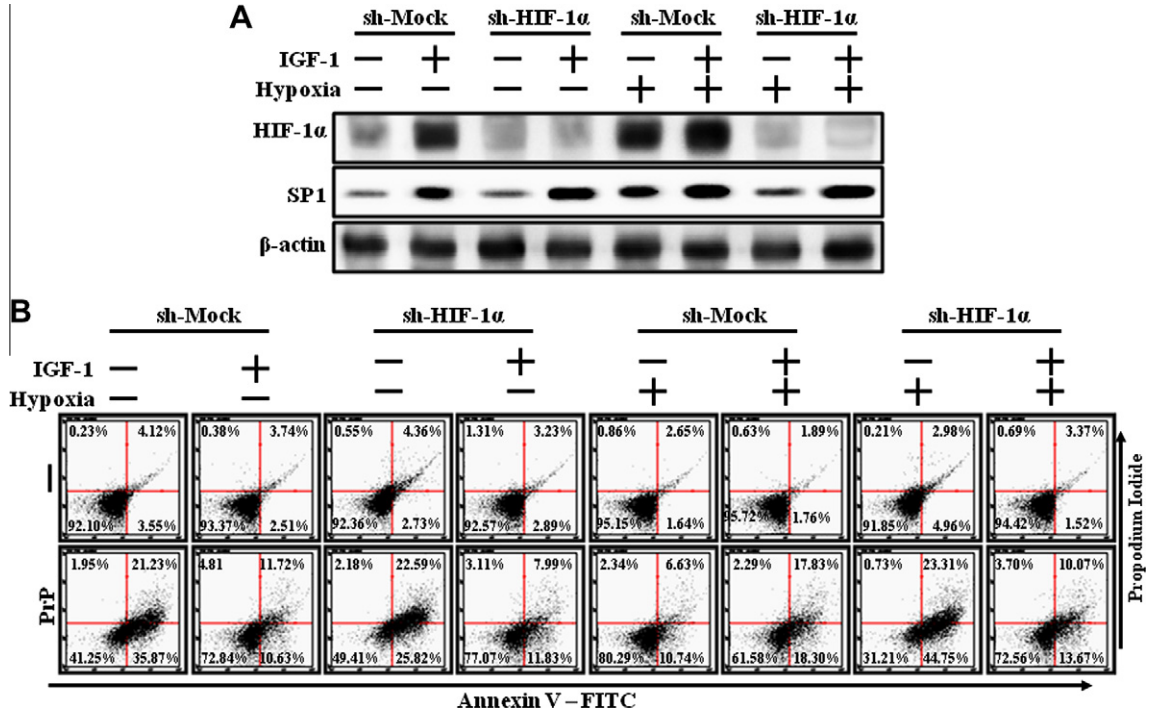


**Fig. 4.** Upregulation of SP1 prevents PrP(106–126)-induced apoptosis in HIF-1α knockdown cells under hypoxic conditions. (A) shHIF-1α- or mock-transfected SH-SY5Y cells exposed to hypoxic conditions and then treated with IGF-1. The cells were assessed for HIF-1α, PrPc and SP1 production by Western Blot analysis. Results were normalized with β-actin and (B) shHIF-1α- or mock-transfected SH-SY5Y cells exposed to hypoxia with or without IGF-1 and then treated with 50 μM PrP(106–126) for 24 h. Cell viability was measured by Annexin V assay.

disease [15]. However, the mechanism of HIF-1α-induced *PRNP* expression in neuron cells has not been reported.

Some reports showed that transcription factor SP1 regulates the expression of many genes involved in various cellular functions, including differentiation, proliferation and apoptosis [29,30]. Expression of *PRNP* is also regulated among neuron cells [22,23]. Consistent with this, the examination of SH-SY5Y cells in which SP1 gene expression had been ablated demonstrated that hypoxia increased HIF-1α activation but did not increase PrPc protein levels (Fig. 3). Also, overstimulation of SP1 caused by IGF treatment increased PrPc protein levels in shHIF-1α-transfected cells (Fig. 4). These results suggest that upregulation of SP1 transcriptional activity may be modulated by HIF-1α-mediated PrPc expression.

Some studies have suggested that upregulation of SP1 transcriptional activity protects against neurodegenerative disorders, including Huntington's disease [31,32]. Mutant huntingtin fragments in the nucleus inhibit the binding of SP1 to the promoters of target genes and thus suppress its transcriptional activity [32]. In the same study, overexpression of SP1 was shown to prevent intranuclear mutant huntingtin-induced neurotoxicity [32]. Also, Ryu et al. demonstrated that inhibition of SP1 transcriptional activity in neurodegenerative diseases, including Huntington's disease, may account for increased susceptibility to oxidative stress [31]. Thus, in the present study, we investigated the influence of SP1 transcriptional activity on HIF-1α-mediated protection against PrP(106–126)-mediated neurotoxicity. We found that depletion of the SP1 gene blocked the protective effect of HIF-1α against PrP(106–126)-induced neurotoxicity (Fig. 1). Also, overexpression of the SP1 gene prevented PrP(106–126)-induced neuron cell death in HIF-1α-shRNA transfected neuron cells (Fig. 4B). Thus, the protective effect of HIF-1α against PrP(106–126)-induced neurotoxicity is thought to be associated with SP1 transcriptional activity.

Future studies will define relationship of SP1 and HIF-1α transcriptional factors to *PRNP* expression in neuron cells. Nevertheless, these results demonstrate that activation of HIF-1α may protect against PrP(106–126)-mediated neurotoxicity by regulation of SP1 transcriptional activity, and HIF-1α-mediated SP1 expression regulates PrPc expression. These results also suggest that activators of the SP1 transcription factor, including IGF-1, may have therapeutic benefits when used as a clinical agent for neurodegenerative disorders, including prion diseases.

## Acknowledgments

This study was supported by the National Research Foundation of the Korea Grant funded by the Korean Government (2012R1A1B3000463) and partially supported by research funds of Chonbuk National University.

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