Neuregulin-1 Protects Neuronal Cells Against Damage due to CoCl2-Induced Hypoxia by Suppressing Hypoxia-Inducible Factor-1α and P53 in SH-SY5Y Cells

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Purpose: Hypoxia-mediated neurotoxicity contributes to various neurodegenerative disorders, including Alzheimer disease. Neuregulin-1 (NRG1) plays an important role in the development and plasticity of the brain. The aim of the present study was to investigate the neuroprotective effect and the regulating hypoxic inducible factor of NRG1 in cobalt chloride (CoCl2) induced hypoxia.
Methods: Hypoxia was induced in SH-SY5Y cells by CoCl2 treatment. SH-SY5Y cells were pretreated with NRG1 and then treated with CoCl2. Western blotting, immunocytochemistry, and lactate dehydrogenase (LDH) release assays were performed to examine neuroprotective properties of NRG1 in SH-SY5Y cells.
Results: Our data showed that CoCl2 induced cytotoxicity and changes of hypoxia-inducible factor-1α (HIF-1α) and p53 expression in SH-SY5Y cells. However, pretreatment with NRG1 inhibited CoCl2-induced accumulation of HIF-1α and p53 stability. In addition, NRG1 significantly attenuated cell death of SH-SY5Y induced by CoCl2.
Conclusions: NRG1 can regulate HIF-1α and p53 to protect neurons against hypoxic damage.

Keywords: Neuregulin-1; Hypoxia; CoCl2; HIF-1α; p53

- *HIGHLIGHTS*
  - CoCl2 induced cytotoxicity and changes of HIF-1α and p53 expression in SH-SY5Y cells.
  - NRG1 inhibited CoCl2-induced accumulation of HIF-1α and p53 stability.
  - NRG1 attenuated SH-SY5Y cell death induced by CoCl2.

INTRODUCTION

Brain requires a continuous supply of the largest amount of oxygen to maintain its normal function. Thus, brain is especially sensitive to hypoxia, a situation where the amount of oxygen is limited. Hypoxia can cause abnormal structural and functional alterations, eventually leading to cell death [1]. Several studies have shown that damage to the brain due to loss of oxygen is

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closely related to neurodegenerative diseases such as vascular dementia, Alzheimer disease, and Parkinson disease [2,3]. In hypoxic brain, accumulation of hypoxia-inducible factor-1α (HIF-1α) can be observed in the several brain regions including the cerebral cortex and the hippocampus [4,5]. HIF-1α is rapidly degraded by the ubiquitin-proteasome pathway [6,7] and kept at very low levels under normoxic conditions. However, it is stabilized due to attenuation of prolyl hydroxylase [8] caused by hypoxia. It is translocated into the nucleus and forms a heterodimeric complex with HIF-1β.

Upon hypoxia, HIF-1α is activated, inducing several genes involved in multiple processes including glucose uptake, glycolysis, angiogenesis, cell survival, and cell invasion [9,10]. The ability of p53 to suppress tumorigenesis is fundamentally linked to its central role in the cellular response to stress. Although normally low or undetectable, p53 level and activity are rapidly increased in response to a wide range of stresses, including hypoxia, DNA damage, oncogene activation, microtubule disruption, and oxidative damage [11,12]. P53-mediated neuronal stress is implicated in neurological damage that occurs following acute insults such as ischemia, traumatic brain injury, and exposure to neurotoxins. Sustained p53-mediated apoptosis is also associated with chronic neuron degenerative pathologies, including Alzheimer's disease and Huntington disease [13,14].

Neuregulin-1 (NRG1) is a member of a family of proteins containing an epidermal growth factor-like motif that mediates important functions in the nervous system [15,16]. It has been shown that NRG1 can regulate nerve cell differentiation, neuron migration, neurotransmission, neurite outgrowth, and synaptic activity [16,17]. NRG1 can protect neurons under various pathological conditions, including ischemia and oxidative stress [18,19]. We have previously reported neuroprotective effects of NRG1 signaling in cell models and Tg2576 mice models [20,21]. Furthermore, we have found that NRG1 is significantly reduced in human AD brains and that it regulates non-amyloidogenic processing in primary cortical neurons. However, no study has reported the role of NRG1 in the expression of hypoxic inducible protein in neuronal cells. Thus, the objective of the present study was to evaluate effects of NRG1 on cobalt chloride (CoCl₂)-induced hypoxic stress in SH-SY5Y cells. In addition, effects of NRG1 on hypoxic inducible factors such as HIF-1α and p53 were characterized.

MATERIALS AND METHODS

Materials and Antibodies
Recombinant human NRG1 was purchased from PROSPEC (East Brunswick, NJ, USA). Cobalt chloride (CoCl₂, C8661) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for HIF-1α and NB100-131 were supplied by Novus Biologicals (Centennial, CO, USA). Antibodies for p53 (sc-126), β-actin (sc-47778), HRP-conjugated anti-rabbit IgG (sc-2004), and HRP-conjugated anti-mouse IgG (sc-2005) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell Culture
SH-SY5Y human neuroblastoma cells were provided from the American Type Culture Collection (Manassas, VA, USA). These cells were cultivated in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplement with a penicillin-streptomycin-amphotericin B solution (Invitrogen) and 10% fetal bovine serum at 37°C with 5% CO_2.

Lactate Dehydrogenase Release Assay
Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) was employed according to the instructions of the manufacturer. Accordingly, 10 μL of the cell supernatants were mixed with 100-μL lactate dehydrogenase (LDH) reaction reagent at room temperature for 30 minutes. Absorbance was determined by using an enzyme-linked immunosorbent assay reader (Victor X3, PerkinElmer, Shelton, CT, USA) with filter for 490 nm.

Immunocytochemistry
SH-SY5Y cells were carried out as previously described [22]. SH-SY5Y cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH, 7.4) at room temperature for 20 minutes. The cells were then washed with PBS 3 times and permeabilized with 0.1% Triton X-100 for 30 minutes. After incubation, cells were blocked with 10% normal horse serum for 30 minutes. The cells were incubated with indirect immunofluorescence using a primary antibody to p53 (1:100) and HIF-1α (1:100) at 4°C overnight. The cells were labeled by fluorescein isothiocyanate-conjugated secondary antibody (1:200; Jackson Immuno Research Laboratories, Inc.) for 2 hours at room temperature. Nuclei were counterstained with 4′-6-diamidino-2-phenylindole (Invitrogen, 10μM) for 30 minutes. Imunostained cells were captured in at least 5 random fields using a Zeiss LSM 5 LIVE con-

focal microscope (Carl Zeiss AG, Oberkochen, Germany).

Western Blot Analysis

Protein samples (30–50 µg) from SH-SY5Y cells lysed in radio immunoprecipitation assay buffer was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a NC membrane (Millipore, Burlington, MA, USA). Membranes were incubated with the appropriate primary antibodies (anti-HIF-1α, 1:1,000, Novus Biologicals; anti-p53, 1:1,000, Santa Cruz Biotechnology; anti-β-actin, 1:5,000, Santa Cruz Biotechnology). After washing with TBS-T (Tris-buffered saline, 0.1% Tween 20) 3 times at RT, membranes were further incubated with horseradish peroxidase-conjugated goat secondary antibodies. The immunoreactive bands were shown using an enhanced chemiluminescent substrate (Bio-Rad Laboratories, Richmond, CA, USA).

Data Analysis

All results are presented as mean ± standard error of the mean. Student t-test and 1-way analysis of variance with Bonferroni *post hoc* test were used to analyze statistical significance (GraphPad Prism 6, CA, USA). $P < 0.05$ was assumed for statistical significance.

RESULTS

CoCl₂ Induces Cytotoxicity and Change of HIF-1α and p53 Protein Expression in SH-SY5Y Cells

CoCl₂ has been widely used to induce hypoxia mimetic condition both *in vitro* and *in vivo*. First, we examined effects of Co-Cl₂-induced cytotoxicity in SH-SY5Y cells. LDH assays showed that CoCl₂ treatment significantly increased cell death in a time-dependent manner. In particular, cell death was significantly increased after exposure to 100µM CoCl₂ for > 12 hours compared with that of the control group. However, LDH release continued to increase in each subsequent time interval (0, 3, 12, 24, 36, 48 hours) (Fig. 1A). As shown in Fig. 1B and C, CoCl₂ induced HIF-1α protein expression in SH-SY5Y cells at 6 hours after treatment. HIF-1α protein expression peaked at 12 hours. This peak sustained through 48 hours after CoCl₂ treatment. Moreover, the expression of p53 was markedly enhanced in CoCl₂-induced SH-SY5Y cells in a time-dependent manner.

NRG1 Pretreatment Reduces CoCl₂-Induced Accumulation of HIF-1α in SH-SY5Y Cells

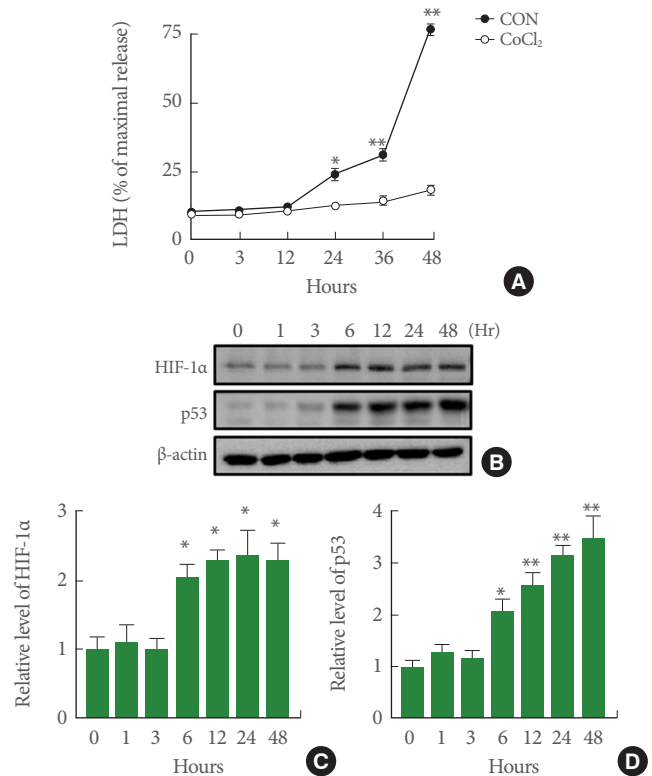The key regulator of hypoxia response is HIF-1. HIF-1 is a com-

Fig. 1. Effects of cobalt chloride (CoCl₂) on cytotoxicity and protein levels of hypoxia-inducible factor-1α (HIF-1α) and p53 in SH-SY5Y cells. (A) Cell death was detected by lactate dehydrogenase (LDH) release assay following treatment with CoCl₂ or serum-free medium for indicated time periods. Effects of CoCl₂ on LDH release in cultured cells for 3, 12, 24, 36, and 48 hours of 100µM CoCl₂ treatment were determined (n = 4, *$P < 0.05$, **$P < 0.01$). (B) Western blotting demonstrated that 100µM CoCl₂ affected HIF-1α and p53 protein expression levels. Both HIF-1α and p53 protein expression were significantly increased by 100µM CoCl₂ treatment in a time dependent manner. (C, D) Quantification of data in B. Densitometry values are shown as ratios relative to values of the control group (n = 6, *$P < 0.05$, **$P < 0.01$).

plex heterodimer including a variety of subunits (HIF-1α and HIF-1β), of which HIF-1α causes hypoxia. To investigate whether NRG1 affected CoCl₂-induced increase in HIF1-1α expression, cells were pretreated with 5nM NRG1 followed by 24 hours of CoCl₂ treatment. Western blotting was then used to examine levels of HIF-1α in SH-SY5Y cells. Results confirmed that treatment of SH-SY5Y cells with both low (100µM) and high (500µM) concentrations of CoCl₂ for 24 hours significantly upregulated levels of HIF-1α (control [CON], 1.00 ± 0.17; 100µM CoCl₂, 2.67 ± 0.26; n = 6, *$P < 0.05$; CON, 1.13 ± 0.09; 500µM CoCl₂, 3.37 ± 0.21, n = 6;
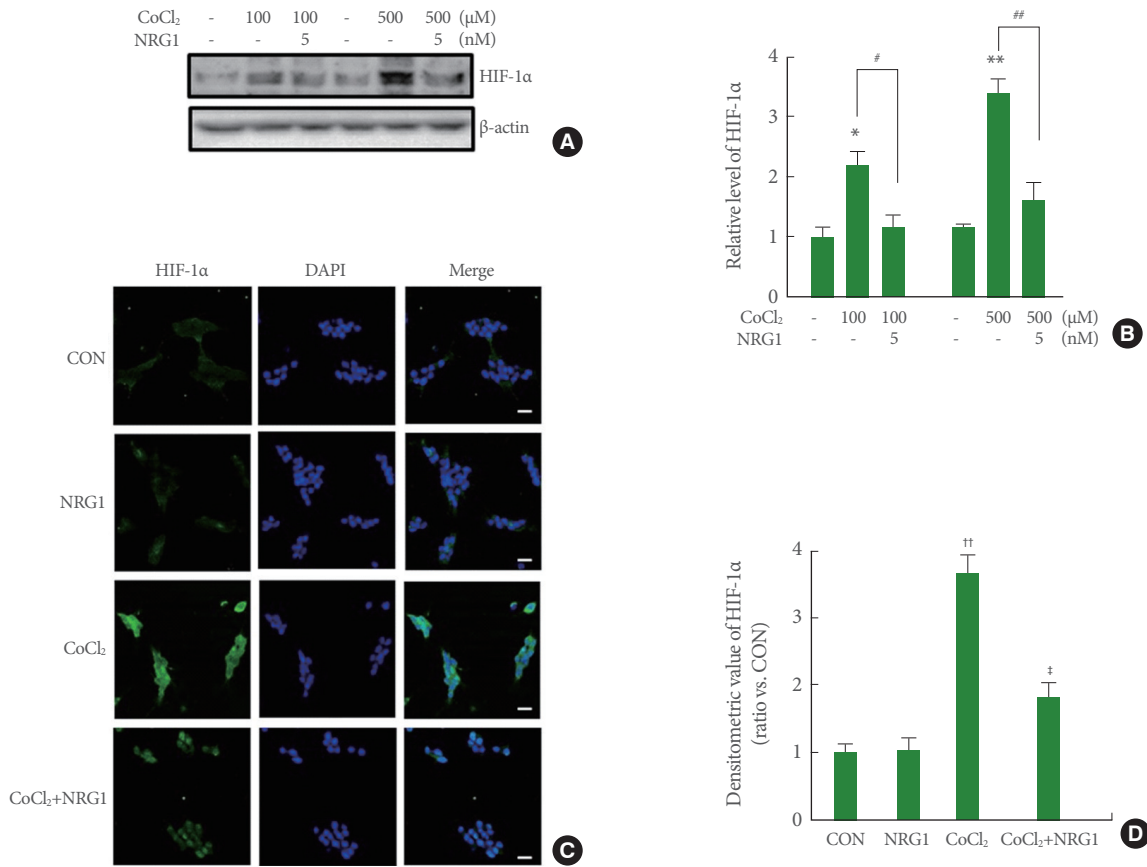
Fig. 2. Neuregulin-1 (NRG1) attenuates cobalt chloride (CoCl$_2$)-induced accumulation of hypoxia-inducible factor-1α (HIF-1α) in SH-SY5Y cells. (A) HIF-1α accumulated upon treatment with 100μM CoCl$_2$ or 500μM CoCl$_2$ and CoCl$_2$-induced HIF-1α accumulation was inhibited by 5nM NRG1 treatment (15 minutes of NRG1 pretreatment followed by 6 hours of CoCl$_2$ treatment). (B) Quantification of data in B. Densitometry values are shown as ratios relative to values of the control (CON) group (n = 6, *P < 0.05, **P < 0.01, #P < 0.05, ##P < 0.01). (C) Expression of HIF-1α after 24 hours of incubation under control, 10nM NRG1, 100μM CoCl$_2$, or treatment with 10nM NRG1 in SH-SY5Y cells by immunofluorescence. SH-SY5Y cells were fixed and immunostained with anti-HIF-1α (green), while 4′-6-diamidino-2-phenylindole (DAPI) (blue) was used as a counterstain. Scale bars represent 20 μm. (D) Fluorescence density of HIF-1α was measured in each group (n = 8, ††P < 0.01, ‡P < 0.05, †compared to the CON group; ‡compared to the CoCl$_2$ group).

**P < 0.01). Moreover, pretreatment with 5nM NRG1 for 24 hours attenuated such increase in HIF-1α expression induced by treatment with CoCl$_2$ (100μM CoCl$_2$, 2.67 ± 0.26; 100μM CoCl$_2$+NRG1, 1.17 ± 0.19, 500μM CoCl$_2$, 3.37 ± 0.21, 500μM CoCl$_2$+NRG1, 1.60 ± 0.31, n = 6; ##P < 0.01) (Fig. 2A, B). The immunoreactivity of HIF-1α in SH-SY5Y cells was measured by immunofluorescence.

To examine effects of NRG1 in SH-SY5Y, cells were pretreated with 10nM NRG1 and then treated with 100μM CoCl$_2$ 15 minutes later. Treatment with 100μM CoCl$_2$ for 24 hours significantly upregulated HIF-1α expression and increased nuclear localization in comparison with that of the control (CON, 1.00 ± 0.12; CoCl$_2$, 3.65 ± 0.28, n = 8; ††P < 0.01). Treatment with

10nM NRG1 for 24 hours attenuated the increase in HIF-1α immunoreactivity induced by treatment with CoCl$_2$ (CoCl$_2$, 3.65 ± 0.28; CoCl$_2$+NRG1, 1.83 ± 0.20, n = 8; ‡P < 0.05) (Fig. 2C, D). These results of immunoreactivity were consistent with western blot assay results.

NRG1 Decreases p53 Stability Induced by CoCl$_2$ in SH-SY5Y Cells

Hypoxic stress could induce stabilization of p53 implicated in regulation of cell fate. To explore the role p53 in response to CoCl$_2$ stress, the expression of p53 protein in SH-SY5Y cells was examined. Results showed a dose-dependent increase inp53 expression by 24 hours of treatment with CoCl$_2$ (50–200μM) (Fig. 3A).
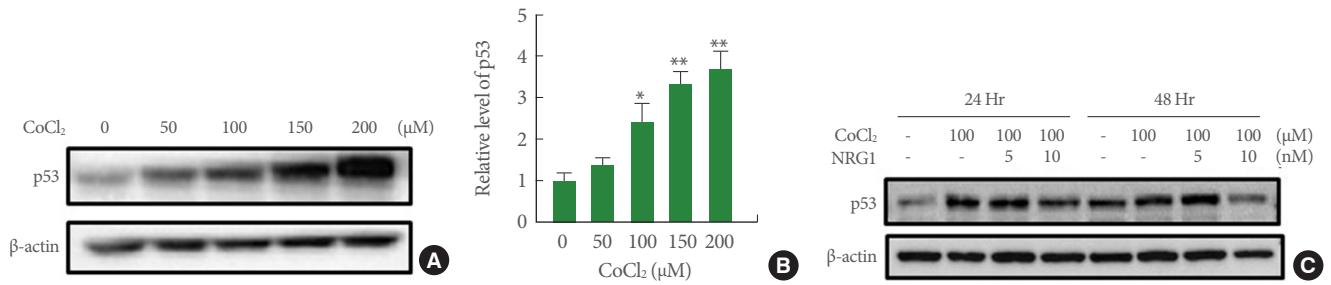
Fig. 3. Neuregulin-1 (NRG1) inhibits cobalt chloride (CoCl₂)-induced p53 stability in SH-SY5Y cells. (A) SH-SY5Y cells were treated with various concentrations (0, 50, 100, 150, and 200μM) of CoCl₂ for 24 hours, which resulted in dose-dependent increase of p53 stability. (B) Quantification of data in A. Densitometry values are shown as ratios relative to values of the control (CON) group (n=6, *P<0.05 vs. control group, #P<0.01 vs. control group). (C) NRG1 decreased CoCl₂-induced p53 stability. SH-SY5Y cells were incubated with or without 100μM CoCl₂ for indicated times, or with pretreatment of NRG1 (5 or 10nM) 15 minutes prior to CoCl₂ treatment. (D) Quantification of data in C. Densitometry values are shown as ratios relative to values of the CON group (n=6, *P<0.05 vs. control group, #P<0.05 vs. CoCl₂-treated group).
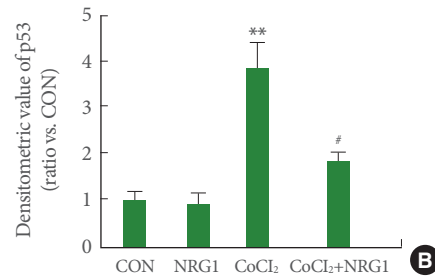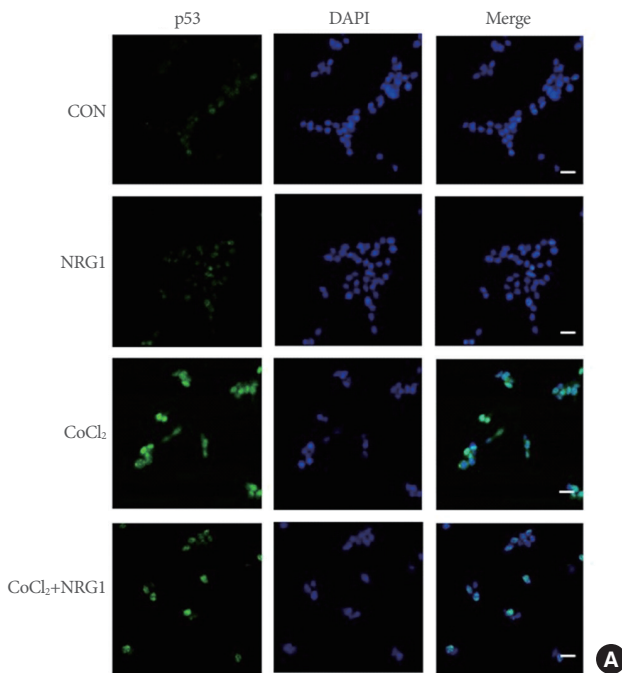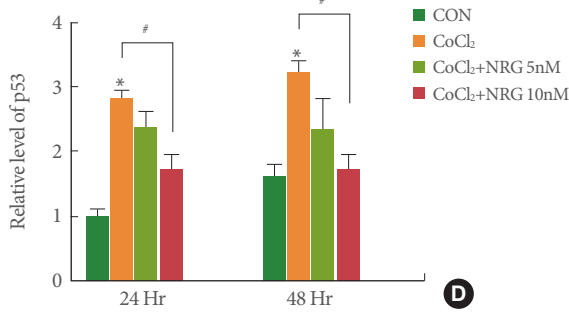

Fig. 4. Neuregulin-1 (NRG1) attenuates the expression and nuclear accumulation of p53 induced by cobalt chloride (CoCl₂) in SH-SY5Y cells. (A) Expression of p53 after 24 hours incubation under control, 10nM NRG1, 100μM CoCl₂, or treatment with 10nM NRG1 in SH-SY5Y cells by immunofluorescence. Cells were double-labeled with 4′-6-diamidino-2-phenylindole (DAPI) (blue) and anti-p53 (green) and then analyzed by fluorescence microscopy. Scale bars represent 20 μm. (B) Fluorescence density of p53 was measured in each group (n=8, **P<0.01, #P<0.05, *compared to the control [CON] group; #compared to the CoCl₂ group).

Quantification demonstrated that CoCl₂ significantly increased the expression of HIF-1α (CON, 1.00±0.20; 50μM CoCl₂, 1.36±0.15; 100μM CoCl₂, 2.40±0.15; 150μM CoCl₂, 3.30±0.30; 200μM CoCl₂, 3.67±0.42, n=6) (Fig. 3B). Effect of NRG1 in SH-SY5Y cells under hypoxic condition was also investigated. As shown in Fig. 3C, treatment with CoCl₂ significantly increased p53 stabiliza-

tion for the indicated time (24 hours: CON, 1.00±0.12; CoCl₂, 2.80±0.15; 48 hours: CON, 1.60±0.21; CoCl₂, 3.23±0.20, n=6) (Fig. 3C, D). Treatment with 10nM NRG1 for 24 or 48 hours attenuated the increase in p53 stabilization induced by treatment with 100μM CoCl₂ (24 hours: CoCl₂, 2.80±0.15; CoCl₂+NRG1, 1.73±0.24; 48 hours: CoCl₂, 3.23±0.20; CoCl₂+ NRG1,
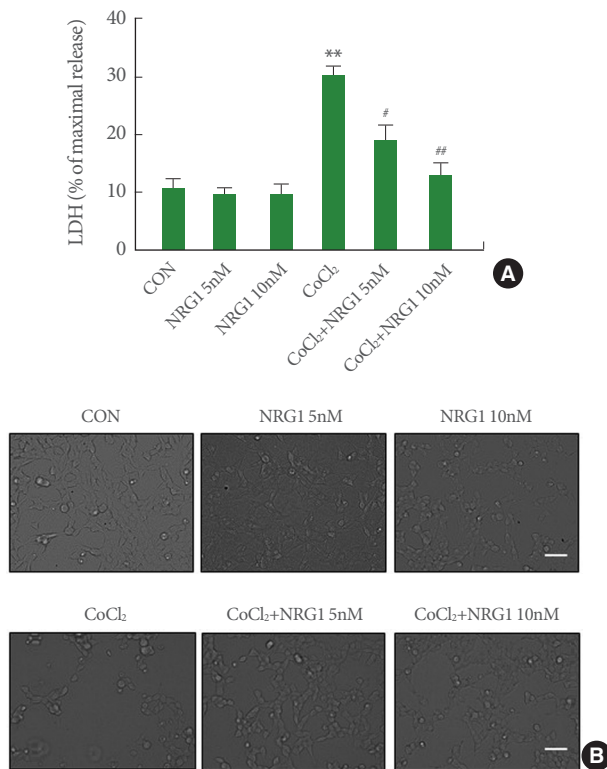
Fig. 5. Neuregulin-1 (NRG1) attenuates SH-SY5Y cell death induced by cobalt chloride (CoCl$_2$). (A) NRG1 (5 or 10nM) attenuated the cell death induced by 100μM CoCl$_2$ in SH-SY5Y cells. After 24 hours, the degree of cell death was assessed by LDH activity in the medium (n = 6, **P < 0.01 vs. control group, [#]P < 0.05 vs. CoCl$_2$ treated group, [##]P < 0.01 vs. CoCl$_2$ treated group). (B) Representative photographs of cells. Scale bar represents 100 μm. CON, control; LDH, lactate dehydrogenase.

1.70 ± 0.25, n = 8; [#]P < 0.05) (Fig. 3C, D).

NRG1 Inhibits CoCl$_2$-Induced Expression and Nuclear Accumulation of p53 in SH-SY5Y Cells

Immunocytochemistry was performed to investigate expression and localization of p53 in SH-SY5Y cells. To investigate effects of NRG1, SH-SY5Y cells were pretreated with 10nM NRG1 and then treated with 100μM CoCl$_2$ 15 minutes later. Treatment with CoCl$_2$ for 24 hours resulted in markedly increased unclear localization of p53 and upregulated p53 expression in comparison with the control (CON, 1.00 ± 0.17; CoCl$_2$, 3.85 ± 0.54, n = 8; **P < 0.01). Moreover, treatment with 10nM NRG1 inhibited the increase in p53 immunoreactivity induced by treatment with CoCl$_2$ (CoCl$_2$, 3.85 ± 0.54; CoCl$_2$+NRG1, 1.81 ± 0.20, n = 8; [#]P < 0.05) (Fig. 4A, B).

NRG1 Prevents SH-SY5Y Cell Death Induced by CoCl$_2$

To determine whether NRG1 could affect the observed cytotoxicity in SH-SY5Y cells induced by CoCl$_2$ treatment, an LDH release assay was performed following 24 hours of CoCl$_2$ treatment. Cells were incubated with different concentrations (5 and 10nM) of NRG1 and then exposed to 100μM CoCl$_2$ for 24 hours. NRG1 at both concentrations (5 and 10nM) attenuated neuronal cell death induced by 100μM CoCl$_2$ treatment in SH-SY5Y cells (CON, 10.61 ± 1.73; 5nM NRG1, 9.55 ± 1.14; 10nM NRG1, 9.89 ± 1.52; CoCl$_2$, 30.49 ± 1.25; CoCl$_2$+NRG1 5nM, 19.20 ± 2.63; CoCl$_2$+ 10nM NRG1, 12.98 ± 1.96, n = 6; **P < 0.01, [#]P < 0.05, [##]P < 0.01) (Fig. 5A). Representative images of cells are shown in Fig. 5B. These results indicate that NRG1 exerts a significant protective effect over cell death induced by CoCl$_2$ treatment.

DISCUSSION

In this study, we determined whether NRG1 was implicated in hypoxic stress induced by CoCl$_2$ using *in vitro* cell models. CoCl$_2$ has been used as a hypoxia-mimicking agent in both *in vivo* and *in vitro* studies to generate hypoxia like environment [23,24] by inducing the accumulation of HIF-1α [25]. It can also up-regulate genes such as p53, p21, and PCNA [26]. Our present study confirmed that CoCl$_2$ treatment increased the cytotoxicity and protein levels of HIF-1α and p53 in SH-SY5Y cells in a time-dependent manner. These observations suggest that the model of hypoxic stress induced by CoCl$_2$ is a suitable tool to investigate mechanisms of neuronal cell injury.

NRG1 is expressed in multiple regions of the adult nervous system on top of the developing brain. NRG1 is a growth factor those signaling plays important roles in the maintenance of brain circuits [27]. Several lines of evidence collectively suggest that NRG1 plays a protective role in neurons against neurotoxic stimuli including ischemic insult, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), and H$_2$O$_2$ [18,28]. In a previous study, we have found that NRG1/ErbB4 signaling exerts neuroprotective effects against the Swedish mutant amyloid precursor protein, amyloid beta-peptide Aβ$_{1-42}$, and C-terminal fragments of amyloid precursor protein [19,21]. We have also shown that NRG1 can prevent Aβ$_{1-42}$-induced impairment of LTP by its ErbB4 receptor [29]. In addition, we have recently reported that NRG1 can attenuate H$_2$O$_2$-indcued oxidative stress [30]. NRG1 is also found to play a key role in the peripheral nerves and skeletal muscle [31]. Glial growth factor 2 (neuregulin-1β3 type II) promotes the recovery of erectile function in a rat mod-

el of radical prostatectomy-associated cavernous nerve injury [32].

To investigate whether NRG1 might affect the increase of HIF-1α expression induced by $CoCl_2$, we evaluated HIF-1α levels via western blotting and immunocytochemistry. We observed that NRG1 attenuated $CoCl_2$-induced accumulation of HIF-1α in SH-SY5Y cells. Especially, NRG1 significantly inhibited the increase of nuclear localization of HIF-1α. These results provide evidence that NRG1 signaling could directly regulate hypoxic dysfunction of neurons.

Under hypoxic conditions, HIF-1α accumulates and triggers the activation of many genes. It has been reported that HIF-1α can cause hypoxia-induced apoptosis by mediating stabilization of p53 and its transactivation under hypoxic conditions. The p53 is a potent transcription factor that activates target genes to initiate apoptosis [33]. In normal cells, p53 is maintained at low levels by ubiquitin ligase mouse double minute 2 homolog (MDM2)-mediated degradation. It is strongly stabilized in response to various types of stress, including hypoxia [34]. HIF-1α can increase the stability of wild-type p53 by directly binding to the p53 ubiquitin ligase MDM2 [33].

Upon exposure to a variety of stress conditions, the stability of p53 increases and the protein accumulates in the nucleus, where it is activated as a transcription factor [35]. To explore the implication of p53 in response to $CoCl_2$ stress, we monitored $CoCl_2$-induced p53 stability. We found that p53 was upregulated by $CoCl_2$ treatment dose-dependently. Moreover, NRG1 ameliorated $CoCl_2$-induced p53 stability in SH-SY5Y. Furthermore, NRG1 markedly decrease unclear localization of p53 and upregulated p53 expression. Taken together, our results suggest that NRG1 can regulate HIF-1α and p53 to protect neurons from hypoxic damage. Further research is needed to clarify the regulation effect of NRG1 on mechanism of under signaling.

AUTHOR CONTRIBUTION STATEMENT

· Full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis: *JHL, RSW*
· Study concept and design: *JHL, RSW*
· Acquisition of data: *SYY, JYY, HBK*
· Analysis and interpretation of data: *RSW*
· Drafting of the manuscript: *SYY, RSW*
· Critical revision of the manuscript for important intellectual

content: *RSW*
· Statistical analysis: *TKB*
· Obtained funding: *JHL, RSW*
· Administrative, technical, or material support: *JYY*
· Study supervision: *RSW*

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