Neuregulin-1 Inhibits CoCl2-Induced Excitatory Amino Acid Carrier 1 Overexpression and Oxidative Stress in SH-SY5Y Cells

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Abstract

Excitatory amino acid carrier 1 (EAAC1) is an important subtype of excitatory amino acid transporters (EAATs) and is the route for neuronal cysteine uptake. CoCl$_2$ is not only a hypoxia-mimetic reagent but also an oxidative stress inducer. Here, we found that CoCl$_2$ induced significant overexpression of EAAC1 in a dose- and time-dependent manner. We further demonstrated that pretreatment with NRG1 rescued the CoCl$_2$-induced upregulation of EAAC1 and tau expression. Neuregulin-1 (NRG1) plays a protective role in the CoCl$_2$-induced accumulation of reactive oxygen species (ROS) and reduction in antioxidative enzyme (SOD and Gpx) activity. Moreover, NRG1 attenuated CoCl$_2$-induced apoptosis and cell death. NRG1 inhibited the CoCl$_2$-induced release of cleaved caspase-3 and reduction in Bcl-X$_L$. Our novel findings suggest that NRG1 may play a protective role in oxidative stress and hypoxia through the regulation of EAAC1.

1. Introduction

Excitatory amino acid carrier 1 (EAAC1, also referred to as EAAT3) is one neuronal subtype of excitatory amino acid transporter (EAAT) that is ubiquitously expressed in the central nervous system (CNS). EAAC1 can also transport cysteine at a rate comparable to that of glutamate and is the primary route for the uptake of neuronal cysteine. Cysteine is a critically important substrate for the synthesis of glutathione (GSH), one of the most important intracellular antioxidants in the brain [1, 2]. Mature neurons utilize cysteine but not cystine for GSH synthesis [3, 4]. EAAC1-mediated uptake may be the major source of cysteine for GSH synthesis in mature neurons [5]. Oxidative stress is a general premonitory hallmark of numerous brain pathologies and largely contributes to the acute and chronic outcomes of CNS disorders, such as epilepsy, ischemic stroke, amyotrophic lateral sclerosis, Alzheimer’s disease and Parkinson’s disease [6]. Modulation of EAAC1 activity correlates with neuronal GSH levels [7]. Knockdown of EAAC1 reduces cysteine uptake and intracellular GSH levels [8].

The intracellular response to hypoxia is regulated by hypoxia inducible factor-1 (HIF-1). HIF-1 is a transcription factor, and a heterodimer consisting of an oxygen-dependent regulatory HIF-1α subunit and a constitutively expressed HIF-1β subunit that acts as a master regulator of adaptation to a low oxygen environment in the cell [9]. Recent evidence suggests that the ROS produced in the mitochondria mediate HIF-1α stabilization during hypoxia [9]. Hypoxia leads to a rapid increase in spontaneous vesicular glutamate release [10] and impaired glutamate uptake [11–13]. EAAC1 was increased at the transcript level in C6 cells by hypoxia [14]. Oxygen-glucose deprivation (OGD) induced the protein expression of EAAC1 in pure and mixed neuronal cultures and promoted EAAT3 activity, which increased glutamate uptake into cultured neurons [15]. EAAC1 transcript levels were transiently upregulated during the reperfusion phase in ischemia-reperfusion models [15]. Ischemia-reperfusion leads to oxidative stress and an accompanying transient increase in EAAT3 immunoreactivity in the hippocampus [16].

Neuregulin-1 (NRG1) is a member of the NRG family of growth factors that play important roles in the developing and adult CNS [17]. Recently, accumulating evidence has collectively shown that NRG1 is a
new regulator of injury and repair with multifaceted roles in neuroprotection, remyelination, and immunomodulation. NRG1 protects against a number of CNS pathological conditions, including ischemia, neurotrauma, and neurodegenerative diseases [18–22]. Our recent work showed that NRG1 regulated hypoxia-inducible factors such as HIF-1α and p53 [23]. NRG1/ErbB4 attenuates neuronal cell damage under OGD in primary hippocampal neurons [24]. These findings suggest a correlation between NRG1 dysfunction and CNS pathology. Therefore, NRG1 may be a potential therapeutic target in the recovery of function after CNS injury.

Herein, we used cobalt chloride (CoCl₂), a hypoxia mimic, to induce oxidative stress in SH-SY5Y cells. Cobalt stimulates reactive oxygen species (ROS) generation through a nonenzymatic, nonmitochondrial mechanism, and CoCl₂ treatment induces HIF-1α accumulation [25].

Our study provides conclusive molecular evidence that CoCl₂ strongly induces EAAC1 expression in SH-SY5Y cells. These changes may alter homeostasis and enhance reactive oxidative stress in neurons. Furthermore, NRG1 rescued the CoCl₂-induced upregulation of EAAC1 and oxidative stress.

**Materials And Methods**

**Reagents and antibodies**

Recombinant β-type NRG1 was purchased from ProSpec (East Brunswick, NJ, USA). Antibodies were obtained from Millipore Corporation (Chemicon, MA, USA) (EAAT3 (EAAC1), MAB1587), Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) (Bcl-X, sc-8392; p53, sc-126; β-actin, sc-47778), Novus Biologicals (Centennial, CO, USA) (HIF-1α, NB100-131; Tau, NBP-25613), and Cell Signaling Technology (CST, MA, USA) (Caspase 3, #9662s; cleaved caspase 3, #9661s; EAAC1, #12179; HRP-conjugated anti-rabbit IgG, #7076s; HRP-conjugated anti-mouse IgG, #7074s). CoCl₂ (C8661) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture**

SH-SY5Y human neuroblastoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) and a penicillin-streptomycin-amphotericin B mixture (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO₂. When the cells grew sufficiently in 100 mm culture dishes (SPL Life Sciences, Gyeonggi-do, Korea), they were subcultured in 6-well or 96-well plates.

**Assessment of cell death**

Cell death after CoCl₂ treatment was assessed by determining the release of lactate dehydrogenase (LDH) into the culture medium, thereby indicating a loss of membrane integrity. LDH activity was measured using a commercial kit (Cytotox 96 nonradioactive cytotoxicity assay kit, Promega, Madison,
according to the manufacturer’s protocol. The absorbance was measured at 490 nm using a VICTOR X3 multilabel plate reader (PerkinElmer, Shelton, USA).

TUNEL staining

In situ DNA fragmentation was assessed using a terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) staining kit (Roche Diagnostics) according to the manufacturer’s instructions. Images were captured after counterstaining with 10 µM 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) for 30 minutes. The number of apoptotic cells was counted in five random fields using a Zeiss LSM 5 LIVE confocal microscope (Carl Zeiss AG, Oberkochen, Germany). The apoptotic cells are expressed as the percentage of TUNEL-positive cells in the total number of DAPI-stained cells.

ROS measurement

ROS generation in SH-SY5Y cells was analyzed using the dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, CA, USA). SH-SY5Y cells were washed three times with Dulbecco’s phosphate-buffered saline (DPBS) and then incubated at 37 °C in DPBS containing 20 µM DCFH-DA for 30 minutes. Once inside the cells, DCFH-DA is hydrolyzed by esterase to form polar DCFH, which then interacts with ROS. Cells were subsequently washed three times with DPBS and visualized with a fluorescence microscope (EVOS M5000, Thermo Fisher Scientific, Eugene, OR, USA) at an excitation wavelength of 488 nm.

Glutathione peroxidase (GPx) activity assay

GPx activity was determined using a Biovision glutathione peroxidase activity assay kit (CA, USA) according to the manufacturer’s protocol. SH-SY5Y cells were homogenized on ice in cold assay buffer and then centrifuged at 10,000 × g for 15 minutes at 4 °C. Then, 50 µl of cell supernatant was added to a 96-well plate with 50 µl of assay buffer. The reaction mixture was added to each sample and incubated for 15 minutes to deplete all GSSG in the samples. Ten microliters of cumene hydroperoxide substrate was subsequently added to initiate the enzymatic reaction. The absorbance was immediately measured at a wavelength of 340 nm using a VICTOR X3 multilabel plate reader (PerkinElmer, Shelton, USA). GPx activity was calculated using an NADPH standard curve.

Superoxide dismutase (SOD) activity assay

SOD activity was measured using a commercially available kit (Cayman Chemical Company, MI, USA) according to the manufacturer’s protocol. SH-SY5Y cells were homogenized in cold 20 mM HEPES buffer (pH 7.2) and centrifuged at 1,500 × g for 5 minutes at 4 °C. Each sample (10 µl) was added to a 96-well plate with 200 µl of the diluted radical detector. Then, 20 µl of diluted xanthine oxidase was added to initiate the enzymatic reaction. The absorbance was immediately measured at a wavelength of 450 nm using a VICTOR X3 multilabel plate reader (PerkinElmer, Shelton, USA).

Immunofluorescence analysis
SH-SY5Y cells were fixed using 4% paraformaldehyde and 4% sucrose in DPBS (pH 7.4) for 20 minutes at room temperature (RT). Next, the cells were permeabilized and blocked using DPBS containing 1% BSA and 0.1% Triton X-100 at RT for 30 minutes, and then primary antibodies (mouse anti-EAAC1 (1:100) and rabbit anti-tau (1:100)) were added and incubated overnight at 4 °C. The cells were then washed three times in PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 595 goat anti-chicken IgG (Jackson ImmunoResearch Laboratories, Inc., 1:200) for 2 hrs at RT. After counterstaining with DAPI (10 µM in PBS), the cells were mounted in Vectorshield (Vector Laboratories). Fluorescent images were acquired with an LSM 5 LIVE confocal system (Carl Zeiss AG, Oberkochen, Germany).

**Western blot analysis**

Western blotting was performed as previously described [22]. Briefly, tissues were homogenized using a modified homogenization buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% sodium-deoxycholate, 1 mM PMSF, 1 mM EDTA, and 1 µg/ml each of aprotinin, leupeptin, and pepstatin protease inhibitors). Samples were then resolved using SDS-PAGE, transferred to nitrocellulose membranes and subsequently blocked with TBS containing 5% fat-free milk and 0.05% Tween-20 for 1 hr. Next, the membranes were incubated overnight at 4 °C with primary antibodies (anti-EAAC1, 1:1,000, Millipore Corporation or Cell Signaling; anti-caspase 3, 1:1,000, 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) and developed using horseradish peroxidase-conjugated secondary antibodies. Immunodetection was performed with a chemiluminescence system (Amersham Pharmacia) and a ChemiDoc TM tough imaging system (Bio-Rad, California, USA).

**Statistical Analysis**

The data are presented as the means ± SEM of three or more independent experiments. For the data of more than two groups, statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. A value of $P < 0.05$ was considered statistically significant.

**Results**

**CoCl₂ increased EAAC1 protein expression in SH-SY5Y cells**

We used CoCl₂ to mimic hypoxia in SH-SY5Y cells. First, we examined whether CoCl₂ altered the protein levels of EAAC1 in SH-SY5Y cells. We found that there was a dose-dependent increase in EAAC1 expression after 24 hrs of CoCl₂ (50–500 µM) treatment (Fig. 1a). Quantification of the data demonstrated that CoCl₂ significantly increased EAAC1 expression (CON, 1.04 ± 0.14; 50 µM CoCl₂, 1.13 ± 0.29; 100 µM CoCl₂, 1.71 ± 0.12; 150 µM CoCl₂, 1.88 ± 0.18; 200 µM CoCl₂, 2.58 ± 0.56; 300 µM CoCl₂, 3.58 ± 0.56; 500 µM CoCl₂, 5.87 ± 0.34; n = 8; ***$P < 0.001$; Fig. 1b). CoCl₂ treatment significantly increased EAAC1 protein expression at each subsequent time point (0, 1, 3, 12, 24, 36, and 48 hrs). EAAC1 protein
expression was significantly increased after exposure to 100 µM CoCl² for >24 hrs compared with that of the controls (n = 6; *P < 0.05, ***P < 0.001; Fig. 1c and d).

NRG1 alleviated CoCl²-induced overexpression of EAAC1 in SH-SY5Y cells

To determine whether NRG1 affected the CoCl²-induced increase in EAAC1 expression, we pretreated cells with NRG1 (5 nM or 10 nM) for 15 minutes before CoCl² administration. Treatment with 100 µM CoCl² for 36 hrs significantly upregulated EAAC1 expression (CON, 0.99 ± 0.21; CoCl², 5.61 ± 0.87, n = 8; ***P < 0.001; Fig. 2a and b). As shown in Fig. 2a, c and d, treatment with 100 µM CoCl² for 36 hrs significantly upregulated HIF-1α (CON, 1.01 ± 0.19; 100 µM CoCl², 4.56 ± 0.41, n = 6; ***P < 0.001) and p53 (CON, 0.92 ± 0.27; 100 µM CoCl², 3.62 ± 0.38, n = 6; ***P < 0.001) expression. Pretreatment with NRG1 for 36 hrs attenuated this increase in HIF-1α accumulation induced by 100 µM CoCl² (CoCl², 4.56 ± 0.41; CoCl² + 10 nM NRG1, 2.55 ± 0.35; CoCl² + 10 nM NRG1, 1.47 ± 0.28, n = 6; #P < 0.01, ###P < 0.001; Fig. 2c). Moreover, pretreatment with 5 nM or 10 nM NRG1 for 36 hrs attenuated the increase in p53 stabilization induced by 100 µM CoCl² (CoCl², 3.62 ± 0.38; CoCl² + 5 nM NRG1, 3.10 ± 0.46; CoCl² + 10 nM NRG1, 1.85 ± 0.15, n = 6; #P < 0.05; Fig. 2d). These results are consistent with those of our previous studies demonstrating the effects of NRG1 on HIF-1α or p53 [23].

NRG1 inhibited CoCl²-induced increases in EAAC1 and Tau immunoreactivity

We examined the immunoreactivity of EAAC1 in SH-SY5Y cells using immunofluorescence staining. To measure the effects of NRG1 on SH-SY5Y cells, cells were pretreated for 15 minutes with 10 nM NRG1 and then treated with 100 µM CoCl² (Fig. 3a). Treatment with 100 µM CoCl² for 24 hrs significantly upregulated EAAC1 expression in comparison to that of the control group (CON, 1.02 ± 0.10; 100 µM CoCl², 4.45 ± 0.64, n = 8; **P < 0.01; Fig. 3b). We also confirmed that the pretreatment of SH-SY5Y cells with 10 nM NRG1 for 24 hrs significantly attenuated EAAC1 overexpression (CoCl², 4.45 ± 0.64; CoCl² + 10 nM NRG1, 1.83 ± 0.37, n = 8, #P < 0.05; Fig. 3b) compared with that of the control group. Interestingly, treatment with 100 µM CoCl² for 24 hrs markedly increased the accumulation of Tau in comparison with that of the control group (CON, 1.00 ± 0.20; CoCl², 2.58 ± 0.27, n = 8; **P < 0.01; Fig. 3c). Furthermore, 10 nM NRG1 attenuated the CoCl²-induced increase in Tau expression (CoCl², 2.58 ± 0.27; CoCl² + NRG1, 1.45 ± 0.15, n = 8; #P < 0.05; Fig. 3c).

NRG1 rescued CoCl²-induced ROS generation and the reduction in antioxidant enzymes in SH-SY5Y cells

We tested the protective effect of NRG1 against CoCl²-induced ROS generation. We found that treatment with 100 µM CoCl² for 24 hrs significantly increased ROS levels (CON, 1.13 ± 0.20; CoCl², 4.46 ± 0.44, n = 6; ***P < 0.001; Fig. 4a and b) compared with the levels in the control group. However, pretreatment with 5 nM or 10 nM NRG1 significantly attenuated CoCl²-induced ROS generation (CoCl², 4.46 ± 0.44; CoCl² +
5 nM NRG1, 2.70 ± 0.37; CoCl2 + 10 nM NRG1, 1.67 ± 0.16, n = 6, #P < 0.05, ##P < 0.01; Fig. 4a and b). To determine whether NRG1 affects the antioxidant defense system, we analyzed the activity of the antioxidant enzymes GPx and SOD. Treatment with 100 µM CoCl2 significantly reduced the activity of GPx (CON, 32.37 ± 1.63; CoCl2, 19.31 ± 1.77, n = 6; **P < 0.01; Fig. 4c) compared with that of the control group. Pretreatment with 5 nM or 10 nM NRG1 for 36 hrs attenuated the CoCl2-induced reduction in GPx activity (CoCl2, 19.31 ± 1.77; CoCl2 + 5 nM NRG1, 34.38 ± 1.94; CoCl2 + 10 nM NRG1, 30.46 ± 1.99, n = 6, ###P < 0.001; Fig. 4c). Moreover, after the cells were exposed to 100 µM CoCl2 in the presence or absence of NRG1 for 36 hrs, SOD activity was measured. We also demonstrated that after the cells were exposed to CoCl2 for 36 hrs, there were distinct decreases in SOD activity (CON, 121.78 ± 2.88; CoCl2, 98.91 ± 5.02, n = 8; ***P < 0.001; Fig. 4d). Moreover, pretreatment of cells with 5 nM or 10 nM NRG1 attenuated the CoCl2-induced decrease in SOD activity (CoCl2, 98.91 ± 5.02; CoCl2 + 5 nM NRG1, 116.74 ± 2.51; CoCl2 + 10 nM NRG1, 114.189 ± 3.76, n = 8, #P < 0.05, ##P < 0.01; Fig. 4d).

**NRG1 rescued CoCl2-induced apoptosis and cell death**

We examined whether NRG1 affects CoCl2-induced apoptosis in SH-SY5Y cells. To detect apoptotic nuclei in SH-SY5Y cells, we used TUNEL staining. Treatment with 100 µM CoCl2 significantly increased the proportion of apoptotic nuclei (CON, 2.00 ± 0.58; CoCl2, 21.33 ± 2.03, n = 6; ***P < 0.01; Fig. 5a and b) compared with that of the control group. Pretreatment with 10 nM NRG1 for 24 hrs reduced the number of CoCl2-induced TUNEL-positive cells (CoCl2, 21.33 ± 2.03; CoCl2 + 10 nM NRG1, 6.33 ± 2.03, n = 6, ##P < 0.01, Fig. 5a and b).

Next, we examined CoCl2-induced cytotoxicity in SH-SY5Y cells. The cells were incubated with 10 nM NRG1 and then exposed to 100 µM CoCl2 for 36 hrs (CON, 10.48 ± 2.10; CoCl2, 31.97 ± 3.21; CoCl2 + 10 nM NRG1, 16.18 ± 2.05, n = 6, #P < 0.05, Fig. 5c).

**Effects of NRG1 on CoCl2-induced changes in apoptotic or antiapoptotic proteins**

We next investigated whether caspase-3 cleavage is increased by CoCl2. SH-SY5Y cells were treated with 100 µM CoCl2 for 24 hrs before fixation and immunofluorescence detection of cleaved caspase-3. We found that CoCl2 increased the cleavage of caspase-3, and quantitative analysis showed that the number of cleaved caspase-3-positive cells was increased (CON, 1.00 ± 0.51; CoCl2, 3.40 ± 0.34, n = 6; **P < 0.01; Fig. 6a and b). Furthermore, pretreatment with 10 nM NRG1 for 24 hrs rescued the CoCl2-induced increase in the number of cleaved caspase-3-positive cells (CoCl2, 3.40 ± 0.34; CoCl2 + 10 nM NRG1, 1.93 ± 0.35, n = 6, #P < 0.05, Fig. 6a and b). To determine whether NRG1 regulates CoCl2-induced caspase-3 cleavage, we performed western blotting. We observed that the level of cleaved caspase-3 (17 and 19 kD) was significantly increased after CoCl2 treatment (CON, 1.28 ± 0.16; CoCl2, 2.56 ± 0.29, n = 6; **P < 0.01; Fig. 6c and d). NRG1 attenuated the CoCl2-induced increase in cleaved caspase-3 (CoCl2, 2.56 ± 0.29; CoCl2 + 5 nM NRG1, 1.52 ± 0.17; CoCl2 + 10 nM NRG1, 1.13 ± 0.10, n = 6; #P < 0.05, ##P < 0.01; P < 0.05; Fig. 6c and
d). Furthermore, the expression of Bcl-xL (an antiapoptotic protein) was decreased in CoCl$_2$-induced cells (CON, 1.02 ± 0.14; CoCl$_2$, 0.4 ± 0.08, n = 6; *P < 0.05; Fig. 6c and e). NRG1 protected against the CoCl$_2$-induced reduction in Bcl-xL protein expression (CoCl$_2$, 0.4 ± 0.08; CoCl$_2$ + 5 nM NRG1, 0.66 ± 0.05; CoCl$_2$ + 10 nM NRG1, 1.01 ± 0.11, n = 6; #P < 0.05, Fig. 6c and e). These results suggest that NRG1 may have a protective role under hypoxic conditions by regulating apoptosis.

**Discussion**

In the present study, we assessed the effects and mechanisms of NRG1 on CoCl$_2$-induced oxidative stress in SH-SY5Y cells. First, we demonstrated that CoCl$_2$ dramatically increased EAAC1 protein expression. EAAT1 and EAAT2 are mainly expressed in glial cells [26–28], whereas EAAT3 is exclusively expressed in neurons [29–32]. The EAAC1 protein is abundantly expressed in the hippocampus, cerebellum, and midbrain areas [30]. In general, EAAC1 activity is considered to be the main mechanism responsible for glutamatergic transmission [2], and EAAC1 also transports cysteine into neurons [33, 34]. Modulation of EAAC1 activity correlates with neuronal GSH levels [7] and the rate-limiting substrate for neuronal synthesis of GSH [35].

EAAC1 may be the major contributor to GSH synthesis [5] in neurons. Interestingly, Rossi et al. reported that glutamate release is largely mediated by reversed activity of the neuronal glutamate transporter in severe brain ischemia. The glutamate transporter plays a key role in generating anoxic depolarizations in hippocampal neurons [36]. These results suggest that the abnormal activity abolished information processing in the CNS within minutes of ischemia [37]. EAAC1-deficient mice showed that the delayed anoxic overexpression of EAAC1 could contribute to the reversed activity in neurons.

Hypoxic conditions have been extensively studied for their potential to regulate glutamate transporters, as this putative regulation could have important consequences for brain pathologies. A previous study reported that chronic hypoxia upregulates EAAC1 expression in PC12 cells [38]. CoCl$_2$ was reported to be a widely used hypoxia mimetic in a large variety of cells and is known to both inhibit prolyl hydroxylases, leading to HIF-1α stabilization, and induce ROS formation under normoxic conditions [39, 40].

Furthermore, we examined the effect of NRG1 on CoCl$_2$-induced EAAC1 and hypoxia-related protein. Several lines of evidence collectively suggest that NRG1 plays a neuroprotective role in the brain against neurotoxic substances related to apoptosis and oxidative damage in neurons [41–44]. In this study, we showed that NRG1 could prevent CoCl$_2$-induced upregulation of EAAC1 levels in SH-SY5Y cells. We also confirmed that NRG1 could attenuate the CoCl$_2$-induced accumulation of HIF-1α and p53 [23]. Immunofluorescence analysis also showed that NRG1 significantly inhibited CoCl$_2$-induced overexpression of EAAC1 in SH-SY5Y cells. Tau protein is a soluble microtubule-associated protein that is abundant in neurons and plays a role in neurite outgrowth and axonal transport [45, 46]. Additionally, the level of Tau increased in cells after CoCl$_2$ treatment, suggesting that hypoxia or oxidative stress can lead to alterations in cell structure. Previously, there was a report showing that hypoxia promoted the
phosphorylation and total expression of tau protein [38, 47]. Moreover, we found that NRG1 prevented the CoCl$_2$-induced upregulation of EAAC1 and Tau.

Next, we examined whether NRG1 protects against CoCl$_2$-induced ROS generation. Numerous studies have suggested that hypoxia induces increased production of ROS in the brain [48–50]. When we treated the cells with CoCl$_2$, ROS levels were increased. According to our results, NRG1 attenuated the CoCl$_2$-induced generation of ROS in SH-SY5Y cells. There is a balance between the generation of ROS and their clearance by antioxidant networks, mainly by GPx, SOD, and catalase under physiological conditions [51, 52]. In the present study, CoCl$_2$ reduced the activity of Gpx and SOD in SH-SY5Y cells. We found that NRG1 had a protective effect on the CoCl$_2$-induced reduction in Gpx and SOD enzymatic activity.

ROS is a powerful initiator of apoptosis, which also contributes to hypoxia-mediated neuronal cell death [53]. We also found that NRG1 significantly reduced CoCl$_2$-induced apoptosis and cell death in SH-SY5Y cells.

In the intrinsic pathway, ROS induce mitochondria-dependent apoptosis. This process can be modulated by the release of cytochrome c and the downstream activation of caspases. We next focused on whether NRG1 could protect SH-SY5Y cells against the activation of caspase-3 after CoCl$_2$ treatment. Our results verified that NRG1 significantly reduced the expression of cleaved caspase-3, which may have prevented hypoxia-induced apoptosis and cell death in SH-SY5Y cells. Immunoblot analysis also confirmed the effect of NRG1 on the CoCl$_2$-induced activation of caspase-3. Bcl-2 family members act as critical regulators of the intrinsic apoptotic pathway. The antiapoptotic Bcl-2 family protein Bcl-x$_L$ predominantly localizes to the outer mitochondrial membrane, whereas other members indirectly interact with mitochondria [54]. We further confirmed that NRG1 inhibited the CoCl$_2$-induced reduction in Bcl-x$_L$ expression.

Taken together, our data suggest that NRG1 protects against CoCl$_2$-induced overexpression of EAAC1.

The abnormal overexpression of EAAC1 by CoCl$_2$ may accelerate ROS generation and hypoxic injury. Pretreatment with NRG1 could activate these cellular defense mechanisms to mimic hypoxic preconditioning. NRG1 exerts its biological effects by activating a family of ErbB tyrosine kinase receptors. NRG1 can trigger signaling pathways such as Raf-MEK-ERK and PI3K-Akt-S6K. Further study is needed to clarify the underlying pathway associated with NRG1 in these effects.

**Conclusion**

Our study suggests that CoCl$_2$ significantly increases EAAC1 expression in SH-SY5Y cells. NRG1 attenuates the CoCl$_2$-induced overexpression of EAAC1 and reduces CoCl$_2$-induced oxidative stress and apoptotic signaling. NRG1 may play a protective role in oxidative stress and hypoxia through the regulation of EAAC1. These results may show a new path toward understanding the pathogenesis and treatment of hypoxia and oxidative stress-related neurological diseases.
Abbreviations

EAAC1: Excitatory amino acid carrier 1 (also referred to as EAAT3).

EAAT: Excitatory amino acid transporter.

CNS: central nervous system.

GSH: Glutathione.

HIF-1: Hypoxia inducible factor-1.

OGD: Oxygen-glucose deprivation.

NRG1: Neuregulin 1.

CoCl2: Cobalt chloride.

ROS: Reactive oxygen species.

LDH: Lactate Dehydrogenase.

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

GPx: Glutathione peroxidase.

SOD: Superoxide dismutase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Please contact author for data requests.

Conflict of Interest

The authors declare no competing financial interests.

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Author contributions

HBK, JYY and SYY performed the experiments. HSK and JHL performed the data analysis. HBK, TKB, and RSW designed the study and wrote the manuscript. All authors critically reviewed the content and approved the final version before submission.

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Figures
Figure 1

The expression of EAAC1 in CoCl2-treated SH-SY5Y cells. 

a SH-SY5Y cells were treated with different concentrations of CoCl2 (0, 50, 100, 150, 200, 300 and 500 µM) for 24 hrs, which resulted in dose-dependent increases in EAAC1 expression. 

b Quantitative analysis of EAAC1 immunoreactivity in a. The results are presented as the means ± S.E.M.; n=8. ***P < 0.001. 

Western blotting demonstrated that 100 µM CoCl2 affected EAAC1 protein expression levels. EAAC1 protein expression was significantly increased by 100 µM CoCl2 in a time-dependent manner. 

d Quantification of the data in c. The
densitometry values are shown as ratios relative to the values of the control group (n=6, *P<0.05, ***P<0.001).
Figure 2

The effects of NRG1 on the CoCl2-induced protein levels of EAAC1. a Representative immunoblots of EAAC1, HIF-1α, and p53 in SH-SY5Y cells in the presence or absence of 5 nM or 10 nM NRG1 following treatment with 100 µM CoCl2 for 36 hrs are shown. b Quantitative analysis of the data in a. Treatment with 100 µM CoCl2 significantly increased the expression of EAAC1. CoCl2-induced EAAC1 overexpression was attenuated by 5 nM or 10 nM NRG1 treatment. The densitometry values are shown as ratios relative to the values of the control group (n=8, *P < 0.05, ***P < 0.001 versus the control group;
c Quantitative analysis of the data in a. CoCl2-induced HIF-1α accumulation was attenuated by 5 nM or 10 nM NRG1 treatment. The densitometry values are shown as ratios relative to the values of the control group (n=6, ***P < 0.001 versus the control group; ##P < 0.01, ###P < 0.001 versus the CoCl2 alone group). d Quantitative analysis of the data in a. CoCl2-induced p53 stability was attenuated by 10 nM NRG1 treatment. The densitometry values are shown as ratios relative to the values of the control group (n=6, ***P < 0.001 versus the control group; #P < 0.05 versus the CoCl2 alone group). Statistical analysis was performed by one-way ANOVA followed by Bonferroni’s post hoc test.
NRG1 attenuated the CoCl2-induced overexpression of EAAC1 and Tau in SH-SY5Y cells. a Immunofluorescence analysis with anti-EAAC1 and anti-Tau was performed 24 hrs after 100 µM CoCl2 treatment in the presence or absence of 10 nM NRG1 in SH-SY5Y cells. The cells were fixed and immunostained with anti-EAAC1 (green) and anti-Tau, while DAPI (blue) was used as a counterstain. The outlined image (upper) is enlarged (bottom). Scale bars, 20 µm. b Bar graph summarizing the data from neurons showing EAAC1 fluorescence (n=8, **P < 0.01 versus the control group; #P < 0.05 versus the control group). c Bar graph summarizing the data from neurons showing Tau fluorescence (n=8, **P < 0.01 versus the control group; #P < 0.05 versus the control group).
CoCl2 alone group). c The fluorescence intensity of Tau was measured in each group (n=8, **P < 0.01 versus the control group; #P < 0.05 versus the CoCl2 alone group).
NRG1 reduced the increase in ROS accumulation and decreased oxidative stress-related enzyme activity induced by CoCl2 in SH-SY5Y cells. a After 24 hrs, intracellular ROS levels were measured by fluorescence microscopy using DCFH-DA dye that was administered to 100 µM CoCl2-treated SH-SY5Y cells that had been pretreated with NRG1 (5 or 10 nM) for 15 minutes. b Bar graph summarizing the data in a (n=6, ***P<0.001 versus the control group; #P<0.05, ##P<0.01 versus the CoCl2 alone group). c SH-SY5Y cells were treated with 100 µM CoCl2 alone or with PBS or NRG1 (5 nM or 10 nM) for 36 hrs. GPx activity was...
measured using a GPx assay kit (BioVision, CA, USA) (n=6, **P<0.01 versus the control group; ###P<0.001 versus the CoCl2 alone group). After the SH-SY5Y cells were exposed to 100 µM CoCl2 in the presence or absence of NRG1 (5 nM or 10 nM) for 36 hrs, SOD activity was evaluated by measuring the inhibition of the reduction of tetrazolium salt by xanthine-xanthine oxidase according to the manufacturer’s instructions (Cayman Chemical Company, MI, USA). The SOD assay measured all three types of SOD (FeSOD, Cu/An, and Mn) (n=8, ***P<0.001 versus the control group; #P<0.05, ##P<0.01 versus the CoCl2 alone group).
NRG1 attenuated CoCl2-induced apoptosis and cell death in SH-SY5Y cells. a TUNEL staining (red) showing the amount of apoptosis that occurred in cells treated with 100 µM CoCl2 and either PBS or 10 nM NRG1 for 24 hrs. DAPI (blue) was used as a counterstain. Scale bars, 20 µm. b Apoptotic cells are expressed as the percentage of TUNEL-positive cells relative to the number of DAPI-stained cells (n=6, ***P < 0.001 versus the control group; ##P < 0.01 versus the CoCl2 alone group). c NRG1 (10 nM) attenuated cell death induced by 100 µM CoCl2 in SH-SY5Y cells. After 36 hrs, the degree of cell death
was measured by LDH activity in the medium (n=6, **P<0.01 versus the control group; #P<0.05 versus the CoCl2 alone group).
Figure 6

The effects of NRG1 on the CoCl2-induced protein levels of cleaved caspase-3 and Bcl-XL. 

a Representative immunofluorescence image of cells after treatment with 100 µM CoCl2 in the presence or absence of 10 nM NRG1 for 24 hrs. Cells were fixed and immunostained with anti-cleaved caspase-3 (red) and counterstained with DAPI (blue). Cleaved caspase-3 staining was significantly higher in CoCl2-only treated cells than in control cells. NRG1 protected against CoCl2-induced expression of cleaved caspase-3.

b The ratio of cleaved caspase-3-positive cells to the total number of cells (n=6, **P<0.01
versus the control group; #P<0.05 versus the CoCl2 alone group). Scale bars, 20 μm. c Protein expression of Bcl-XL (30 kDa), cleaved caspase-3 (17, 19 kDa), caspase-3 (35 kDa) and β-actin (43 kDa) was analyzed by western blotting. Representative immunoblots showing SH-SY5Y cells treated with 100 μM CoCl2 and either PBS or NRG1 (5 nM or 10 M) for 36 hrs. d Quantitative analysis of the data in a. Treatment with 100 μM CoCl2 significantly increased the expression of cleaved caspase-3. NRG1 attenuated the increase in cleaved caspase-3 expression, as shown by the densitometric values, which are shown as ratios relative to the values of the untreated control group (n=6, **P<0.01 versus the control group; #P<0.05, # #P<0.01 versus the CoCl2 alone group). e Quantitative analysis of the data in a. Treatment with 100 μM CoCl2 significantly decreased the expression of Bcl-XL in SH-SY5Y cells. NRG1 inhibited the reduction in Bcl-XL expression, as shown by the densitometric values, which are shown as ratios relative to the values of the untreated control group (n=6, *P<0.01 versus the control group; #P<0.05 versus the CoCl2 alone group).