Huwe1 is a novel mediator of protection of neural progenitor L2.3 cells against oxygen-glucose deprivation injury

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Abstract. Hypoxic-ischemic encephalopathy is one of the most notable causes of brain injury in newborns. Cerebral ischemia and reperfusion lead to neuronal damage and neurological disability. In vitro and in vivo analyses have indicated that E3 ubiquitin protein ligase (Huwe1) is important for the process of neurogenesis during brain development; however, the exact biological function and the underlying mechanism of Huwe1 remain controversial. In the present study, neural progenitor cells, L2.3, of which we previously generated from rat E14.5 cortex, were used to investigate the role of Huwe1 and its effects on the downstream N-Myc-Delta-like 3-Notch1 signaling pathway during oxygen-glucose deprivation (OGD). To evaluate the role of Huwe1 in L2.3 cells, transduction, cell viability, lactate dehydrogenase, 5-bromo-2'deoxyurine incorporation, western blotting and immunocytochemical assays were performed. The results of the present study indicated that Huwe1 rescued L2.3 cells from OGD-induced insults by inhibiting proliferation and inducing neuronal differentiation. In addition, Huwe1 was suggested to mediate the survival of L2.3 cells by inhibiting the activation of the N-Myc-Notch1 signaling pathway. Of note, the effects of Huwe1 on Notch1 signaling were completely abolished by knockdown of N-Myc, indicating that Huwe1 may require N-Myc to suppress the activation of the Notch1 signaling in L2.3 cells. The determination of the neuroprotective function of the Huwe1-N-Myc-Notch1 axis may provide insight into novel potential therapeutic targets for the treatment of ischemic stroke.

Introduction

Hypoxic-ischemic encephalopathy is a type of brain injury within newborns, in which cerebral ischemia and reperfusion lead to neuronal damage and neurological disability (1,2). Increasing evidence has suggested that neuronal injury induces the proliferation and differentiation of neural stem cells/progenitor cells (NSCs/NSPs) and adult neurogenesis (3-5). NSPs possess a notable ability to self-renew and differentiate into glia and functional neurons (6,7). Animal and clinical studies (8) have suggested that NSPs are a powerful potential treatment for numerous diseases of the central nervous system (9-11). Specifically, it has been reported that NSPs are involved in the regeneration of neurons and glia during perinatal hypoxia/ischemia (H/I) (12).

Numerous signaling pathways are involved in the regulation of NSCs (13-16). N-Myc, which is mainly distributed in the developing nervous system, is predominantly expressed in NSCs and neuroectodermal progenitors under normal conditions (17,18). In addition, N-Myc is required for the development of the nervous system; loss of N-Myc function results in the failure of neural progenitor cell expansion (19). Notch signaling has been associated with the regulation of NSCs; the Notch signaling pathway promotes the survival and proliferation of normal and tumor-derived NSCs, and prevents their differentiation (20-22). Binding of the ligands Delta or Jagged to Notch receptors induces the intramembranous proteolytic cleavage of the Notch receptor, yielding an activated Notch intracellular domain (NICD), which is necessary for the regulation of the transcription of several target genes, such as fibroblast growth factor 5 (23).

By using mass spectrometry, E3 ubiquitin protein ligase (Huwe1), containing a HECT domain, was purified from H1299
cells transfected with a plasmid expressing HA-ARF-Flag (24). Huwel has numerous substrates, including N-Myc, p53 and cell division cycle 6 (25-29). It has been demonstrated that Huwel is important for the transition from NSCs/NSPs to differentiated neurons (27). Loss of Huwel severely disrupts neurogenesis and the laminar organization in the cortex and, interestingly, the N-Myc-Delta-like 3 (DLL3)-Notch1 signaling cascade has been reported to be involved in this process (27,30). In vitro and in vivo research has indicated that Huwel is a key factor for neurogenesis; however, the exact biological function and underlying mechanism of Huwel remains controversial: Huwel affects the accumulation of many substrates, including N-Myc, c-Myc and Mcl1, which are involved in carcinogenesis. However, Huwel also promotes the accumulation of p53 protein, which inhibits the growth and development of multiple cancer types.

We previously generated neural progenitor L2.3 cells from a rat E14.5 cortex with the ability to differentiate into neurons, astrocytes, oligodendrocytes and other neuronal cell types (31,32). In the present study, radial glial L2.3 cells were used to investigate the role of Huwel and the downstream N-Myc-DLL3-Notch1 signaling pathway during oxygen-glucose deprivation (OGD) and the restoration of normal conditions.

Materials and methods

Cell culture. The culturing conditions of L2.3 cells were described previously (32). Briefly, L2.3 cells were cultured as neurospheres for 3 to 4 days in culture medium: Dulbecco's modified Eagles medium/F12 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 25 mM glucose (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin (both Invitrogen; Thermo Fisher Scientific, Inc.), 10 ng/ml fibroblastic growth factor 2 (FGF2; BD Biosciences, San Jose, CA, USA), 2 µg/ml heparin (Sigma-Aldrich; Merck KGaA) and 1X B27 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were propagated as neurospheres and passaged by mild trypsinization (0.025% trypan blue staining technique, the viability of cells after cell sorting was higher than 95%). (33) (x4 magnification, 488 nm excitation). L2.3 cells were transduced with lentiviral particles (Huwe1 shRNA or Huwe1 shRNA and N-Myc shRNA) diluted (2X, 5X) in serum free medium containing 10 µg/ml of Polybrene* (Merck KGaA) at a multiplicity of infection (MOI) of 10, all carrying green fluorescent protein (GFP). After 6 h, an equal amount (1 ml) of culture medium was added to each well. The transduction cocktail was removed and replaced with complete medium containing puromycin (2 µg/ml) the following day, and western blot analysis for Huwel and N-Myc expression in cell lysates was performed to select for Huwe1 shRNA-transfected cells or Huwe1 and N-Myc shRNA-transfected cells. Control shRNA-transfected cells and untransfected cells were used as the controls.

Cell viability assay. A Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess cell viability: 100 µl of the L2.3 cell suspension (in PBS) was seeded in 96-well plates (50,000 cells/well). Untransfected cells were used as the control. The kit was utilized according to the manufacturer's protocols; the absorbance at 450 nm was measured with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Cell viability was calculated by setting the absorbance of the normoxic control to 100%.

Lactate dehydrogenase (LDH) release assay. LDH release in the culture medium was measured using an LDH diagnostic kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols. LDH activity was calculated in 96-well plates (5x10^4 cells/well) by measuring the absorbance at 490 nm with a microplate reader (Bio-Rad Laboratories, Inc.).
5-bromo-2-deoxyurine (BrdU) assay. Cells were treated with 10 µM BrdU (Roche Diagnostics, Sussex, UK) for 24 h. Spheres were dissociated with 10 mg/ml collagenase for 10 min at room temperature and ~2x10^4 cells were fixed with a formalin solution for 15 min at 37˚C on a Matrigel-coated coverslip (incubated overnight with Matrigel at 37˚C). Following permeabilization with PBS containing Triton X-100 (0.1%) at room temperature for 30 min, DNA denaturation was performed with 2N HCl for 10 min at room temperature, followed by neutralization with 0.1 M sodium tetra-borate for 10 min at room temperature. The subsequent steps conducted were described below in ‘Immunocytochemistry’. Genome-integrated BrdU was detected using an anti-BrdU antibody (1:200; cat. no. 555627; BD Biosciences) and a Cy3 conjugated anti-mouse secondary antibody (1:1,000; cat. no. 115-165-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The proportion of BrdU positive cells relative to the total number of cells was estimated using a fluorescent microscope (x10 magnification, 550 nm excitation). Three fields of view were analyzed.

Immunocytochemistry. L2.3 cells underwent expansion for 5 days under normal conditions or following OGD. Cells (2x10^4) were fixed in paraformaldehyde (4%) at room temperature for 15 min. The primary antibodies employed were: Mouse anti-nestin (1:200; cat. no. MAB5326; Merck KGaA) or rabbit anti-microtubule-associated protein 2 (MAP2; 1:100; cat. no. M3696; Sigma-Aldrich; Merck KGaA). Following incubation at 4˚C overnight, the cells were washed and incubated with the appropriate secondary antibodies at 37˚C for 1 h, including fluorescein isothiocyanate goat anti-mouse secondary antibody (cat. no. ab6785; 1:1,000; Abcam, Cambridge, USA), Cy3 goat anti-rabbit secondary antibody (cat. no. BA1032; 1:500; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 1:10,000, USA, cat. no. D9564) at 37˚C for 30 min, using a fluorescent microscope (x100 magnification, 359 nm excitation). Three fields of view were analyzed using a fluorescent microscope (Nikon Tii).

Western blot analysis. Cells were lysed with SDS lysis buffer [50 mM Tris (pH 8.1), 1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na3VO4 and 0.5 µg/ml leupeptin] and heat-denatured at 95°C for 5 min. Protein concentration was measured with a bicinchoninic acid protein assay. Cellular proteins (20 µg/lane) were separated via SDS-PAGE (10%) and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h with blocking buffer at room temperature (1X PBS with 0.1% Tween 20 and 5% non-fat dry milk) and then incubated with primary antibodies against Huwe1 (1:500; cat. no. ab701612; Abcam), Notch1 (1:200; cat. no. sc6014; Santa Cruz Biotechnology, Inc., Dallas TX, USA), NICD

Figure 1. Huwe1 rescues L2.3 cells from OGD-induced injury. (A) Representative western blot analysis of Huwe1 expression in L2.3 cells. 1, Prior to OGD exposure; 2. OGD for 6 h; 3, following OGD for 6 h and restoration for 8 h; 4, following OGD for 6 h and restoration 16 h; and 5, following OGD for 6 h and restoration for 24 h. (B) Quantification of Huwe1 protein expression levels, which were normalized to GAPDH. *P<0.05 vs. prior to OGD exposure (line 1). (C) Huwe1 protein expression levels were detected in control L2.3 cells and L2.3 cells transduced with viral particles containing control shRNA or Huwe1 shRNA. (D) Cell viability and (E) LDH levels in culture medium. The data were presented as the mean ± standard error. Data were obtained from three independent experiments. *P<0.05 vs. control and control shRNA groups. Huwe1, E3 ubiquitin protein ligase; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation; shRNA, short hairpin RNA.
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Statistical analysis. For comparisons among multiple groups, one-way analysis of variance followed by Fisher's Least Significant Difference method was performed to determine the statistical differences between groups by using the SPSS 15.0 software. All data were expressed as the mean ± standard deviation of three experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Huwe1 knockdown decreases cell viability after 6 h. We previously reported that, upon the onset of OGD, the viability of L2.3 cells progressively decreased; the cell number was significantly lower after 6 h (P<0.001, n=3) (34). Accordingly, in the present study, 6 h was selected as the duration of OGD. To investigate whether Huwe1 serves a key role in OGD, the expression levels of Huwe1 in L2.3 cells exposed to OGD for 6 h were determined, followed by the restoration of normal conditions at 8, 16 and 24 h (Fig. 1A). Quantification of Huwe1 protein expression levels were normalized to GAPDH (Fig. 1B). The results revealed that the expression levels of Huwe1 were significantly increased in OGD-treated L2.3 cells compared with cells unexposed to OGD.

To evaluate the role of Huwe1 in L2.3 cells, GIPZ shRNA lentiviral vectors were employed to knockdown Huwe1 expression in L2.3 cells (Fig. 1C) as aforementioned. Accordingly, knockdown of Huwe1 expression was associated with significant reductions in the viability of L2.3 cells exposed...
to OGD (Fig. 1D); LDH release was significantly elevated compared with in OGD control and OGD control shRNA cells (Fig. 1E). The data indicated that Huwe1 may be required to maintain L2.3 cell viability following the onset of OGD. To further evaluate whether N-Myc is involved in Huwe1-mediated survival of L2.3 cells during OGD, the cell viability and LDH release in N-Myc-silenced L2.3 cells were determined. The results revealed that the downregulation of N-Myc expression reduced LDH release and increased cell viability compared with in the normal control and OGD control shRNA groups (Fig. 1D and E).

**Huwe1 knockdown induces nestin expression and the proliferation of L2.3 cells.** The present study analyzed the expression of nestin and MAP2 in L2.3 cells to determine whether cell proliferation led to cell differentiation. L2.3 cells underwent expansion for 5 days under normal condition or following OGD, and were stained with neural precursor cell marker nestin (green), neuronal marker MAP2 (red) and DAPI (blue). As presented in Fig. 2A and B, a significant increase in the number of MAP2+ cells was detected following OGD exposure compared with in the normal control conditions. Knockdown of Huwe1 expression induced a significant increase in the percentage of nestin+ cells and a decrease in MAP2+ precursor cells (35) under normal and OGD conditions.

To determine whether the reduced viability of Huwe1-silenced L2.3 cells was due to the inhibition of proliferation, BrdU incorporation assays were conducted. The results of the present study revealed that BrdU+ cells were more abundant in the control than under the OGD condition; notably fewer BrdU+. A significantly greater number of Huwe1-silenced cells were observed in OGD condition compared with in normal conditions (Fig. 2C). This indicated that, under normal conditions, a higher percentage of actively dividing cells may be detected. The results were consistent with the onset of cell proliferation, suggesting that Huwe1 may inhibit the proliferation in L2.3 cells.

**OGD/restoration (OGD/R) inhibits N-Myc-DLL3-Notch signaling in L2.3 cells.** The potential mechanisms underlying the effects of Huwe1 in the neuronal differentiation of L2.3 cells were investigated in the present study. As presented in Fig. 1A, the expression levels of Huwe1 began to increase significantly after 16 h following the restoration of normal conditions. Therefore, 16 h was selected as the duration of restoration for the normal culture conditions. The results demonstrated that the expression levels of Notch1, NICD, DLL3 and N-Myc were significantly increased following exposure to OGD for 6 h and decreased after restoration for 16 h (OGD/R) compared with in the normal control (Fig. 3A and B). The results suggested that OGD/R may inhibit Notch signaling.
Additionally, Notch1-transcriptional activity was analyzed via a luciferase reporter assay, which revealed a significant decrease in Notch1 activation when L2.3 cells were exposed to OGD/R compared with the normal control (Fig. 3C). These results further indicated that Notch signaling may be associated with cell fate.

Huwe1 mediates the survival of L2.3 cells via the N-Myc-Notch1 signaling pathway during oxygen-glucose deprivation and restoration. To evaluate whether Huwe1 affects the Notch1 signaling pathway, Huwe1 was downregulated in L2.3 cells, which were exposed to OGD/R with normal control (Fig. 3C). These results further indicated that Notch signaling may be associated with cell fate.

Discussion

To the best of our knowledge, the present study is the first to report the protective role of Huwe1 in L2.3 cells under the OGD condition. Specifically, Huwe1 was observed to rescue L2.3 cells from OGD-induced injury by inhibiting proliferation and inducing neuronal differentiation. Subsequently, its effects on the downstream N-Myc-Delta-like 3-Notch1 signaling pathway were investigated to explore the underlying mechanisms. Mechanistically, it was demonstrated that Huwe1 mediated
the survival of L2.3 cells via inhibition of the N-Myc-Notch1 signaling axis in the present study. Thus, in response to OGD and the restoration of normal conditions, Huwe1 was proposed to inhibit the N-Myc-Notch1 signaling axis and induce the differentiation of L2.3 cells in the present study. In addition, the effects of Huw1 on Notch1 signaling were notably abolished via the knockdown of N-Myc. Therefore, the findings of the present study indicated that the Huw1-N-Myc-Notch1 axis may be a novel neuroprotective pathway associated with ischemic stroke.

Huw1 is important for neurogenesis (30); however, the biological function of Huw1 in neurological diseases remains unknown (36). The present study reported that Huw1 served a role in the survival of L2.3 cells under the OGD condition, which suggests Huw1 as a potential target in the treatment of ischemic stroke. Consistent with the role of Huw1 on the transition from NSCs to differentiated neurons, Huw1 may improve the neurobehavioral outcomes following ischemic brain injury by enhancing neuronal differentiation and rescuing cells from OGD-induced insults. Previous studies have reported that Huw1 acts via the N-Myc-DLL3-Notch1 signaling pathway during neural development (27,30). Huw1 was reported to suppress the N-Myc-DLL3 cascade associated with the initiation of neurogenesis in the developing brain, and negatively regulated the expression of DLL3 in an N-Myc-dependent manner, and the activation Notch1, a transmembrane receptor on the surface of neural progenitors, was proposed to be involved in NSC maintenance (37,38). When activated by the ligands of neighboring cells, NICD translocates to the nucleus and activates transcription (39). In the developing brain, activated Notch signaling maintains the state of NSCs by promoting the proliferation of neural progenitors and inhibiting the differentiation progenitor cells into neurons (40). In support of this, a study indicated that attenuation of Notch signaling promoted the differentiation of neural progenitors in the subacute phase following ischemia (41). In conclusion, Huw1 may protect neural progenitors L2.3 cells from injury associated with OGD and the restoration of normal conditions; however, further investigation is required to evaluate the pathological mechanism underlying the protective effects of Huw1 in the acute phase of stroke.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

XI, JY and WX performed the experiments and analyzed the data. HL and YQ conducted the cell culture and molecular pathway experiments. HY and YT analyzed the resulting data and drafted the manuscript. All authors were responsible for drafting the manuscript, and read and approved the final version.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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