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Original Article

HIF1α and HIF2α mediated UCHL1 upregulation in hypoxia-induced neuronal injury following neuronal hypoxic ischemic encephalopathy

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Abstract: Background: Recent studies have reported elevated UCHL1 concentration in many neurological diseases, including neonatal hypoxic ischemic encephalopathy (HIE), but with unrevealed mechanism of this alteration. This study aimed to explore the reason for UCHL1 level change in neuronal apoptosis following neonatal HIE. Methods: Serum UCHL1 levels were detected by ELISA assays and compared in neonatal patients with HIE and healthy ones. UCHL1 expression was analyzed by RT-PCR and western blot assays. Apoptosis and cell proliferation were detected by using Annexin V/PI staining and CCK-8 method respectively. Hypoxia-induced neuronal apoptosis was introduced to simulate neonatal HIE *in vitro*. And, CHIP experiments were performed to verify putative HRE sites within UCHL1 promoter. Results: Elevated serum UCHL1 level was confirmed in neonatal HIE patients. UCHL1 stimulation *in vitro* aggravated cell apoptosis, while cell proliferation was improved by UCHL1 interference, demonstrating its regulation on neuronal cell apoptosis. Further hypoxia-induced analysis showed that UCHL1 expression was positively regulated by HIF1α and HIF2α under hypoxic conditions, via HRE-mediated UCHL1/HIFα complex binding. Conclusion: This study revealed a new insight into the regulatory mechanism underlying hypoxia-induced apoptosis during neonatal HIE development, involving UCHL1 upregulation mediated by HIF1α and HIF2α. Additionally, this study provided more evidence for UCHL1 to be a potential pro-apoptotic factor in neuronal injury, and also its therapeutic value for the treatment or prognosis of neonatal HIE.

Keywords: Hypoxic ischemic encephalopathy, neuronal cell apoptosis, UCHL1, HIF1α, HIF2α

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is the most frequent source of brain injury among term infants, resulting from perinatal asphyxia that caused by various factors, and showing injuries induced by partial or complete hypoxia or the reduction or suspension of cerebral blood flow [1, 2]. And, HIE still acts as one leading cause of abnormity and lethality during neonatal neurodevelopment, with high incidence rate [3]. Former researches have reported the involvement of various mechanisms in the pathogenesis of HIE, including the reduction of cerebral blood flow, change of energy metabolism in brain tissues, damage of inflammatory mediators and oxygen free radicals, toxicity of abnormal amino acids on neurons, calcium influx and brain cell apoptosis [4, 5]. Normally, severe hypoxia ischemia often leads to the necrosis of brain cells, while relatively mild hypoxia ischemia can result in delayed cell death that regulated by apoptosis [6]. However, due to the limitations in sensitivity and specificity, neuronal apoptosis is often difficult to identify and diagnose for clinical patients [7]. Thus, to investigate new targets underlying the mechanism of HIE-induced neuron apoptosis might help to identify irreversible neuron apoptosis in neonatal neurodevelopment.

Ubiquitin (Ub) carboxyl-terminal hydrolase L1 (UCHL1), also known as PGP 9.5, a kind of cysteine protease, is a unique cytoplasmic enzyme in neurons and nervous tissues, and expressed in nearly all brain regions [8]. It has been indicated that the increase of UCHL1 in cerebrospinal fluid or blood is associated with the damage

of neurons and the destruction of blood brain barrier [9-11]. And, recent studies have also reported elevated UCHL1 concentration in many neurological diseases, such as aneurysmal subarachnoid hemorrhage, traumatic brain injury, stroke, anoxic encephalopathy and neonatal HIE [12-16]. However, it is still unclear about the reason for elevated serum level of UCHL1 and also the mechanism of this alteration in HIE-induced neuronal apoptosis.

In the present study, elevated serum UCHL1 levels in neonatal HIE were verified by compared with those in healthy ones. And, due to the presence of a close relationship in UCHL1 and hypoxia-induced apoptosis [17], further experiments were also performed to examine the potential function of UCHL1 in neuronal apoptosis involving hypoxia-inducible factors (HIFs) mediated pathways. This study suggested that UCHL1 might potentially facilitate assessment of neuronal apoptosis following HIE. This study investigated the mechanism of hypoxia-induced apoptosis in human neuronal cell lines AGE1.HN.

Materials and methods

Samples and UCHL1 measurement

Total twenty cases of neonates with HIE and corresponding normal neonates who were treated in the neonatal department from Jan 2013 to Mar 2014 were analyzed in this study. Blood samples from these subjects were subjected to serum preparation for the measurement of UCHL1 levels by using a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [9]. This study was approved by the hospital board of ethics and informed written consent was obtained from all of the subjects.

Cell culture and reagents

Human neuronal cell line, AGE1.HN [18], was obtained from Probiogen AG (Berlin, Germany), and propagated in the maintenance medium (1:1 Dulbecco's modified Eagle's medium: Ham's F-12 mix) supplemented with 5% fetal bovine serum (Invitrogen, Shanghai, China), 1% antibiotic-antimycotic (Invitrogen), 10 mg/mL human transferring and 30 mmol/L sodium selenite (Sigma-Aldrich, Shanghai, China). Recombinant human UCHL1 was purchased from ProSpec-Tany TechnoGene (PRO-576; Rehovot, Israe) that ready for use. 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1), as HIFs inhibitor, was obtained from Sigma-Aldrich (Shanghai, China) and dissolved in dimethyl sulfoxide for use.

Hypoxia treatment

For hypoxia treatment, cells were maintained in Anoxomat hambers (Mart Microbiology, Lichtenvoorde, Netherlands) for physiological hypoxia (1% O_2) or normoxia (20% O_2) at 37°C for indicated time intervals.

Small interference RNA and cell transfection

Small interference RNAs (siRNAs) were used here for knockdown experiments. Three UCHL1 siRNAs against UCHL1 mRNA and those specific for HIF1α and HIF2α mRNA were all designed and synthesized by Ribobio (Guangzhou, China), as well as scrambled negative control siRNA (si-NC). Cells were pre-incubated and transfected with siRNAs with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At indicated time points, cells were harvested for qRT-PCR and western blot analysis.

Apoptosis assay

Annexin V/PI Apoptosis Detection Kit (Roche, Shanghai, China) was used to measure the percentage of apoptotic cells. According to the manufacture's protocol, cells were collected, washed with cold PBS and resuspended in the binding buffer (Roche). Then, cells were mixed with Annexin V (1:20) for 5 min, and incubated with propidium iodide (PI, 1 mg/mL) for 15 min. Finally, samples were immediately analyzed by BD FACSCalibur (BD Biosciences, Heidelberg, Germany) with Cell Quest software supplied by the manufacturer. All experiments were carried out in triplicate.

Cell proliferation assay

Cells were seeded at a concentration of 2×10^4 cells/well onto a 96-well plate with 100 μL medium, and cultured at 37°C. At indicated time points, cell viability was measured by using a commercial Cell Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Briefly, CCK8 reagents were added to a subset of wells and incubated

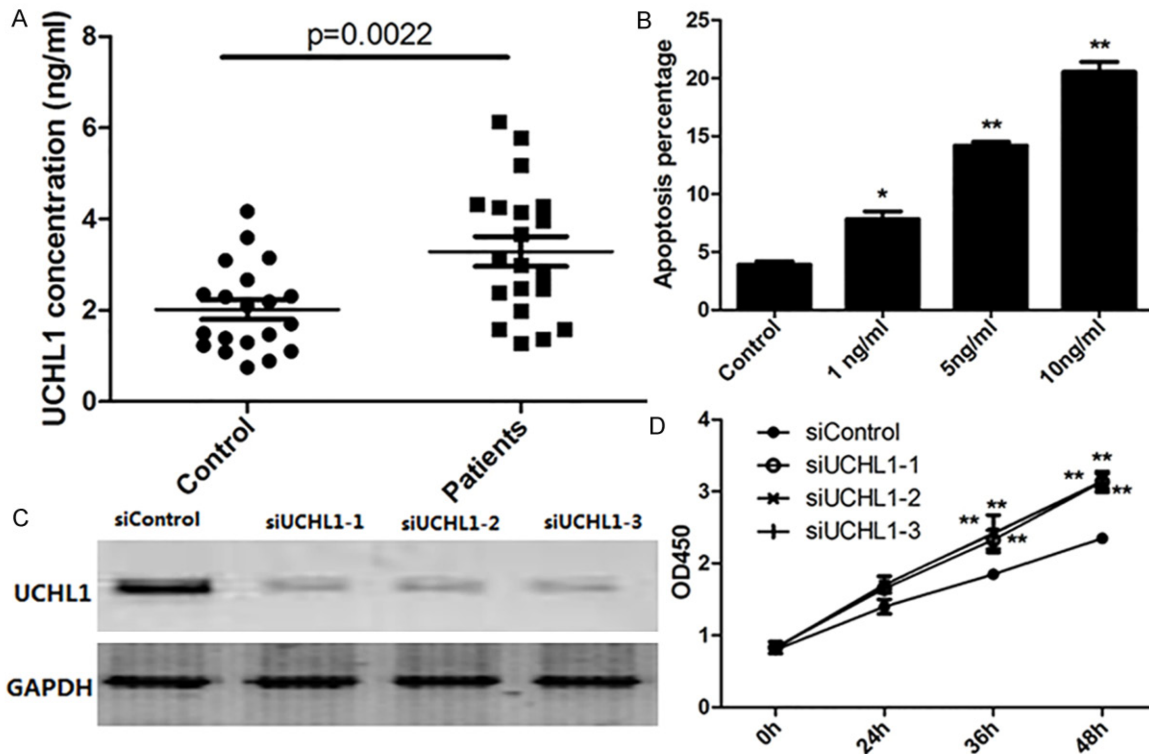

Figure 1. Detection for UCHL1 levels in HIE patients and its regulation on neuronal apoptosis. A. Peripheral blood samples from twenty HIE infants and corresponding healthy ones were collected for the detection of UCHL1 levels. Results were compared by one way AVOID analysis. B. Analysis for the apoptotic percentage of AGE1.HN cells 48 h after the treatment of UCHL1 at indicated dosage. C. Western blotting analysis for UCHL1 expression in cells 48 h after the transfection of three separated si-UCHL1s. D. CCK8 analysis for the cell proliferation in cells under UCHL1 interference. *P<0.05; **P<0.01.

for 2 h. The optical absorbance at 450 nm (OD450) was measured using an automated plate reader (INFINITE M200, Tecan, Switzerland). Data were collected from at least triple experiments for each sample.

Western blot analysis

Cell lysates were prepared by using RIPA protein extraction reagent (Beyotime, Beijing, China) and protein concentrations were determined by bicinchoninic-acid-based (BCA) method (Pierce Chemical; Rockford, IL, USA). Samples containing equal amounts of protein were separated by 10% SDS-PAGE and electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore; Bedford, MA, USA). Then, membranes were incubated with specific antibodies (purchased from Santa Cruz Biotechnology; Santa Cruz, CA, USA) for UCHL1 and GAPDH, according to the manufacturer's recommendations. ImageQuant LAS 4000 System (GE Healthcare) was used to acquire the immunoblot images.

Quantitative real-time PCR

Total RNA from cells was extracted and purified with TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer's instructions. Then, RNA was reverse transcribed into cDNA using random hexamers with the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real time PCR was performed using the Sybgreen I real-time PCR kit (TaKaRa, Dalian, China) in an ABI PRISM 7500 Real-time PCR System (Applied Biosystems). The specific primers used here were: UCHL1-F: 5'-CCTGAAGACAGAGCAAAA-TGC-3', UCHL1-R: 5'-CCTGAAGACAGAGCAAA-ATGC-3'; β-actin-F: 5'-GTGGCATCCACGAAACTA-CC-3', β-actin-R: 5'-GTACT TGCGCTCAGGAGG-AG-3'. Results were analyzed from at least triple experiments, with β-actin used as internal control.

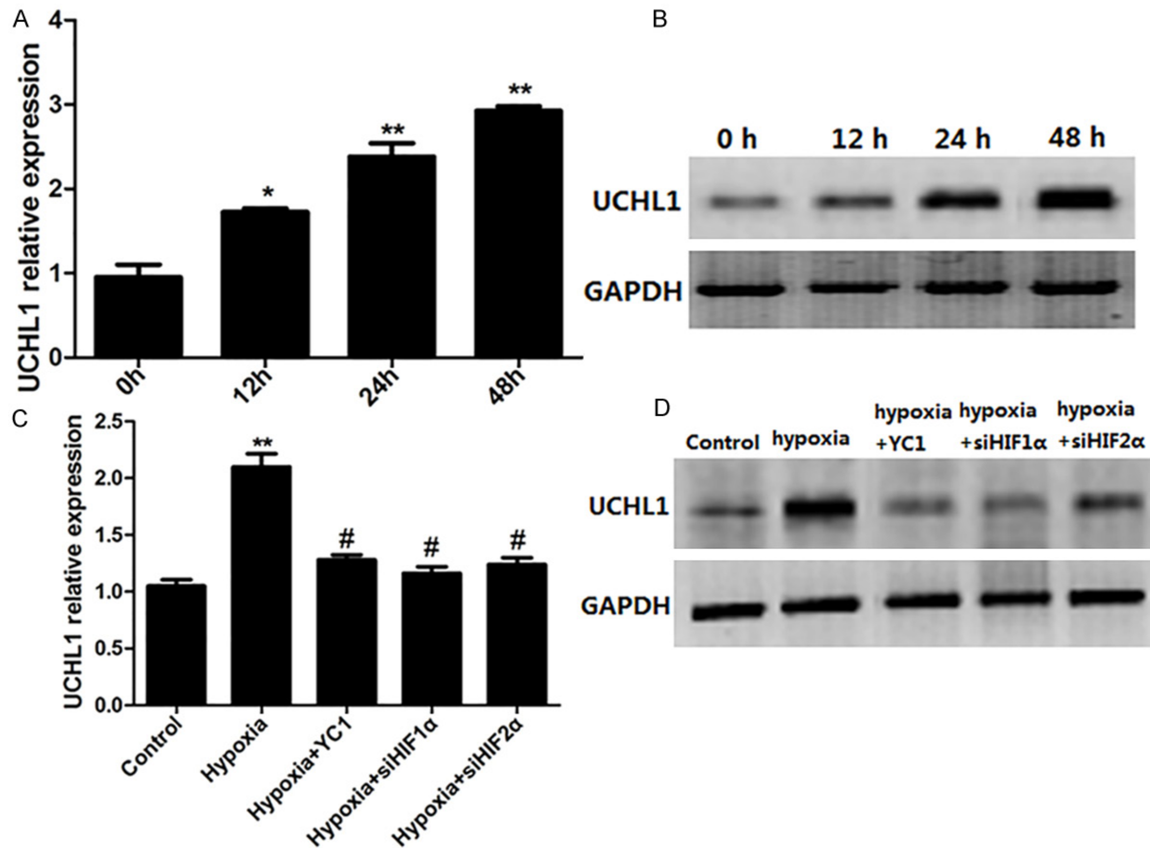

Figure 2. Analysis for hypoxia-induced UCHL1 expression in AGE1.HN cells. (A) and (B) UCHL1 expression in AGE1.HN cells under hypoxia treatment for indicated time intervals was detected through RT-PCR (A) and western blotting (B) analysis respectively. *P<0.05; **P<0.01. (C) and (D) AGE1.HN cells were pretreated with YC-1 for 6 h or transfected with siHIF1α or siHIF2α for 12 h, and then subjected to hypoxia treatment for 24 h. RT-PCR (C) and western blotting (D) analysis for UCHL1 expression were performed accordingly. *P<0.05 vs. control; **P<0.01 vs. control; #P<0.05 vs. hypoxia treatment.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) assay was performed to verify the binding of HIF1α or HIF2α protein to the promoter of UCHL1 by using a Chromatin Immunoprecipitation Kit (Upstate Biotechnology, NY, USA) as described before [19]. After the isolation of DNA-protein complexes from cells, cross-linked chromatin DNA were prepared for immunoprecipitation using specific antibodies against HIF1α, HIF2α or IgG (Santa Cruz Biotechnology) combined with quantitative PCR analysis. Primers spanning the hypoxia response element (HRE) site of the UCHL1 promoter were F: 5'-AATTAGCCGGGTGTGGT-3' and R: 5'-ATTTCCCTCATGCCGTTT-3'. Primers for the amplification of non-HRE site were F: 5'-TGGCAGCATAAGAAATAC-3' and R: 5'-GAAGTAGGGGGTGATT-3'. Three or more independent experiments were repeated for data presentation.

Statistical analysis

The results were expressed as the mean ± SD. Student's *t* test (two-tailed) or one-way ANOVA analysis (with Bonferroni adjustment) was performed to calculate the statistical significance of the means among multiple groups, by using SPSS 17.0 software (SPSS Inc., Chicago, IL). And, differences were considered to be statistically significant at P<0.05.

Results

Serum UCHL1 levels showed a significant increase in neonatal HIE

In order to verify the increased UCHL1 concentration in neonatal HIE reported in a polity study [12], serum UCHL1 level was also detected here in neonates with HIE and healthy ones. As shown in **Figure 1A**, individual UCHL1 concen-

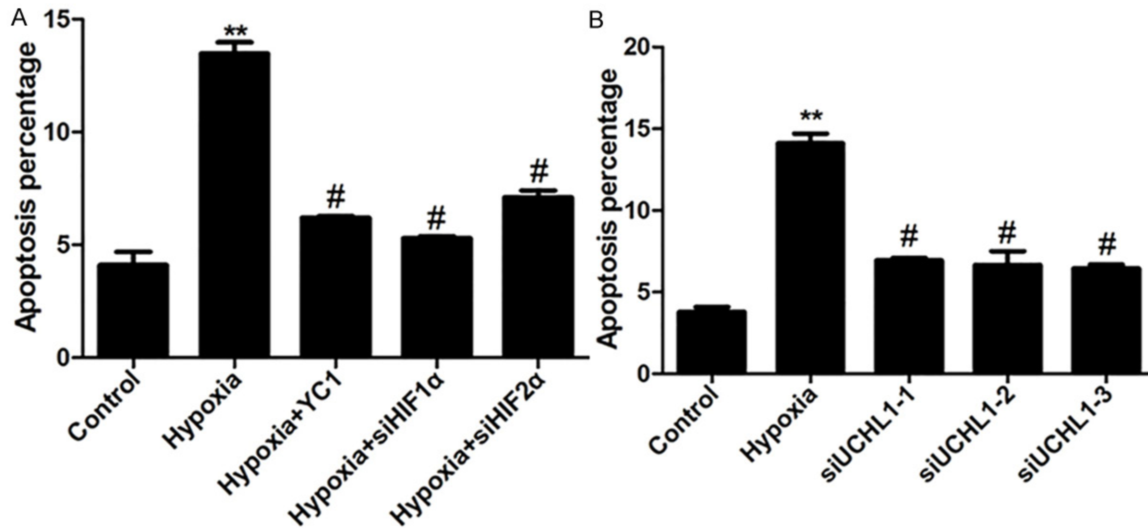

Figure 3. Detection for hypoxia induced apoptosis in HIF or UCHL1 inhibiting cells. A. Flow cytometry analysis for the apoptosis of AGE1.HN cells that were pretreated with YC-1 for 6 h or transfected with siHIF1α or siHIF2α for 12 h, and then subjected to hypoxia treatment for 48 h. B. Apoptotic analysis for AGE1.HN cells, separately transfected with three UCHL1 siRNAs (siUCHL1-1, siUCHL1-2, siUCHL1-3) for 12 h, under hypoxia treatment for 48 h. $^{**}P<0.01$ vs. control; $^{\#}P<0.05$ vs. hypoxia treatment.

trations were plotted, with a mean value at 3.3 ng/ml or 2.0 ng/ml for HIE or healthy subjects respectively. Further data analysis showed that serum UCHL1 levels were significantly elevated in neonates with HIE as compared with healthy ones (P=0.0022), providing more evidence for the potential function of UCHL1 in HIE development.

UCHL1 induced apoptosis in neuronal cells

In order to investigate the mechanism of increased UCHL1 levels in HIE pathogenesis, the effect of UCHL1 on neuronal cell apoptosis was detected in a human neuronal cell line, AGE1.HN. After the incubation with UCHL1 for 48 h, the percentage of apoptotic cells increased at a dosage dependent manner, with a maximum of 5-fold increase after treated with 10 ng/ml UCHL1 (**Figure 1B**). In the contrary, compared to normal cells, better cell growth was observed in cells with downregulated UCHL1 expression that obtained by the transfection of UCHL1 siRNAs (**Figure 1D**), while the decrease of UCHL1 protein levels was also confirmed through western blot analysis (**Figure 1C**).

UCHL1 expression was upregulated by hypoxia treatment involving HIFα mediated mechanism

As hypoxia-induced neuronal apoptosis has been a major part in HIE pathogenesis [20], the expression of UCHL1 was also analyzed in neuronal cells under hypoxia treatment. In line with results from *in vivo* analysis, UCHL1 expression was also upregulated by hypoxia at both mRNA and protein levels, in a time independent manner (**Figure 2A** and **2B**). Protein levels of hypoxia-inducible α-subunits (HIFα), including HIF1α and HIF2α, are rapidly degraded in normoxia but highly induced by hypoxia [21], thus HIFα mediated mechanism was presumed to contribute to hypoxia-induced upregulation of UCHL1. As shown in **Figure 2C** and **2D**, the upregulated UCHL1 expression induced by hypoxia was counteracted in HIFα inhibiting cells by pretreated with YC-1 or transfected with siHIF1α or siHIF2α. These results implied that UCHL1 expression was induced by hypoxia via HIFα mediated pathway.

Hypoxia-induced cell apoptosis was regulated by HIFα and UCHL1

Since HIFα showed contribution to hypoxia-induced upregulation of UCHL1, the effect of HIFα inhibition on hypoxia-induced cell apoptosis was also investigated, with UCHL1 inhibition as control. As shown in **Figure 3**, the percentage of apoptotic cells was strongly increased by hypoxia treatment, which could be attenuated in both HIFα inhibiting cells and UCHL1 silencing cells. Taken together, it could be concluded that the regulatory role of UCHL1 in hypoxia-

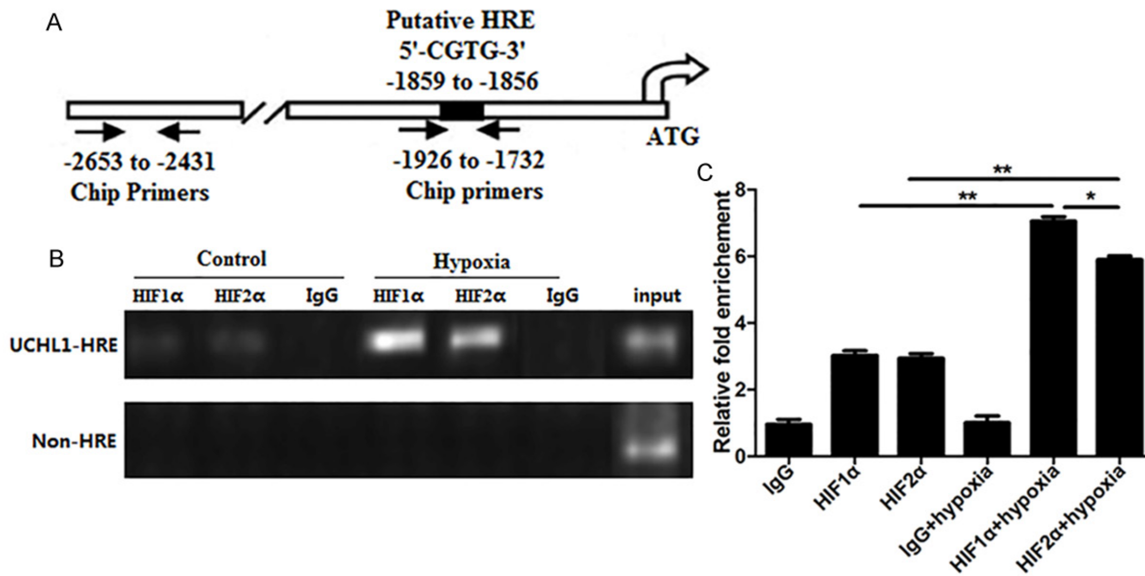

Figure 4. Analysis for the putative HRE sites within UCHL1 promoter. A. Putative HRE locus in UCHL1 promoter with primers used for CHIP analysis. HRE: hypoxia response element. B. DNA binding analysis about HIF1α/HIF2α and UCHL1 HRE by CHIP in AGE1.HN cells under hypoxia treatment for 12 h. "HIF1α": with anti- HIF1α; "HIF2α": with anti- HIF2α; "IgG": with anti-IgG as negative control; "Input": Chromosome. C. qRT-PCR was performed for HIF1α and HIF2α occupancy on the UCHL1 HRE in ChIP samples as described above. Relative promoter enrichment compared with IgG is plotted. *P<0.05; **P<0.01.

induced neuronal apoptosis was mediated by both HIF1α and HIF2α.

HIF1α and HIF2α promoted UCHL1 transcription by binding potential HRE sites within UCHL1 promoter

Because HIF1α or HIF2α is a transcription factor that works by binding to the hypoxia response element (HRE) of gene promoters (Semenza, 2003), and the promoter region of the UCHL1 gene contains a putative HRE sites (**Figure 4A**), it was speculated that HIF1α or HIF2α upregulated UCHL1 expression by binding to its promoter. Indeed, chromatin immunoprecipitation (ChIP) assay confirmed recruitment and direct binding of HIF1α or HIF2α to the UCHL1 promoter in hypoxia-treated cells, but not in normoxia incubated cells (**Figure 4B**). Quantitative real time PCR analysis demonstrated that HIF1α or HIF2α was significantly enriched on the proximal UCHL1 promoter with putative HRE sites, compared to a distal region of the promoter without HRE in AGE1.HN cells (**Figure 4C**).

Discussion

UCHL1 is a highly abundant neuronal deubiquitinase, and it has been identified as potential biomarker for neuronal cell injury under different neurological conditions [12, 13, 15], including newly reported neonatal HIE, but with unrevealed regulatory mechanism. However, some studies support that UCHL1 expression can regulate neuronal apoptosis [22, 23], one important part of neonatal HIE pathogenesis [20]. Thus, this study was designed to explore the regulatory mechanism of UCHL1 expression in hypoxia-induced neuronal apoptosis following neonatal HIE. Firstly, serum UCHL1 level was detected to be significantly upregulated in neonates with HIE, in line with the elevation in UCHL1 concentration that consistent with the severity of neuronal apoptosis as previously reported [17]. After the confirmation for the relationship between elevated UCHL1 level and neonatal HIE, a human neuronal cell line, AGE1.HN [18], was chosen here to be cultured with different concentrations of UCHL1 *in vitro*, simulating neuronal cells surrounded by elevated UCHL1 concentration in the serum. Remarkably, the apoptosis of neuronal cells was strongly aggravated by UCHL1 addition, of note, at a dosage dependent manner. Oppositely, UCHL1 interfering AGE1.HN cells showed increased cell proliferation as compared to normal cells. These results implied

that UCHL1 level and its expression indeed showed regulation on neuronal apoptosis following neonatal HIE.

To further investigate the reasons for upregulated UCHL1 expression in neonatal HIE, UCHL1 expression was also analyzed under hypoxia-induced neuronal apoptosis, simulating neonatal HIE *in vitro*. Similarly, UCHL1 expression was strongly upregulated at both mRNA and protein levels in neuronal cells at low O_2 tension. Since HIFs are essential for cellular responses to hypoxia [24], including HIF1α and HIF2α, two best characterized HIFα-subunits and involved in HIE pathogenesis [25, 26], the potential roles of HIF1α and HIF2α in regulating UCHL1 expression were both explored. The expression of HIF1α or HIF2α was inhibited by the addition of YC-1, one HIFα inhibitor that can reduce the protein levels of both HIF1α and HIF2α under hypoxic conditions [27], or by using siRNAs that specifically targeted HIF1α or HIF2α. As indicated, hypoxia-induced upregulation of UCHL1 was all strongly inhibited by YC-1, siHIF1α or siHIF2α. Meanwhile, hypoxia-induced apoptosis was also attenuated under HIF1α and HIF2α inhibition, similar to that by UCHL1 interference, implying the correlation between HIF1α and HIF2α mediated pathways and UCHL1 expression under hypoxic-induced apoptosis.

Canonically, HIF1α or HIF2α function by binding to the hypoxia response elements (HREs) within the promoters of hypoxia-regulated genes during hypoxic transcriptional response, modulating their expression and governing survival under stress [28]. For example, vascular endothelial growth factor and glucose transporter-1 are regulated by HIFαs to induce angiogenesis and glycolysis respectively at low O_2 conditions [29]. Thus, putative HRE sites within the promoter region of UCHL1 gene were speculated, and CHIP experiments were conducted to verify the potential HIFα/UCHL1 complex binding. Results showed that HIF1α and HIF2α indeed promoted UCHL1 expression by binding one identified HRE site within its promoter under hypoxic conditions. Therefore, it was concluded that UCHL1 expression was positively regulated by HIF1α and HIF2α through HREs-mediated HIFα/UCHL1 complex binding under hypoxia-induced mechanism.

In conclusion, a new insight into the regulatory mechanism underlying hypoxia-induced apoptosis during neonatal HIE development was described involving the upregulation of UCHL1 mediated by HIF1α and HIF2α. More importantly, UCHL1 was characterized as a hypoxia-regulated gene with HREs, expanding the mechanism of hypoxia-induced apoptosis especially for neonatal HIE pathology. Furthermore, UCHL1 acted as a potential pro-apoptotic factor in hypoxic-induced neuronal injury following neonatal HIE. More studies could focus on the potential therapeutic value of UCHL1 for the treatment or prognosis of neonatal HIE.

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Disclosure of conflict of interest

None.

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