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Roles of HIF1α- and HIF2α-regulated BNIP3 in hypoxia-induced injury of neurons

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ABSTRACT

Background: To explore the roles of HIF1α- and HIF2α-regulated BNIP3 in hypoxia-induced injury of neurons.
Methods: The sera of neonates with hypoxic-ischemic encephalopathy (HIE) within 24 h after birth and full-term healthy newborns (n = 40) were collected. The BNIP3 levels were detected by ELISA. AGE1.HN cells were cultured in 1% O_2 at 37 °C. The apoptosis of cells treated with 1, 5 and 10 ng/ml BNIP3 for 48 h was detected by flow cytometry. The proliferation of cells transfected with siBNIP3 was detected by CCK-8 assay. The mRNA level of BNIP3 in cells under hypoxic conditions was measured by RT-PCR. The protein level of BNIP3 in cells cultured under hypoxic conditions after pretreatment with HIF1α or HIF2α inhibitor was measured by Western blot.
Results: The serum BNIP3 concentration of HIE neonates ((4.5 ± 2.1) ng/ml) was significantly higher than that of healthy neonates ((1.2 ± 0.5) ng/ml) ($P < 0.001$). Compared with untreated group, the number of apoptotic AGE1.HN cells treated with BNIP3 significantly increased ($P < 0.05$). Under hypoxic conditions (1%), the mRNA and protein levels of BNIP3 increased significantly with prolonged time. After pretreatment with HIF1α or HIF2α inhibitor and hypoxic culture, BNIP3 expression was significantly lower than that of cells hypoxically cultured only. Inhibiting the expression of HIF1α or HIF2α or transfecting with siBNIP3 before hypoxic treatment significantly reduced the number of apoptotic cells. Under hypoxic conditions, HIF1α or HIF2α bound BNIP3 promoter, which did not occur under normal culture conditions. HIF1α or HIF2α was significantly enriched near the hypoxia response element (HRE) site of BNIP3 promoter.
Conclusions: BNIP3 was involved in the apoptosis of cells undergoing HIE. The HRE site of BNIP3 promoter bound HIF to promote its transcription.

1. Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is the most common cause for brain injury in full-term infants, which is induced by perinatal asphyxia due to various factors and manifested as damage resulting from partial or complete hypoxia and reduction of cerebral blood flow [1]. Besides, HIE is also the leading cause for abnormal and fatal injuries in neonatal neural development [2]. A variety of mechanisms have been associated with HIE pathology, including decreased cerebral blood flow, changes in energy metabolism in brain tissue, destruction of inflammatory regulatory factors and oxygen free radicals, toxic effects of abnormal amino acids on neurons, calcium influx and brain apoptosis [3,4]. In general, severe hypoxia and ischemia often lead to brain cell necrosis, while mild ones trigger apoptosis-regulated delayed cell death. However, it is often difficult to identify and to diagnose neuronal apoptosis in clinical practice owing to limited sensitivity and specificity. Therefore, studying the potential molecular mechanisms of HIE-induced neuronal apoptosis may help identify irreversible neuronal apoptosis in neonatal neural development.

BNIP3 is a protein that can interact with Bcl-2 protein and adenovirus E1B19KD [5]. BNIP3 is a BH3-only subfamily containing only the BH3 homology domain in the Bcl-2 family. It has different structural characteristics, special activities and regulatory mechanisms from those of other members in the Bcl-2 family, and plays an important role in regulating apoptosis [6]. The NH2 domain and carboxy-terminal transmembrane region of BNIP3 dominate in the formation of dimers to promote apoptosis [7]. In addition, BNIP3 has high concentrations in a

Abbreviations: ChIP, chromatin immunoprecipitation; HIE, hypoxic-ischemic encephalopathy; HRE, hypoxia response element

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variety of neurological diseases [8], such as aneurysmal subarachnoid hemorrhage, traumatic brain injury, stroke, hypoxic encephalopathy and neonatal HIE [9,10]. Nevertheless, the mechanism by which the rise in BNIP3 level is associated with neuronal apoptosis induced by HIE remains unclear.

Hypoxia and ischemia are most commonly responsible for brain damage, leading to serious neurological diseases by disturbing brain development. When hypoxia occurs, a series of regulatory mechanisms are triggered, such as enhancement of the glycolysis pathway to supplement the energy under hypoxic conditions. Among various regulatory pathways, HIFs, which are activated as "main regulators", participate in the growth and development of organisms and the pathogenesis of diseases [11]. HIFs are heterodimeric transcription factors composed of two subunits, i.e. α and β. The HIF-α subunit is regulated by oxygen content and a post-translational modification sensitive to oxygen content, and HIF-β is continuously expressed in the nucleus [12]. At present, three HIF-α subunits have been identified: HIF-1α, HIF-2α and HIF-3α of which the former two have been mostly studied [13]. Activation of the HIF pathway allows cells to survive in a hypoxic environment [14,15], whereas adaptive responses may accelerate disease progression in some cases [16]. Accumulating evidence has verified that HIFs play crucial roles in the pathogenesis of HIE. By using brain cells with HIF-1α knock-out from adult mice, Helton et al. found that HIF-1α predominantly facilitated apoptosis in an acute hypoxic environment. In contrast, HIF-1α has neuroprotective effects on a stroke model of moderate hypoxia-reperfusion in neonatal rats [17]. Thus, the dual roles of HIF ought to be further clarified. Zhang et al. established a rat model of acute myocardial infarction, and found that BNIP3 was activated by HIF-1α, which played a key role in ischemia-reperfusion injury by inducing mitochondrial dysfunction [18]. Additionally, Wu et al. reported that BNIP3 was regulated by HIF-1α and induced apoptotic cell death after focal cerebral ischemia in rats. Until now, the interactions between HIF and BNIP3 in hypoxia-induced neuronal damage remain unclear. Accordingly, we herein detected the expressions of BNIP3 in peripheral blood of HIE and healthy neonates by ELISA. Subsequently, siRNA technology was used to assess the effects of BNIP3 on the apoptosis and proliferation of human neuronal cell line AGE1.HN, and the influence of inhibiting the expression of HIF-1α or HIF-2α on the level and function of BNIP3 in AGE1.HN cells cultured under hypoxic conditions. HIF-α regulated the transcription of BNIP3 by binding hypoxia response element (HRE).

2. Materials and methods

2.1. Clinical tissues and cells

From January 2015 to January 2017, 40 neonates with HIE delivered in our hospital and 40 full-term healthy newborns in the same period were selected. HIE was diagnosed according to diagnostic criteria and clinical grading criteria developed by the Neonatal Group of the Chinese Medical Association Pediatrics Branch. This study has been approved by the ethics committee of our hospital, and written consent has been obtained from the guardians of all neonates.

Human neuronal cell line AGE1.HN was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (China).

2.2. Collection of blood samples

Briefly, 2 ml of blood was collected from the femoral vein within 1 day after birth, and quickly placed in a non-anticoagulant tube. After being left still for 15 min at 4 °C, the blood sample was centrifuged at 4000 rpm for 5 min. The upper serum was stored in an EP tube in a −20 °C refrigerator, and then the expression level of BNIP3 protein was measured by ELISA.

2.3. Cell culture and hypoxic treatment

AGE1.HN cells were cultured in DMEM and Ham's F-12 medium mixed at a ratio of 1:1, which also contained 5% fetal bovine serum, 100 units/ml penicillin, 100 ng/ml streptomycin, 10 mg/ml human transferrin and 30 mmol/L sodium selenite. Normally, the cells were cultured in an incubator at 37 °C in the presence of 20% O_2. For hypoxic treatment, the cells were cultured in the presence of 1% O_2 at 37 °C.

2.4. Construction of interference sequences with inhibited BNIP3, HIF1α and HIF2α expressions

Three siRNAs targeting BNIP3, HIF1α and HIF2α were synthesized by Shanghai GenePharma Co., Ltd. (China) based on their sequences, which were siBNIP3, siHIF1α and siHIF2α, respectively. The mismatch sequence was siControl.

2.5. Cell culture

Briefly, 2.5×10^4 AGE1.HN cells were seeded in 24-well plates with a total medium volume of 0.45 ml. After 24 h, transfection was performed when the confluence reached 30%–40%. The cells were washed once with PBS before transfection to ensure serum-free status. A siRNA-lipofectamine 2000 mixture was added to the above culture wells, shaken and mixed gently. The culture plate was placed in a 37 °C incubator with 5% CO_2. After 4–6 h of culture, the medium containing the siRNA-lipofectamine 2000 mixture was removed, and complete medium containing serum and antibiotics was used for further culture.

2.6. RT-PCR

Total RNA was extracted from cells and purified with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Then RNA was reverse-transcribed into cDNA using random hexamers with SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed using Sybgreen I real-time PCR kit (TaKaRa, Dalian, China) on ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA). Primer sequences: BNIP3-F: 5′-GGATGCAGGAGAGAGCCT-3′, BNIP3-R: 5′-CGAGGTGGCTGTCACAGT-3′; β-actin-F: 5′-CTCCATCCTGGCCTCGC TGT-3′, β-actin-R: 5′-GCTGTCACCTTCACCGTTCC-3′. Results were analyzed from at least triplicate experiments, using β-actin as internal control.

2.7. Western blot

Cell lysates were prepared by using RIPA protein extraction reagent (Beyotime Institute of Biotechnology, Beijing, China), and protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL, USA). Samples containing equal amounts of protein were separated by 10% SDS-PAGE and electronically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Afterwards, the membranes were incubated with specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against BNIP3 and GAPDH according to the manufacturer's instructions.

2.8. Detection of cell apoptosis

The treated cells were collected and centrifuged at 2000 rpm for 5 min to discard the supernatant. The cells were thereafter washed twice with PBS, centrifuged at 2000 rpm for 5 min, and prepared into a suspension of 1×10^6/ml with 1× binding buffer. Then 100 μl of the cell suspension was placed into a 5 ml culture tube, added with 2 μl of annexin V-FITC and 4 μl of PI at 50 μg/ml in dark to react for 5–15 min, and mixed with 100 μl of 1× binding buffer for flow cytometry within

1 h.

2.9. Detection of cell proliferation

The cells were seeded in a 96-well plate at 2×10^4/well with the medium volume of 100 μL/well. The plate was pre-incubated in a 37 °C incubator with 5% CO_2, into which 10 μL of CCK-8 solution was then added to each well. The cells were incubated for another 4 h. The absorbance at 450 nm was measured with a microplate reader.

2.10. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed to verify the binding of HIF1α or HIF2α protein to the promoter of BNIP3 by using a ChIP kit (Upstate Biotechnology, New York, NY, USA) as described before [19]. After isolation of DNA-protein complexes from cells, cross-linked chromatin DNA was prepared for immunoprecipitation using specific antibodies against HIF1α, HIF2α or IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in combination with quantitative PCR analysis. Primers spanning the HRE site of BNIP3 promoter: F: 5′-AATTAGCCGGGTGT GGT-3′ and R: 5′-ATTTCCCTCATGCCGTTT-3′. Primers for amplification of non-HRE site: F: 5′- GAAGAGAAGUUGAAAGUAUTT-3′ and R: 5′-AUACUUUCAACUUCUCUUCTT-3′. Three or more independent experiments were repeated for data presentation.

2.11. Statistical analysis

All data were analyzed by SPSS16.0 software (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation. Inter-group comparisons were conducted with the *t*-test or one-way analysis of variance. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Serum BNIP3 protein expression levels

The mean concentrations of BNIP3 in sera of HIE and healthy neonates were (4.5 ± 2.1) ng/ml and (1.2 ± 0.5) ng/ml, respectively. The serum BNIP3 concentration in HIE neonates was significantly higher ($P < 0.001$) (Fig. 1).

3.2. Effects of BNIP3 on neuronal apoptosis

Compared with untreated group, the number of apoptotic cells in AGE1.HN cell group treated with BNIP3 significantly increased ($P < 0.05$). After treatment with 10 ng/ml BNIP3, the number of apoptotic cells was 4.8-fold that of untreated group ($P < 0.05$) (Fig. 2A).

Then BNIP3 expression in AGE1.HN cells was suppressed by using siBNIP3, an siRNA targeting BNIP3. The interference efficiency was detected by Western blot 48 h after transfection. As exhibited in Fig. 2B, siBNIP3 significantly decreases the BNIP3 protein expression level, indicating that siRNA interference was successful. The viability of AGE1.HN cells transfected with siBNIP3 was significantly augmented compared with that of cells transfected with siControl ($P < 0.01$) (Fig. 2C). Collectively, inhibiting BNIP3 expression promoted the proliferation of neurons.

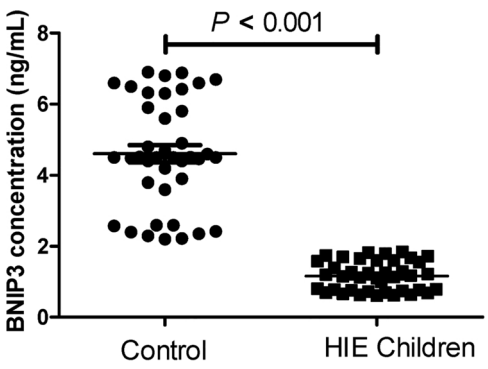

Fig. 1. BNIP3 protein expression levels in sera of HIE and healthy neonates.

3.3. Effects of HIF1α and HIF2α on hypoxia-induced up-regulation of BNIP3 expression

Under hypoxic conditions (1%), the mRNA (Fig. 3A) and protein (Fig. 3B) levels of BNIP3 increased significantly with prolonged time. After 48 h, the mRNA level of BNIP3 increased by 1.8-fold.

After pretreatment with HIF1α or HIF2α expression inhibition and culture under hypoxic conditions, BNIP3 expression was significantly lower than that of AGE1.HN cells only undergoing hypoxic culture (Fig. 3C and D).

3.4. Hypoxia induced cell apoptosis through HIFα and BNIP3

Inhibiting the expression of HIF1α or HIF2α (Fig. 4A) or transfecting with siBNIP3 before hypoxic treatment (Fig. 4B) significantly reduced the number of apoptotic cells compared with that of cells undergoing hypoxic treatment only.

3.5. HIF1α and HIF2α promoted BNIP3 transcription by binding HRE site

When AGE1.HN cells were cultured under hypoxic conditions, HIF1α or HIF2α bound BNIP3 promoter [20], which did not occur under normal culture conditions (Fig. 5A). HIF1α or HIF2α was significantly enriched near the HRE site of BNIP3 promoter (Fig. 5B).

4. Discussion

Neonatal HIE can be characterized by abnormal crying, difficulty falling asleep, breathing changes, no crying or moving, low consciousness, convulsions, etc. For newborns, HIE is the main cause of seizures. The types of epilepsy are commonly microsmall, clonic, tonic, focal, multifocal or generalized myoclonus [21,22]. Perinatal hypoxic-ischemic or other lossy gas exchange factors often lead to asphyxia, therefore, neonatal HIE is considered to be an acute delivery event, which can cause different degrees of neonatal brain damage, fetal umbilical arterial blood metabolic acidosis, spasm paralysis or dyskinesia, or cerebral palsy [23]. Eliminating the influencing factors in neonatal HIE is also important to prevent diagnostic delays and neuroprotective interventions [24].

As the current methods for treating neonatal HIE have certain limitations, it is particularly important to determine the effective biomarkers of brain injury to improve the consequences of perinatal brain injury. They can provide a basis for identifying patient risk in neurotherapeutic interventions and provide prognostic information for early home interventions. In particular, rapid measurement of serum markers can help to objectively assess the accuracy of current methods for determining the extent of brain damage, such as clinical examinations, brain MRI and EEG data. These methods are subjective or require specific equipment technology/professional knowledge, and not all hospitals have the conditions. Therefore, early and reliable laboratory tests that reflect the severity of brain damage play a key role in the treatment of these high-risk newborns.

BNIP3 is a member of the Bcl-2 family of proteins that contain only atypical BH3 domains [25]. It is known that BNIP3 is involved in the regulation of various cell biological behaviors, with its specific effects varying with cell types and conditions. In addition, BNIP3 also plays an important role in tumor development, myocardial ischemia, hypertrophy and atrophy, cerebral ischemia and hypoxia damage [26–30].

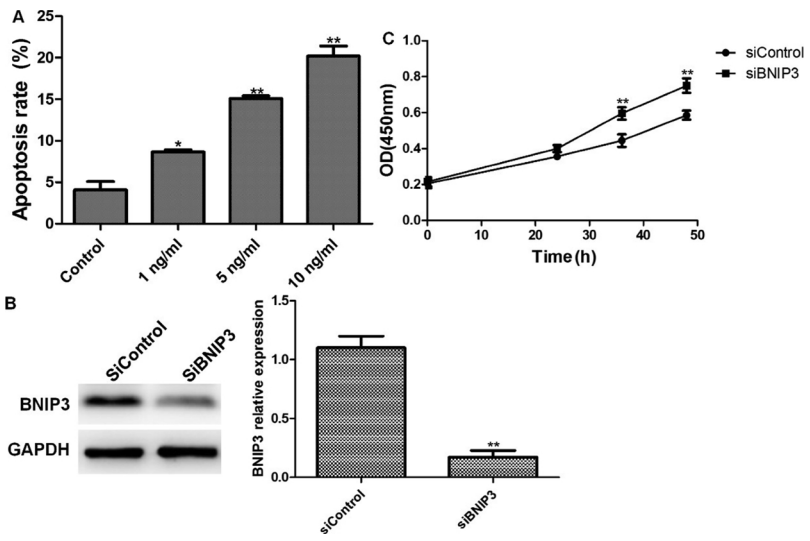

Fig. 2. Effects of BNIP3 on neuronal apoptosis. A: Apoptosis of AGE1.HN cells after treatment with different concentrations of BNIP3 for 48 h; B: Western blotting results 48 h after transfection with siBNIP3; C: cell proliferation after transfection detected by CCK-8 assay. *P < 0.05, compared with control group; **P < 0.01, compared with control or siControl group.

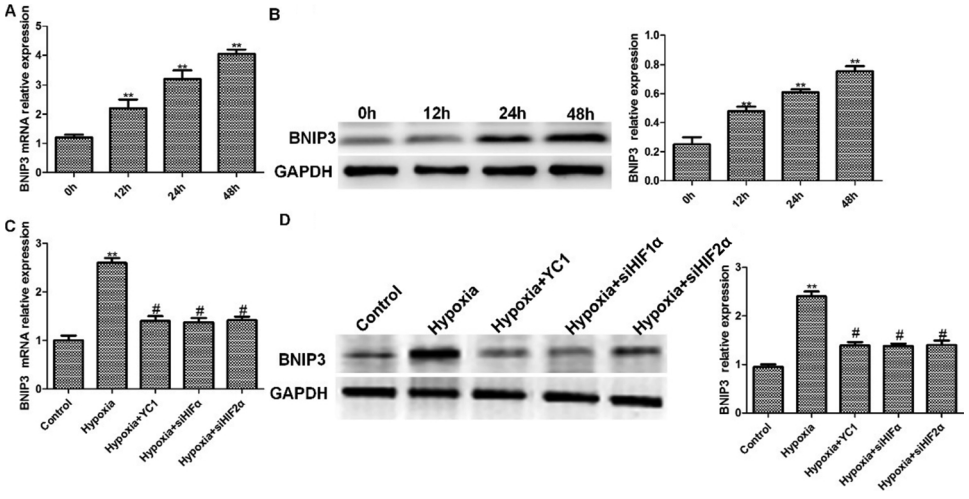

Fig. 3. Effects of HIF1α and HIF2α on hypoxia-induced up-regulation of BNIP3 expression. A: BNIP3 mRNA expressions after 12, 24 and 48 h of treatment under hypoxic conditions (1%) detected by RT-PCR; B: BNIP3 protein expressions after 12, 24 and 48 h of treatment under hypoxic conditions (1%) detected by Western blotting; C: BNIP3 mRNA expressions after 6 h of pretreatment with HIFα inhibitor YC-1 or 12 h of treatment with siHIF1α or siHIF2α and 24 h of hypoxic treatment detected by RT-PCR; D: BNIP3 protein expressions after 6 h of pretreatment with YC-1, or 12 h of treatment with siHIF1α or siHIF2α and 24 h of hypoxic treatment detected by Western blotting. **P < 0.01, compared with control group; #P < 0.05, compared with hypoxic treatment group.

To our knowledge, fewer studies have reported the role of BNIP3 in neonatal brain injury. In this study, we used ELISA to detect BNIP3 levels in serum of newborn HIE children and healthy newborns. The results showed that the average concentration of BNIP3 in serum of newborn HIE children was significantly higher than that of healthy newborns.

Since neuronal apoptosis is an important change in the pathological process of HIE, we examined the effect of BNIP3 on neuronal apoptosis in the human neuronal cell line AGE1.HN. The AGE1.HN cells were treated with BNIP3 at 1 ng/ml, 5 ng/ml and 10 ng/ml for 48 h, respectively. The results of flow cytometry showed that the number of apoptotic cells was significantly increased in the BNIP3 treated group compared with the untreated group. And the higher the treatment concentration, the more the number of apoptotic cells. The above results indicate that BNIP3-treated neuronal cells can promote the apoptosis of neuronal cells. And the CCK8 assay has shown that inhibition of BNIP3 expression can promote the proliferation of neuronal cells.

Hypoxia is one of the important causes of brain damage in neonates, and apoptosis caused by hypoxia is regulated through the coordination of HIF and many other factors. HIF-1α and HIF-2α are the two major subtypes of HIF-α and are key genes in the regulation of the body's

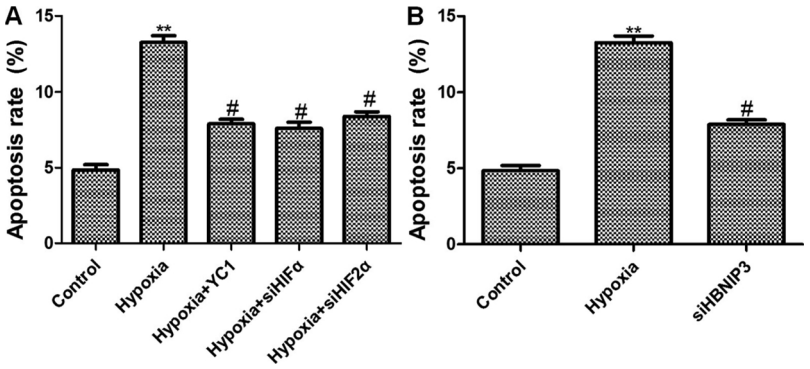

Fig. 4. Effects of HIFα and BNIP3 on hypoxia-induced AGE1.HN cell apoptosis. A: Apoptosis after 6 h of pretreatment with HIFα inhibitor YC-1 or 12 h of treatment with siHIF1α or siHIF2α and 24 h of hypoxic treatment detected by flow cytometry; B: apoptosis after 12 h of treatment with siBNIP3 and 48 h of hypoxic treatment detected by flow cytometry. **P < 0.01, compared with control group; #P < 0.05, compared with hypoxic treatment group.

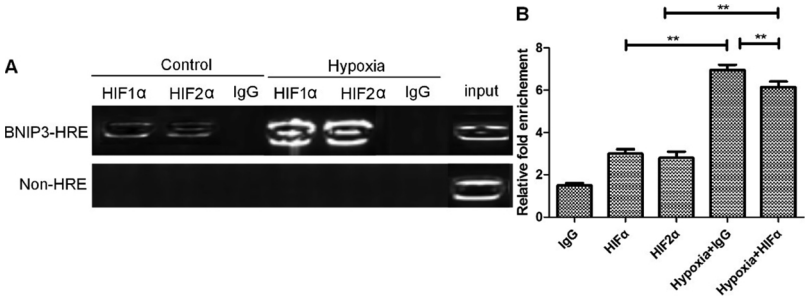

Fig. 5. HIF1α and HIF2α promoted BNIP3 transcription by binding HRE site. A: Binding of HIF1α or HIF2α to BNIP3 after 12 h of treatment in the presence of 1% or 20% O_2 detected by ChIP assay; B: enrichment of HIF1α or HIF2α in HRE site of BNIP3 promoter detected by RT-PCR. **P < 0.01.

response during hypoxia. In this study, in order to explore the effect of HIF-α on BNIP3 under hypoxic conditions, AGE1.HN cells were first cultured under hypoxia (1%) for 12 h, 24 h or 48 h, and then detected by real-time PCR. A significant increase in m RNA levels and protein levels of BNIP3 was found. The above results indicate that hypoxic culture conditions can induce up-regulation of BNIP3 expression in neuronal cells. Through hypoxia treatment and inhibition of HIF-1α or HIF-2α expression, real-time fluorescent quantitative PCR and Western blot showed that after pretreatment with inhibition of HIF-1α or HIF-2α expression and then hypoxic culture, the AGE1.HN cells' ability of BNIP3 expression was significantly lower than that of AGE1.HN cells treated only with hypoxic culture; flow cytometry revealed that inhibition of HIF-1α and HIF-2α expression in neuronal cells reversed hypoxia-induced apoptosis. All these results indicate that inhibition of HIF-1α and HIF-2α expression in neuronal cells can reverse hypoxia-induced BNIP3 up-regulation, and HIF-α can also regulate hypoxia-induced apoptosis.

Studies have found that HIF-α can regulate the expression of multiple genes, such as cell proliferation genes, erythropoietin-producing genes, angiogenic genes, and genes that promote glucose metabolism. These genes are involved in angiogenesis and glucose metabolism of hypoxic ischemic cells, which is extremely important for the survival of cells. However, the role of HIF-α in neurons during hypoxia-ischemia remains controversial, which is believed to have a dual role in promoting death and anti-apoptosis. When HIF-1α expression is up-regulated by hypoxic pretreatment, it has neuroprotective effects in cerebral ischemic injury [31], but cerebral ischemia can also up-regulate HIF-1α expression. Inhibition of HIF-1α expression in adult rat ischemia model can damage its neuroprotective effect [32]. In hypoxia-inducible mice with knockout of HIF-1α in the brain, HIF-1α level reduction can induce neuroprotective effects. The regulation of HIF-1α in the nervous system seems to depend on the duration of action of the stimuli, the type of pathological stimuli and the type of cells. Vangeison et al. indicated that inhibition of HIF-α function in astrocytes could protect cells from hypoxia, while inhibition of HIF-α function in neurons could increase neuronal sensitivity to hypoxia-induced damage [33], indicating HIF-α action is cell specific.

HIF-α typically regulates gene expression at the transcriptional level by binding to a hypoxia response element of the target gene. In this study, we found that the promoter region of BNIP3 gene contains a potential hypoxia response element site, and we speculate that HIF-1α or HIF-2α upregulates BNIP3 expression by binding to the hypoxia response element of the BNIP3 promoter region. To confirm this hypothesis, chromatin immunoprecipitation analysis revealed that HIF-1α or HIF-2α could bind to the BNIP3 promoter when AGE1.HN cells were cultured under hypoxic conditions, while HIF-1α and HIF-2α could not bind to the BNIP3 promoter under normal oxygen culture conditions. Fluorescence quantitative PCR showed that HIF-1α or HIF-2α was significantly enriched near the site of the hypoxia response element on the BNIP3 promoter, but there was very few at the site of the non-hypoxia responsive element at the distal end. The above results indicate that HIF-1α and HIF-2α can promote BNIP3 transcription by binding to hypoxia response element sites.

5. Conclusion

In summary, we used ELISA to detect that the serum BNIP3 concentration in newborn HIE children was higher than that in healthy newborns. BNIP3 treated neuronal cells can promote neuronal cell apoptosis, while inhibition of BNIP3 expression can promote the proliferation of neuronal cells. Hypoxic culture conditions can induce BNIP3 expression in neuronal cells, while further inhibition of HIF-1α and HIF-2α expression can reverse hypoxia-induced BNIP3 expression up-regulation. Inhibition of HIF-α or BNIP3 expression in neuronal cells can reverse hypoxia-induced apoptosis. Finally, chromatin immunoprecipitation analysis revealed that HIF-1α and HIF-2α promoted BNIP3 transcription by binding to the hypoxia response element site of the BNIP3 promoter. On the one hand, BNIP3 promotes the occurrence and development of multiple nervous system damage diseases by mediating apoptosis and inflammatory reaction; on the other hand, it exerts neuroprotective effects by regulating the expression of related protective target genes. We will conduct a more in-depth study of BNIP3 and its signal pathway in the future.

Conflict of interest

None.

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