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Original Article

Lentivirus-mediated silencing of HSP70 aggravated hypoxia-induced apoptosis in SH-SY5Y cells

Chunyu Xie[1], Chun Guan[2], Suxin Qu[3], Qingshu Li[2], Dan Hu[2], Yun Wang[1], Yan Qu[2]

[1]Medical College of Qingdao University, Qingdao, Shandong, China; [2]Department of Critical Care Medicine, Affiliated Qingdao Municipal Hospital of Qingdao University, Qingdao, Shandong, China; [3]Women and Children Hospital, Qingdao, Shandong, China

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Abstract: Heat shock protein 70 (HSP70) is a molecular chaperone, which plays critical roles in the neuroprotection against hypoxia. It was reported that abnormal up-regulation of HSP70 was one of the major hallmarks of hypoxia. We previously identified that over expression of HSP70 could effectively protected cells against hypoxic-induced apoptosis. In this study, we established cell model of hypoxia-ischemia in vitro and HSP70 lentiviral shRNA vector. Then we observed the effect of HSP70 silencing on hypoxic-induced apoptosis in SH-SY5Y cells and elucidated the possible mechanisms underlying this neuroprotection. Results showed that silencing of HSP70 in SH-SY5Y cells significantly decreased the cell viability after exposing to hypoxia, and aggravated hypoxia-induced apoptosis. Further studies showed that lentivirus-mediated silencing of HSP70 increased the protein levels of NF-κB and caspase-3 in hypoxic SH-SY5Y cells. These results provide evidence that down-regulation of HSP70 aggravated hypoxia-induced apoptosis by increasing the expression of NF-κB and caspase-3 in SH-SY5Y cells.

Keywords: HSP70, BAG-1, caspase-3, NF-κB, ischemia and hypoxia, apoptosis

Introduction

Hypoxic encephalopathy is one of the most common causes of death and disability. The incidence of hypoxic-ischemic encephalopathy is increasing. However, studies have showed that many aspects were involved in pathophysiologic mechanisms of hypoxic ischemic encephalopathy. This complex pathogenesis induced difficulty in treatment of hypoxic-ischemic encephalopathy. Novel strategies of promising neuroprotection will be a major direction to improve the cure rate of patients with hypoxic-ischemic encephalopathy and therefore urgently required.

From many experimental findings, it was clear that heat shock protein 70 (HSP70) was directly implicated in almost all kinds of cancers and reached an extreme level in the stress condition. Increasing evidence showed that HSP70 could provide a primary protective effect against damage of neurons. In the model of neurodegeneration, over-expression of HSP70 could protect cellular proteins from misfolding, aggregation and toxicity [1, 2]. Many experimental findings have proved that over-expression of HSP70 could protect neurons and HSP70 silence could increase the sensitivity of neurons to hypoxia [3-5]. However, the mechanisms of the neuroprotection of HSP70 on hypoxia-induced damage of neurons were not fully understood. Evidence has showed that hypoxia-induced apoptosis contributed to the cell damage in hypoxic-ischemic encephalopathy. And neurons undergo caspase-dependent apoptosis in response to hypoxia.

BAG-1 was a multifunctional anti-apoptotic gene, which was discovered in 1995 firstly as a BCL-2 binding protein. It was confirmed that BAG-1 could enhance its anti- apoptotic activity by binding with BCL-2 [6]. There were many subtypes of BAG-1, which could be combined with a variety of molecular targets in the cells, and play a role in inhibiting apoptosis. The human BAG-1 gene was located on chromosome 9, which mainly expressed four BAG-1 isoforms,

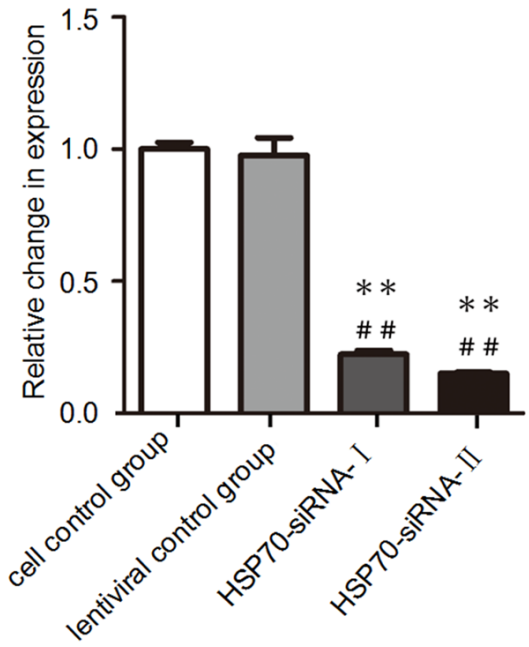

Figure 1. HSP70 silence decreased HSP70 mRNA expression in SH-SY5Y cells. SH-SY5Y cells were exposed to hypoxia. Treatment of SH-SY5Y cells with HSP70 lentiviral shRNA decreased the expression of HSP70 mRNA. $^{\#\#}P<0.05$, compared with the cell control group, $^{**}P<0.05$, compared with the lentiviral control group.

including BAG-1L, BAG-1M, BAG-1S, and BAG-1 [7]. Different subtype showed different anti-apoptotic effects. Among them, BAG-1M had the strongest anti-apoptotic effect, while the anti-apoptotic effect of BAG-1S was not observed.

All BAG-1 subtypes had BAG-1 functional domain, and this domain may be related to the anti-apoptotic effect of BAG-1. BAG-1 domain contained 110-124 amino acids [8, 9], consisting of 3 anti-parallel α-helices. Each helix had about 30-40 amino acids. The first and second helices bind to serine/threonine kinase Raf-1, the second and third helices bind to the ATP domain of HSP70, form a functional active conformation to regulate the function of HSP70 [8-10]. Studies have shown that BAG-1 could play a role in the cerebral ischemia by up-regulating the level of HSP70 [11]. It was found that BAG-1 mutation lost its protective effect against cyclosporine-induced apoptosis. These results suggested that the anti-apoptotic effect of BAG-1 might be achieved by combing ATP domain of HSP70.

Nuclear transcription kappa B (NF-κB), an important transcriptional activator, exists in various eukaryotic cells [12]. In the nervous system, NF-κB was widely distributed in neurons, astrocytes and microglia. NF-κB could regulate many genes which related to immune function and inflammation, and play an important role in physiological and pathological condition. It have been shown that the function of NF-κB was involved in many pathological processes, such as immune response, thymus development, embryogenesis, inflammation and acute reaction, cell proliferation, apoptosis, viral infection and so on. In the model of transient global ischemia which occlusion of 4 vessels in the brain for 30 minutes, NF-κB was selectively activated at 72 h after reperfusion in hippocampal CA1 neuron, and the activation time was similar to that of neuron apoptosis. It was speculated that the increase of NF-κB in the dying CA1 neurons may be related to the mechanism of apoptosis or necrosis [13].

Cysteinyl aspartate specific proteinase (caspase), was a group of proteases which have similar structures in the cytoplasm. Caspase was closely related to the apoptosis of eukaryotic cells and was involved in cell growth, differentiation. According to the homology of the protease sequence, caspase could be divided into 3 subgroups, caspase-1, caspase-2, and caspase-3. Caspase-3, one of the most important apoptosis executors in the caspase family, was the main factor in the process of apoptosis. Its activation was a sign of the irreversible phase of apoptosis. Moreover, the functional roles of caspase-3 in neurons have also been recognized gradually. In the mice which caspase-3 was over-expressed, cerebral ischemia and reperfusion in the brain showed a larger trauma and increased apoptosis compared with wild mice, although the phenotype was normal [14], suggesting that caspase-3 might be involved in ischemia-induced apoptosis in neurons.

Therefore, in this study, we investigated the possible role of down-regulation of HSP70 in caspase-dependent apoptosis after ischemia and reperfusion. Our findings showed that HSP70 RNAi could effectively reduce the expression of HSP70, and then decreased the cell viability and increased the apoptosis of SH-SY5Y cells in the condition of hypoxia via

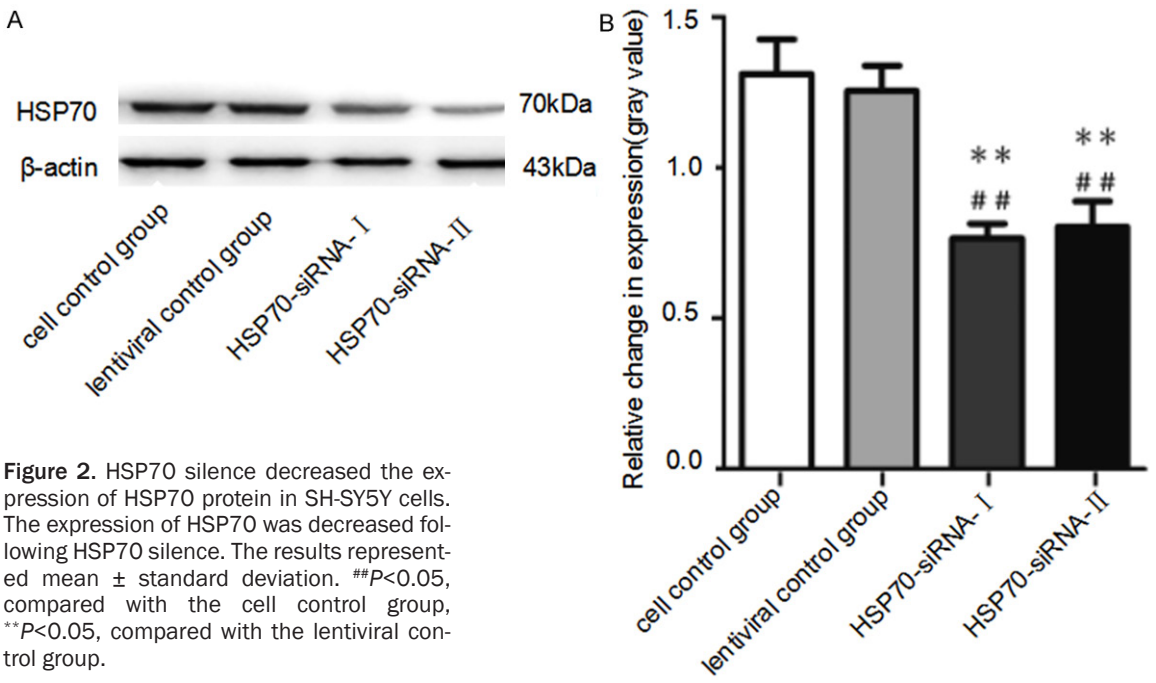

Figure 2. HSP70 silence decreased the expression of HSP70 protein in SH-SY5Y cells. The expression of HSP70 was decreased following HSP70 silence. The results represented mean ± standard deviation. $^{\#\#}P<0.05$, compared with the cell control group, $^{**}P<0.05$, compared with the lentiviral control group.

regulation of NF-κB and caspase-3, but not BAG-1.

Materials and methods

Materials and cell culture

SH-SY5Y cells were obtained from Department of physiology, medical College of Qingdao University. They were cultured in DMEM medium (GIBco, USA) containing 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in a humidified atmosphere with 5% carbon dioxide. Cells at logarithmic growth phase were used for experiments.

Lentiviral infection of shRNA

HSP70 shRNA sequence I (GGACGAGTTTGAGCACAAG, HSP70-siRNA-I), and shRNA sequence II (GCCATGACGAAAGACAACAAT, HSP70-siRNA-II) were cloned into the GV248 vector. Lentivirus was produced by Shanghai Ji Kai Gene Technology Corporation. SH-SY5Y cells were seeded in 24-well plates. Cells were infected by lentivirus containing GFP. Puromycin was used to screen the stable infected cells.

Cell culture model of ischemia-hypoxia/reoxygenation

SH-SY5Y cells were cultured in low glucose DMEM medium without fetal bovine serum. Cells were then incubated in a hypoxic environment (approximately 95% nitrogen and 5% carbon dioxide) at 37°C for 4 h, 8 h, 12 h, and then cells were cultured with DMEM medium containing 10% fetal bovine serum, and then re-oxygenation in aerobic environment at 37°C in a 5% CO_2 humidified incubator for 24 h.

Cell viability assay

5×10^3 cells/well were seeded into 96-well plates and incubated for 24 h, then cells were exposed to hypoxia for 4 h, 8 h, 12 h, and then re-oxygenation for 24 h, followed by addition of Cell Counting Kit-8 (CCK-8) solution (Wuhan Biological Engineering Corporation, 10 μl/well). After incubation for 1 h, plates were measured at 450 nm using a microplate absorbance reader.

Cell apoptosis analysis

Cells were placed on 6-well plates at a density of 8×10^4 cells per well, maintained in hypoxia device for 8 h, and re-oxygenation for 24 h. Collected cells were washed with ice-cold PBS and centrifuged at 800 g for 5 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 100 μl 1× Binding Buffer, followed by addition of 2.5 μl Annexin V-APC and 2.5 μl 7-AAD (BD Biosciences, USA) and incubation for 15 minutes at room temperature in the dark. Finally, 400 μl

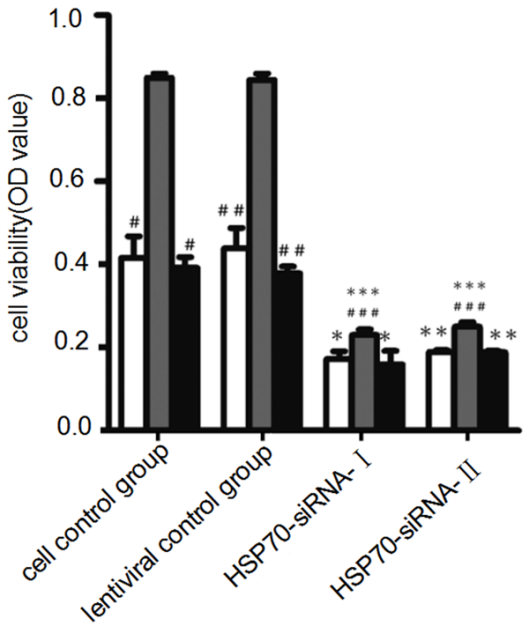

Figure 3. The cell viability of SH-SY5Y cells decreased response to hypoxia. White represents hypoxia 4 h, gray represents hypoxia 8 h, black represents hypoxia 12 h. SH-SY5Y cells were exposed to hypoxia (4, 8, 12 h). With the prolongation of time of hypoxia, the cell viability in all groups showed an increased followed by a weak, and peaked at 8 h. $^{\#}P<0.05$, compared with the control group exposed to hypoxia for 8 h, $^{\#\#}P<0.05$, compared with the lentiviral control group exposed to hypoxia for 8 h, $^{*}P<0.05$, compared with lentiviral infection group I exposed to hypoxia for 8 h, $^{**}P<0.05$, compared with lentiviral infection group II exposed to hypoxia for 8 h. SH-SY5Y cells were exposed to hypoxia for 8 h. The cell viability was assessed by CCK-8 assay. The results represented mean ± standard deviation. $^{\#\#\#}P<0.05$, compared with the cell control group, $^{***}P<0.05$, compared with the lentiviral control group.

1× Binding Buffer was added. Cells were analyzed per sample using a flow cytometry (BD Biosciences, USA).

Western blotting

After treatment with infection and hypoxia, cells were harvested. Cells were lysed with RIPA and PMSF within 20 minutes. Proteins were resolved on 10% SDS-PAGE gels, transferred to PVDF membranes, blocked with 5% milk and incubated with primary antibodies for two hours at room temperature. Primary antibodies HSP70 (1:1000, Abcam, UK), BAG-1 (1:1000, Abcam, UK), caspase-3 (1:5000, Abcam, UK), NF-κB (1:80000, Abcam, UK) and β-actin antibody (1:2000, Wuhan Biological Engineering Corporation) were used. Membranes were then induced with corresponding secondary antibodies to horseradish peroxidase for 1 h at room temperature. The gray values of proteins were analyzed by the Bio-Rad Quantity one software.

qRT-PCR

Total RNA was extracted using TRIzol reagent (Gibco, USA), and the quality and quantity of RNA were determined by spectrophotometry. Reverse transcription was carried out using primer. cDNA was then amplified by qRT-PCR using the primers β-actin: 5'-GATGAGATTGGCATGGCTTT-3' (forward), 5'-CACCTTCACCGTTCCAGTTT-3' (reverse); HSP70: 5'-CAAGAAGAAGGTGCTGGACA-3' (forward), 5'-GTACAGTCCGCTGATGATGG-3' (reverse); BAG-1: 5'-GAGCTCTAATTTGCCTTGGG-3' (forward), 5'-CCTCCTGGTGATTCTGGTTT-3' (reverse). Primers were synthesized by GenScript Biological Science and Technology Service Corporation (Nanjing, China). PCR amplification products were separated by 1.5% agarose gel electrophoresis, and visualized using gel imaging system to verify product size. The $2^{-\Delta\Delta Ct}$ method was used to calculate gene expression changes relative to control samples.

Statistical analysis

All experimental dates were analyzed by using SPSS17.0 statistics software. All results were recorded as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by LSD-test was used to compare difference between means in more than two groups. $P<0.05$ was considered statistically significant.

Results

Vector-mediated RNAi inhibits the expression of HSP70

The expression of HSP70 was silenced using two specific HSP70 lentiviral shRNAs. Results showed that the mRNA expression of HSP70 was reduced by more than 80% following transduction of HSP70 lentiviral shRNAs separately in SH-SY5Y cells compared to the control group and the lentiviral control group (**Figure 1**). Accordingly, the expression of HSP70 protein in HSP70 lentiviral shRNAs-transduced SH-SY5Y cells were also decreased significantly com-

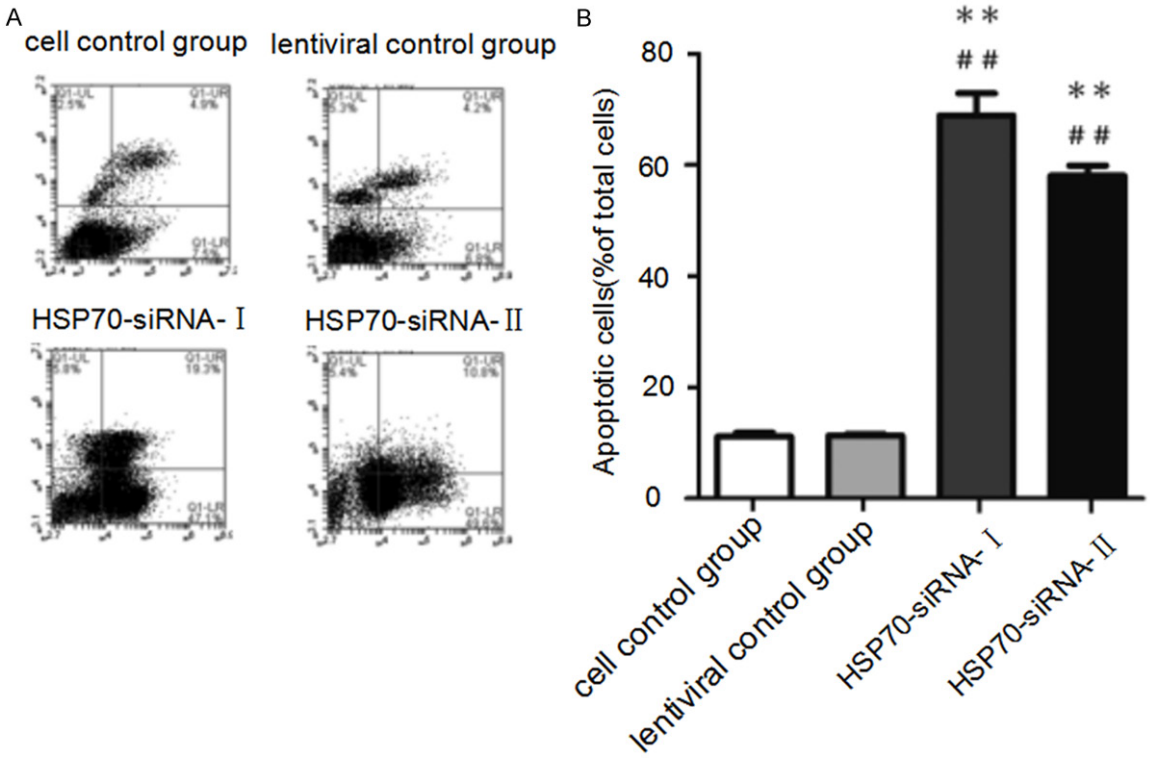

Figure 4. HSP70 silence promoted SH-SY5Y cell apoptosis. In (A) the left lower quadrant represents normal cell, the other quadrants represent apoptosis cells. SH-SY5Y cells were exposed to hypoxia for 8 h, and measured apoptosis by flow cytometry. The results represented as mean ± standard deviation. $^{\#\#}P<0.05$, compared with the cell control group, $^{**}P<0.05$, compared with the lentiviral control group. (B) Represents the rate of apoptosis in each group. SH-SY5Y cells were exposed to hypoxia for 8 h and re-oxygenation 24 h, the percentage of apoptotic cells increased in lentivirus infected group.

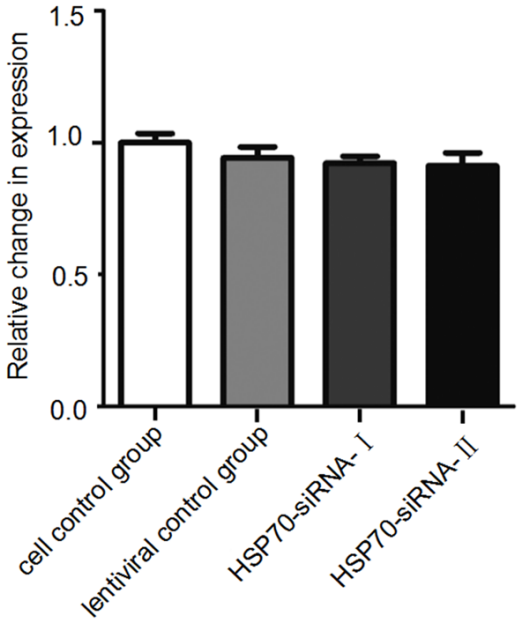

Figure 5. HSP70 silence had no effect on BAG-1 mRNA expression in SH-SY5Y cells. SH-SY5Y cells were exposed to hypoxia for 8 h. The results represented mean ± standard deviation. Compared with each other, there was no statistically significant ($P>0.05$).

pared to the control group and the lentiviral control group (**Figure 2**).

Gene-silence of HSP70 inhibits cell viability of SH-SY5Y cells

In order to examine the effect of HSP70 gene silence on cell viability of SH-SY5Y cells, we infected SH-SY5Y cells with HSP70 lentiviral shRNA. After treatment with hypoxia for 4 h, 8 h, 12 h, respectively, and re-oxygenation for 24 h, CCK-8 assays were performed and cell viability was illustrated according to OD values. With the prolongation of time of hypoxia, the cell viability in all groups appeared peak at 8 hours; followed by a declining tendency (**Figure 3**). Cell viability in HSP70 lentiviral infected cells decreased compared to those cells in the control group and the lentiviral control group ($P<0.05$, **Figure 3**).

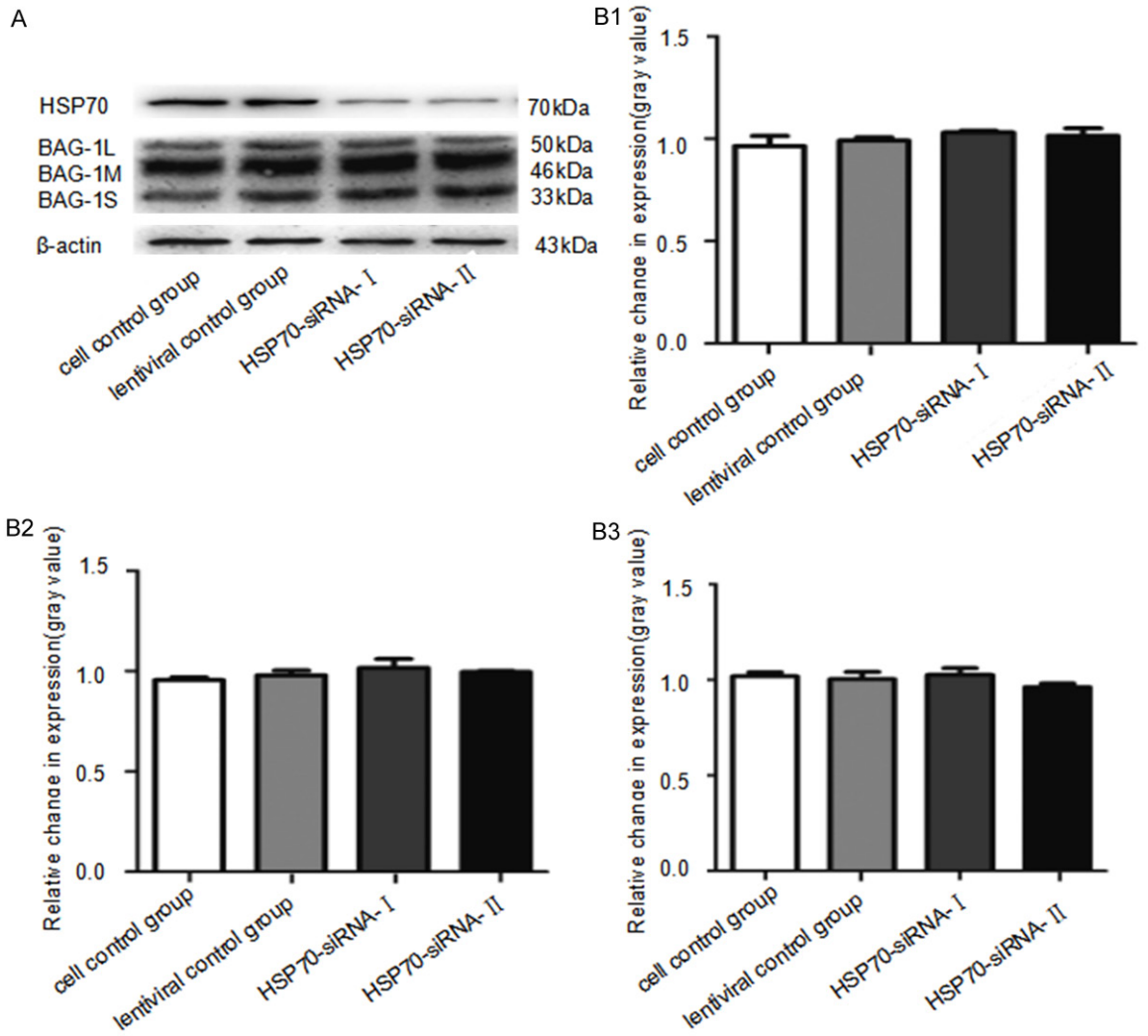

Figure 6. HSP70 silence had no influence to BAG-1 protein expression. (A) Represents the protein expression levels in each group. The (B1) represents the gray value of BAG-1L, the (B2) represents the gray value of BAG-1M, the (B3) represents the gray value of BAG-1S. The expression of BAG-1 unchanged following HSP70 silencing. The results represented mean ± standard deviation. Compared with each other, there was no statistically significant (P>0.05).

Down-regulation of HSP70 sensitizes SH-SY5Y cells to hypoxia-mediated apoptosis

We then investigated whether HSP70 down-regulation could sensitize SH-SY5Y cells to hypoxia-mediated apoptosis. The flow cytometric studies showed that RNA interference of HSP70 caused an apparent increase in the apoptotic rate of SH-SY5Y cells response to hypoxia compared to the control group and the lentiviral control group (P<0.05, **Figure 4**).

Silence of HSP70 had no effect on expression of BAG-1

qRT-PCR and western blotting were performed to confirm the effect of HSP70 silence on BAG-1 expression. We exposed SH-SY5Y cells to hypoxia for 8 h, and re-oxygenation for 24 h. The results showed that treatment of SH-SY5Y cells with HSP70 silence had no effect on BAG-1 mRNA and protein expression (P>0.05, **Figures 5**, **6**).

Silence of HSP70 promotes the expression of NF-κB and caspase-3

Further study is conducted to determine whether down-regulation of HSP70 could enhance NF-κB and caspase-3 expression. Result showed that down-regulation of HSP70 also led to increased expression of NF-κB and caspase-3 (**Figure 7**).

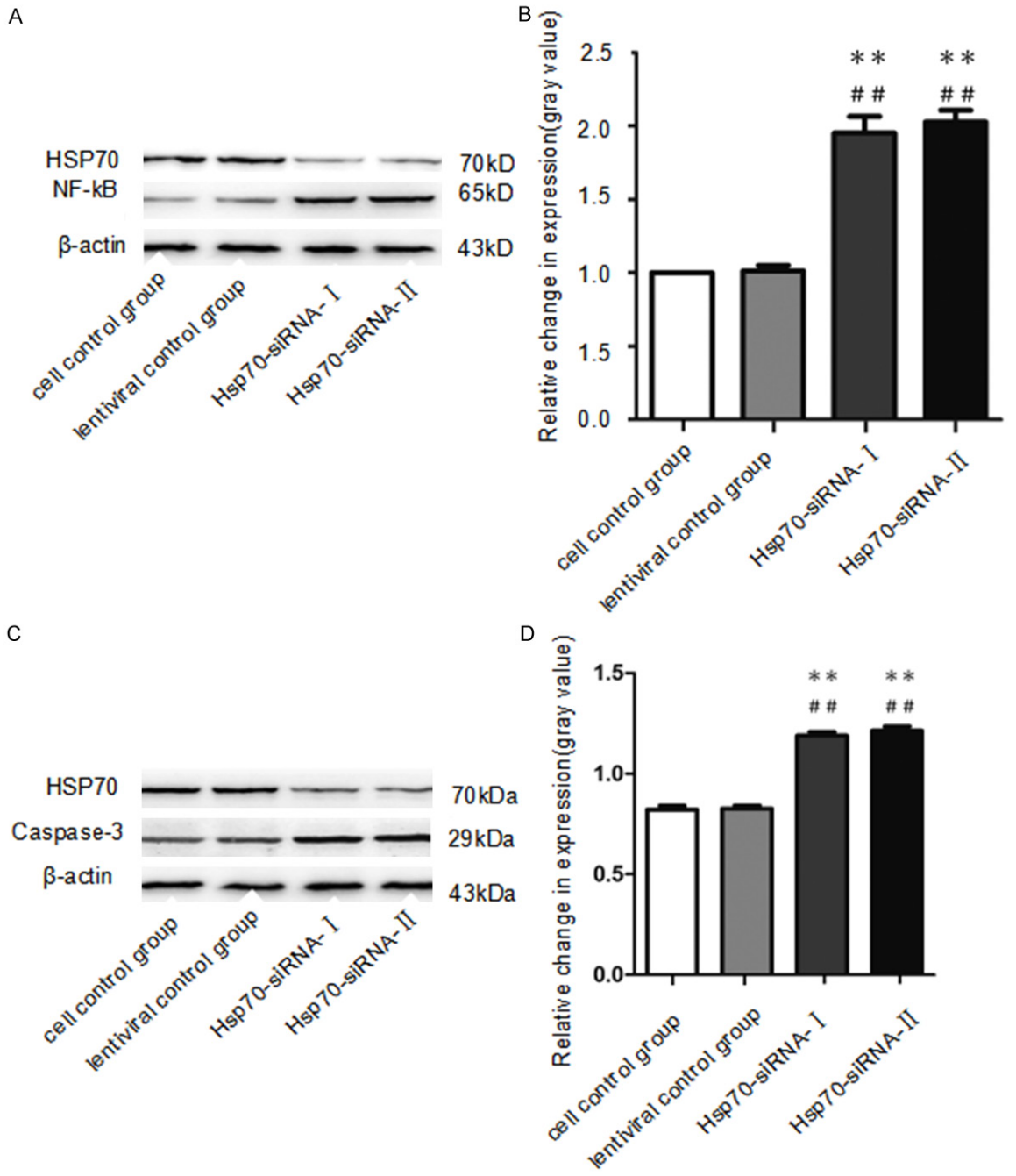

Figure 7. HSP70 silence increased the expression of NF-κB and Caspase-3. The expression of NF-κB and Caspase-3 was increased following HSP70 silence. The results represented as mean ± standard deviation. $^{\#\#}P<0.05$, compared with the cell control group, $^{**}P<0.05$, compared with the lentiviral control group.

Discussion

In the present study, we showed that HSP70 RNAi could effectively reduced the expression of HSP70, and then aggravated the decrease in the cell viability and enhanced apoptosis of SH-SY5Y cells in the condition of hypoxia. These results suggested that reduced expression of HSP70 could intensify the injury of SH-SY5Y cells induced by hypoxia. SH-SY5Y cells are derived from SK-N-SH cell line, and have the ability of adhesion and proliferation [6-8]. Compared with primary cell culture, SH-SY5Y is easy to cultivate and is often used as in vitro

models of neuronal function. Therefore, in the present study, SH-SY5Y cells were used to establish the experimental model [15, 16].

As a multifunctional anti-apoptosis gene, the properities of BAG-1 were intriguing. At present, the research on BAG-1 was mainly focused on the role of tumors cells [17-19]. A large number of experimental results support the involvement of anti-apoptotic effect of BAG-1 in cancer cells [17, 19]; However, the involvement of BAG-1 in neuron apoptosis in the brain were seldom reported. Experiments in vivo showed that there was a high expression of BAG-1 protein in immature neurons during brain development. With the maturation of neurons, the expression of BAG-1 protein decreased gradually [20]. However, the expression of BAG-1 was decreased in the apoptotic cells after neuron injury [21]. It was suggested that BAG-1 may play a potential role in neuron survival. In fact, high expression of BAG-1 could inhibit the activation of caspase and reduce the generation of reactive oxygen species [22]. Therefore, BAG-1 played a role in anti-apoptosis in cultured neurons. Recent studies showed that high expression of BAG-1 could protect neurons against injury of neurotoxin in Parkinson's disease, prompting the neuroprotective effect of BAG-1 and its potential application value [23]. The neuroprotection of BAG-1 was associated with the C-terminal domain which was combined with the ATP domain of HSP70/HSC70 [10]. Therefore, we detected the expression of BAG-1 in HSP70 silencing SH-SY5Y cells. Results showed that HSP70 silencing had no effect on the expression of BAG-1. Our results demonstrated that the anti-apoptotic effect of HSP70 was not associated with BAG-1.

NF-κB participated in a variety of biological processes, including innate and adaptive immune responses, tissue injury and stress as well as cell proliferation and apoptosis. Activated NF-κB could enter the nucleus and execute its fundamental function as a transcription factor. In the cardiac muscle cells model of ischemia reperfusion injury, HSP70 over-expression could inhibit the translocation of NF-κB from cytoplasm to nucleus, thus decrease the release of inflammatory factors and reduce apoptosis [24]. Previously reports have shown that up-regulation of HSP70 can restrain the activation of NF-κB and increase the vitality of Hela cells [25]. NF-κB was activated in neurons and glial cells after brain injury and induced production of pro-poptotic protein and gene in neurons (e.g. COX-2, iNOS, P53, C-myc/Fas ligand, etc.); this then activated caspase and lead to neuronal apoptosis [26]. It was found that NF-κB specific inhibitors can inhibit the expression of inflammatory factors, but also inhibits the apoptosis of neurons and improved the neurobehavioral score of rats [27]. In this study, we measured the expression of NF-κB in SH-SY5Y cells under hypoxia condition. We found that silencing HSP70 reduced HSP70 expression and increased the total NF-κB expression. Considering the function of NF-κB, we evaluated the viability and the proliferation of SH-SY5Y cells via CCK-8 and the flow cytometric studies. We found that the viability was decreased and the apoptotic rate was increased. This result indicated that HSP70 silencing might increase the expression of NF-κB, and then lead to cell apoptosis.

Furthermore, caspase-3 is the most important enzymes in the process of cell apoptosis, which was related to DNA repair and gene integrity monitoring. Fas/FasL way or granular enzyme pathway could activate caspase-3, and then caused cell apoptosis. In this study, we found that the expression of caspase-3 was increased in SH-SY5Y cells after the HSP70 silence treatment. Similar results were also reported by Shukla et al [28-30]. These results indicated that HSP70 silencing might increase the expression of NF-κB and caspase-3, and then lead to cell apoptosis.

In conclusion, our results showed that HSP70 silencing might lead to cell apoptosis by increasing the expression of NF-κB and caspase-3 in the condition of ischemia reperfusion injury. And we also showed the anti-apoptotic effect of HSP70 was not associated with BAG-1. These results provided the experimental basis for the study of hypoxic encephalopathy and were helpful to understand the mechanisms underlying the neuroprotective effect of HSP70 against ischemia reperfusion injury.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Qu, Department of Critical Care Medicine, Affiliated Qingdao Municipal Hospital of Qingdao University, Qingdao, Shandong, China. Tel: 86-13668892506; Fax: 86-0532-88905392; E-mail: qdquyan@aliyun.com

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