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Hypertonic saline protects brain endothelial cells against hypoxia correlated to the levels of estimated glomerular filtration rate and interleukin-1β

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Abstract

Objective: The aim of this study was to verify the protective effect of hypertonic saline (HS) on brain endothelial cells under hypoxic conditions and the relevant underlying mechanism.

Methods: bEnd.3 cells were treated with oxygen-glucose deprivation (OGD)-induced injury. To measure HS performance, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay, and cell apoptosis was assessed by flow cytometry and Terminal deoxynucleotidyl transferase UTP nick-end labeling staining. RNA-seq was performed to assess the expression profiles and screen the candidate genes that participated in OGD-induced injury and the HS protective effect. Quantitative real-time polymerase chain reaction (qPCR) and western blot analysis were used to confirm the expression of candidate genes, and enzyme-linked immunosorbent assay was used to measure the level of interleukin (IL)-1β. Overexpression analyses were performed to confirm the functions of the differentially expressed genes.

Results: HS with a concentration of 40 mmol/L NaCl had an obvious protective effect on bEnd.3 cells after OGD-induced injury, resulting in increased cell viability and a smaller percentage of apoptotic cells. According to the RNA-seq results, epidermal growth factor receptor (EGFR) was chosen as the differentially expressed gene target in this study. The qPCR and western blot analyses further confirmed that the levels of EGFR/phosphorylated epidermal growth factor receptor and IL-1β were enhanced after OGD-induced injury, but attenuated after treatment with 40 mmol/L of NaCl HS. Overexpressed EGFR reversed the protective effect of HS that caused low viability and high rates of apoptosis in cells.

Conclusion: HS can protect endothelial cells against OGD-induced injury, but is affected by the expression of EGFR/p-EGFR and IL-1β.

Abbreviations: BBB = blood-brain barrier, BSS = balanced salt solution, DMEM = Dulbecco modified Eagle's medium, HS = hypertonic saline, MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt, NC = normal control, OGD = oxygen-glucose deprivation, PVDF = polyvinylidene difluoride, qPCR = quantitative real-time PCR, TBST = Tris buffered saline/Tween, TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Keywords: blood-brain barrier, EGFR, endothelial cells, hypertonic saline, IL-1β, oxygen-glucose deprivation

1. Introduction

Hypoxia and hypercapnia are commonly detected during asphyxiation and chronic obstructive pulmonary disease, and they usually induce brain damage.[1] The brain injury is also correlated to high rates of neonatal and adult morbidity and mortality.[2] Brain edema, which is defined as an increase in brain

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water content, is usually related to the brain injury and is caused by leakage owing to a damaged blood-brain barrier (BBB), dysfunction of capillary permeability, and an accumulation of osmolytes in the interstice, resulting in lysis, necrosis, and ischemia.[3,4] Brain edema is characterized by cell swelling, which alters the concentrations of cellular metabolites and subsequently affects brain functions.[5] During this period, the intracranial pressure is usually increased, which is followed by headache, coma, and life-threatening herniation.[6] In addition, the innate immune cells in the central nervous system, especially the microglia, are usually activated in response to hypoxia. Under such conditions, some cytokines are released, such as tumor necrosis factor (TNF)-α and interleukin (IL)-β,[7,8] which are closely related to brain edema because they can disrupt endothelial cell tight junctions.[8,9] Therefore, the development of an effective therapy and understanding of the mechanism of hypoxia-induced brain edema are required to limit its occurrence.

Currently, various neuroprotective agents have been applied for the treatment of brain edema, such as oxygen-free radical scavengers, calcium channel blockers, and excitatory amino acid antagonists, among others. These drugs have been shown to be effective, but are usually associated with serious side effects in human clinical trials.[10–12] Hypertonic saline (HS) has been widely used as the first-line medicine for the treatment of patients with traumatic shock, brain edema, and elevated intracranial pressure resulting from cerebral infarction, hemorrhage, or traumatic brain injury.[13,14] Infusion of HS causes an increase in the osmotic gradient, drawing the fluid back to the intracellular area owing to the impermeability of the BBB to sodium and mannitol.[15,16] Moreover, it has been reported that HS has a beneficial inhibitory effect on microglial activation because it attenuates inflammation by suppressing neutrophil activation.[17–19] However, the role of HS is not completely understood and thus remains to be established with regard to usage and dose methods. Understanding the underlying mechanism, especially the mechanism by which HS regulates brain endothelial cells of the BBB, will be beneficial for the treatment these patients.

In this study, we prepared murine brain endothelial cells (b. End.3 cells) and subjected them to oxygen-glucose deprivation (OGD) injury to simulate hypoxic injury to the BBB. The cells were treated with different concentrations of HS to determine its ideal usage and dose. RNA-seq was used to evaluate cellular expression profiles to identify the potential underlying protective molecular mechanism of HS against hypoxic injury. Candidate genes or molecules were chosen and tested to confirm their roles in this protective effect.

2. Materials and methods

2.1. Cell culture and treatment

Murine brain endothelial cells (bEnd.3 cells, ATCC, VA, USA) were purchased from the ATCC and cultured in Dulbecco Modified Eagle's Medium (DMEM, ATCC, VA) supplemented with 10% (v/v) fetal bovine serum (Gibc) in tissue culture flasks at 37°C in a humidified atmosphere in the presence of 5% CO_2. The cells were passaged every 3 to 4 days, and the culture medium was changed after 24 hours of passaging and every 2 days thereafter. For this study, bEnd.3 cells were used at passage 13.

The cell culture was diluted to 1×10^5 cells/mL and seeded into 96-well culture plates in a volume of 100 μL per well. After overnight culture, the bEnd.3 monolayer was subjected to OGD injury. For OGD treatment, the cells were transferred to an anaerobic chamber (Forma 311, Thermo Scientific) (O_2 tension, 0.1%) and then washed 3 times with phosphate buffered saline (PBS). Next, the culture medium was replaced with deoxygenated, glucose-free balanced salt solution (BSS). Following OGD injury, the cells were incubated under normal growth conditions with or without treatment. The present study consisted of 8 groups: normal control (NC), bEnd.3 cells cultured with normal medium without OGD injury; OGD, bEnd.3 cells exposed to untreated BSS and 6 hours of OGD injury, followed by culturing in untreated DMEM for 24 h; saline solution, bEnd.3 cells treated with BSS containing 10/20/40/80/160 mM NaCl and 6 hours of OGD injury. The cells were then cultured in DMEM together with 10/20/40/80/160 mmol/L NaCl, respectively, for 24 hours; blank, DMEM without bENd.3 cells.

2.2. Measurement of cell viability

The cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega, Melbourne, Australia). Each group of bEnd.3 cells was subsequently incubated with MTS solution for 1.5 hours at 37°C. The absorbance was then detected at 490 nm using a DK-200BS luminometer (Diatek, Jiangsu, China).

2.3. Quantification of apoptosis by flow cytometry

The bEnd.3 cells were harvested, washed twice with PBS, and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Subsequently, they were incubated with Annexin V-PE and 7-ADD (BD bioscience) for 15 minutes at room temperature in the dark. After addition of 400 μL binding buffer and incubation for 1 hour, the stained bEnd.3 cells were analyzed within 1 hour by Accuri C6 flow cytometry (BD science).

2.4. Terminal deoxynucleotidyl transferase UTP nick-end labeling staining

The bEnd.3 cells from the NC group, OGD group, and OGD + 40 mmol/L NaCl group were selected to carry out Terminal deoxynucleotidyl transferase UTP nick-end labeling (TUNEL) staining using the DeadEnd Fluorometric TUNEL System (Promega, Melbourne, Australia) according to the manufacturer's protocol. Briefly, the cells were fixed with fresh 4% paraformaldehyde in PBS and then incubated in membrane lysis buffer (0.1% Triton X-100 in 0.1% sodium citrate) twice for 5 minutes each. Next, the cells were washed with PBS and treated with equilibrium buffer at room temperature for 10 minutes, followed by the TUNEL reaction mixture (terminal deoxynucleotidyl transferase and modified nucleotides) for 60 minutes at 37°C. The cells were incubated with SSC buffer for 15 minutes at room temperature and then washed twice with PBS. The cells were developed with Hoechst solution and washed 3 times. The stained cells were detected using optical microscopy.

2.5. Total RNA preparation and quantitative real-time PC,R

Total RNA from bEnd.3 cells from the NC group, OGD group and OGD+40 mM NaCl group was extracted using TRIzol (Invitrogen, Paisley, Scotland) according to the manufacturer's protocol. DNA was digested using DNase I. Total RNA was

Table 1

The sequences of the primers used in this study.

	Sequences
M-Rtn4 F	5′-GGTGCCTTGTTCAATGGTTTG-3′
M-Rtn4 R	5′-TGGCATCCTTAACGCTCTTGT-3′
M-Cav1 F	5′-GATGTGATTGCAGAACCAGAAGG-3′
M-Cav1 R	5′-AGTAAATGCCCCAGATGAGTGC-3′
M-EGFR F	5′-CCATCTCTGACTCCCCTCTCA-3′
M-EGFR R	5′-GCACTAGCAGTAGCCATCACA-3′
M-Eef2 F	5′-AAACTTCCCCGCACCTTCT-3′
M-Eef2 R	5′-CCTTGTCTTTGTCCTCGCTGT-3′
M-β-Actin F	5′-GGCTGTATTCCCCTCCATCG-3′
M-β-Actin R	5′-GGCTGTATTCCCCTCCATCG-3′

quantified by the absorption of light at A260, and the quality was analyzed by the A260/280 and A260/230 ratio using a NanoDrop 2000 (Thermo fisher, USA). cDNA was generated from 2 μg of total RNA using M-MLV Reverse Transcriptase (Promega, Melbourne, Australia), and PCR amplification was performed using GoTaq quantitative real-time PCR (qPCR) Master Mix (Promega, Melbourne, Australia). The specific primers are shown in Table 1.

2.6. Western blot analysis of p-EGFR and estimated glomerular filtration rate

After pretreatment, OGD injury and restoration, the cells were washed with PBS and collected. The cell pellets were lysed with ice-cold RIPA buffer and collected in microtubes. The lysates were sonicated 3times for 0.8 seconds at 37% intensity. The protein content was quantified according to the Bradford method. Protein was separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk prepared in Tris-buffered saline/Tween (TBST; 20 nmol/L Tris [pH 7.2]; 150 mmol/L NaCl; 0.1% Tween 20), the immunoblots were incubated overnight at 4°C with primary antibodies specific to phosphorylated epidermal growth factor receptor (p-EGFR), epidermal growth factor receptor (EGFR), and GAPDH (1:2000, Cell Signaling, MA). The blots were then washed twice with TBST and incubated with HRP-linked IgG antibodies for 1 hour at room temperature (1:1000, Forevergen, Guangzhou, China). Enhanced chemiluminescence was performed by ECL (Forevergen, Guangzhou, China).

2.7. Measurement of IL-1β by enzyme-linked immunosorbent assay

Cytokine concentrations in the culture supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit for IL-1β (Boster Biotech, Wuhan, China). Briefly, 96-well plates were coated with the IL-1β-specific monoclonal murine antibody, and murine IL-1β was used for the standard titration curve. The samples were added and incubated for 90 minutes at 37°C. Biotin-labeled IL-1β-specific antibody was added and incubated for 60 minutes at 37°C. TMB substrate was added and incubated for 25 minutes at 37°C. Photospectrometry was performed at 450 nm.

2.8. Transcriptome library construction and sequencing

Libraries were prepared using the VAHTS mRNA-seq V2 library Prep kit (Illumina, CA). Briefly, mRNA was captured using mRNA Capture beads. Fragmentation buffer was added to the RNA samples to generate short mRNA fragments. Random hexamer primers were used to synthesize first-strand cDNA. Second-strand cDNA was synthesized using 2nd Strand Buffer and 2nd Strand Enzyme Mix. The double-stranded cDNA was purified using VAHTS DNA Clean Beads followed by end repair, a-addition and adaptor ligation. The products were purified by VAHTS DNA Clean Beads for the second time and amplified for 15 cycles using specific primer mix and amplification mix. VAHTS DNA Clean Beads were used again to purify the amplified products, and an Agilent 2100 was used to profile the distribution of the insert size. The constructed libraries were sequenced on an Illumina Hiseq 2000 according to the manufacturer's protocol (Illumina). Hiseq control software (v1.1.37) with RTA (v1.7.45) was used to assess the management and execution of the experimental runs.

Images generated using Hiseq 2000 were converted to nucleotide sequences and stored in FASTQ format. To obtain clean reads, several steps were performed as follows. reads containing >9N bases were removed; reads with >36 low-quality bases (<Q20) were removed; adaptor contamination was removed; duplicates were removed. The clean reads were mapped to the reference mouse genome (mm10) using SOAP2. Mismatches were set at ≤5 bases. Reads that were matched to the reference rRNA sequences were also removed. The fragments per kb per million reads (FPKM) values of each gene were calculated. The gene was considered a differentially expressed gene (DEG) if the FPKM value difference between the 2 samples was ≥2-fold (log2 ration >1). For expression pattern analysis, hierarchical clustering analysis was performed using the Agilent GeneSpring 7.3 program.

2.9. Gene over-expression of Egfr

The *Egfr* expression vector (LV003 vector, Forevergen, Guangzhou, China) was designed. The bEnd.3 cells were transfected with the *Egfr* expression vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The performance of the transfected cells was checked by qPCR. The cells were exposed to OGD injury and 40 mmol/L NaCl treatment, and cell viability and apoptosis were detected using the MTS assay and flow cytometry. The expression levels of p-EGFR and EGFR were detected by western blot analysis, and the expression of IL-1β was determined by ELISA, as described above.

2.10. Statistical analysis

Comparisons were performed using independent *t* tests. SPSS 19.0 (SPSS Inc, Chicago, IL) software was used for the statistical analysis. Data are expressed as the mean ± standard error. Significance was established as $P < 0.05$.

3. Results

3.1. HS attenuates the cell death of bEnd.3 Cells after OGD injury

To confirm the protective effect of HS on OGD injury, the MTS assay was performed to check the cell viability in all groups (Fig. 1A). The results showed that the OGD injury group exhibited decreased cell viability compared with the NC group (100% cell viability in the NC group; 68% cell viability in the OGD group). The cell viability in the 10 to 160 mmol/L

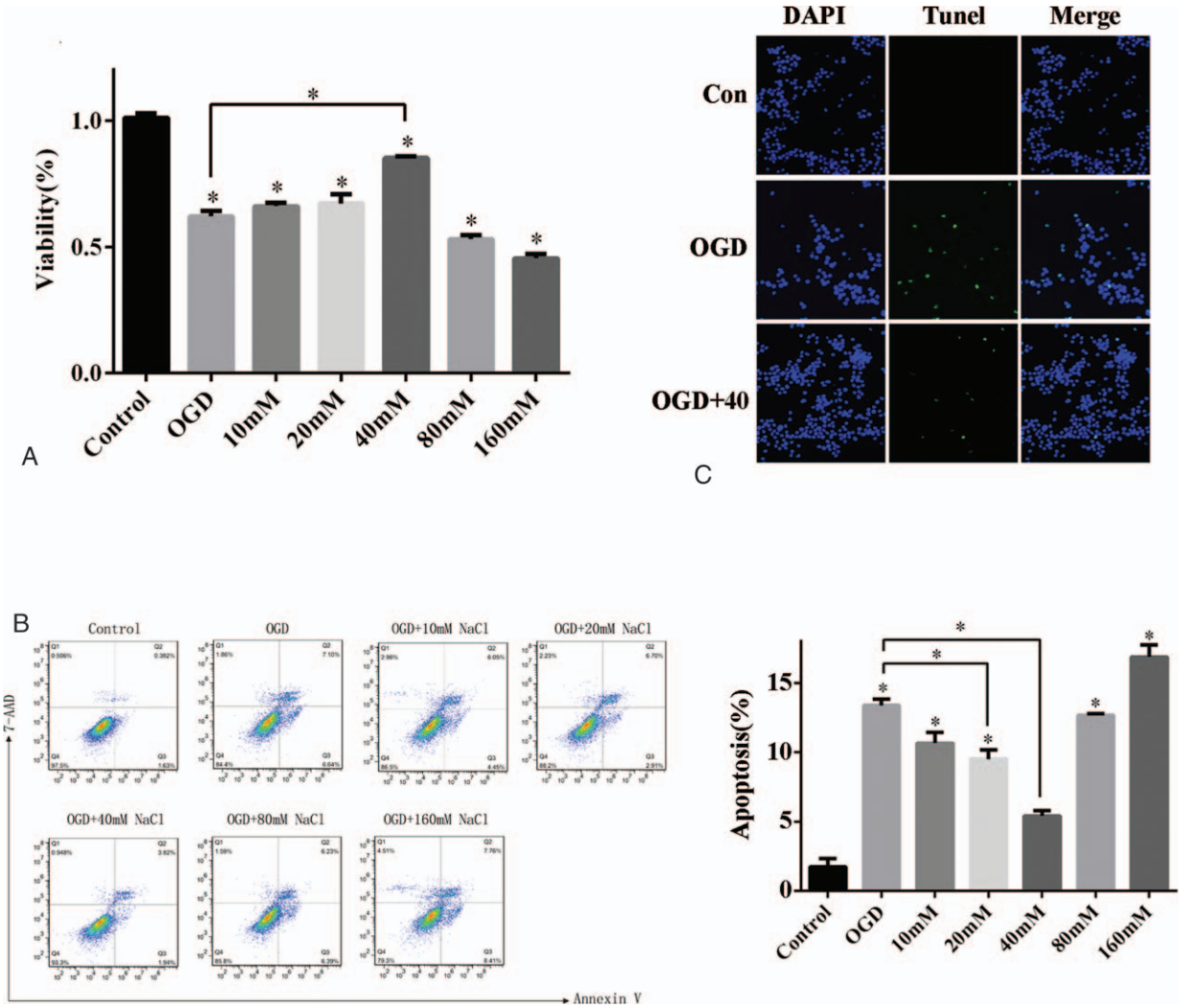

Figure 1. The 40 mmol/L NaCl HS protected bEnd.3 cells against OGD-induced injury. (A) Cell viability determined by the MTS assay; (B) flow cytometry was used to quantify cell apoptosis; (C) cell apoptosis detected by TUNEL; $^{*}P < 0.05$. HS = hypertonic saline, MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt, OGD = oxygen-glucose deprivation, TUNEL = Terminal deoxynucleotidyl transferase dUTP nick end labeling.

NaCl treatment group was then evaluated. The cell viability in the 10, 20, and 40 mmol/L NaCl group was clearly increased after OGD injury in comparison to the OGD group (86% cell viability in the 40 mmol/L NaCl treatment group). However, cell viability was almost no different from the OGD injury group in the 80 and 160 mmol/L NaCl group. It displayed a slight decrease in cell viability followed by an increased concentration of HS. In addition, we evaluated cell apoptosis by flow cytometry (Fig. 1B). The apoptosis ratio was 2.01% in the NC group but 13.74% in the OGD injury group. The apoptosis ratio was decreased in the 10, 20, and 40 mmol/L NaCl treatment groups by 5.76%. However, in the 80 and 160 mmol/L NaCl treatment group, the apoptosis ratio was not significantly decreased but even increased in the 160 mmol/L NaCl treatment group by 16.17%. Considering the cell viability and flow cytometry data, we selected a HS concentration of 40 mmol/L NaCl to compare the effect of HS. We conducted a TUNEL assay to identify the dead cells in those groups (Fig. 1C). DAPI/TUNEL staining images revealed that TUNEL-positive cells were significantly increased in the OGD injury group compared with the NC group. However, 40 mmol/L NaCl HS treatment promoted cell survival and inhibited cell death following OGD injury. According to the results, these findings suggested that OGD injury induced damage to brain endothelial cells, but HS with a concentration of 40 mmol/L NaCl had a protective effect against OGD injury.

3.2. Identification of DEGs by transcriptome sequencing and qPCR

To identify candidate genes that were differentially expressed and related to the protective effect of HS, transcriptome sequencing was performed. The reads were matched to the murine genome (mm10), and the average match ratio of raw reads was ~77%, whereas the average match ratio of the clean reads was ~97.37%. The FPKM value was then subjected to 2 comparisons based on the gene expression profile to assess differentially expressed genes: OGD group versus NC group; 40 mmol/L NaCl treatment group versus NC group. The genes with an absolute fold change ≥2 (log2 fold change ≥1) were regarded as significantly expressed. In assessments of the protective effect, the 40 mmol/L NaCl treatment could attenuate or enhance the genes that were

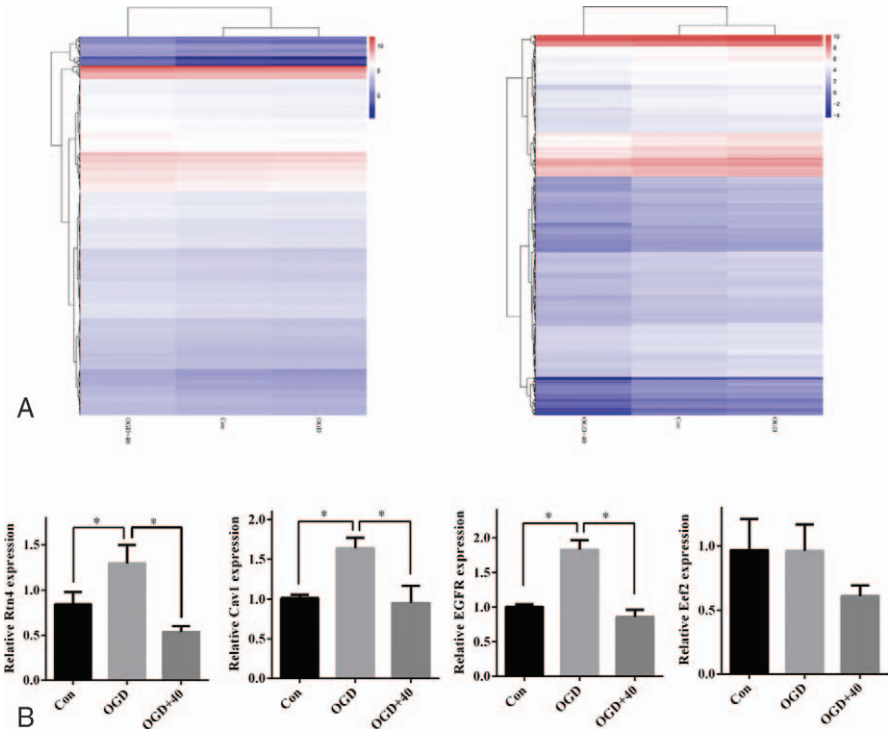

Figure 2. Expression profiles to screen DEGs in OGD-induced injury and the protective effect of HS. (A) The results of hierarchical clustering analysis: A1. The DEGs were upregulated in the OGD group but downregulated in the HS treatment group; A2. The DEGs were downregulated in the OGD group but upregulated in the HS treatment group. (B) Gene expression level of the DEGs detected by qPCR; $^{*}P < 0.05$. DEG = differentially expressed gene, HS = hypertonic saline, OGD = oxygen-glucose deprivation, qPCR = quantitative real-time polymerase chain reaction.

enhanced or inhibited by OGD injury. Therefore, significant DEGs should be detected in both comparisons, but the expression patterns were opposite (up- or downregulated). Based on this principle, the hierarchical clustering analysis was conducted for candidate DEGs as the similarity measurement of gene expression (Fig. 2A). To understand the biological significance of the differentially expressed genes, we preliminarily carried out the GO analysis and pathway analysis. A total of 14 DEGs were eventually selected for validation using qPCR. The details of the DEGs are shown in Table 2.

The genes were chosen from different functional categories to verify their gene expression patterns by qPCR using amplified cDNA derived from bEnd.3 cells. The results obtained from qPCR experiments confirmed the expression pattern of a portion of the genes. The data showed that *Rtn4*, *Cav1*, *Egfr*, and *Eef2* were differentially expressed. Among them, *Rtn4*, *Cav1*, and *Egfr* were significantly differentially expressed in both comparisons (Fig. 2B). Depending on our understanding of the gene functions of these genes and the results, we eventually chose Egfr, which had the greatest fold-change, for functional verification.

Table 2

The details of the DEGs screened by the expression profiles.

Gene ID	Gene Length	log2 Ratio (OGD/Con)	log2 Ratio (OGD + 40/Con)	Up-Down-Regulation (OGD/Con)	Up-Down-Regulation (OGD + 40/Con)
Rtn4	6165	5.974760	−0.662717	Up	Down
Plet1	1846	2.721098	−1.021245	up	Down
Cav1	2622	2.338977	−1.130107	Up	Down
Rpl22l1	477	1.687320	−2.292670	Up	Down
Ncapg	3698	1.452969	−1.007761	Up	Down
Ddias	3449	1.368229	−1.003845	Up	Down
St8sia4	5413	1.230595	−2.445167	Up	Down
Egfr	5965	1.141122	−2.576965	Up	Down
Smad7	4441	−4.477248	3.123629	Down	Up
Crlf1	1646	−3.667326	2.436339	Down	Up
Ccne2	3113	−2.510340	1.373329	Down	Up
Xpo1	5144	−1.751097	1.751097	Down	Up
Plb1	4853	−1.135460	2.568517	Down	Up
Eef2	3100	−0.936949	0.004534	Down	Up

DEG = differentially expressed gene, OGD = oxygen-glucose deprivation.

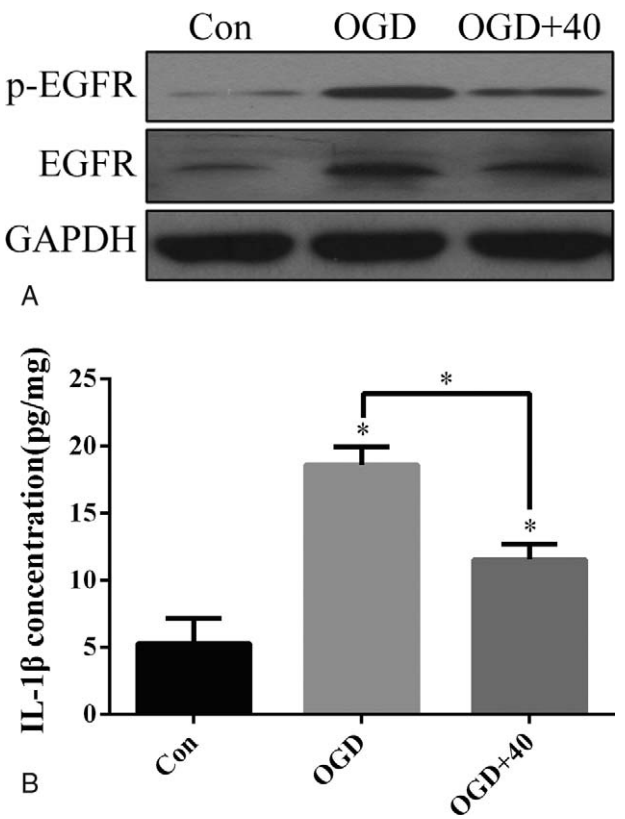

Figure 3. HS downregulated the expression of EGFR/p-EGFR and decreased the level of IL-1β. A. The expression of EGFR/p-EGFR detected by western blot analysis. B. The level of IL-1β detected by ELISA; $^{*}P < 0.05$. EGFR = epidermal growth factor receptor, ELISA = enzyme-linked immunosorbent assay, HS = hypertonic saline, IL-1β = interleukin-1β, p-EGFR = phosphorylated epidermal growth factor receptor.

3.3. HS protects bEnd.3 cells followed by decreased IL-1β and EGFR expression

To investigate whether EGFR signaling was activated during OGD-induced stress, we first measured the phosphorylation status of EGFR by western blot analysis (Fig. 3A). According to our current understanding of the function of EGFR, the phosphorylation of EGFR is associated with the activation of EGFR signaling. Our results suggest that phosphor-EGFR/EGFR protein expression in the OGD injury group was enhanced compared with the NC group. However, phosphor-EGFR/EGFR protein expression was attenuated in the 40 mmol/L NaCl treatment group compared with the OGD injury group. Considering that EGFR activation is often correlated to proinflammatory cytokine, we also examined the expression of IL-1β by ELISA (Fig. 3B). The expression of IL-1β was increased by OGD injury. However, treatment with 40 mmol/L NaCl resulted in a clear suppression of IL-1β after OGD-induced injury. These results suggest that OGD-induced injury could be correlated to the increased expression of IL-1β and the activation of EGFR signaling, whereas HS could have a protective effect that is followed by decreased IL-1β and EGFR expression.

3.4. EGFR signaling enhances cell apoptosis after OGD injury

To confirm the correlation between EGFR signaling and OGD injury, we designed an EGFR expression vector and transfected bEnd.3 cells to increase the expression of EGFR. The performance of the EGFR expression vector was assessed by qPCR (Fig. 4A). The data showed that the expression of EGFR was approximately 4-fold greater compared with the NC group after transfection with the EGFR expression vector. The cell viability of the bEnd.3 cells was then detected by the MTS assay (Fig. 4B). Similar results were obtained for the NC group, OGD injury group, and 40 mmol/L NaCl treatment group, as the cell viability of the OGD group was significantly decreased compared with the NC group, but was increased after treatment with 40 mmol/L NaCl HS compared with the OGD injury group. However, after transfection of the EGFR expression vector, OGD-induced injury clearly attenuated cell viability compared with the NC group, but the cell viability of the 40 mmol/L NaCl HS remained almost unchanged compared with the OGD injury group and was even slightly decreased. Cell apoptosis was then determined by flow cytometry (Fig. 4C). The protective effect of 40 mmol/L NaCl HS was apparent in normal bEnd.3 cells after OGD-induced injury, in which cell apoptosis was significantly suppressed. However, after the bEnd.3 cells were transfected with the EGFR expression vector, cell apoptosis was not suppressed after OGD-induced injury. In comparison to the 40 mmol/L NaCl treatment group of normal bEnd.3 cells, EGFR vector-transfected bEnd.3 cells exhibited a significantly higher apoptosis ration even after treatment with 4 mmol/L NaCl HS.

The p-EGFR/EGFR protein expression was then determined by western blot analysis (Fig. 4D). The results showed that after transfecting bEnd.3 cells with the EGFR expression vector, 40 mmol/L NaCl HS almost did not attenuate the expression of p-EGFR and EGFR. The expression of p-EGFR and EGFR was elevated compared with normal bEnd.3 cells treated with 40 mmol/L NaCl HS after OGD-induced injury. In this case, the expression of IL-1β detected by ELISA was higher in the EGFR expression vector-transfected compared with the normal bEnd.3 cells, although both bEnd.3 cells were treated with 40 mmol/L NaCl HS after OGD-induced injury. These results indicated that the overexpression of EGFR signaling attenuates cell viability and enhances cell apoptosis; 40 mmol/L NaCl HS has a protective effect by suppressing GFR signaling.

4. Discussion

In this study, 10, 20, and 40 mmol/L NaCl HS ameliorated cell viability and attenuated cell apoptosis of bEnd.3 cells after OGD-induced injury. The protective effect of HS might be closely correlated to the expression of EGFR/p-EGFR and IL-1β.

HS has recently received considerable attention because of its efficiency in ameliorating cerebral edema. Compared with mannitol, which is widely used as a medicine for the treatment of cerebral edema, HS was more effective than an equal volume of 20% mannitol.[20] A previous study focusing on patients with cerebral edema also confirmed that 3% HS showed better performance than mannitol.[21] Those findings are consistent with our results. According to the studies conducted to date, the protective effect of HS is attributed to its functions in decreasing the cerebral water content and reducing intracranial pressure.[22–25] However, the usage and dose of HS are important and should be considered before treatment. Our results demonstrated that 40 mmol/L NaCl HS was comparatively the most suitable concentration for enhancing cell viability and attenuating cell apoptosis, whereas higher NaCl concentrations led to lower cell viability and increased cell apoptosis. The potential root of the phenomenon might be correlated to the side effects of HS. Hypertonicity is a stressful condition and usually

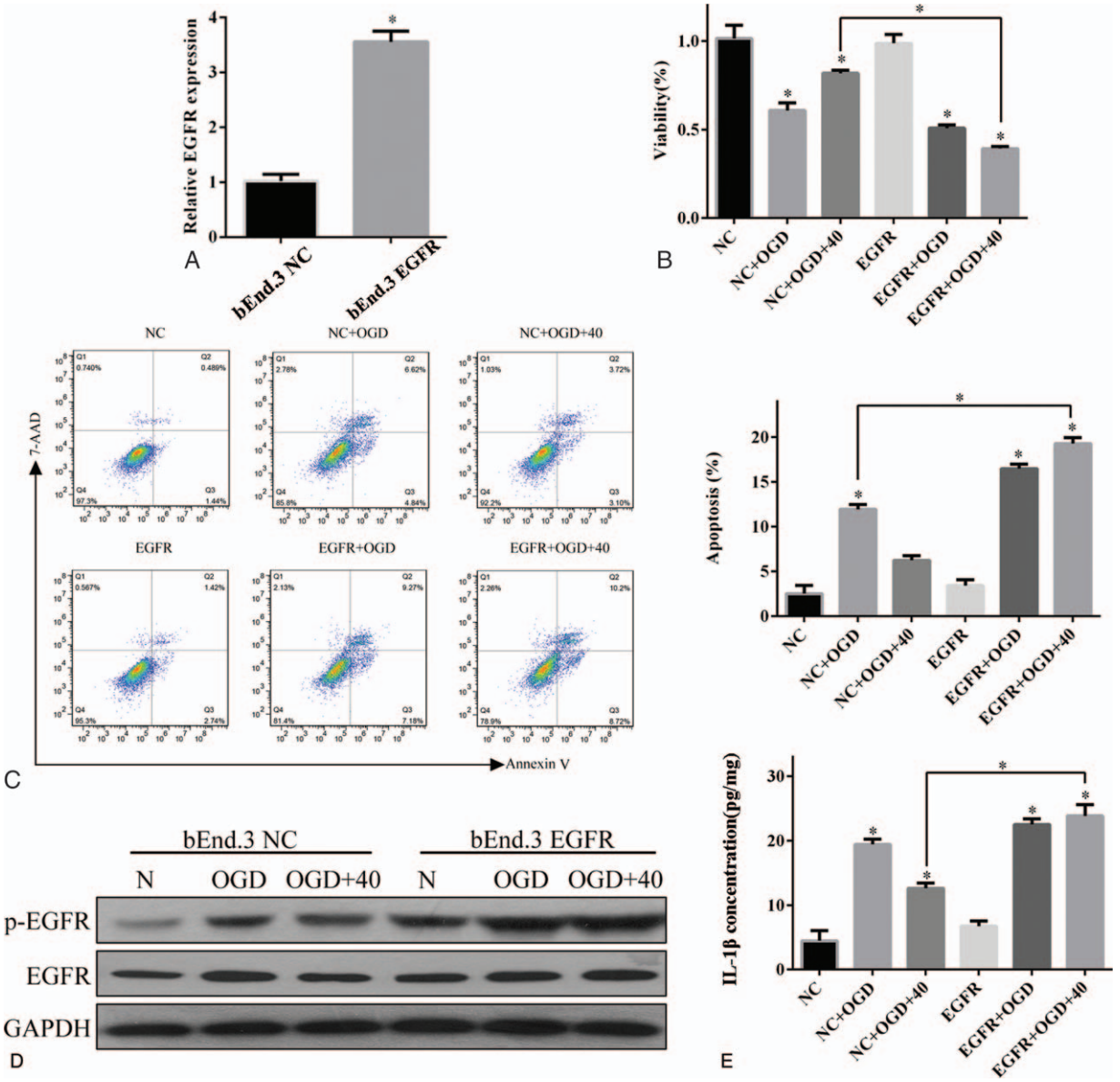

Figure 4. Overexpressed EGFR reversed the protective effect of HS in bEnd.3 cells after OGD-induced injury. (A) qPCR verified the performance of the EGFR expression vector transfection. (B) Cell viability determined by the MTS assay. (C) Flow cytometry for quantification of cell apoptosis. (D) The expression of EGFR/p-EGFR determined by western blot analysis. (E) The level of IL-1β detected by ELISA; $^{*}P < 0.05$. EGFR = epidermal growth factor receptor, ELISA = enzyme-linked immunosorbent assay, HS = hypertonic saline, IL-1β = interleukin-1β, MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt, OGD = oxygen-glucose deprivation, p-EGFR = phosphorylated epidermal growth factor receptor, qPCR = quantitative real-time polymerase chain reaction.

leads to cell shrinkage, increased intracellular ionic strength, and macromolecule damage. A high osmolarity disturbs a variety of cell functions, such as the growth cycle and proliferation, leading to oxidative stress.[26] However, as a compensatory mechanism of cells to counteract cell shrinkage or the associated deleterious consequences of hypertonicity,[27,28] HS in specific NaCl concentrations will never have deleterious effects on cells.

IL-1β, an inflammatory cytokine, is frequently detected during brain edema. Because microglia are extremely sensitive to hypoxic-ischemia,[29] IL-1β is activated and eventually releases a variety of inflammatory cytokines such as TNF-α and IL-1β.[7] These cytokines disrupt the BBB by destroying endothelial tight junctions and thus leading to cerebral edema.[9] Previous studies have shown that the reduced TNF-α and IL-1β resulted in a marked reduction in cerebral edema.[30,31] These findings are consistent with our results showing that HS can reduce the level of IL-1β and protect cells against OGD-induced injury. However, the underlying mechanism of the inhibitory effect on inflammation of HS is not clear. It is well known that P38 and JNK signaling are closely related to cytokine production. Using HS, hypertonicity might have an effect on the inhibition on P38.[18,32] After hypoxia for 4 hours, the levels of phosphorylated P38 and JNK levels were significantly upregulated, whereas they were downregulated after HS treatment.[33] Therefore, the high level of IL-1β resulted from microglia under conditions of hypoxia, but this phenomenon was abrogated by HS to protect the cells against damage.

Apart from IL-1β, we proved that EGFR/p-EGFR was correlated with cell viability and cell apoptosis after OGD-induced injury. A high expression level of EGFR is frequently observed in carcinoma cells and tumor tissues.[34,35] Activation of

EGFR pathways is usually followed by the activation of downstream MAPK/ERK and PI3K/AKT pathways that lead to cell proliferation.[36,37] According to a previous study, EGFR acts as an antiapoptotic agent under hypertonic conditions, and inhibition of EGFR has a strong proapoptotic effect that induces cell death.[38] However, our results showed that EGFR had different functions when the cells were exposed to OGD-induced injury. The level of EGFR and p-EGFR increased after OGD-induced injury. During this period, cell viability was decreased by ~60%, and cell apoptosis percentage increased by ~15%. Interestingly, when we attempted to overexpress EGFR, the apoptosis percentage of the bEnd.3 cells was slightly increased, although it was not very significant. The results indicated that the functions of EGFR in bEnd.3 might differ from the causes of apoptosis in brain endothelial cells. The underlying reason might be correlated to the level of IL-1β. EGFR phosphorylation induced IKK complex activation via the regular EGFR pathway and thus activated the NF-kB pathway.[39] NF-kB is an important molecule in the Toll-like receptor pathway. The activation of NF-kB subsequently resulted in the release of inflammatory cytokines such as TNF-α and IL-1β.[39,40] As described previously, the cytokines interrupted the endothelial junctions and led to cell damage. However, the inflammatory cytokines regularly activated the JNK and P38 pathway and therefore induced cell apoptosis.[39,41] Under these conditions, the cell regulatory functions were destroyed, and OGD-induced injury was more likely to induce a higher apoptosis percentage, which is consistent with our results. Impairment of the cells owing to OGD-induced injury and overexpressed EGFR led to regulatory system defects that might become more sensitive and vulnerable. Therefore, the hypertonicity caused by 40 mmol/L NaCl HS was no longer tolerable to bEnd.3 cells, resulting in decreased cell viability and increased proportions of cell apoptosis. Taken together, we concluded that under hypoxic conditions, the increased level of EGFR and IL-β induced inflammatory-related pathways and led to cell death and apoptosis. Higher EGFR expression will deteriorate the cell regulatory system to not only facilitate increased cell apoptosis but also to provide a defense against the protective effect of HS. Thus, it is worthwhile to determine the levels of IL-β and EGFR/p-EGFR before using HS.

Here, we showed that 40 mmol/L NaCl HS can obviously protect bEnd.3 cells against OGD-induced injury, and that the levels of IL-1β and EGFR should be correlated to the performance of HS and apoptosis. However, some features must be improved for further study. Our current conclusion is based on an in-vitro experiment using bEnd.3 cells and OGD treatment to simulate the hypoxic conditions. However, in-vivo studies are still needed to further confirm the actual performance of the HS for the treatment of hypoxia ischemia and brain edema. Moreover, the expression profiles indicated that the EGFR/p-EGFR pathway was only a part of the mechanism responsible for OGD-induced injury and hypertonic saline. Further investigation of the remaining DEGs is meaningful to understand the mechanism more comprehensively and therefore benefit the development of relevant treatments and therapies.

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