Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Correlation of sLOX-1 and CSF1 with the pathological progression of hypoxic-ischemic encephalopathy in neonatal rats

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ARTICLE INFO

Original paper

Article history:
Received: May 02, 2023
Accepted: August 31, 2023
Published: October 31, 2023

Keywords:

hypoxic-ischemic encephalopathy, low-density lipoprotein receptor-1, colony stimulating factor-1, apoptosis of neuronal cells, seahorse district injuries

ABSTRACT

To provide clinical evidence for the management of hypoxic-ischemic encephalopathy (HIE) by analyzing the role of soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) and colony-stimulating factor-1 (CSF1) in the disease. We purchased 15 Sprague-Dawley (SD) rat pups and randomized them into five groups (n=3), of which one group was untreated as the control group and the other four were modeled by HIE. After modeling, a group was treated as a model group without any treatment, another group was injected with sLOX-1-silencing lentiviral vector (sLOX-1-si group), and the third and fourth were injected with CSF1-silencing lentiviral vector (CSF1-si group) and an equal amount of normal saline (blank group), respectively. After the corresponding intervention, the rat tissue in each group was obtained to observe the pathological injury by HE and TUNEL staining. In addition, sLOX-1, CSF1, 5-hydroxytryptamine (5-HT), dopamine (DA), and norepinephrine (NE) levels in brain tissue of each group were determined. The model group showed more severe pathological damage of the hippocampus and higher neuronal apoptosis than the control group. Besides, higher sLOX-1 and CSF1 levels and lower 5-HT, DA and NE contents were identified in the model group versus the control group (P<0.05). Compared with the blank group, sLOX-1-si and CSF1-si groups showed significantly alleviated hippocampal damage, inhibited neuronal apoptosis, reduced 5-HT, DA, NE, Bax, and cl-caspase-3, and increased Bcl-2 (P<0.05). Silencing sLOX-1 and CSF1 expression ameliorated the pathological injury of HIE and inhibited neuronal apoptosis.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.10.21 Copyright: © 2023 by the C.M.B. Association. All rights reserved.

Introduction

Hypoxic-ischemic encephalopathy (HIE), a brain hypoxia and ischemia-induced cerebral disease, as well as a very prevalent high-risk encephalopathy, is common in infants and children and is currently one of the main causes of infant death and disability (1, 2)). According to statistics, there are approximately 1500-2000 HIE cases per 1 million live births on average (3). With the continuous growth of the world's population, the incidence of HIE also presents a rising trend year by year, increasing by about 3.4 times compared with 2010 (4). Because of this, HIE has always been a key disease in clinical research, and an in-depth understanding of its pathogenic mechanism is the key to ensuring the life safety of children with HIE in the future. Although the mechanism has not been fully defined, the pathological progression of HIE has been found to be associated with many biological functional changes such as oxidative stress (OS), mitochondrial damage, neuronal apoptosis, and autophagy (5).

Recent evidence has shown that serum biomarkers like calcium-binding protein B can indicate early-stage HIE and are related to the onset and development of brain injury (6). In addition, soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) can reflect the body's vascular endothelial injury and inflammatory reaction to a certain extent and is a biomarker for a variety of ischemic cardio-cerebrovascular diseases such as acute coronary syndrome and acute cerebral infarction; however, its relationship with hypoxia and ischemia-induced HIE remains unclear (7, 8). Furthermore, colony-stimulating factor-1 (CSF1) is shown to reduce neuronal apoptosis and neuroinflammation after HIE-induced brain injury by binding to its receptor CSF1R (9). sLOX-1, which belongs to the same colony factor as CSF1, has been considered by researchers as a promising therapeutic target to relieve cognitive disorders, memory impairment and other pathological injuries in the future (10, 11). Therefore, this study establishes a rat model of HIE to analyze the role of sLOX-1 and CSF1, aiming at understanding their roles in HIE and providing a reference for finding new diagnostic and therapeutic plans for HIE.

Materials and Methods

Animal data

We purchased 15 10-day-old Sprague-Dawley (SD) rat pups (16-22g) from Jiangsu Nantong Jingqi Biotechnology Co., Ltd. (SYXK [Su] 2023-0028) for experiments. The animals were acclimated to a 12:12-h light: dark cycle with free feeding and drinking. This study, approved by the Animal Ethics Committee of our hospital, strictly fol-

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lowed the 3R (Reduction, Replacement, and Refinement) principle.

Grouping and treatment

The purchased SD rats were randomized into five groups (n=3), among which 4 groups underwent HIE modeling and the other group was fed normally without treatment as a control group. Modeling was conducted with reference to the study of Groves AM et al. (12): Following anesthesia, the common carotid artery was separated by a skin incision in the posterior middle of the neck, after which double ligation with non-invasive sutures was performed. The vessels were then cut from the middle of the ligation line, sterilized, and sutured. After 2 hours of postoperative recovery, the rats were placed in an anoxic chamber where a mixture of 92% nitrogen and 8% oxygen was introduced for 2 hours. After modeling, one group of model rats was randomly selected and injected with 2 μg of SLOX-1-silencing lentivirus vector into bilateral ventricles and set as sLOX-1-si group; another group was injected with CSF1-silencing lentivirus vector, which was used as CSF1-si group; the third group was injected with the same amount of normal saline and marked as blank group; the last group received no treatment and was set as a model group. sLOX-1 and CSF1 abnormal expression vectors were commissioned to be designed and constructed by Shanghai Biomics Biotech.

Sample collection

After the above treatment, all rat pups were killed by cervical dislocation under anesthesia, and the whole brain tissue was separated for subsequent detection.

Polymerase chain reaction (PCR) quantification of sLOX-1 and CSF1 mRNA expression

Total RNA from tissues was extracted by Trizol and reverse transcribed into cDNA. The PCR reaction system was then configured according to SYBR kit instructions. The reaction conditions were 40 cycles of 95°C for 10min, 95°C for 10s, 60°C for 30s, and 72°C for 10s. sLOX-1 and CSF1 mRNA levels relative to GAPDH were calculated based on the $2^{-\Delta\Delta CT}$ formula.

Western blot determination of sLOX-1 and CSF1 protein expression

The total protein of the tissue was extracted by RIPA lysate, and the supernatant was obtained by centrifugation after a 30-minute incubation on ice. After quantitative protein analysis by bicinchoninic acid (BCA), the samples were placed on a polyacrylamide gel, separated by electrophoresis and transferred to a PVDF membrane, followed by sealing with 5% skimmed milk powder and overnight incubation with sLOX-1 and CSF1 antibody (1:1,000) at 4°C. After PBST washing, the membrane was treated with room temperature incubation with a secondary antibody (1:5,000) for 1 hour. It was developed by ECL following another PBST rinsing. Gray value analysis was performed using Image J software to calculate the target proteins' expression.

ELISA of 5-hydroxytryptamine (5-HT), dopamine (DA), and norepinephrine(NE) contents

With kits all supplied by Shanghai Lianzu Biotech, the levels of 5-HT, DA and NE in the homogenate of rat hippocampus were detected in strict accordance with the kit instructions in a sterile environment.

Observation of histopathological changes by hematoxylin-eosin (HE) staining

The rat hippocampus fixed with 4% paraformaldehyde was dehydrated by gradient alcohol and transparentized with xylene, followed by paraffin embedding, slicing (5 μm), and drying. Then, xylene dewaxing, gradient alcohol hydration, and distilled water washing were performed successively. The slices were then stained with hematoxylin and eosin for 3 min, dehydrated with gradient ethanol, transparentized with xylene, and mounted with neutral gum. They were finally observed under an optical microscope and photographed.

Neuronal apoptosis detection by TUNEL staining

Paraffin sections of the brain hippocampus were dewaxed by xylene twice, soaked in gradient ethanol, added with 20 g/L DNase-free protease K to incubate at 37°C for 30 min, and washed with PBS buffer. After slicing, the samples were added with 50 μL TUNEL solution to incubate in the dark, followed by 15 min of cultivation with DAPI. Thereafter, the slices were sealed with an anti-fluorescence quenching solution and observed and photographed with a fluorescence microscope to calculate the neuronal apoptosis rate.

Statistical analyses

Data were statistically analyzed using SPSS22.0 and presented in the form of ($\bar{\chi}\pm s$). The statistical methods used for between-group and multi-group comparisons were independent sample t-tests and analysis of variance plus LSD post-hoc tests, respectively. A minimum significance threshold of $P<0.05$ was used.

Results

Observation of pathological changes in HIE

In the control group, the morphology and structure of hippocampus neurons were clear and complete, with clear nucleoli, no edema, and no glial cell infiltration. In contrast, the hippocampus in the model group showed neuronal swelling, nuclei disorientation, disordered arran-

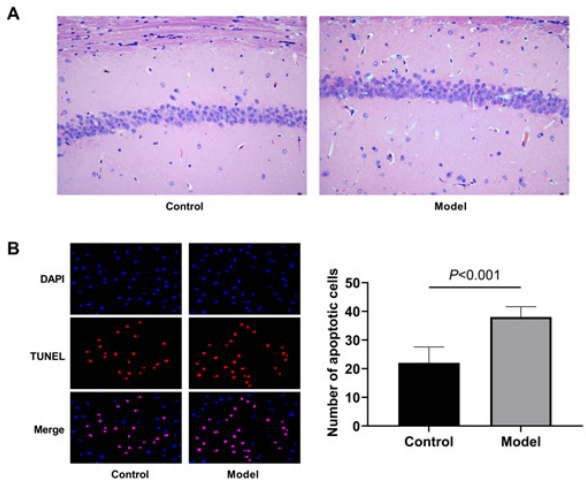

Figure 1. Observation of pathological changes in HIE. (A) HE staining of tissues in the hippocampal region (200×). (B) Observation of neuronal apoptosis by TUNEL staining (100×).

gement, deep staining and decreased number, as well as glial cell proliferation. According to TUNEL staining, the apoptotic neuron number in the model group was about (38.00±3.61), which was markedly elevated compared with the control group (P<0.05) (Fig. 1).

Comparison of nerve injury markers

The concentrations of 5-HT, DA, and NE in the brain of the model group were (21.53±2.45) μg/L, (0.12±0.02) μg/L and (8.40±0.73)μg/L, respectively, all lower compared with the control group (P<0.05) (Fig. 2).

sLOX-1 and CSF1 expression in HIE

After detection, the mRNA and protein levels of sLOX-1 and CSF1 in the brain tissue were found to be higher in model rats than in control rats (P<0.05), suggesting that sLOX-1 and CSF1 are highly expressed in HIE (Fig. 3).

Impacts of sLOX-1 and CSF1 on the pathological progression of HIE

First, sLOX-1 and CSF1 levels in blank, sLOX-1-si, and CSF1-si groups were detected and found to be lower in sLOX-1-si and CSF1-si groups compared with the blank group (P<0.05), confirming the success of the intervention of the silencing expression vectors; while no evident differences were determined in sLOX-1 and CSF1 between sLOX-1-si and CSF1-si groups (P>0.05) (Fig. 4).

Effects of sLOX-1 and CSF1 on pathological changes of HIE

HE staining showed that the pathological manifestations of the hippocampus in the blank group were basically the same as those in the model group, but the injury was serious. In contrast, hippocampal neurons in sLOX-1-si and CSF1-si groups had complete morphological structure, ameliorated edema, orderly arrangement, and improved glial cell proliferation, with the damage repaired to a certain extent. In addition, the apoptotic neurons number in sLOX-1-si and CSF1-si groups were about (29.33±2.08) and (30.00±3.61), respectively, which were also significantly reduced compared with the blank group (P<0.05) (Fig. 5).

Influences of sLOX-1 and CSF1 on apoptosis-related proteins

Further detection of apoptosis-related protein expression showed that Bax, cl-caspase-3 and Bcl-2 protein levels were not statistically different between sLOX-1-si and CSF1-si groups (P>0.05); however, sLOX-1-si and CSF1-si groups had lower Bax and cl-caspase-3 than the blank group and higher Bcl-2 than the control group (P<0.05) (Fig. 6).

Effects of sLOX-1 and CSF1 on nerve injury markers

Finally, the nerve injury markers (5-HT, DA and NE) of each group of rats were detected, and higher concentrations of these markers were determined in sLOX-1-si and CSF1-si groups as compared to the blank group (P<0.05) (Fig. 7).

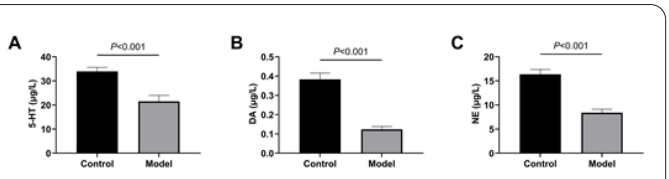

Figure 2. The results of nerve injury markers. (A) Comparison of 5-HT. (B) Comparison of DA. (C) Comparison of NE.

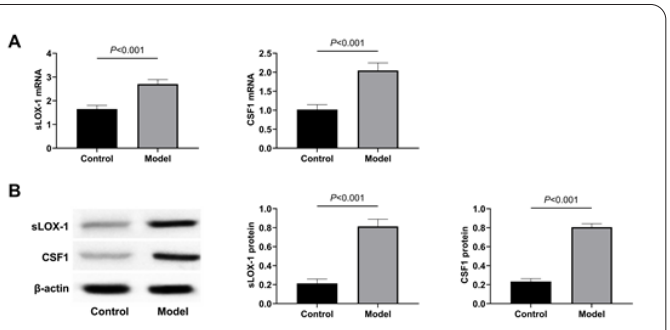

Figure 3.sLOX-1 and CSF1 expression in HIE. (A) PCR was performed to detect the expression of sLOX-1 and CSF1. (B) Western blot was performed to detect the expression of sLOX-1 and CSF1.

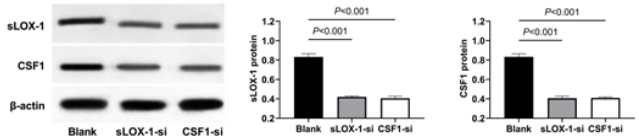

Figure 4. Western blot was performed to detect the expression of sLOX-1 and CSF1.

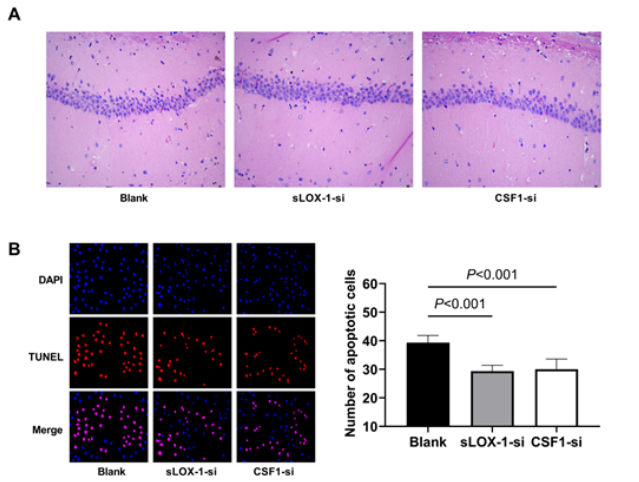

Figure 5. Observation of pathological changes in HIE. (A) HE staining of tissues in the hippocampal region (200×). (B) Observation of neuronal apoptosis by TUNEL staining (100×).

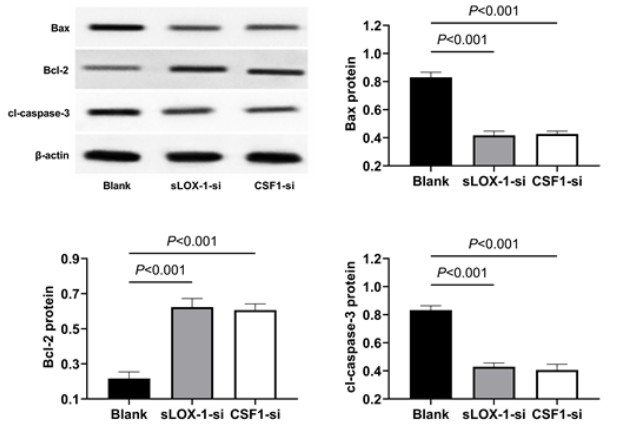

Figure 6. Western blot to detect the expression of apoptosis-related proteins.

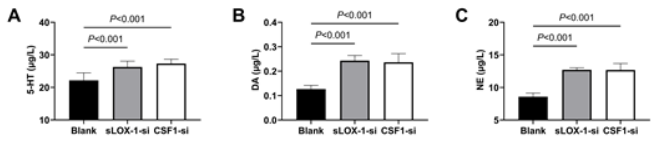

Figure 7. The results of nerve injury markers. (A) Comparison of 5-HT. (B) Comparison of DA. (C) Comparison of NE.

Discussion

In this study, we found that sLOX-1 and CSF1 were increased in HIE rats and that inhibiting their expression delayed the pathological progression of HIE, indicating the potentially important role of sLOX-1 and CSF1 in the future diagnosis and treatment of HIE.

According to HE and TUNEL staining and nerve injury detection, the hippocampus of rats in the model group was seriously damaged, with a large number of apoptotic neurons and reduced DA and NE levels, which is also in line with the pathological manifestations of HIE (13, 14), confirming the success of modeling on the one hand and the serious nerve injury of HIE on the other. In addition, sLOX-1, an acute-phase reactant, usually presents a low expression level in cells, but its expression will increase rapidly in the case of increased OS, inflammation, etc. (15). In previous studies, sLOX-1 has been hailed as an important prognostic marker for recurrent ischemic stroke with great clinical implications (16). Herein, we also identified an increase in sLOX-1 levels, which also demonstrates the important relationship between sLOX-1 and HIE. This is consistent with previously reported results (17, 18), which can support our experimental results. We believe that there may be serious OS in the pathogenesis of HIE, during which large amounts of oxygen free radicals are produced and low-density lipoproteins are oxidized. As reported by Yan P et al., sLOX-1 can bind to oxidized low-density lipoproteins to produce a large number of degradation of low-density lipoproteins, resulting in lipid metabolism disorders (19). Therefore, we believe that the increase in sLOX-1 levels in HIE is a feedback regulation mechanism that promotes disease exacerbation.

CSF1 is a cytokine of the mononuclear phagocytic system, which plays a vital role in promoting the migration, proliferation, differentiation, survival, and polarization of macrophage cell lines (20). It has been repeatedly confirmed that the increase of CSF1 level can promote the inflammatory reaction and stress injury in the body (21, 22), which is basically consistent with our findings. Marzan DE et al. reported that the activation of CSF1 could drive the demyelination of microglia cells, accelerate the apoptotic cycle and cause pathological tissue damage (23). We speculate that this is also the mechanism underlying the involvement of CSF1 in HIE development, as more obvious neuronal apoptosis was observed in model rats.

However, after the intervention of HIE rats with sLOX-1- and CSF1-silencing expression vectors, the pathological injury of rats was significantly ameliorated compared with the blank group, the apoptosis of neurons was decreased, and the levels of 5-HT, DA and NE were increased, which indicates that the pathological injury process of HIE can be effectively reversed by inhibiting sLOX-1 and CSF1 expression. Similarly, in multiple previous studies, silencing sLOX-1 and CSF1 has been shown to reverse the malignant progression of diseases such as malignant meningioma and stroke (10, 20), which is in line with our research. It also suggests that future molecular therapies with targeted silencing of sLOX-1 and CSF1 may become new choices for HIE treatment. However, more research is needed to realize its clinical application.

However, in view of the differences between animals and humans, we need to include clinical cases to confirm sLOX-1 and CSF1 expression in humans. Moreover, more in vitro experiments should be carried out to analyze the role of sLOX-1 and CSF1 in HIE and neurons, so as to more comprehensively understand their clinical significance in HIE.

SLOX-1 and CSF1 are highly expressed in HIE, and silencing their expression can alleviate pathological damage, inhibit neuronal apoptosis, and reverse the malignant development of HIE. In the future, molecular therapies targeted to silence sLOX-1 and CSF1 may be novel choices for HIE treatment.

Consent for publications

The author read and proved the final manuscript for publication.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of Chaohu Hospital Affiliated to Anhui Medical University(No.2021-0251)

Competing interests

The authors report no conflict of interest.

Funding

Not applicable.

Acknowledgements

Not applicable.

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