Biochemical and Biophysical Research Communications xxx (2018) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

LncRNA FIRRE/NF-kB feedback loop contributes to OGD/R injury of cerebral microglial cells

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ARTICLE INFO

Article history:
Received 20 April 2018
Accepted 25 April 2018
Available online xxx

Keywords:
FIRRE
NF-kB
NLRP3
Stroke

ABSTRACT

Stroke is one of the leading causes for serious long-term neurological disability. LncRNAs have been investigated to be dysregulated in ischemic stroke. However, the underlying mechanisms of some specific lncRNAs have not been clearly clarified. To determine lncRNA-mediated regulatory mechanism in ischemic stroke, we constructed OGD/R injury model of cerebral microglial cells. Microarray analysis was carried out and analyzed that lncRNA functional intergenic repeating RNA element (FIRRE) was associated with OGD/R injury. Based on the molecular biotechnology, we demonstrated that FIRRE could activate NF-kB signal pathway. Meanwhile, the activated NF-kB promoted FIRRE expression in OGD/R-treated cerebral microglial cells. Therefore, FIRRE and NF-kB formed a positive feedback loop to promote the transcription of NLRP3 inflammasome, thus contributed to the OGD/R injury of cerebral microglial cells. All findings in this study may help to explore novel and specific therapeutic target for ischemic stroke.

1. Introduction

Stroke has gradually become one of main causes for serious long-term neurological disability in developed countries. Stroke-induced mortality has risen to 30% [1]. Among all subtypes of stroke, ischemic stroke accounts for 80%–85% [2]. Accumulating evidence has revealed that ischemic stroke occurs accompanied by a series of neurological events, including inflammatory response [3]. Ischemic stroke eventually leads to acute necrosis and apoptosis of neuronal cell [4]. Despite the efforts have been made in developing the pharmacology and surgery, the effective treatment method for ischemic stroke remains unsatisfied. Therefore, it is necessary and crucial to develop the underlying molecular mechanism and pathological of ischemic stroke.

During the past decades, more and more human genomes have been uncovered to be transcribed into noncoding RNAs [5]. Among non-coding RNAs, lncRNAs have been gradually studied in human diseases, including ischemic stroke [6,7,8]. Here, we investigated the biological function and molecular mechanism of lncRNA FIRRE in OGD/R injury of cerebral microglial cells. Firstly, we constructed OGD/R injury model of cerebral microglial cells. Next, lncRNA FIRRE was chosen to do study by applying microarray analysis. LncRNA FIRRE can anchor the inactive X chromosome through maintaining H3K27me3 methylation [9]. The biological role of FIRRE has been reported previously [10,11]. In this study, we demonstrated that FIRRE can activate NF-kB signaling pathway. Moreover, the activated NF-kB contribute to FIRRE upregulation in OGD/R-treated cerebral microglial cells. They could form a positive feedback loop to promote the transcription of NLRP3 inflammasome, and finally contributed to the OGD/R injury of cerebral microglial cells. All these data indicated that FIRRE regulated the transcription of inflammatory genes in the innate immune system.

2. Materials and methods

2.1. Primary microglial cell culture

Primary microglial cells were extracted under the guidance of previously reported study. Briefly, cells were extracted from the cerebrum of one-day-old SD rat pups produced by Laboratory Animal Center of Shanghai Institute of Materia Medica (Shanghai, China), and then were seeded in the 75 cm^2 flasks and cultured

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https://doi.org/10.1016/j.bbrc.2018.04.194
0006-291X/

under the condition with 5% CO_2 at 37 °C. Once the cells were confluent, we isolated the cells from the mixed glial population by shaking the flasks at 200 rpm for 1 h. The cells were resuspended in DMEM added with 10% FBS, 4.5 g/L glucose at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Construct oxygen and glucose deprivation/reoxygenation (OGD/R) model

The microglial cells treatment with OGD/R was conducted as previously reported study [12]. Briefly, cell were cultured in a hypoxia condition containing 95% N2 and 5% CO2 in serum/glucosefree DMEM medium at 37 °C and maintains for 4 h or 8 h, and terminated by replacing the medium with high-glucose DMEM containing 10% FBS, and then transferred into the normoxic incubator with 95% air and 5% CO_2 for 12 h.For NF-kB inhibition, 5 μM JSH-23 dissolved in DMSO was added into the medium after OGD/R injury, and treatment with the solvent DMSO alone was used as control.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR).

Total RNA was extracted from culturing cells by TRIzol reagent (Invitrogen) under the guidance of the manufacturer's introduction. The cDNA was produced by reverse transcription with the M-MLV Reverse Transcriptase (Invitrogen). qRT-PCR was performed with the SYBR Premix Ex Taq II (Takara, Dalian, China) by an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The target genes expression level was evaluated by comparative Ct method and GAPDH was utilized as an endogenous control for lncRNA and mRNA.

2.3. Construction of vectors

Full length transcript of FIRRE was polymerase chain reaction (PCR) amplified from cDNA using PrimeSTAR HS DNA polymerase (Takara). The PCR amplified product was inserted into the Kpn I and BamH I sites of pcDNA (Invitrogen) vector, termed pcDNA/FIRRE. FIRRE or NLRP3 promoter region containing the p50 binding sites was PCR amplified from genomic DNA using PrimeSTAR HS DNA polymerase (Takara) and inserted into the Kpn I and Hind III sites of the luciferase reporter pGL3-Basic (Promega, Madison, WI) vector, termed pGL3-FIRRE-promoter or pGL3-NLRP3-promoter. Two independent shRNAs specifically targeting FIRRE were designed (GenePharma, Shanghai, China). A scrambled shRNA was used as negative control for lncRNA-FIRRE shRNAs.

2.4. Cell transfection

pcDNA3.1 vector (Invitrogen) was used for overexpression. siRNAs or shRNAs for knockdown of specific gene were synthesized by GenePharma. Cells transfection was finished by using the Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's protocol.

2.5. Cell viability assay

A volume of 10 mm MTT solution (Roche Applied Science, Basel, Switzerland) was added to each culture well. After 2 h incubation, the cell viabilities were monitored by measuring the OD value of the converted dye at 450 nm wave length.

2.6. Cell apoptosis

AnnexinV/propidium iodide detection kit (Keygene, Nanjing, China) was applied for cell apoptosis assay.

2.7. Subcellular fractionation assay

RNA was isolated by using Cytoplasmic and Nuclear RNA Purification Kit (Norgen, Thorold, ON, Canada). In short, cells were lysed on ice for 5 min followed by centrifugation (12000g, 3min). The cytoplasmic RNA was measured by collecting the supernatant, whereas the nuclear RNA was evaluated by using the nuclear pellet.

2.8. Chromatin immunoprecipitation assay

LoVo cells were used to undergo chromatin immunoprecipitation (ChIP) assay by using a p50 specific antibody (Millipore) and the EZ-Magna ChIP A/G Kit (Millipore) based on the manufacturer's protocol. The retrieved DNA was quantified by qRT-PCR as previously described, which spanning the p50 binding sites in FIRRE or NLRP3 promoter region.

2.9. Luciferase reporter assay

pcDNA, pcDNA-FIRRE, or si-p50 or si-p65 was co-transfected with pGL3-FIRRE-promoter and pRL-TK (Renilla luciferase expression vector, Promega) into Lovo cells. Forty-eight hours after transfection, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega) under the guidance of the manufacturer's protocol. The relative firefly luciferase activity was normalized to Renilla luciferase activity.

2.10. Western blot

The proteins were retrieved from cells by using RIPA buffer and were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, following transferred to nitrocellulose membrane, and then was blocked with 5% bovine serum albumin. The membranes were incubated with primary antibodies specific for p50 (Abcam), p65 (Abcam), IkB (Abcam), p-IκBα (Abcam), SMAD3 (Abcam), NF-kB1 (Abcam), and TCF4 (Abcam) or GAPDH (Cell Signaling Technology), and IRDye 800CW goat anti-rabbit IgG or IRDye 700CW goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE), and being detected with an Odyssey infrared scanner (Li-Cor Biosciences).

2.11. Statistical analysis

Data are exhibited as mean ± SD of more than two independent experiments. Comparisons between two groups were evaluated with Student's *t*-test. Data were considered to be statistically significant when p value less than 0.05.

3. Results

3.1. FIRRE was up-regulated in OGD/R-treated microglial cells

To determine the function and mechanism by which lncRNA regulates OGD/R injury, OGD/R-induced injury model was firstly constructed. According to the previous report [12], microglial cells were treated with oxygen and glucose deprivation for 4 h or 8 h. Then, the microglial cells were recovered under normoxic culture conditions for 12 h. To construct control group, cells without OGD/R injury were maintained in regular culture medium. Subsequently, proliferation and apoptosis of cells (Non-OGD and OGD) were examined. As illustrated in Fig. 1A, with the treatment of OGD, cell viability was significantly inhibited, whereas, cell apoptosis was obviously increased. Additionally, the expression levels of several inflammation factors (TNF-a, IL-1β, IL-6 and mcp-1) was increased by OGD in a time-dependent manner (Figure 1A). Based on the results

Please cite this article in press as: Y. Zang, et al., LncRNA FIRRE/NF-kB feedback loop contributes to OGD/R injury of cerebral microglial cells, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.04.194