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Research article

Foxo1-mediated inflammatory response after cerebral hemorrhage in rats

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HIGHLIGHTS

- Foxo1 expression peaked at 12 h post-intracerebral hemorrhage (ICH) and in the ipsilateral corpus striatum.
- Intracerebroventricular injection of Foxo1 siRNA effectively inhibited Foxo1 mRNA and protein expression.
- Foxo1 siRNA significantly increased neurological function and decreased brain water content after ICH injury.
- Foxo1 siRNA obviously reduced inflammatory factors release.
- Inhibition of Foxo1 may inhibited the TLR4/NF-κB pathway after ICH injury.

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ABSTRACT

The forkhead box O (Foxo) family of transcription factors plays a crucial role in cell apoptosis, immune regulation, and tissue development. Foxo1, as the foremost member of the Foxo family, regulates a wide range of molecular signals in many tissues, including tumor, liver, and brain. This study investigated Foxo1 expression at different time points and in different brain areas, and the role of Foxo1 *in vivo* in regulating inflammatory injury in a rat model of autologous blood-injected cerebral hemorrhage injury. We found that Foxo1 expression peaked at 12 h post-intracerebral hemorrhage (ICH) and in the ipsilateral corpus striatum. Foxo1 knockdown by Foxo1 siRNA decreased ICH injury, improved neurological function, and decreased the expression of inflammatory factors downstream of the Foxo1 pathway, including TLR4, NF-κB, TNF-α, IL-1β, and IL-18. Foxo1 knockdown also decreased the expression and activity of myeloperoxidase, IL-1β, and IL-18. In conclusion, our findings demonstrate that Foxo1 is a key regulator of inflammatory injury in rats after ICH. By identifying the molecular mechanisms of Foxo1/TLR4/NF-κB signaling, we provide a novel rationale for therapeutic approaches to managing inflammatory injury after ICH.

1. Introduction

Intracerebral hemorrhage (ICH), an important clinical classification of stroke, induces neuronal apoptosis, formation of perihematomal edema, inflammatory lesions, neurological impairment, and energy metabolism disorder [1]. ICH remains a tremendous challenge for which there is no effective therapy at present [2]. Cerebral edema starts with an inflammatory reaction following introduction of a hematoma. In particular, blood-derived leukocytes and neutrophils infiltrate the brain parenchyma, disrupting the blood–brain barrier and causing edema formation as well as deterioration of neurobehavioral function [3]. These

Abbreviations: Foxo1, the forkhead box O1; ICH, intracerebral hemorrhage; siRNA, small interfering RNA; TLR4, toll-like receptor 4; NF-κB, nuclear factor-k-gene binding; TNF-α, tumor necrosis factor; IL-1β, interleukin-1β; IL-18, interleukin-18; MPO, myeloperoxidase; PASMSs, pulmonary artery smooth muscle cells; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; WW, wet weight; DW, dry weight; Ipsi-CS, ipsilateral corpus striatum; Ipsi-CX, ipsilateral cortex; Cont-CS, contralateral corpus striatum; Cont-CX, contralateral cortex.

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responses are consequences of increased levels of myeloperoxidase (MPO), IL-1β, and IL-18 [4–6]. Therefore, it is important to find a key upstream transcription factor in the inflammatory reaction.

The forkhead box O (Foxo) family of transcription factors plays a crucial role in cell apoptosis, inflammation, and tissue development. Foxo1, a member of the Foxo family, regulates a wide range of molecular signals in many tissue types, such as tumor, liver, and brain [7,8]. Previous studies have suggested that under inflammatory conditions, Foxo1 expression is upregulated, which could induce the production of cultured macrophage inflammatory factors [9]. Immunoregulation of Foxo1 was demonstrated by enhancing TLR4-mediated inflammation in lung and liver tissue, among others, but its immunoregulation function in brain tissue after ICH in rats was uncertain [10,11].

In this study, we investigated the role of Foxo1 in ICH using an experimental ICH model with Foxo1 knockdown. We evaluated whether downregulated Foxo1 could reduce brain damage. Furthermore, we hypothesized that Foxo1 could regulate inflammatory injury via the TLR4/NF-κB signaling pathway and modulate inflammatory factor activity.

2. Material and methods

2.1. Animals

The animal study was approved by the Institutional Animal Care and Use Committee at Chongqing Medical University. Eight-week-old male Sprague–Dawley rats (weight 280–320 g, sourced from the Chongqing Medical Animal Experimentation Center) were housed in a temperature- and light-controlled environment under pathogen-free conditions, and provided unlimited access to food and water, 12/12 light/dark with humidity 60 ± 5% and 22 ± 3 °C. All rats were randomly allocated to the following group: sham surgery group, ICH group, Foxo1 siRNA group and control siRNA of Foxo1 group in blind manner.

2.2. ICH rat model

ICH was induced with a single infusion model of autologous blood (50 μL). The rats were randomly assigned to an experimental group, anesthetized with 10% chloral hydrate (1 mL/100 g) by intraperitoneal injection, and stabilized prone in a stereotactic head frame. The autologous blood was collected from the arteria femoralis on the operation side and transferred into an anticoagulation syringe with a microinjection pump. The blood was infused into the right corpus striatum anterior-posterior 0.2 mm, mediolateral 3.0 mm, and dorsoventral 5.8 mm. An injection of 10 μL blood was delivered 5.0 mm dorsoventral and retained for 2 min; the final 40 μL blood was given as a total injection at dorsoventral 5.8 mm and retained for 15 min. Sham-operated rats were only given a needle insertion.

2.3. Preparation of siRNA

The siRNA against Foxo1 was designed using RNA oligochemical synthesis (Genepharma, Shanghai, China). Four Foxo1-rat-siRNA fragments were used: Foxo1-rat-1285 (sense: 5′-GCAGACACCUUGCUAUUCATT-3′, antisense: 5′-UGAAUAGCAAGGUGUCUGCTT-3′); Foxo1-rat-1434 (sense: 5′-GAGGAUUGAACCAGUAUAATT-3′, antisense: 5′-UUAUACUGGUUCAAUCCUCTT-3′); Foxo1-rat-1612 (sense: 5′-CCAGGCACCUCAUAACAAATT-3′, antisense: 5′-UUUGUUAUGAGGUGCCUGGTT-3′); and stable negative contol (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCGGAGAATT-3′). The most significant fragment screened by western blot was Foxo1-rat-1612, and the scramble siRNAs of Foxo1-rat-1612 were 5′-GCCAAGACCCACAUAAUCATT-3′ (sense) and 5′-UGAUUAUGUGGGUCUUGGCTT-3′ (antisense). All siRNA was synthesized, purified, desalted, and shipped in the 2′-deprotected, duplexed form.

2.4. Injection of Foxo1-rat-siRNA

Each tube of Foxo1-rat-siRNA was dissolved in 20 μL DEPC, oscillated, centrifuged, and oscillated again, then infused into the right paracele at anterior–posterior 1 mm, mediolateral 2 mm, and dorsoventral 3.5 mm (8 μL per rat), and retained for 15 min. The ICH model was established 24 h later. The stable negative control and scramble siRNA of Foxo1-rat-1612 were administered using the same procedure.

2.5. Brain water content assay

ICH rats were decapitated under deep anesthesia, and the brains were immediately removed. Each brain was divided into two halves: hemorrhagic (ipsilateral) and contralateral. Each part was weighed on an electronic analytical balance to give the wet weight (WW) and dried at 100 °C for 24 h in the oven to determine the dry weight (DW). Brain water content was then calculated as $[(WW - DW)/WW] \times 100\%$.

2.6. Garcia neurological score assay [12]

ICH rats were given a score between 0 and 18 on the Garcia test, which consists of six sections: spontaneous activity, axial sensation, vibrissae proprioception, limb outstretching, lateral turning, and forelimb walking, with a possible score of 0–3 for each section (0 = worst, 3 = best).

2.7. Western blot

Amounts of 30 μg of protein from each brain sample were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Monoclonal rabbit anti-rat Foxo1 (C29H4, Cell Signaling Technology, Danvers, USA, 1:1000, AB_2106495, Rabbit Anti-Foxo1), TLR4 (BS3489, Bioworld Technology, Minnesota, USA, 1:500, AB_1662746, Rabbit Anti-TLR4), NF-κB (sc-372, Santa Cruz Biotechnology, Texas, USA, 1:1000, AB_632037, Rabbit Anti-NF-κB), TNF-α (BS1857, Bioworld Technology, Minnesota, USA, 1:1000, AB1662107, Rabbit Anti-TNF-α), IL-1β (BS3506, Bioworld Technology, Minnesota, USA, 1:500, AB_1661842, Rabbit Anti-IL-1β), IL-18 (sc-7954, Santa Cruz Biotechnology, Texas, USA, 1:200, AB_1564060, Rabbit Anti-IL-18), and β-actin (13E5, Cell Signaling Technology, Danvers, USA, 1:1000, AB_2223169, Rabbit Anti-β-actin) were used. Relative protein quantities were determined by densitometry and expressed in absorbance units.

2.8. Quantitative RT-PCR

Total RNA was extracted from frozen brain using Reagent Kit(TaKaRa Biotechnology, Dalian, China). A total of 40 μL RNA was reverse-transcribed into cDNA. Quantitative PCR was performed as described [13]. Primer sequences for the amplification of Foxo1 and β-actin. Target gene expressions were calculated by their ratios to β-actin.

2.9. Enzyme-linked immunosorbent assay

The presence of MPO was used as an index of neutrophil accumulation in the post-ICH brain tissue homogenate. The presence of IL-1β and IL-18 was used as an index of inflammatory injury in