DJ-1 exerts anti-inflammatory effects and regulates NLRX1-TRAF6 via SHP-1 in stroke

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Abstract

Background: Acute inflammation developed by reactive astrocytes after cerebral ischemia/reperfusion (I/R) injury is important in protecting the resultant lesion. Our previous study demonstrated the abundant expression of DJ-1 in reactive astrocytes after cerebral I/R injury. Here, we show that DJ-1 negatively regulates the inflammatory response by facilitating the interaction between SHP-1 and TRAF6, thereby inducing NLRX1 dissociation from TRAF6.

Methods: We used oxygen-glucose deprivation/reoxygenation (OGD/R) in vitro in primary astrocyte cultures and transient middle cerebral artery occlusion/reperfusion (MCAO/R) in vivo to mimic ischemic reperfusion insult.

Results: The inhibition of DJ-1 expression increased the expression of the inflammatory cytokines TNF- α , IL-1 β , and IL-6. DJ-1 knockdown facilitated the interaction of NLRX1 with TRAF6. However, the loss of DJ-1 attenuated the interaction of SHP-1 with TRAF6. In subsequent experiments, an SHP-1 inhibitor altered the interaction of SHP-1 with TRAF6 and facilitated the interaction of NLRX1 with TRAF6 in DJ-1-overexpressing astrocytes..

Conclusion: This finding suggests that DJ-1 exerts an SHP-1-dependent anti-inflammatory effect and induces the dissociation of NLRX1 and TRAF6 in cerebral I/R injury. Thus, DJ-1 may be an efficacious therapeutic target for the treatment of I/R injury.

Introduction

Pathophysiological cascade responses involving inflammation are triggered by cerebral ischemia/reperfusion (I/R)-induced neuronal death [1, 2][[2019 #179]]. Cerebral ischemia induces an inflammatory response characterized by the activation of astrocytes and microglia and the elevated production and release of inflammatory cytokines and chemokines that aggravate tissue damage [3, 4]. Although microglia are the first cells to respond to inflammation of cerebral ischemia, the responses of astrocytes to proinflammatory cytokines may also be relevant to tissue damage [5, 6]. Astrocytes respond to inflammation by losing vital effects or acquiring injurious functions that aggravate inflammation and delay ischemic recovery [7]. The acute inflammation developed by reactive astrocytes after insult is an important response to protect and repair the lesion [8]. In addition, the secretome of reactive astrocytes was determined as a therapeutic approach to reduce inflammation [9]. Knowledge of the mechanism of the astrocytic inflammatory response may allow the development of an efficacious therapeutic strategy to alleviate brain injury in stroke.

NLRX1 is a recently characterized member of the NOD-like family that is widely expressed in the mitochondria of all tissues [10]. NLRX1 as a anti-inflammatory regulator attenuates antimicrobial immune responses [11–13] and sterile inflammation [14] by inhibiting the NF- κ B and RIG–1-MAVs signaling pathways. NLRX1 was also recently shown to exert negative effects on inflammatory responses to the central nervous system (CNS) [15]. NLRX1 $^{-/-}$ mice show upregulated NF- κ B signaling that contributes to neural tissue damage [16]. In unstimulated cells, NLRX1 associates with TRAF6. However, after cell

stimulated by lipopolysaccharide (LPS), NLRX1 rapidly dissociates with TRAF6 and inhibits TLR-mediated NF- κ B activation and proinflammatory cytokine release [17]. Therefore, NLRX1, an anti-inflammatory agent that dissociates from TRAF6, is critical in regulating inflammation.

DJ-1 [also known as PARK7] has been linked to an early-onset autosomal recessive form of PD [18] and is abundantly expressed in reactive astrocytes in Parkinson's disease [19]. Our previous study showed that upregulated astroglial DJ-1 in infarct regions plays a critical role in astrocyte neuroprotection after stroke [20], suggesting that DJ-1 affects the function of astrocytes. DJ-1-knockout (KO) astrocytes increased the LPS-induced expression of proinflammatory mediators, which aggravated inflammatory damage induced by IFN- γ [21]. Tumor necrosis factor- α (TNF- α) was found to be increased in injured DJ-1-KO brains [21]. DJ-1 regulates TRAF6 signals in bone marrow macrophages (BMMs) via Src homology region 2 domain-containing phosphatase-1 (SHP-1) [22]. In addition, astroglial DJ-1 exerts anti-inflammatory effects by promoting the interaction of SHP-1 with STAT1 [23]. However, to our knowledge, the detailed mechanisms by which DJ-1 plays this anti-inflammatory role in cerebral I/R injury remain unclear.

Previously, we reported that astroglial DJ-1 plays a critical role in neuroprotection in ischemic injury [20]. These findings led us to further study the protective mechanism of astroglial DJ-1. In this study, we demonstrate that DJ-1 plays anti-inflammatory roles in astrocytes. DJ-1 induces the dissociation of NLRX1 from TRAF6 by facilitating the interaction of SHP-1 with TRAF6. Thus, the anti-inflammatory function of astroglial DJ-1 in I/R insult is required.

Methods

Experimental Animals and Reagents

Adult male Sprague-Dawley (SD) rats (between 250–280 g in weight) were obtained from the Animal Experimental Center of Chongqing Medical University and used for the in vivo study. Primary astrocytes were extracted from the cerebral cortices of newborn SD rats and cultured.

Glucose-free Dulbecco's modified Eagle's medium, Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Trypsin and Hank's solution were obtained from HyClone (Logan, UT, USA). Penicillin/streptomycin (Pen/Strep) and phosphate-buffered saline solution (PBS) were obtained from Beyotime (Shanghai, China). Poly-L-lysine was purchased from Sigma-Aldrich (Milan, Italy). IL-1 β , IL-6 and TNF- α enzyme-linked immunosorbent assay (ELISA) kits were obtained from Boster (Wuhan, China), and a CCK-8 kit was obtained from Dojindo (Kumamoto, Japan). TPI-1 was purchased from MedChem Express (New Jersey, USA).

Middle Cerebral Artery Occlusion/Reperfusion (MCAO/R) Model and TPI-1 Dosing

Transient MCAO/R was performed as previously described by our laboratory [24]. Briefly, adult male SD rats were deprived of feed and water for 8 h before operation, anesthetized with 3.5% chloral hydrate (350 mg/kg,

intraperitoneal injection) and then placed on a heating pad to maintain their body temperatures at 37 ± 0.5 °C for operation. A nylon filament (Beijing Cinontech Co., Ltd., Beijing, China) was inserted into the left middle cerebral artery. Then, the filament was removed after 1 h of ischemia to allow reperfusion. The local cerebral blood flow (CBF) was monitored by a laser Doppler flowmeter (Periflux System 5000, Perimed, Sweden) during the operation. After reperfusion for 24 h, neurological deficit score assays were used to evaluate the success of the MCAO model, and brain tissue was subjected to Western blot analysis, immunoprecipitation and immunohistochemistry. Sham-operated animals were received the same surgery as the MCAO/R group, except they were not subjected to MCAO.

TPI-1 was obtained from MedChem Express. The SHP-1 inhibitor TPI-1 (HY-100463, MedChem Express) was dissolved in 10% DMSO and diluted to its final concentration in 90% saline (20% SBE- β -CD in saline). We intraperitoneally injected 1 mg/kg TPI-1 into MCAO rats after waking. An identical volume of DMSO was injected intraperitoneally as a control.

All the analyses conducted in SD rats (N = 353) were divided in two sets of experiments; the first part was used to study the effect of DJ-1 on inflammation after cerebral I/R, and the second part was used to study the regulatory mechanism of DJ-1 on the NLRX1-TRAF6 interaction. The first part comprised 4 groups, and the second part comprised 6 groups. The groups in the first part were as follows: the (1) sham (n = 31, 1 died), (2) MCAO (n = 33, 3 died), (3) NC (n = 34, 4 died): MCAO + negative control siRNA, and (4) DJ-1 siRNA (n = 36, 6 died): MCAO + DJ-1 siRNA groups. The groups in the second part were as follows: the (1) sham (n = 24, 0 died), (2) MCAO (n = 26, 2 died), (3) Scramble (n = 28, 4 died): MCAO + scramble, (4) Overexpression (n = 28, 4 died): MCAO + DJ-1 overexpression, (5) DMSO (n = 27, 3 died): MCAO + DMSO, (6) TPI-1 (n = 27, 3 died): MCAO + TPI-1, (7) Overexpression + DMSO (n = 30, 6 died): MCAO + DMSO + DJ-1 overexpression, and (8) Overexpression + TPI-1 (n = 29, 5 died): MCAO + TPI-1 + DJ-1 overexpression groups. In our study, 6 rats were used in per experiment group, and at least duplicates-triplicates were carried out for each technique.

Treatment with DJ-1 siRNA or DJ-1 Adeno-associated Virus (AAV)

DJ-1 siRNA was obtained and used as described in our previous studies [20]. AAV was purchased from Neuron Biotech (Shanghai, China). The following RNA sequences were used to overexpress the rat DJ-1 gene (Gene ID: NM_117287): 5-CCGGACGGCAGTCACTACAGCTACTCAAGAGA and TAGCTGTAGTGACTGCCGTTTTTTTG-3 (overexpression). Scramble AAV did not contain inserts. One month before MCAO, AAV was injected into the left lateral cerebral cortices of the rats. Overexpression efficiency of DJ-1 AAV was confirmed by Western blot analysis.

Evaluation of neurological deficit

To assess neurological deficit of the rats on days 1 after MCAO/R, a modified scoring system was applied as described in our previous studies [20]. As follows: grade 0, no neurological damage; grade 1, failure to extend

contralateral forelimb fully; grade 2, circling to right; grade 3, falling to the right; grade 4, no spontaneous autonomic activity or loss of consciousness; grade 5, dead.

HE and Nissl Staining

After neurological examination, the rats were sacrificed with an overdose of 3.5% chloral hydrate after MCAO/R, and their brains were perfused transcardially with 4% paraformaldehyde. The brains were dehydrated and embedded in paraffin after immersion in paraformaldehyde for 24 h. Then, 5- μ m coronal sections were stained with hematoxylin and eosin (HE) or 0.1% cresyl violet (Nissl staining) based on standard protocols and prepared for microscopic mounting.

Primary Rat Cortical Astrocyte Culture and Oxygen and Glucose Deprivation/Reoxygenation (OGD/R) Treatment

Astrocytes were cultured as previously described[20]. Briefly, cortical astrocytes were cultured onto 75cm² flasks at a density of 2.0×10^7 /well in DMEM/F12 medium with 10% Fetal Bovine Serum and 1% Pen/Strep and cultured in a humidified incubator in 5% CO₂ at 37 °C. After incubating the cells in culture for 2–3 weeks, microglia were removed from flasks with mild shaking, the remaining adherent astrocytes were detached by trypsin, and recovered cells were plated directly on the bottom of 6-well plates. Astrocytes spread over the bottom of the plates (7–9 days) were used for further study. Astrocyte purity was identified using the astrocyte-specific marker GFAP.

OGD/R was conducted as previously described. Briefly, astrocytes were established in glucose-free DMEM and were transferred to an incubator with 94% N₂, 1% O₂, and 5% CO₂ at 37 °C for 5 h. The glucose-free DMEM was then changed back to normal culture medium (DMEM/F12 medium containing 10% Fetal Bovine Serum and 1% Pen/Strep), and then TPI-1 (10ng/ml) was added into the culture medium of 6-well plates. TPI-1 was dissolved in 10% DMSO and diluted to its final concentration in PBS. An identical volume of DMSO and PBS was added into the culture medium as a control. Next, cells were grown in an incubator with 5% CO₂ for 24 h at 37 °C. All the analyses conducted in astrocytes were divided in two sets of experiments; the first part was used to study the effect of DJ-1 on inflammation after cerebral I/R, and the second part was used to study the regulatory mechanism of DJ-1 on the NLRX1-TRAF6 interaction. The first part comprised 4 groups, and the second part comprised 6 groups. The following groups were used in the first part: the (1) Control, (2) OGD/R, (3) NC: OGD/R + negative control siRNA, and (4) DJ-1 siRNA: OGD/R + DJ-1 siRNA groups. The following groups were used in the second part: the (1) Control, (2) OGD/R, (3) Scramble: OGD/R + scramble (4) Overexpression: OGD/R + DJ-1 overexpression (5) DMSO: OGD/R + DMSO, (6) TPI-1: OGD/R + TPI-1, (7) Overexpression + DMSO: OGD/R + DMSO + DJ-1 overexpression, and (8) Overexpression + TPI-1: OGD/R + TPI-1 + DJ-1 overexpression groups. Astrocytes were cultivated with 100 nM TPI-1 after OGD. In our study, 6 cultures were performed in per experiment group, and at least duplicates-triplicates were carried out for each technique.

DJ-1 Interference and Overexpression in Astrocytes

Lentivirus was obtained and used as described in our previous studies[20]. The following sequence used to knock down DJ-1 was designed and synthesized: 5-CCGGACGGCAGTCACTACAGCTACTCAAGAGATAGCTGTAGTGACTGCCGTTTTTTTG-3. The control sequence for DJ-1 knockdown was 5-CCGGTTCTCCGAACGTGTCACGTTTCAAGAGACGTGACACGTTCCGAGAATTTTTTG-3. The sequence used to overexpress DJ-1 was 5-ATGGACTACAAGGATGACGATGACAAGGATTACAAAGACGACGATGATAAGGACTATAAGGATGATGACGACAAA-3. Scramble lentivirus did not contain inserts. 3 days before OGD/R, Lentivirus was added into the culture medium. OGD/R was performed after astrocyte transfection. Western blot was used to assess transfection efficiency of Lentivirus.

Cell Viability

To assess astrocyte viability, we used a CCK-8 assay kit (Dojindo, Kumamoto, Japan). Astrocytes were cultured in 96-well plates. Three days before OGD/R, the astrocytes were treated with lentivirus for 72 h. Then, cells were subjected to OGD/R, and a CCK-8 assay was performed. CCK-8 solution was added to the medium (10 µl/mL). After the astrocytes were cultured with the medium at 37 °C for up to 2 h, astrocytes were measured on a microplate reader (Bio-Rad, Foster City, CA, USA) at the absorbance of 450 nm.

Immunocytochemistry and Immunohistochemistry

Frozen sections and cells coverslips were fixed in 4% paraformaldehyde and then washed with PBS. After blocked with 5% FBS/0.01% Triton X-100 in PBS at 37 °C for 1 h, the cells were incubated with the following primary antibodies: NLRX1 (1:100, Abcam, ab105412), Hsp60 (1:100, Proteintech, 66041-1-1g) DJ-1 (1:50, Abcam, ab76008), NeuN (1:100, Abcam, ab104224), and GFAP (1:100, Servicebio, GB12096) and IBA-1 (1:100, Servicebio, GB12105) overnight at 4 °C. The next day, cells and sections were incubated in goat anti-mouse IgG antibody DyLight 488 (1:200, Abbkine, green, A23210) and goat anti-rabbit IgG antibody DyLight 549 (1:200, Abbkine, red, A23320) at 37 °C for 30 min. Vectashield containing DAPI was used to mount coverslips and sections. A laser scanning confocal microscope (LSCM) was used to visualize sections and glass slides.

Western Blot Analysis

Total protein from cultured astrocytes and ischemic brain tissue was prepared using RIPA buffer with the protease inhibitor PMSF. A total of 50 µg of protein (per lane) were separated using SDS-PAGE (different percentage of PAGE used to separate and analyze the different proteins) and electrotransferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). The membranes were then blocked with 5% nonfat milk in TBST buffer for 2.0 h at 37 °C and incubated in the following primary antibodies overnight at 4 °C: DJ-1 (1:1000, Abcam, ab76008), anti-β-actin (1:3000, Proteintech, 66009-1-Ig), IL-1β

(1:200, Boster, PB0055), IL-6 (1:200, Boster, PB0061), TNF- α (1:200, Boster, PB0082), NLRX1 (1:200, Finetest, FNab05759), TRAF6 (1:200, Santa Cruz Biotechnology, sc-8409), and SHP-1 (1:200, Abcam, ab227503). The next day, the membranes were incubated with secondary antibodies at 37°C for 2 h. The densities of the bands were detected using an imaging densitometer (Bio-Rad, Foster City, CA, USA), and the gray values of the bands were quantified using ImageJ. Relative protein expression was normalized to that β -actin staining intensity.

Immunoprecipitation and Immunoblot Analyses

In the immunoprecipitation experiments, whole-tissue and cell lysates were prepared after I/R and incubated with the indicated antibodies together with protein A/G beads (MedChem Express, New Jersey, USA) overnight. NLRX1 (1:200, Finetest, FNab05759), TRAF6 (1:200, Santa Cruz Biotechnology, sc-8409) and SHP-1 (1:200, Abcam, ab227503) antibodies were used to immunoprecipitate NLRX1, TRAF6 and SHP-1, respectively. The beads were then washed 4 times with lysis buffer, and the immunoprecipitates were separated using SDS-PAGE. The proteins were transferred to PVDF membrane (Millipore, Boston, MA, USA) and then incubated with the indicated antibodies.

ELISA Measurements of TNF- α , IL-1 β , IL-6

After MCAO for 1 h and OGD for 5 h followed by reoxygenation for 24 h, total protein from ischemic penumbra of the cerebral cortex and cultured astrocytes was prepared using RIPA buffer with the protease inhibitor PMSF. We used TNF- α , IL-1 β , IL-6 ELISA kits (Boster Institute, Wuhan, China) to measure the levels of TNF- α , IL-1 β , and IL-6 expression in vivo and in vitro. We assessed the expression of these inflammatory cytokines in collected tissue and cell lysates according to the manufacturer's instructions. The signal was measured at 450 nm.

Statistical Analysis

All data are shown as the means $\pm$ standard errors of the means, and statistical analysis was conducted using GraphPad Prism software (version 6.0). Statistical comparisons were assessed with one-way analysis of variance (ANOVA) followed by Tukey's test. A value of $P < 0.05$ was indicated statistically significant.

Results

DJ-1 Interference Aggravated Injury After Cerebral I/R Injury

HE and Nissl staining were conducted to assess morphological changes in the ischemic lesions 24 h after reperfusion (Fig. 1A,B). HE staining demonstrated that neurons in the MCAO and NC groups adopted a more disorderly arrangement than in the sham group and exhibited loosened cytoplasm and karyopyknosis. However, transfection with DJ-1 siRNA increased the level of diffuse vacuolization and edema in the interstitial area and decreased the number of intact neurons. The results from Nissl staining were similar to

those from HE staining. In contrast, a large number of atrophic neurons that showed damaged nuclei and shrunken cytoplasm were observed in the MCAO and NC groups. Most neurons showed a loss of Nissl bodies in the DJ-1 interference group than in the MCAO groups. As shown in Fig. 1C, compared with the MCAO group, neurological deficit scores increased after rats were infected with DJ-1 siRNA.

The CCK-8 assay was conducted to evaluate the roles of DJ-1 in astrocyte viability after exposure to OGD/R. Cells in the OGD/R group and NC group were less viable than those in the control group, and viability was further decreased in the DJ-1 siRNA group [Fig. 1D]. These results suggest that DJ-1 interference can aggravated cerebral I/R injury.

DJ-1 Interference Increased the Expression of TNF- α , IL-1 β , and IL-6 After Cerebral I/R Injury

To determine whether DJ-1 is involved in inflammation, we analyzed the expression of inflammatory cytokines after interfering with DJ-1. Western blot analysis and ELISA were conducted to detect the expression levels of TNF- α , IL-1 β , and IL-6. In vivo TNF- α , IL-1 β , and IL-6 expression in MCAO group was increased compared with that in the sham group, and the expression of these cytokines was further increased in the DJ-1 siRNA group (Fig. 2A, C, E-G). Similar results were observed in vitro (Fig. 2B, D, H-J).

DJ-1 Regulated NLRX1, TRAF6 ,

NLRX1, a recently characterized member of the NOD-like family, is widely expressed in the mitochondria. We assessed mitochondrial NLRX1 expression using the mitochondria-specific marker HSP60. As shown in Fig. 3E, F, NLRX1 was expressed in the mitochondria. NLRX1, which is known to negatively regulate inflammation, also negatively regulated TLR-induced NF- κ B signaling by dissociating from TRAF6[17]. Due to DJ-1 is also an anti-inflammatory regulator, we investigated whether DJ-1 regulates the expression of NLRX1, TRAF6 after cerebral I/R injury. The expression of NLRX1 was significantly upregulated following cerebral I/R injury compared to that in control samples. However, changes in DJ-1 had no effect on the levels of NLRX1 in vivo and in vitro (Fig. 3A-D). After MCAO, the expression of TRAF6 increased compared with that in the sham group and was further increased in the DJ-1 siRNA group (Fig. 3A, C). Similar results were observed in vitro (Fig. 3B, D). Similarly, SHP-1 (Src-homology 2 domain-containing phosphatase-1) acts as a critical negative regulator of inflammatory response [26], we thus assessed the expression of SHP-1. After MCAO, the expression of SHP-1 was higher than that in the sham group, and after DJ-1 knockdown, the expression of SHP-1 was lower than that in the MCAO group (Fig. 3A, C). Similar results were observed in vitro (Fig. 3B, D).

DJ-1 Regulated the Interaction of TRAF6 with NLRX1 and SHP-1 After Cerebral I/R Injury

NLRX1 inhibits TLR-mediated NF- κ B signaling by dissociating from TRAF6[17], we thus investigated whether DJ-1 regulates the interaction between NLRX1 and TRAF6 after cerebral I/R injury. NLRX1 interacted with TRAF6 in the sham group; however, NLRX1 disassociated from TRAF6 after cerebral I/R injury. Interestingly,

DJ-1 interference promoted the interaction of NLRX1 with TRAF6 (Fig. 4A, C). These results indicate that NLRX1 may associate with TRAF6 in rested cells. After cerebral I/R injury, NLRX1 may disassociate from TRAF6 and thus inhibit inflammation. However, DJ-1 interference prevented the dissociation of NLRX and TRAF6 and thus enhanced inflammation. Since TRAF6 plays a prominent role in inflammation by NF- κ B activation, and a previous study demonstrated the association between SHP-1 and TRAF6 in RANKL-stimulated BMMs[25]. Therefore, we hypothesized that TRAF6 interacts with SHP-1. We assessed the interaction between SHP-1 and TRAF6 after astroglial DJ-1 interference in cerebral I/R injury. SHP-1 was associated with TRAF6 in the MCAO group but not in the sham group. These interactions were inhibited significantly after treatment with DJ-1 siRNA (Fig. 4B, D).

As shown in Fig. 4A, C, DJ-1 interference promoted the interaction of NLRX1 with TRAF6. These results suggest that DJ-1 facilitate the dissociation of TRAF6 from NLRX1 and may be related to the interaction of TRAF6 with SHP-1.

DJ-1 Inhibited the Expression of TNF- α , IL-1 β , and IL-6 After Cerebral I/R Injury via SHP-1

SHP-1 has strong negative regulator of inflammation effects, and SHP-1-deficient mouse brains show enhanced inflammation [26]. To determine whether SHP-1 is involved in inflammation, TPI-1 was used to inhibit the expression of SHP-1. As shown in Fig. 6E-H, inhibition of SHP-1 increased the expression of TNF- α , IL-1 β , and IL-6 compared with that in the DMSO group in vivo and vitro. However, inhibition of SHP-1 had no effect on the level of DJ-1. Interestingly, DJ-1 can regulate SHP-1, we evaluated whether DJ-1 inhibits cytokine levels after stroke via SHP-1. Virus and TPI-1 was used to overexpress the DJ-1 and inhibit the SHP-1. Overexpression of DJ-1 reduced the expression of TNF- α , IL-1 β , and IL-6 compared with that in the MCAO group (Fig. 5A, C, I, K, M). After treatment with SHP-1 inhibitor, the expressions of TNF- α , IL-1 β , and IL-6 were increased. Similar results were obtained in vitro (Fig. 5B, D, J, L, N). Thus, DJ-1 exerts anti-inflammatory effects in astrocytes in cerebral I/R injury via SHP-1.

DJ-1 Regulated the Disassociation of NLRX1 and TRAF6 After Cerebral I/R Injury via SHP-1

To determine whether the DJ-1-induced dissociation of NLRX1 and TRAF6 occurs via SHP-1, Western blotting was conducted to assess the expression of NLRX1, TRAF6, and SHP-1, and immunoprecipitation was used to detect the interactions between NLRX1 with TRAF6 and TRAF6 with SHP-1. As shown in Fig. The overexpression of DJ-1 facilitated the interaction of SHP-1 with TRAF6, which was inhibited after treatment with SHP-1 inhibitor. However, NLRX1 disassociated from TRAF6 with DJ-1 overexpression, and SHP-1 inhibition promoted the interaction of NLRX1 with TRAF6 (Fig. 6E, F). Thus, SHP-1 plays a vital role in the disassociation of NLRX1 and TRAF6, which is regulated by DJ-1.

Discussion

The present study showed the important role of DJ-1 in reducing brain injury by inhibiting the levels of inflammatory cytokines in astrocytes. siRNA-induced DJ-1 knockdown increased cell death in astrocytes. Reduced DJ-1 expression led to the upregulation of TNF- α , IL-1 β , and IL-6. The loss of DJ-1 expression decreased SHP-1 expression and suppressed the interaction between SHP-1 and TRAF6. Conversely, the interaction between NLRX1 and TRAF6 was facilitated. Indeed, DJ-1 induced the disassociation of NLRX1 and TRAF6, which was reversed after the loss of SHP-1. Thus, the anti-inflammatory effects of DJ-1 in astrocytes in cerebral ischemic injury may be due its induction of NLRX1 and TRAF6 dissociation via SHP-1.

Pathophysiological cascade responses involving inflammation are triggered by cerebral ischemia/reperfusion (I/R)-induced neuronal death [1, 2]. Cerebral ischemia induces acute inflammatory response aggravate tissue damage by the elevated production and release of inflammatory cytokines [3, 4]. The acute inflammation developed by reactive astrocytes after insult is an important response to protect and repair the lesion [8]. In addition, the secretome of reactive astrocytes was determined as a therapeutic approach to reduce inflammation [9]. Previously, we reported that astrocytes plays a critical role in neuroprotection in ischemic injury and it is involved in DJ-1 [20]. Compared with neuron and microglia, DJ-1 significantly increased 24h after reperfusion in reactive astrocytes in vivo. In addition, in our present study, we showed the important role of astroglial DJ-1 in reducing brain injury.

As a multifunctional protein, DJ-1 possesses many functions, such as its roles in transcriptional regulation [27], antioxidative stress [28], and antiapoptosis [29]. Many previous studies have demonstrated these functions, and new evidence suggests that DJ-1 also has an anti-inflammatory effect. Activated astrocytes produce the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 [30], and DJ-1 is increased in reactive astrocytes in cerebral I/R injury [20]. In our study, we detected changes in the expression of the cytokines TNF- α , IL-1 β , and IL-6 to assess the effect of DJ-1 on inflammation in astrocytes exposed to ischemic injury. The levels of TNF- α , IL-1 β , and IL-6 were higher in the DJ-1 knockdown group than in the sham group. High levels of these cytokines can be detrimental to ischemic recovery because they directly induce apoptosis in neuronal cells [31, 32]. In addition, DJ-1 deficiency in other diseases aggravates injury by inducing inflammation [33, 34]. Thus, DJ-1 plays a clear anti-inflammatory role in ischemic injury.

NLRX1, a member of the NLR family of proteins, is a recently characterized protein that is expressed widely in the mitochondria [10, 12]. NLRX1 acts as a anti-inflammatory regulator and inhibits the activation and production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-6 [15]. A previous report showed increased proinflammatory cytokine production in Nlr1-/- mice and aggravated tissue damage during experimental autoimmune encephalomyelitis (EAE) [35]. The anti-inflammatory function of NLRX1 was also shown in sterile CNS inflammation, such as that in traumatic brain injury [16]. Because DJ-1 is also an anti-inflammatory regulator, we assessed the relationship between DJ-1 and NLRX1 and found NLRX1 expression in the mitochondria. Changes in DJ-1 had no effect on NLRX1 levels despite the upregulation of NLRX1 under disease conditions. This may be because DJ-1 does not directly regulate NLRX1. NLRX1 associates with TRAF6 in rested cells, but NLRX1 rapidly dissociates from TRAF6 and inhibits NF- κ B activation and proinflammatory cytokine release after cell stimulation [17]. Thus, NLRX1 is an anti-inflammatory agent that dissociates from TRAF6 and is critical in regulating inflammation. In our study, DJ-1 induced NLRX1 dissociation from TRAF6. Because DJ-1 had no effect on NLRX1 levels, the molecular

mechanisms responsible for the disassociation of NLRX1 and TRAF6 may depend on other molecules or complexes.

SHP-1 is a member of a family of cytosolic protein tyrosine phosphatases [36] that negative regulates the innate and acquired immune responses [37]. The IFN- γ - and LPS-induced activation of NF- κ B was shown to be enhanced in SHP-1-deficient astrocytes [26]. We found that DJ-1 facilitates SHP-1 expression, however, inhibition of SHP-1 had no effect on the level of DJ-1. Since both DJ-1 and SHP-1 play anti-inflammatory role in ischemic injury. It is maybe that DJ-1 exerts anti-inflammatory effects in astrocytes in cerebral I/R injury via SHP-1. Previous work also demonstrated that astroglial DJ-1 exerts anti-inflammatory effects by facilitating the interaction of SHP-1 with STAT1 [23]. The interaction between SHP-1 and TRAF6 is very important to inhibit inflammation. SHP-1 can also inhibit the production of cytokines induced by Tir by binding to TRAF6 [38]. In addition, SHP-1 was directly interacted with TRAF6 in RANKL-stimulated BMMs, which inhibited downstream signaling molecules such as NF- κ B and MAP kinases [25]. We showed the association between SHP-1 and TRAF6 in ischemic injury. DJ-1 promotes the interaction of SHP-1 with TRAF6. Similarly, DJ-1-deficient significantly suppresses the association of SHP-1 and TRAF6 in RANKL-mediated BMMs [22]. Thus, DJ-1 exerts anti-inflammatory effects in astrocytes in cerebral I/R injury by facilitating the interaction between SHP-1 and TRAF6.

TRAF6, a member of the TNF receptor-associated factor (TRAF) family, is an adaptor that mediates signaling for a host of TNF-R superfamily receptors, including TLRs and NOD-like receptors (i.e., NLRX1), among others [39]. TRAF6 is also known to regulate innate immune responses and proinflammatory cytokines, including NF- κ B and IL-6 [40]. We showed that DJ-1 inhibits the expression of TRAF6. In our study, TRAF6 acted as a vital intermediate adaptor involved in a dynamic interaction with SHP-1 or NLRX1. In DJ-1-overexpressing astrocytes, an SHP-1 inhibitor altered the interaction between SHP-1 and TRAF6; therefore, the remaining TRAF6 served as an adaptor scaffold bound to NLRX1. The TRAF6 protein has 4 types of distinct domains: 4 zinc finger (ZF) domains, an N-terminal RING finger domain, a TRAF-C domain, and a coiled-coil domain [41]. A previous study demonstrated that the TRAF6 ZF domain plays a role in SHP-1 recruitment, and the binding of SHP-1 and TRAF6 led to the deubiquitination of TRAF6 and inhibited NF- κ B activity in RANKL-activated BMMs [38]. TRAF6 is a ubiquitin (Ub) ligase that activates signaling via the attachment of K63-linked Ub chains, and a class of deubiquitinating enzymes that disassemble these chains to negatively modulate signaling has been identified [42]. In our study, the binding of SHP-1 and TRAF6 may have led to the deubiquitination of TRAF6 and the subsequent disassembly of the TRAF6 and SHP-1 complex. These results suggest that the dynamic interaction between TRAF6 and NLRX1 or SHP-1 may be more complicated than previously believed. Thus, further studies to determine whether the binding of SHP-1 and TRAF6 leads to the deubiquitination of TRAF6 and whether the deubiquitination of TRAF6 affects the binding of TRAF6 and NLRX1 are needed.

Conclusion

The findings presented here identify a previously unrecognized role for DJ-1 in exerting anti-inflammatory effects in astrocytes in cerebral I/R injury by regulating the interaction between SHP-1 and TRAF6, thereby

mediating the disassociation of NLRX1 and TRAF6. Thus, DJ-1 may be an efficacious therapeutic target to treat I/R injury.

Abbreviations

I/R: cerebral ischemia/reperfusion; SHP-1: Src homology region 2 domain-containing phosphatase-1; TRAF6: TNF receptor-associated factor 6; TPI-1: SHP-1 inhibitor; TNF- α : Tumor necrosis factor- α

Declarations

Acknowledgements

Not applicable.

Authors' contributions

LP, SY, and YZ designed the experiments. LP, YZ, NJ, JZ, YJ and LL contributed to the execution of the entire experiments, LP, TW and JZ performed the statistical analysis. LP, SY and YZ drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

All experiments were authorized by the Institutional Animal Ethics Committee of Chongqing Medical University, Chongqing, China, and procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals. Maximum efforts were made to minimize animal suffering.

Consent for publication

Not applicable.

Competing interests

The authors have no conflicts of interest to declare.

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Control, (2) OGD/R, (3) NC: MCAO + negative control siRNA, (4) DJ-1 siRNA. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 6$ per group.

Figure 2

DJ-1 interference increased the expression of TNF- α , IL-1 β , and IL-6 after cerebral I/R injury. A and C Western blot to detect the DJ-1 and cytokines TNF- α , IL-1 β , and IL-6 in rats. B and D Western blot to detect the DJ-1 and cytokines TNF- α , IL-1 β , and IL-6 in astrocytes. E-G Quantification of TNF- α , IL-1 β , and IL-6 in rats by ELISA. H-J Quantification of TNF- α , IL-1 β , and IL-6 in astrocytes by ELISA. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 6$ per group.

Figure 3

DJ-1 regulated the expression of SHP-1, TRAF6 and SHP-1 after cerebral I/R injury. A and C Western blot to detect NLRX1, TRAF6, and SHP-1 in rats. B and D Western blot to detect NLRX1, TRAF6, and SHP-1 in astrocytes. E Immunohistochemistry was used to measure NLRX1 expression in the mitochondria in rats after DJ-1 knockdown, Original magnification, 400 $\times$ F Immunocytochemistry was used to measure NLRX1 expression in the mitochondria of astrocytes, Original magnification, 600 $\times$. Fluorescence microscopy was used to assess NLRX1 expression, which is indicated by red fluorescence. HSP60 expression is indicated by

green fluorescence. Cell nuclei were stained with DAPI. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 6$ per group.

Figure 4

DJ-1 regulated the interactions of SHP-1/TRAF6 and NLRX1-TRAF6 and after cerebral I/R injury. A and C Immunoprecipitation and immunoblot analyses of NLRX1-TRAF6 in rats. NLRX1 disassociated from TRAF6 after MCAO/R, NLRX1 interacted with TRAF6 after treatment with DJ-1 SiRNA. B and D Immunoprecipitation and immunoblot analyses of SHP-1/TRAF6 in rats. SHP-1 was associated with TRAF6 in the MCAO group, SHP-1 disassociated from TRAF6 after treatment with DJ-1 SiRNA. $n = 6$ per group.

Figure 5

DJ-1 inhibited the expression of TNF- α , IL-1 β , and IL-6 after cerebral I/R injury via SHP-1. A and C After virus and TPI-1 was used to overexpress the DJ-1 and inhibit the SHP-1, western blot to detect the cytokines IL-1 β , IL-6, and TNF- α in rats. B and D After virus and TPI-1 was used to overexpress the DJ-1 and inhibit the SHP-1, western blot to detect the cytokines IL-1 β , IL-6, and TNF- α in astrocytes. E and G After treatment with SHP-1 inhibitor, western blot to detect the cytokines IL-1 β , IL-6, and TNF- α in rats. F and H After treatment with SHP-1 inhibitor, western blot to detect the cytokines IL-1 β , IL-6, and TNF- α in astrocytes. I, K, M Quantification of TNF- α , IL-1 β , and IL-6 in rats by ELISA. J, L, N Quantification of TNF- α , IL-1 β , and IL-6 in astrocytes by ELISA. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. the sham group; # $p < 0.05$ vs. the MCAO group; & $p < 0.05$ vs. the Overexpression group; $\boxtimes p < 0.05$ vs. the DMSO group; $\triangle p < 0.05$ vs. the DMSO group; $\boxtimes p < 0.05$ vs. the TPI-1 group. $n = 6$ per group. Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the OGD/R group; & $p < 0.05$ vs. the Overexpression group; $\boxtimes p < 0.05$ vs. the DMSO group; $\triangle p < 0.05$ vs. the DMSO group; $\boxtimes p < 0.05$ vs. the TPI-1 group. $n = 6$ per group. The groups of A and B corresponding groups of statistical graphs is as follows: Sham(--), MCAO(+), Scramble(+), Overexpression(++), Overexpression+DMSO(+++), Overexpression+TPI-1(+++). The groups of E and F corresponding groups of statistical graphs is as follows: DMSO(+), TPI-1(++), Overexpression+DMSO(++), Overexpression+TPI-1(+++).

Figure 6

DJ-1 regulated the disassociation of NLRX1 and TRAF6 after cerebral I/R injury via SHP-1. A and G After virus and TPI-1 was used to overexpress the DJ-1 and inhibit the SHP-1, western blot to detect NLRX1, TRAF6, and SHP-1 in rats. B and H After virus and TPI-1 was used to overexpress the DJ-1 and inhibit the SHP-1, western blot to detect NLRX1, TRAF6, and SHP-1 in astrocytes. C and I After treatment with SHP-1 inhibitor, western blot to detect the cytokines IL-1β, IL-6, and TNF-α in rats. D and J After treatment with SHP-1 inhibitor, western blot to detect the cytokines IL-1β, IL-6, and TNF-α in astrocytes. E Immunoprecipitation and immunoblot analyses of NLRX1-TRAF6 in rats. F Immunoprecipitation and immunoblot analyses of SHP-1/TRAF6 in rats. Data are expressed as the mean ± SEM. *p<0.05 vs. the sham group; #p<0.05 vs. the MCAO group; &p<0.05 vs. the Overexpression group; ⌘p<0.05 vs. the DMSO group; Δp<0.05 vs. the DMSO group. n=6 per group. Data are expressed as the mean ± SEM. *p<0.05 vs. the control group; #p<0.05 vs. the OGD/R group; &p<0.05 vs. the Overexpression group; ⌘p<0.05 vs. the DMSO group; Δp<0.05 vs. the DMSO group. n=6 per group.

Figure 7

Mechanisms of DJ-1 regulating inflammation. DJ-1 exerts an anti-inflammatory effect on decreasing the expression cytokines IL-1 β , IL-6, and TNF- α in cerebral I/R injury. DJ-1 facilitated the dissociation of NLRX1 and TRAF6. And DJ-1 upregulated SHP-1 expression and facilitated the interaction of NLRX1 with TRAF6. DJ-1 induces the dissociation of NLRX1 and TRAF6 via SHP-1.