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Original Paper

Edaravone Attenuates the Proinflammatory Response in Amyloid-β-Treated Microglia by Inhibiting NLRP3 Inflammasome-Mediated IL-1β Secretion

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Key Words
Amyloid-β • Microglia • NLRP3 inflammasome • Manganese superoxide dismutase • Edaravone

Abstract
Background/Aims: Microglial activation is an important pathological feature in the brains of patients with Alzheimer's disease (AD), and amyloid-β (Aβ) peptides play a crucial role in microglial activation. In addition, edaravone (EDA) was recently shown to suppress oxidative stress and proinflammatory cytokine production in APPswePS1dE9 (APP/PS1) mice. However, the mechanism by which EDA inhibits the Aβ-induced proinflammatory response in microglia is poorly understood. ***Methods:*** The mitochondrial membrane potential (Δψm) was evaluated using JC-1 staining. Intracellular reactive oxygen species (ROS) and mitochondrial ROS levels were detected using CM-H2DCFDA and MitoSOX™ Red, respectively. The levels of CD11b, NLRP3, pro-caspase-1 and manganese superoxide dismutase (SOD-2) were observed by western blotting, and the levels of interleukin-1beta (IL-1β) in culture supernatants were quantified using an ELISA kit. ***Results:*** Aβ induced microglia activation and mitochondrial dysfunction. In addition, mitochondrial dysfunction was associated with ROS accumulation and activation of the NLRP3 inflammasome. Importantly, Aβ induced activation of the NLRP3 inflammasome, leading to caspase-1 activation and IL-1β release in microglia. Moreover, EDA obviously attenuated the depolarization of Δψm, reduced mitochondria-derived ROS production and increased SOD-2 activity, resulting in the suppression of NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. ***Conclusion:*** EDA is a mitochondria-targeted antioxidant and exhibits anti-inflammatory effects on Aβ-treated microglia.

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Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by neuronal degeneration, intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques composed of accumulated amyloid-β (Aβ) peptides [1-4]. Although the precise etiology of AD remains unknown, accumulating evidence indicates that neuroinflammation is involved in the pathogenesis of AD [1, 3, 5]. Microglial activation is a pathological feature in patients with AD. Activated microglia are capable of releasing proinflammatory molecules, such as tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), reactive oxygen species (ROS), and nitric oxide (NO), which contribute to the progression of AD. Additionally, oxidative stress has also been shown to be involved in the pathogenesis of AD [6]. Oxidative stress contributes to neuronal damage and cognitive deficits in patients with AD. The mitochondria are the major source of ROS in the cell under physiological conditions. Meanwhile, mitochondria contain antioxidant enzyme systems that are sufficient to neutralize ROS. Manganese superoxide dismutase (SOD-2) is exclusively located in the mitochondrial matrix and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, which are further detoxified by catalase or glutathione peroxidase. In addition, SOD-2 expression is decreased in mature APP/PS1 neurons [7]. Moreover, SOD-2 activity is significantly diminished in the brains of patients with AD and mouse models of AD [8-10]. SOD-2 overexpression has recently been shown to prevent the Aβ-induced death of cultured neuronal cells. SOD-2 overexpression has previously been shown to alleviate the amyloid plaque burden, reduce oxidative stress, and improve memory deficits in mouse models of AD [11, 12]. In addition, mitochondrial dysfunction is increasingly recognized as one of the early pathological features in the brains of patients with AD. Abnormalities in mitochondrial function, including reduced mitochondrial respiration, decreased $\Delta\psi m$, and enhanced ROS production, are observed in patients with AD and in mouse models of AD. Interestingly, mitochondrial dysfunction is associated with increased ROS production [13]. Based on these data, mitochondrial dysfunction is involved in the pathogenesis of AD.

Additionally, elevated levels of NLRP3 and IL-1β are observed in the brains of patients with AD, indicating that the NLRP3 inflammasome is involved in the etiology of AD. The NLRP3 inflammasome is also activated in patients with AD and NLRP3 inflammasome deficiency decreases Aβ deposition in APP/PS1 mice. Furthermore, activation of the NLRP3 inflammasome has important roles in tissue damage and cognitive dysfunction in patients with AD [1, 14]. The NLRP3 inflammasome is a multiprotein complex consisting of NLRP3, pro-caspase-1, and apoptosis-associated speck-like protein (ASC). The NLRP3 inflammasome is activated by an array of environmental and endogenous molecules. In response to various NLRP3-activating stimuli, NLRP3 recruits ASC and pro-caspase-1, leading to caspase-1 activation and the subsequent secretion of proinflammatory cytokines, including IL-1β and interleukin-18 (IL-18). Importantly, mitochondria-derived ROS play a pivotal role in activating the NLRP3 inflammasome. Therefore, downregulation of mitochondria-derived ROS may attenuate the progression of AD by inhibiting NLRP3 inflammasome activation.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, EDA, Fig. 1), a free radical scavenger, exerts neuroprotective effects on cerebral infarction, traumatic brain injury and AD [15, 16]. EDA has recently been shown to attenuate microglial activation and the release of proinflammatory cytokines *in vitro* and *in vivo* [17, 18]. Based on accumulating evidence, EDA inhibits Aβ-induced oxidative damage and neurotoxicity in PC12 cells and an Aβ-induced rat model of AD [19, 20]. Additionally, in the study by Zhou et al., EDA not only improved cognitive deficits but also reduced oxidative stress in rats that received an intracerebroventricular streptozotocin injection [21]. Furthermore, EDA was recently shown to suppress oxidative stress and the production of proinflammatory cytokines, including interferon-gamma (IFN-γ), TNF-α, IL-1β, and

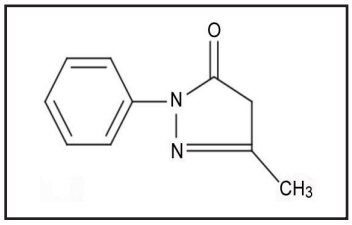

Fig. 1. Chemical structures of edaravone (EDA).

interleukin-6 (IL-6) in APP/PS1 mice. Interestingly, EDA obviously improves cognitive impairments in APP/PS1 mice [22]. However, the mechanism by which EDA inhibits the Aβ-induced proinflammatory response in microglia remains unclear.

The depolarization of the Δψm is associated with increased mitochondrial ROS generation. In the present study, Aβ induced mitochondrial dysfunction, including depolarization of the Δψm and increased mitochondrial ROS generation. In addition, Aβ reduced SOD-2 activity, indicating that Aβ decreased the antioxidant capacity of the mitochondria in microglia. Furthermore, Aβ induced the activation of microglia and the NLRP3 inflammasome, resulting in enhanced caspase-1 activity and IL-1β release. Based on these results, mitochondria-derived ROS played a critical role in activating NLRP3 inflammasome in Aβ-treated microglia. Importantly, mitochondrial dysfunction may contribute to the proinflammatory response by inducing NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. We then tested the ability of EDA to alleviate the inflammatory response in Aβ-treated microglia. EDA attenuated the depolarization of the Δψm, increased SOD-2 activity and reduced mitochondria-derived ROS production, leading to the suppression of NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. Thus, EDA acts as a ROS scavenger.

Based on these results, EDA inhibits the proinflammatory response by downregulating NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. The ability of EDA to inhibit the NLRP3 inflammasome may directly correlate with its ability to increase the Δψm and SOD-2 activity, resulting in reduced mitochondria-derived ROS production.

Materials and Methods

Antibodies and Reagents

Dulbecco's Modified Eagle Medium (DMEM), Neurobasal medium, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). Lipopolysaccharide (LPS), human $A\beta_{1-42}$, the lactate dehydrogenase (LDH) toxicity assay and mouse anti-β-actin primary antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). The rabbit anti-SOD-2 and rabbit anti-NLRP3 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The mouse anti-CD11b, rabbit anti-caspase-1, secondary anti-mouse and anti-rabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-VDAC1 antibody was purchased from Abcam (Cambridge, MA, USA). CM-H2DCFDA, MitoSOX™ Red and 5, 5′,6, 6′-tetrachloro-1, 1′,3, 3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR, USA). Edaravone was obtained from Simcere Pharmaceuticals (Nanjing, Jiangsu, China).

Preparation of Aβ oligomers

Aβ was prepared and aggregated into oligomers according to a published protocol [23]. The Aβ peptide was initially dissolved in hexafluoroisopropanol to a concentration of 1 mM and the solvent was evaporated. For oligomer preparation, the peptide film was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 5 mM and then diluted in F12 medium to a concentration of 100 μM. After a 24 h incubation at 4°C, the preparation was centrifuged at 14, 000 g for 10 min at 4°C to eliminate any formed fibrils and the supernatant containing soluble Aβ oligomers was used for experiments.

Primary microglia cultures

Primary microglia cultures were isolated from postnatal day 1 C57BL/6J mice and cultured as previously described [24]. The brains were removed, and cortices were freed from meninges and blood vessels. Cortices were then digested with 0.25% Trypsin/EDTA at 37°C. Cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The culture medium was changed every 3 days. After 14 days, microglia were selectively harvested in culture medium by shaking the flasks. The purity of microglia cultures was greater than 95%, as determined by CD11b staining. Unless indicated otherwise, microglia were primed with 500 ng/ml LPS for 4 h, washed three times and then exposed to different treatments [25, 26]. All animal experiments were reviewed and approved by the Institutional Animal Use and Care Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital.

Preparation of microglia-conditioned medium

LPS-primed microglia were incubated with Aβ or Aβ+EDA for 24 h, washed with PBS, and cultured in fresh medium for an additional 24 h. Then, the conditioned medium was collected and centrifuged to remove cells and debris. Medium from LPS-primed microglia was used as a control. Microglia-conditioned medium was added to Neurobasal medium to obtain a final ratio of 1:1.

Primary cortical neuron cultures

Primary cortical neurons were prepared from E16 mouse embryos (C57BL/6J mice) as previously described [27]. Cells were grown in Neurobasal medium supplemented with 2% B27 and GlutaMAX. After 3 days *in vitro*, cortical neurons were exposed to microglia-conditioned medium for 24 h.

Caspase-1 assay

Caspase-1 activity was measured using the Caspase-1 Colorimetric Assay Kit from BioVision (Milpitas, CA, USA). Each experiment was conducted in triplicate.

Detection of IL-1β levels in the cell supernatant

IL-1β levels in culture supernatants were quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Assays were performed in triplicate.

Measurement of the mitochondrial membrane potential (Δψm)

The Δψm was evaluated using JC-1 staining. Cells were incubated with 10 μg/ml JC-1 for 15 min at 37°C and images were captured with a fluorescence microscope. The ratio of JC-1 aggregates (red fluorescence) to monomers (green fluorescence) was measured. Mitochondrial depolarization was indicated by a decrease in the ratio of the red/green fluorescence intensity.

Measurement of intracellular ROS levels

Cells were incubated with 10 μM CM-H2DCFDA for 30 min. Intracellular ROS levels were then detected using a fluorescence spectrometer. Each experiment was conducted in triplicate.

Detection of mitochondria-derived ROS levels

Mitochondrial ROS levels were detected using MitoSOX™ Red. Cells were stained with 5 μM MitoSOX™ Red for 10 min and the fluorescence intensity was measured using a fluorescence spectrometer with an excitation wavelength of 510 nm and an emission wavelength of 580 nm. Each experiment was conducted in triplicate.

Malondialdehyde (MDA) levels and Superoxide Dismutase (SOD) activity

Levels of MDA, a sensitive indicator of lipid peroxidation, were measured using a commercial assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's manual. SOD-2 activity was determined using the Superoxide Dismutase Assay Kit from Jiancheng Bioengineering Institute. Each sample was tested in triplicate.

MTT assay

Cell viability was assessed using MTT assay as previously described [28, 29]. Briefly, primary cortical neurons were treated with microglia-conditioned medium in a 96-well plate for 24 h. MTT reagent (0.5 mg/ml) was then added to each well. After a 4 h incubation at 37°C, the formazan crystals were dissolved in DMSO and the absorbance was recorded at 570 nm using a microplate reader.

Lactate dehydrogenase (LDH) Assay

LDH levels in cell supernatants were measured using a commercial LDH detection kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol.

Preparation of mitochondrial fractions

Mitochondrial fractions were isolated by differential centrifugation using a mitochondria isolation kit (Beyotime, Jiangsu, China). The protein concentration was determined using a BCA Protein Assay kit. Aliquots were stored at -80°C until SOD-2 expression was analyzed.

Western blots analysis

After treatment, cells were harvested and lysed in lysis buffer containing a protease inhibitor cocktail. Equivalent amounts of protein were electrophoresed on 10% SDS–PAGE gels and transferred to PVDF membranes. Membranes were blocked with TBST buffer containing 5% nonfat milk and then incubated with the primary antibody overnight at 4°C. After washing, the membrane was subsequently incubated with a secondary antibody for 1 h at room temperature and examined using an ECL detection reagent. β-actin was used as a loading control.

Statistical analysis

Results are presented as the means ± SEM from at least three independent experiments. When appropriate, the Mann-Whitney U test was used to compare quantitative variables between two groups (JC-1 staining, caspase-1 activity, IL-1β level, SOD-2 activity, MDA level, and LDH release). Otherwise, statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. A P value <0.05 was considered statistically significant.

Results

Aβ reduces the mitochondrial membrane potential and increases ROS levels in microglia

Mitochondrial dysfunction and oxidative stress have been reported to contribute to progressive neurodegeneration in patients with AD. Moreover, Aβ peptides have been shown to trigger mitochondrial depolarization, an indicator of mitochondrial dysfunction. Therefore, we first evaluated the effects of Aβ on the Δψm in LPS-primed microglia. The Δψm was monitored by JC-1 staining, and a loss of the Δψm was indicated by a reduction in the ratio of red/green fluorescence intensity. Treatment with 5-10 μM Aβ dose-dependently reduced the Δψm in microglia (Fig. 2A and B), whereas 1 μM Aβ did not alter the Δψm in microglia.

We next investigated the effects of Aβ on intracellular and mitochondrial ROS levels in LPS-primed microglia. Intracellular ROS production was detected using a fluorescent

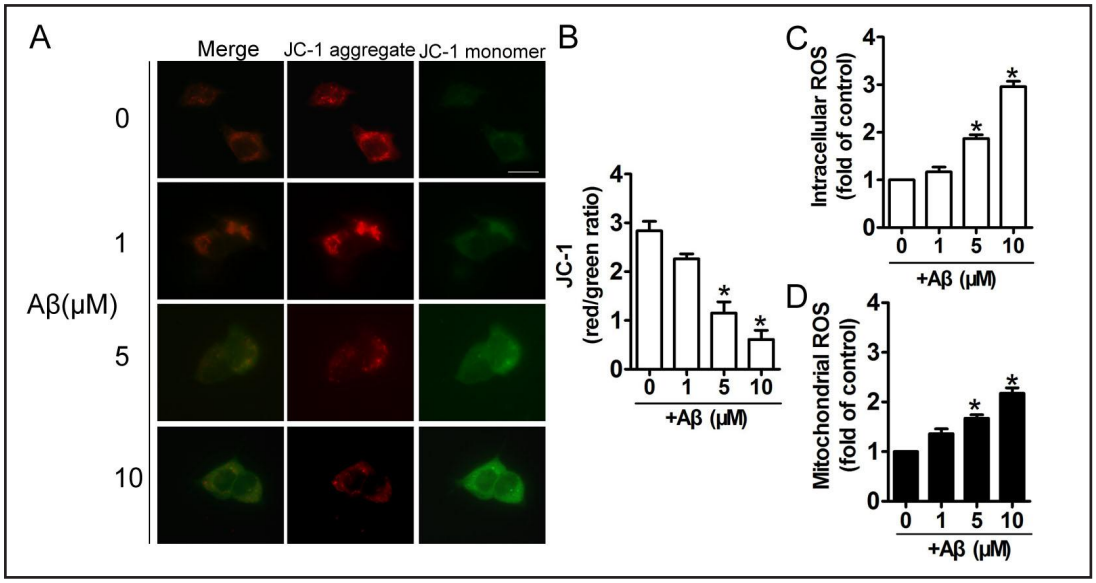

Fig. 2. Aβ induces mitochondrial dysfunction in microglia. (A)LPS-primed microglia were untreated or treated with Aβ (1-10 μM) for 24 h, the mitochondrial membrane potential was evaluated using JC-1 staining. Scale bar, 25 μm. (B)The results shown are representative of three independent experiments, *P<0.05 compared with untreated group. (C and D) Intracellular ROS (C) and mitochondrial ROS levels (D) were measured using CM-H2DCFDA and MitoSOX™ Red, respectively. The results shown are representative of three independent experiments, *P<0.05 compared with untreated group.

H2DCFDA assay, whereas mitochondrial ROS production was measured using MitoSOX™ Red staining. Interestingly, treatment with 5-10 μM Aβ dose-dependently increased intracellular and mitochondrial ROS levels (Fig. 2C and D). However, the low-dose Aβ treatment (1 μM) failed to alter the ROS levels in microglia.

Mitochondrial ROS promote NLRP3 inflammasome activation and IL-1β secretion in Aβ-treated microglia

Aβ is generally accepted to induce microglial activation and the release of proinflammatory molecules, leading to neuronal death and synaptic damage, which are critical for the ongoing process of neurodegeneration in patients with AD. In addition, Aβ has been shown to activate the NLRP3 inflammasome in microglia. Caspase-1 expression is also increased in the brains of patients with AD [30]. Notably, increased IL-1β expression is observed in activated microglia surrounding Aβ plaques in patients with AD and in mouse models of AD. Furthermore, Aβ has been consistently shown to trigger IL-1β secretion *in vitro* and *in vivo* [1, 24]. Importantly, the activation of the NLRP3 inflammasome is a critical upstream regulator of caspase-1 activation, which results in the maturation and secretion of IL-1β [31]. Therefore, we next investigated NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. The IL-1β precursor (pro-IL-1β) is not constitutively expressed in microglia and requires a priming signal to induce the transcription and synthesis of pro-IL-1β. Therefore, microglia were primed with 500 ng/ml LPS for 4 h and then exposed to different treatments. Consistent with the results from other studies [32, 33], IL-1β was detected in culture supernatants from LPS-primed microglia. LPS-primed microglia were untreated or incubated with Aβ (1-10 μM) for 24 h. As shown in Fig. 3A, the 5-10 μM Aβ treatments increased the levels of CD11b (a microglial activation marker) and NLRP3 in a dose-dependent manner. However, the low-dose Aβ treatment (1 μM) failed to increase the CD11b and NLRP3 levels. Consistent with the CD11b and NLRP3 levels, caspase-1 activity and IL-1β secretion were increased in the presence of 5-10 μM Aβ (Fig. 3B and C). Although Aβ obviously increased caspase-1 activity, it did not alter pro-caspase-1 expression. Based

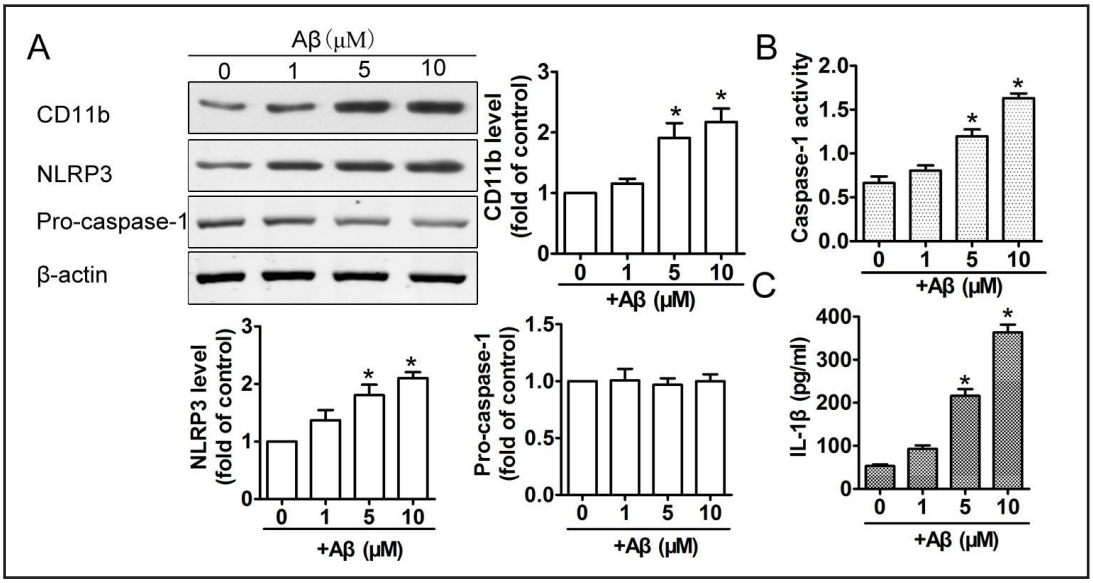

Fig. 3. Aβ activates microglia and increases level of NLRP3, caspase-1 activity and IL-1β secretion. LPS-primed microglia were untreated or treated with Aβ (1-10 μM) for 24 h, the levels of CD11b, NLRP3 and pro-caspase-1 were observed by western blotting. Band density values of CD11b, NLRP3 and pro-caspase-1 were normalized to β-actin and expressed as a ratio to the untreated microglia. Values represent means ± SEM from three independent experiments, *P<0.05 compared with untreated group. (B and C) Caspase-1 activity (B) and IL-1β levels (C) were assessed by ELISA. The results shown are representative of three independent experiments, *P<0.05 compared with untreated group.

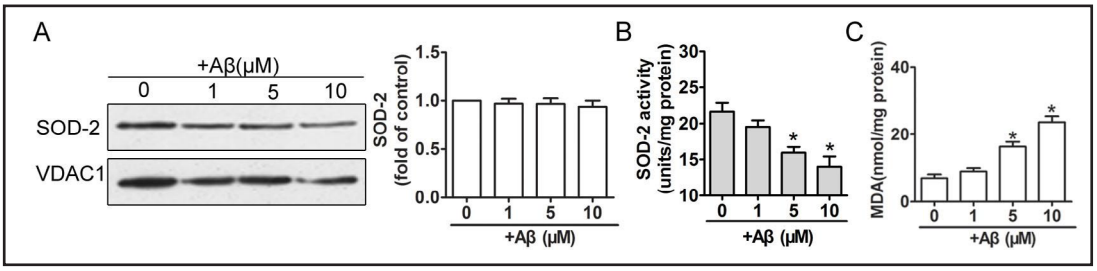

Fig. 4. Aβ reduces SOD-2 activity and enhances MDA levels in microglia. LPS-primed microglia were untreated or treated with Aβ (1-10 μM) for 24 h, SOD-2 expression was observed by western blotting. Band density value of SOD-2 was normalized to VDAC1 and expressed as a ratio to the untreated microglia. VDAC1 was used as a loading control for mitochondrial fractions. Values represent means ± SEM from three independent experiments, *P<0.05 compared with untreated group. (B) SOD-2 activity was determined by ELISA. The results shown are representative of three independent experiments, *P<0.05 compared with untreated group. (C) MDA levels were tested by ELISA. The results shown are representative of three independent experiments,*P<0.05 compared with untreated group.

on these data, the activation of the NLRP3 inflammasome is required for caspase-1 activation and subsequent IL-1β secretion in Aβ-treated microglia.

SOD-2 activity is decreased in Aβ-treated microglia

Based on our results, Aβ increased mitochondria-derived ROS generation in microglia. Oxidative stress has been shown to play a critical role in the pathogenesis of AD. Although the mitochondria are the main source of ROS in the cell, SOD-2, a primary antioxidant enzyme, is located in the mitochondria and catalyzes the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen. Moreover, in the study by Massaad et al., SOD-2 overexpression in a mouse model of AD decreased amyloid plaque deposition and prevented memory deficits [12]; however, a consensus regarding SOD-2 protein expression and SOD-2 activity in patients with AD and transgenic mice has not been reached. The expression of the SOD-2 protein was recently shown to be increased in patients with AD and mouse models of AD [34, 35], whereas the expression of the SOD-2 protein expression was not changed in wild-type (WT) and APP/PS1 transgenic mice in another study [8]. Nevertheless, SOD-2 activity is significantly decreased in patients with AD and APP/PS-1 mice [8, 35]. Therefore, we next evaluated SOD-2 expression and SOD-2 activity in LPS-primed microglia after the Aβ treatment. As shown in Fig. 4A, SOD-2 expression was not altered in the presence of 5-10 μM Aβ. However, the 5-10 μM Aβ treatments remarkably decreased SOD-2 activity in microglia (Fig. 4B). Moreover, the low-dose Aβ treatment (1 μM) had no effect on SOD-2 activity in microglia. Additionally, lipid peroxidation is used as an indicator of oxidative stress in cells and MDA, a marker of lipid peroxidation, is elevated in the brains of patients with AD. Thus, we also observed the MDA levels in Aβ-treated microglia. Consistent with the SOD-2 activity data, MDA levels were significantly increased in the presence of 5-10 μM Aβ (Fig. 4C). However, the low-dose Aβ treatment (1 μM) did not increase the MDA levels. Thus, Aβ increases oxidative stress in microglia.

EDA reduces mitochondrial depolarization and ROS levels in Aβ-treated microglia

Aβ induced mitochondrial depolarization and increased mitochondria-derived ROS production in microglia in the present study. In addition, EDA, a ROS scavenger, inhibits the release of proinflammatory cytokines from activated microglia [18]. Hence, we hypothesized that EDA inhibits mitochondrial depolarization and reduces ROS production in Aβ-treated microglia. We first observed the effect of EDA on the Δψm in LPS-primed microglia after the Aβ treatment. As shown in Fig. 5A and B, EDA obviously inhibited the Aβ-induced mitochondrial depolarization. We subsequently investigated the effects of EDA on intracellular and mitochondrial ROS levels in LPS-primed microglia after the Aβ treatment. EDA strongly

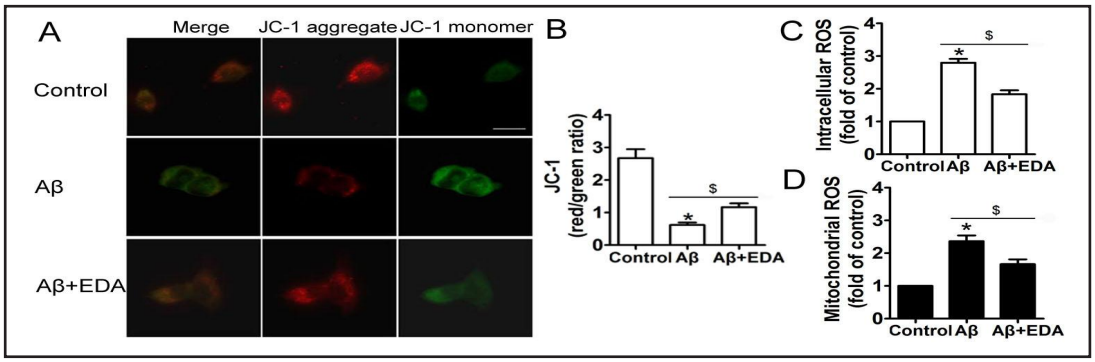

Fig. 5. EDA inhibits mitochondrial dysfunction in Aβ-treated microglia. LPS-primed microglia were untreated or treated with 10 μM Aβ for 24 h or pretreated with EDA for 30 min and then exposed to 10 μM Aβ for 24 h, the mitochondrial membrane potential was evaluated using JC-1 staining. Scale bar, 25 μm. (B) The results shown are representative of three independent experiments, *P<0.05 compared with control; $P<0.05 compared with Aβ-treated group. (C and D) Intracellular ROS (C) and mitochondrial ROS levels (D) were measured using CM-H2DCFDA and MitoSOX™ Red, respectively. The results shown are representative of three independent experiments, *P<0.05 compared with control; $P<0.05 compared with Aβ-treated group.

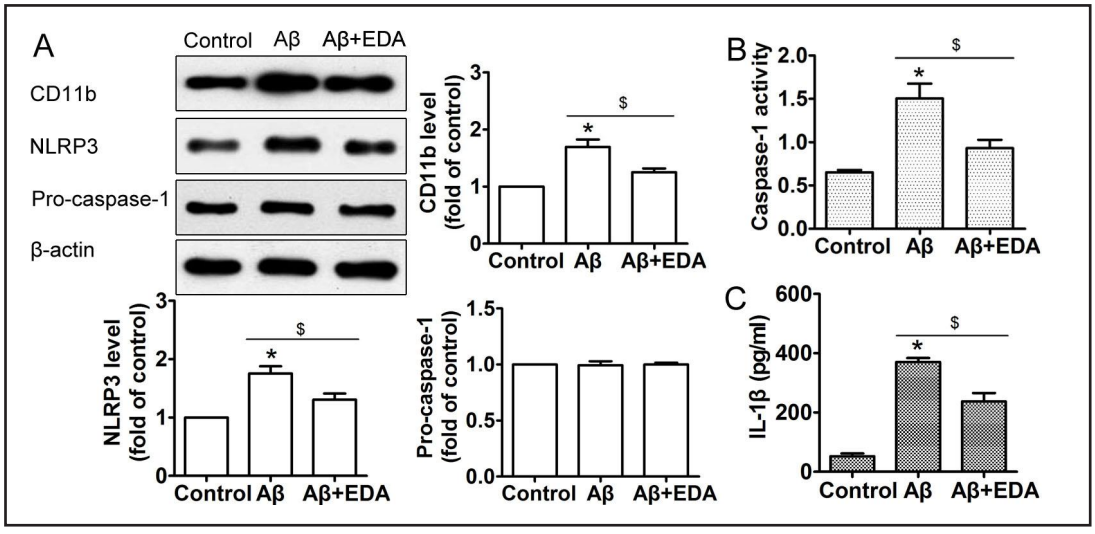

Fig. 6. EDA attenuats level of NLRP3, caspase-1 activity and IL-1β secretion in Aβ-treated microglia. (A) LPS-primed microglia were untreated or treated with 10 μM Aβ for 24 h or pretreated with EDA for 30 min and then exposed to 10 μM Aβ for 24 h. The levels of CD11b, NLRP3 and pro-caspase1 were observed by western blotting. Band density values of CD11b, NLRP3 and pro-caspase1 were normalized to β-actin and expressed as a ratio to the untreated microglia. Values represent means ± SEM from three independent experiments,*P<0.05 compared with control; $P<0.05 compared with Aβ-treated group. (B and C) Caspase-1 activity (B) and IL-1β levels (C) were determined by ELISA. The results shown are representative of three independent experiments, *P<0.05 compared with control; $ P<0.05 compared with Aβ-treated group.

suppressed the Aβ-induced increase in the intracellular and mitochondrial ROS levels (Fig. 5C and D). Taken together, these data support the hypothesis that EDA is a ROS scavenger.

EDA reduces NLRP3-mediated IL-1β secretion in Aβ-treated microglia

EDA has been shown to suppress microglial activation. On the other hand, the inhibition of mitochondria-derived ROS production abolishes NLRP3 inflammasome activation and

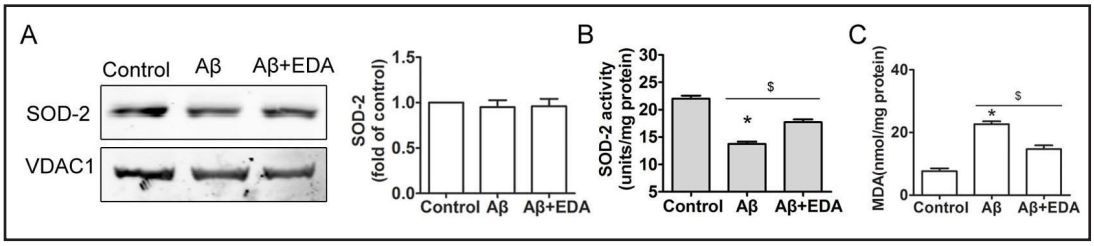

Fig. 7. EDA increases SOD-2 activity and reduces MDA levels in Aβ-treated microglia. LPS-primed microglia were untreated or treated with 10 μM Aβ for 24 h or pretreated with EDA for 30 min and then exposed to 10 μM Aβ for 24 h. SOD-2 expression was observed by western blotting. Band density value of SOD-2 was normalized to VDAC1 and expressed as a ratio to the untreated microglia. VDAC1 was used as a loading control for mitochondrial fractions. Values represent means ± SEM from three independent experiments. (B) SOD-2 activity was determined by ELISA. The results shown are representative of three independent experiments, *P<0.05 compared with control; $P<0.05 compared with Aβ-treated group. (C) MDA levels were tested by ELISA. The results shown are representative of three independent experiments, *P<0.05 compared with control; $P<0.05 compared with Aβ-treated group.

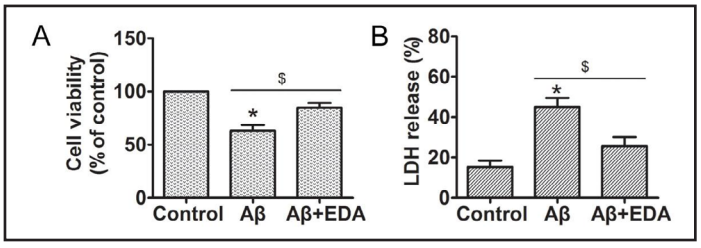

Fig. 8. EDA protects neurons from the toxic effects of conditioned media from Aβ-treated microglia. LPS-primed microglia were untreated or treated with 10μM Aβ for 24 h or pretreated with EDA for 30 min and then exposed to 10μM Aβ for 24 h. Microglia were washed with sterile PBS and then incubated with fresh medium for another 24 h. Culture supernatants from untreated, Aβ-treated or Aβ plus EDA-treated microglia were added to primary cortical neurons for 24 h, then neuronal viability and cell death were measured by MTT assay (A) and LDH release (B), respectively. The results shown are representative of three independent experiments, *P<0.05 compared with control; $P<0.05 compared with Aβ-treated group.

NLRP3-mediated IL-1β secretion. Moreover, in our study, EDA reduced the mitochondrial ROS levels in Aβ-treated microglia. Therefore, we observed the effects of EDA on CD11b, NLRP3 and pro-caspase-1 levels in LPS-primed microglia after the Aβ treatment. As shown in Fig. 6A, EDA suppressed the levels of CD11b and NLRP3, but it did not affect pro-caspase-1 expression in Aβ-treated cells. Similarly, EDA obviously attenuated the Aβ-induced increase in caspase-1 activity and IL-1β secretion (Fig. 6B and C).

EDA alters SOD-2 activity and MDA levels in Aβ-treated microglia

We next examined the potential role of EDA in regulating SOD-2 activity in LPS-primed microglia after the Aβ treatment. As shown in Fig. 7A, although EDA did not alter SOD-2 expression, it obviously increased SOD-2 activity in the presence of Aβ (Fig. 7B). Similarly, EDA strongly inhibited the Aβ-induced increase in the MDA levels (Fig. 7C).

EDA protects neurons from the toxic effects of conditioned media from Aβ-treated microglia

Activated microglia are known to exert toxic effects on neurons by inducing the release of proinflammatory molecules. We further examined whether EDA protected neurons from the toxic effects of conditioned media from Aβ-treated microglia. Culture supernatants from untreated, Aβ-treated or Aβ plus EDA-treated microglia were added to primary cortical neurons for 24 h. Cell viability and cell death were then assessed using the MTT assay and LDH release, respectively. Exposure to the conditioned medium from Aβ-treated microglia significantly reduced neuronal viability, which was inhibited by EDA (Fig. 8A). Similarly, EDA

obviously reduced neuronal death induced by the conditioned medium from Aβ-treated microglia (Fig. 8B).

In summary, Aβ induces the accumulation of mitochondrial ROS, ultimately leading to NLRP3-mediated caspase-1 activation and IL-1β secretion in microglia (Fig. 9). Our findings support the hypothesis that mitochondria-derived ROS are involved in Aβ-induced NLRP3 inflammasome activation. Importantly, EDA exerts an anti-inflammatory effect on Aβ-treated microglia by reducing ROS production.

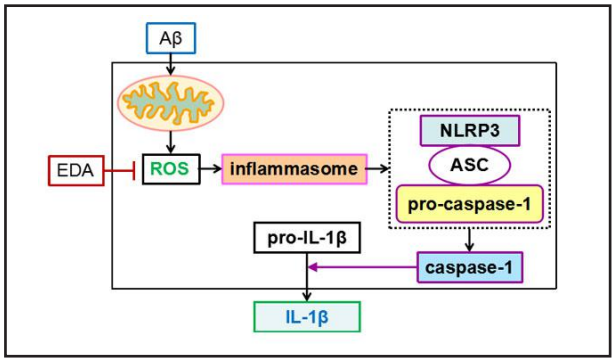

Fig. 9. Mitochondrial ROS is responsible for NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia.

Discussion

Microglia, the resident macrophages of the central nervous system (CNS), are activated in response to injury, neurotoxins, or immunological stimuli. Microglia are key regulators of neuroinflammation in the CNS. In the brains of patients with AD, Aβ plaques are surrounded by activated microglia, and the persistent activation of microglia enhances neurodegenerative processes via oxidative stress and the release of proinflammatory mediators. Moreover, mitochondrial dysfunction has been observed in patients with AD [11, 12]. Indeed, mitochondrial dysfunction occurs in patients with early AD. Altered mitochondrial function, including altered mitochondrial properties, reduced adenosine triphosphate (ATP) synthesis, decreased mitochondrial respiration and increased ROS production, contribute to the progressive neurodegeneration in patients with AD. Additionally, a decrease in the Δψm, another indicator of mitochondrial dysfunction, is detected in the brains of patients with AD and mouse models of AD. Although mitochondrial dysfunction and neuroinflammation are involved in the pathogenesis of AD, the precise relationship between Aβ-induced mitochondrial dysfunction and neuroinflammation in microglia remains elusive.

We examined alterations in the Δψm using JC-1 staining to observe the effects of Aβ on mitochondrial function. Aβ exposure diminished the Δψm, which was confirmed by increased green fluorescence and decreased red florescence. In addition, a loss of the Δψm increases the ROS levels in the mitochondrial matrix. Therefore, we next investigated intracellular and mitochondrial ROS levels in cells treated with Aβ. Consistent with the Δψm loss, Aβ exposure increased both intracellular and mitochondrial ROS levels in microglia. Based on these results, Aβ induced mitochondrial dysfunction, which was confirmed by the loss of the Δψm and increased mitochondrial ROS generation. Excessive ROS production damages proteins, lipids, and nucleic acids, contributing to the progression of AD. However, mitochondria are equipped with a variety of antioxidant enzyme systems that are sufficient to eliminate excessive ROS production. SOD-2 is a major mitochondrial ROS scavenging enzyme. In addition, Aβ has been shown to enter the mitochondria and inhibit SOD-2 activity, resulting in increased superoxide levels [8]. Therefore, we subsequently observed the effects of Aβ on SOD-2 expression and SOD-2 activity in microglia. SOD-2 expression was not altered by the Aβ treatment, whereas SOD-2 activity was reduced by the addition of Aβ, indicating that the antioxidant capacity of the mitochondria is impaired by Aβ treatment. Similarly, Aβ significantly increased the levels of the oxidative damage marker MDA, suggesting that Aβ induces oxidative stress in microglia. Based on our findings, the Aβ-induced accumulation of intracellular and mitochondrial ROS might be associated with mitochondrial depolarization and reduced SOD-2 activity.

Moreover, the NLRP3 inflammasome plays a role in the pathogenesis of AD [1, 14, 25, 26]. Upon activation, NLRP3 interacts with pro-caspase-1 through ASC, which then

activates caspase-1. Active caspase-1 then cleaves pro-IL-1β into mature IL-1β. The NLRP3 inflammasome is activated in patients with AD and increased amounts of cleaved caspase-1 are detected in the brains of patients with AD. Similarly, increased IL-1β expression has been observed in the microglia surrounding Aβ plaques in patients with AD and in animal models of AD. Additionally, Aβ-induced mitochondrial dysfunction plays an important role in the pathogenesis of AD and mitochondrial ROS are required for NLRP3 inflammasome activation. Studies clarifying the precise relationship between Aβ-induced mitochondrial dysfunction and the proinflammatory response may provide a potential therapeutic strategy for AD.

Therefore, we detected levels of the NLRP3 and its downstream targets in cells treated with Aβ. Aβ triggered formation of the NLRP3 inflammasome and caspase-1 activation, leading to the release of IL-1β in microglia. Thus, NLRP3 inflammasome-mediated IL-1β secretion played a crucial role in the proinflammatory response induced by Aβ. In addition to the activation of the NLRP3 inflammasome, Aβ increased the CD11b level, indicating that Aβ induced the activation of microglia, which strongly supports the hypothesis that activated microglia produce proinflammatory molecules in response to neurotoxins. Importantly, mitochondrial dysfunction is associated with ROS accumulation and the activation of the NLRP3 inflammasome, ultimately resulting in caspase-1 activation and IL-1β secretion. Based on these data, mitochondrial ROS-mediated activation of the NLRP3 inflammasome is required for caspase-1 activation and IL-1β secretion in Aβ-treated microglia.

EDA, a ROS scavenger, is widely used to treat ischemic cerebrovascular disease. In addition, EDA has been reported to reduce amyloid deposition, improve spatial learning and memory, and decrease oxidative injury in APP transgenic mice. Moreover, EDA has recently been shown to protect neurons against Aβ-induced cell death, indicating that EDA is a promising therapeutic drug for AD. However, the mechanism by which EDA inhibits Aβ-induced ROS production and the inflammatory response in microglia remains unclear.

In the present study, EDA not only prevented Aβ-induced mitochondrial depolarization but also increased the Aβ-induced reduction in SOD-2 activity, leading to a reduction of ROS generation and suppression of NLRP3-mediated IL-1β secretion. Consistent with these findings, EDA obviously reduced the levels of the oxidative damage marker MDA in Aβ-treated microglia. Based on these data, the inhibition of mitochondrial ROS by EDA may directly correlate with its ability to increase SOD-2 activity, and EDA exerts its anti-inflammatory effect by inhibiting NLRP3-mediated IL-1β secretion in Aβ-treated microglia. Indeed, EDA suppresses proinflammatory cascades by inhibiting ROS-mediated NLRP3 inflammasome activation. On the other hand, EDA protects against mitochondrial dysfunction. In addition to suppressing IL-1β production, EDA drastically inhibits the neurotoxicity of conditioned medium from Aβ-treated microglia. Thus, EDA indirectly decreases neuronal loss by inhibiting microglial activation and proinflammatory cytokine secretion.

In conclusion, mitochondrial ROS are responsible for NLRP3 inflammasome-mediated IL-1β secretion in Aβ-treated microglia. Additionally, EDA acts as a ROS scavenger at least partially by upregulating SOD-2 activity, resulting in the suppression of NLRP3 inflammasome activation. Importantly, EDA is a mitochondria-targeted antioxidant and exerts anti-inflammatory effects on Aβ-treated microglia.

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Disclosure Statement

The authors declare no Disclosure Statement.

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