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Yoda1 Activates Piezo1 in Vitro to Simulate the Upregulation of Piezo1 in the Infected Brain: Piezo1 Participates in the Immune Activation of Microglia.

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Abstract

Aim: The expression of Piezo1 in reactive glial cells in the peripherally infected patient's brain was upregulated. This study aimed to determine whether Piezo1 is involved in the immune activation of microglial cells induced by bacterial lipopolysaccharides.

Materials and methods: BV2 cells were used as a model of brain microglia. In vitro, Yoda1 was used to activate Piezo1 in BV2 cells, and Piezo1 was simulated for LPS-induced Piezo1 activation to evaluate the role of Piezo1 in microglial inflammatory activation.

Key findings: In vitro, LPS upregulates the expression of Piezo1 in microglial cells through TLR4. In the absence of LPS, Yoda1 treatment of microglia produced similar immune function changes as LPS treatment. This indicates that Piezo1 plays a role in LPS-induced microglial immune activation. Specifically, Piezo1-mediated Ca^{2+} signals are involved in the immune activation of microglia. Piezo1-mediated Ca^{2+} regulates multiple signaling mechanisms downstream of TLR4**,** including the JNK1, mTOR and NF-κB signaling pathways, which are related to the immune activation of microglia.

Significance: Piezo1 is involved in the immune response of microglia to LPS. Changes in Piezo1 activity may play an indispensable role in the immune response of microglia, and mechanical environmental changes may affect neuroinflammatory progression through Piezo1.

Introduction

Alzheimer's disease (AD) is mainly manifested by chronic neuroinflammation and progressive cognitive decline[1]. Lipopolysaccharide (LPS) levels in brain samples from patients with advanced AD are elevated, indicating that LPS may be involved in cognitive impairment in AD. Under normal circumstances, the plasma LPS concentration is shallow, and infection will cause the plasma LPS level to rise, which leads to brain inflammation and microglial activation. The systemic inflammatory response induced by a single LPS can trigger memory loss and a process that may lead to long-term neurodegeneration[2]. María Velasco-Estevez et al. [3] found that peripheral infection caused a significant increase in the expression of Piezo1 in the cerebral cortex and hippocampus. The Piezo1 channel is a newly discovered mechanosensitive ion channel that gives cells the ability to convert mechanical signals into intracellular signals[4]. A large amount of evidence supports that Piezo1 is involved in regulating inflammation induced by mechanical stress [5–8]. Piezo1 is mainly located in the plasma membrane, and it has also been reported in the endoplasmic reticulum, cytoplasmic compartment and nuclear envelope near the nucleus[9–13]. Mechanical stimulation activates Piezo1 channel opening and induces the cytoplasmic Ca^{2+} peak. Ca^{2+} is the second messenger in the cell and plays an essential role in regulating cell function[14]. Yoda1 is a new class of synthetic small molecule compounds that can specifically activate the Piezo1 channel. Yoda1 at micromolar concentrations induces a strong Ca^{2+} response in cells transfected with human or mouse Piezo1[15].

Piezo1 was first discovered to play a role in astrocytes in the center of infection. The expression of Piezo1 was enhanced in primary mouse cortical astrocytes and BV2 microglia exposed to LPS in vitro, indicating that mechanical stimulation is not the only means of Piezo1 activation[16]. The migration of astrocytes stimulated by LPS exposure to Yoda1 was much slower than that of reactive astrocytes treated with GsMTx4. Additionally, Yoda1 reduced the release of IL-1β, TNFα and CX3CL1 from astrocytes stimulated by LPS. In an inflammatory state, Piezo1 may potentially regulate the signal transduction of reactive astrocytes, thereby affecting the phenotype of astrocytes [16].

Microglia are the innate immune cells of the brain. They mediate the brain's natural immune response to injury, inflammation and neurodegenerative diseases. LPS mainly induces inflammation by activating TLR4 on the microglial surface, which leads to the transcriptional activation of hundreds of inflammatory genes, including proinflammatory cytokines such as TNFα IL-6 and IL-1β[17–19]. In addition to its role in disease, microglia are also involved in developing neuronal networks and maintaining brain tissue homeostasis. Increasing evidence shows that the executive function of microglia is coupled with intracellular Ca^{2+} signaling. For example, the increase in Ca^{2+} is related to the release of proinflammatory and anti-inflammatory cytokines, nitric oxide or nutritional factors[20–23]. The role of Piezo1 in LPS immune activation of microglia is not exact. Therefore, we investigated the effect of Piezo1 on the immune activation of microglia. Specifically, Piezo1-mediated Ca^{2+} may play an essential role in the immune activation of microglia.

Materials And Method

Reagents and antibodies

LPS (L2630), Yoda1 (SML2397) and TAK-242 (614316) were purchased from Sigma-Aldrich. ELISA kits including TNFα (DY410), IL-1β (DY401), IL-6 (DY406), IL-1β (70-EK981-96), and IL-10 (70-EK210/4-96) were purchased from MULTI Science (Suzhou, China), and TGFα (MU30305) was purchased from Bioswamp (Wuhan, China). TLR4 antibody (bs-20595R) and NF-κB p65 antibody (BSM-33117 M) were purchased from Bioss. PIEZO1 antibody (NBP1-78537) was purchased from Novus Biologicals. MTOR antibody (66888-1-LG) and JNK1 antibody (66210-1-LG) were purchased from Proteintech. Phospho-mTOR (Ser2448) antibody (AF3308) was purchased from Affinity Biosciences. P-JNK1 (Thr183) antibody was purchased from ImmunoWay Biotechnology. GAPDH antibody (ab8245) and phospho-NF-κB p65 (s536) (ab28856) were purchased from Abcam. A Ca^{2+} fluorescence detection kit (F3015) was purchased from US EVERBRIGHT INC. Unless otherwise stated, other commercially available reagents are from Sigma-Aldrich.

Cell culture

BV2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). The cells were cultured in DMEM (Gibco, USA) high glucose medium containing 10% fetal bovine serum plus 100 U/mL streptomycin in an incubator at 37°C and 5% CO_2.

Cell proliferation assay

BV2 cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and cultured overnight; then, the old medium was replaced with fresh medium containing Yoda1 or LPS. After 24 hours, CCK-8 was used to test cell proliferation according to the manufacturer's instructions.

Cell wound healing assay

The cells grow to confluence in 6-well plates. At 0 hours, a 2 mm scraping wound was created with a pipette tip, and dead cells and floating cells were washed out with PBS. The remaining cells were cultured continuously for 24 hours in complete medium containing LPS or Yoda1. The progress of cell migration was recorded at 0 and 24 hours, and the distance between the two edges of the scratch was measured and calculated.

Real-time quantitative PCR

According to the manufacturer's instructions, total RNA was extracted from BV2 cells using TRIzol reagent (Invitrogen, Carlsbad, California, USA). The PrimeScript™ RT kit (TaKaRa, Tokyo) was used for reverse transcription. SYBR Select Master Mix for CFX (Invitrogen) and CFX Connect Real-time PCR system (BioRad) were used to perform quantitative real-time PCR (qRT-PCR) at 95℃ for 15 s, and then 40 cycles at 95℃ for 5 s, Lasting 34 s at 60℃. The primer sequences are shown in Table S1. GAPDH is used as an internal reference gene. Each test was performed in triplicate, and the data were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Elisa

BV2 cells were treated with Yoda1 and LPS for 24 hours, and the supernatant was collected. A commercially available ELISA kit was used to quantitatively detect the protein content of TNFα, IL-1β, IL-6, TGFα, TGFβ, and IL-10. Perform according to the instruction method, and finally read the absorbance at 450 nm on a microplate reader (PerkinElmer, USA), and the wavelength is corrected at 570 nm.

Western blot analysis

Total protein was extracted from cells by RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein sample (20 μg) was applied to a 10% gel and separated by SDS-PAGE. The isolated protein was transferred to a PVDF membrane by electrophoresis. The blot was blocked with 5% skim milk in TBS with Tween-20 at room temperature for 1 hour and then incubated with the primary antibody to assess protein levels. GAPDH was used as an internal control. The secondary antibody was used to detect the blot, and the Odyssey Fc system (LI-COR Biosciences) was used.

Ca^{2+} fluoroscopy

The precultured cells were removed, the medium was removed, and the cells were washed three times with PBS solution. Fluo-3 AM working solution was added to the cells and incubated at 37°C for 10-60 min. An Olympus IX73 vertical optical microscope (Tokyo, Japan) was used to observe the fluorescence effect; pictures were obtained using a DP73 camera. Ca^{2+} detection was performed on the microplate reader.

Statistical Analysis

SPSS 24.0 software (IBM, USA) was used for statistical analysis, and ImageJ1.52 was used for image analysis. The data are expressed as the mean ± standard deviation. Student's t-test or one-way analysis of variance was used, and then multiple comparison tests were performed to determine significant differences between groups. $P < 0.05$ was considered to be statistically significant.

Result

LPS upregulates Piezo1 expression via TLR4 receptor

First, the reasons for LPS upregulation of Piezo1 were analyzed. TAK-242 is an inhibitor of the TLR4 receptor, and microglia were treated with 1 μM TAK-242 for 24 hours to inhibit TLR4 channel activity (Figure 1A, B). LPS did not upregulate the expression of Piezo1 after using TKA-242 to inhibit the TLR4 channel compared with LPS treatment alone (Figure 1A, C). This indicates that LPS upregulates Piezo1 expression not directly but through TLR4 signal transduction mechanism activation.

Yoda1 simulated Piezo1 activation induced by LPS in vitro.

To investigate the relationship between Piezo1 channel upregulation and microglial cell activation, Yoda1 was used to simulate Piezo1 channel activation in vitro. Yoda1 was dissolved in DMSO, and Bv2 cells were incubated with Yoda1 at 2 μM or 5 μM for 24 hours. DSMO had no significant cytotoxicity to cells (Figure 2A). The expression of Piezo1 was significantly upregulated in both Yoda1 at 2 μM and 5 μM (Figure 2B), and both the Ca^{2+} fluorescence assay (Figure 2C) and the Ca^{2+} content assay (Figure 2D) showed that Yoda1 induced intracellular Ca^{2+} signal enhancement. A concentration of 5 μM was selected for subsequent experiments.

Ca^{2+} mediated by Piezo1 is involved in LPS-induced immune activation of microglial cells.

The effect of Piezo1 on the inflammatory activation of microglial cells was further examined. In vitro, microglia activated by LPS produce morphological, proliferation, migration, and cytokine secretion changes. BV2 microglial cells were stimulated with Yoda1 or LPS for 24 hours, and characteristics related to cellular immune activation were detected. As shown in Figure 3A, both LPS and Yoda1 triggered intracellular Ca^{2+} increases. From the perspective of cell morphology, microglial cells cultured in vitro in the resting state tended to be oblate or spindle-shaped with long antennae extending to the surrounding environment (Figure 3B). After the activation of BV2 cells induced by LPS, the morphology of BV2 cells

showed a significant increase in pseudopodia and protrusions, similar to that of amoebae (Figure 3B). Yoda1 treatment had no significant effect on cell morphology (Figure 3B). Compared with the untreated group, both LPS- and Yoda1-treated BV2 cells significantly increased the number of cells (Figure 3C) and the migration rate (Figure 3D). After the activation of microglia by LPS, the expression of the M1-type inflammatory cytokines TNFα, IL-1β and IL-6 was significantly increased (Figure 3E, F). In contrast, the expression of the M2-type cytokines TGFα, TGFβ and IL-10 was inhibited (Figure 3E, F). Yoda1 significantly increased the mRNA levels of the cytokines IL-6, CD86, TGFα, TGFβ, IL-10, and CD206 (Figure 3E) and increased the protein expression of IL-6, TGFα, TGFβ, and IL-10 (Figure 3F). We speculated that Piezo1-mediated Ca^{2+} is involved in LPS-induced microglial immune activation.

Ca^{2+} mediated by Piezo1 is involved in early signaling in microglial immune activation.

The NF-κB, mTOR and JNK1 signaling pathways are downstream signaling pathways of TLR4 that have been proven to be the main molecular mechanisms involved in microglial cell activation, and these pathways are regulated by intracellular Ca^{2+} signaling. As shown in Figure 4A, the expression levels of p-NF-κB, p-mTOR and p-JNK1 were increased after LPS-activated microglia. Compared with the LPS treatment group, the expression levels of p-NF-κB, p-MTOR, p-JNK1, p-NF-κB/NF-κB, p-MTOR/mTOR and p-JNK1/JNK1 in microglia treated with Yoda1 showed similar changes (Figure 4A). This suggests that the Piezo1-mediated Ca^{2+} peak is involved in microglial cell immune activation-related signaling pathway activation. Previous results showed that Piezo1-mediated Ca^{2+} is involved in the functional regulation of BV2 cells. TLR4 expression was upregulated in BV2 cells treated with Yoda1, and the expression of TLR4 and Piezo1 was further increased after BV2 cells were treated with Yoda1 and LPS (Figure 4B). These results indicate that Piezo1 signaling plays a vital role in TLR4 signal transduction.

Discussion

Coste et al. [9] identified Piezo1 in the expression profile analysis of the candidate gene and RNA knockdown. They described how it forms a stretch-activated calcium osmotic channel in the neuroblastoma cell line Neuro2A. In a healthy human brain, Piezo1 mRNA is expressed primarily in neurons [3]. However, postmortem analysis of AD brains showed that Piezo1 mRNA was downregulated in neurons and upregulated in astrocytes [4]. In addition, peripheral infection promotes increased expression of Piezo1 in reactive astrocytes and reactive microglia[3]. Why is the expression of Piezo1 upregulated in the subgroup of reactive glial cells? Our in vitro studies may explain part of the mechanism. LPS triggers the upregulation of Piezo1 through the Toll receptor cascade, while non-LPS directly activates the Piezo1 ion channel[24]. LPS binds to the TLR4 receptor and induces cytoplasmic signal transduction and nuclear events[25, 26]. Furthermore, through the ligand-receptor system and its cytoplasmic effector cascade, the opening of calcium channels (including Piezo1) is regulated to promote extracellular calcium ions, which leads to the enhancement of Ca^{2+} in microglia. Piezo1-mediated Ca^{2+} ion peaks may have the following roles in microglial activation: At early stages, LPS triggers piezo1 activation-mediated transient Ca^{2+} to promote microglial activation (Figure 5E).

Activated microglia in aged and diseased brains exhibit abnormal morphology, producing and secreting large amounts of proinflammatory and anti-inflammatory cytokines. The data accumulated in vitro show that microglial activation is related to the continuous increase in Ca^{2+}[27]. For example, a chronic increase in Ca^{2+} was observed in microglia cultured in vitro treated with LPS[28]. Increased mean Ca^{2+} in microglia of rats and human fetuses exposed to amyloid[29] [30]. Similarly, the basal Ca^{2+} level of microglia isolated from AD patients' brains is higher than that of individuals without dementia, and there are apparent abnormalities in Ca^{2+}-mediated signal transduction[30].

Yoda1 triggered the increase of Ca^{2+} in microglia. Piezo1 activation not only activates the influx of extracellular Ca^{2+} but also promotes the release of Ca^{2+} from internal storage (endoplasmic reticulum, ER). Additionally, the consumption of Ca^{2+} in internal storage can activate calcium release-activated channels (CRACs) in the outer membrane, thereby enhancing the influx of extracellular Ca^{2+} and promoting the replenishment of internal Ca^{2+} storage[31]. Indeed, in the presence of extracellular Ca^{2+}, we found that Yoda1 initially enhances the level of intracellular Ca^{2+} (most likely inflow through the membrane-bound Piezo1 channel), which may cause Ca^{2+}-induced Ca^{2+} release process (CICR) from intracellular stores (Ca^{2+} in cells). CICR can also manipulate calcium channels (SOCs) (such as TRP cation channels) through membrane-expressed storage to trigger further Ca^{2+} entry, leading to cytoplasmic Ca^{2+} peaks.

Ca^{2+} represents an important cytoplasmic signaling molecule because it can affect almost all cellular processes [32]. The basis of Ca^{2+} as a cell messenger is that there is a concentration gradient between cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores or extracellular Ca^{2+}. When a specific stimulus significantly increases the intracellular Ca^{2+} concentration, it plays a role in transmitting signals [33]. Piezo1 mediates various functions, such as protein synthesis, secretion, migration, proliferation, vitality and apoptosis, of cells under mechanical pressure[14]. Piezo1-mediated Ca^{2+} regulates microglial function. The evidence in Figure 3 shows that the increase in Ca^{2+} is necessary for microglial activation characteristics (such as cell proliferation, migration, and cytokine release) [21]. Moreover, Piezo1-mediated Ca^{2+} may promote the overall function of microglia rather than just the release of proinflammatory mediators.

The activation of Piezo1 channels is crucial to many intracellular Ca^{2+}-dependent signaling pathways. The JNK1, mTOR and NF-κB signaling pathways, as downstream mechanisms of TLR4, are the main signaling pathways involved in the activation of microglia and are regulated by Ca^{2+} signaling [34-36]. The results in Figure 4 further indicate that Ca^{2+} acts in the earliest step of the induction cascade rather than in subsequent stages of the interference release performance. The results explain the study's significance; on the one hand, Piezo1 activation may play an essential role in infection-induced immune regulation. On the other hand, pharmacological control of Piezo1 activity may have important prospects in the regulation of microglial immunity.

Conclusion

In summary, our results suggest that Piezo1-mediated Ca^{2+} plays a vital role in the immune activation of microglial cells. More research is needed to investigate the role of Piezo1 channel opening in the infected brain as a mechanically induced cation channel. Whether the opening of regulatory channels protects or exacerbates the disease state remains unclear. More importantly, Piezo1 is located in the plasma membrane, and Piezo1 may be a novel drug target for regulating the immune response of microglial cells.

Declarations

Ethics approval and consent to participate: Not Applicable.

Consent for publication: All named authors have been seen and agree with the contents of the manuscript.

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests: The authors declares they have no conflict of interest.

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