ORIGINAL ARTICLE

Pinitol Prevents Lipopolysaccharide (LPS)-Induced Inflammatory Responses in BV2 Microglia Mediated by TREM2

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Abstract

Microglia-involved neuroinflammation in the central nervous system (CNS) has been shown to aggravate brain damage and is associated with the pathogenesis of various neurodegenerative diseases. Thus, suppression of microglial activity has the potential to be a strategy for the treatment of neurodegenerative diseases. Pinitol, a methylated product of D-chiro-inositol, has been used as a treatment for blood-sugar metabolism and as an anti-tumor agent via its anti-inflammatory effects in cancer. However, whether or not pinitol can inhibit microglia-associated neuroinflammation is still unknown. This study aims to determine the effects of pinitol on inflammatory responses in BV2 microglia induced by LPS. Here, we found that the presence of pinitol ameliorates LPS-induced oxidative stress by reducing the production of ROS. Pinitol suppresses the expression and secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Notably, pinitol prevents the production of NO and PGE_2 by inhibiting the expression of iNOS and COX-2. Mechanistically, our findings demonstrate that pinitol inhibits the phosphorylation and degradation of IκBα and subsequent activation of NF-κB. Furthermore, we show that pinitol increases the expression of TREM2 in BV2 microglia, and silencing of TREM2 abolished the anti-inflammatory effects of pinitol. These findings suggest that TREM2 mediates the protective effects of pinitol against LPS in microglia. In summary, our results display that pinitol possesses a robust and beneficial effect against the LPS-induced inflammatory response in microglia.

Keywords Neuroinflammation · Microglia · TREM2 · Pinitol · Cytokines · Mediator

Introduction

Inflammation is an organism's host defense response to harmful pathogens, stimulation, and injury (Aggarwal et al. 2009). It plays a vital role in innate immunity by repairing damaged tissue, protecting the organism, and initiating the healing process (Biber et al. 2016). Specifically, neuroinflammation is the inflammation that occurs in the central nervous system (CNS) (More et al. 2013). In neurodegenerative diseases, neuroinflammation can clear up infections to control disease development.

However, persistent and excessive neuroinflammation in the CNS can accelerate many neurodegenerative diseases, including Alzheimer's disease (AD) (Eikelenboom et al. 2002) and Parkinson's disease (PD) (Marinova-Mutafchieva et al. 2009). Lipopolysaccharide (LPS), a central component of the membrane in Gram-negative bacteria, is a recognized endotoxin that induces inflammation. Microglia are essential immune cells in the brain and have a critical role in regulating immune system responses to brain injuries and immunological stimuli (Lee et al. 2012). In acute inflammation, microglia release substantial neurotrophic factors to rescue neurons. However, in chronic inflammation, overly activated microglia secrete various pro-inflammatory cytokines and cytotoxic mediators including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), prostaglandin E2 (PGE2), and nitric oxide (NO) (Kumagai et al. 2007). These factors are related to the progression and development of neurodegenerative diseases (Block and Hong 2005a, b). Also, the production of reactive oxygen

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species (ROS) in the inflammatory process leads to oxidative stress and takes on an essential role in neuron damage (Shi et al. 2017). The release of these pro-inflammatory cytokines is closely related to the mobilization of the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways (Fujioka et al. 2004; Ramachandiran et al. 2002). Triggering receptor expressed on myeloid cells 2 (TREM2) is expressed on the surface of microglia and mediates pivotal functions of microglia, including clearance of apoptotic neurons (Takahashi et al. 2005) and inhibition of pro-inflammatory responses (Hamerman et al. 2006; Zhong et al. 2015). The inhibition of abnormal microglial activation has become a therapeutic strategy for the treatment of neuroinflammation-related disorders.

Pinitol, first extracted from legumes, is a type of methylated product of D-chiro-inositol (Streeter 1980). Concerning pinitol transforming into D-chiro-inositol in the body, it has been shown to exert insulin-like effects (Bates et al. 2000). Previous research regarding pinitol has focused primarily on its regulative impact on blood-sugar metabolism (Joseph 2010), its protective effect on pancreatic tissue (Sivakumar and Subramanian 2009), and its use as an anti-tumor agent (Rengarajan et al. 2012). Moreover, pinitol has displayed diverse biological capacities such as suppression of the T helper cell-1 response, and anti-oxidant, anti-viral, larvicidal, anti-hyperlipidemic, and cardioprotective effects (Sethi et al. 2008; Sivakumar et al. 2010). Importantly, pinitol has a robust anti-inflammatory activity by blocking the activation of NF-κB abrogation of IKK activation and IκBα phosphorylation (Kumar et al. 2004). However, few studies have researched the effects of pinitol in neuroinflammation. The purpose of this study is to investigate the impacts of pinitol on inflammatory responses in BV2 microglia induced by lipopolysaccharide (LPS).

Materials and Methods

Cell Culture, siRNA Transfection, and Treatment

BV2 microglial cells were maintained in DMEM buffer supplemented with 15% FBS and 1% penicillin/streptomycin. For all experiments, the cells were pretreated with 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. For the TREM2 silencing experiment, BV2 cells were transfected with TREM2 siRNA for 12 h, followed by the indicated treatment.

Dihydroethidium Staining

To determine the level of ROS production, we used the dye dihydroethidium (DHE) (Sigma-Aldrich, USA)

dissolved in anhydrous dimethyl sulfoxide (DMSO). Briefly, the cells were probed with 5 μmol/L DHE in Krebs buffer for 20 min at a temperature of 37 °C in darkness. The cells were then washed 3 times with PBS buffer, and a fluorescence microscope (Olympus, Japan) was used to determine the fluorescence intensity. The intracellular levels of ROS were calculated using the software ImageJ. Briefly, we defined regions of interest (ROI) in the fluorescent image and counted the average number of cells presented in ROI: intracellular levels of ROS = the integrated density value (IDV)/the average number of cells.

Real-time Polymerase Chain Reaction Analysis

After the indicated treatment, RNA was extracted from BV2 cells using QIAzol reagent (Qiagen, USA). A NanoDrop spectrophotometer was used to assess the extracted RNA. cDNA was synthesized using a Bio-Rad cDNA synthesis kit following the manufacturer's instructions. Real-time polymerase chain reaction (PCR) analysis was performed on an ABI 7500 platform using the SYBR Green–based method. Quantification of the target genes was performed using the $2^{-\Delta\Delta Ct}$ method. The following primers were used in this study: TNF-α: For: 5′-CCAGACCCTCACACTCAGATC-3′, Rev: 5′-CACTTGGTGGTTTGCTACGAC-3′; IL-1β: For: 5′-TGGGCCTCAAAGGAAAGAAT-3′, Rev: 5′-CAGG CTTGTGCTCTGCTTGT-3′; IL-6 For: 5′-TGGA GTCACAGAAGGAGTGGCTAAG-3′, Rev: 5′-TCTG ACCACAGTGAGGAATGTCCAC-3′; iNOS: For: 5′-CAGGAGGAGAGAGATCCGATTTA-3′, Rev: 5′-GCAT TAGCATGGAAGCAAAGA-3′; COX-2: For: 5′-TGCA TGTG-GCTGTGGATGTCATCAA-3′, Rev: 5′-CACT AAGACAGACCCGTCATCTCCA-3′; TREM2: For: 5′-TGTGGTCAGAGGGCTGGACT-3′, Rev: 5′-CTCC GGGTCCAGTGAGGA-3′; GAPDH: For: 5′-ACTC CCACTCTTCCACCTTC-3′, Rev: 5′-TCTT GCTCAGTGTCCTTGC-3′.

Western Blot Analysis

To determine the protein expression of the target genes, cell lysates were prepared with cell lysis buffer. To measure nuclear levels of NF-κB p65, nuclear fractions were prepared using a commercial Nuclear Protein Extraction Kit (Thermo Fisher Scientific, USA). Briefly, equal amounts of cell lysates or nuclear extractions (20 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electronically transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was then blocked with 5% non-fat dry milk followed by probing with primary antibodies overnight at 4 °C. The membrane was washed 3 times and then probed with HRP-
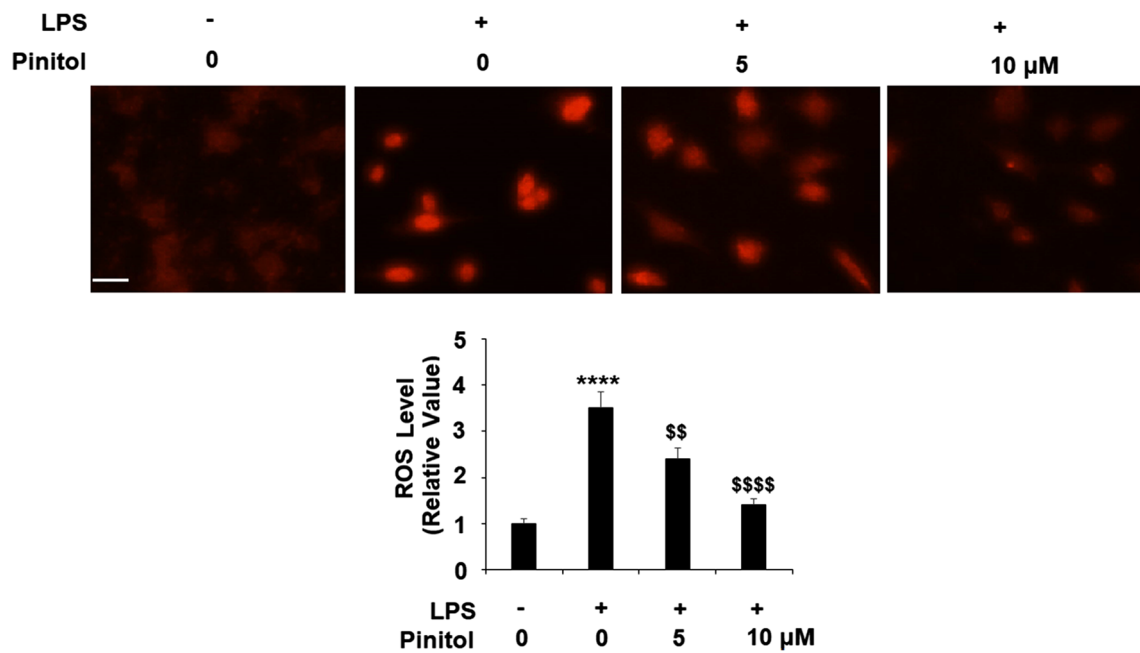
Fig. 1 Pinitol reduced LPS-induced generation of ROS in BV2 microglia. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. Intracellular ROS was measured by dihydroethidium (DHE) staining; scale bar, 100 μm (****$P < 0.0001$ vs. control group; $^{\$\$, \$\$}P < 0.01, 0.0001$ vs. LPS treatment group)

linked secondary antibody for 1 h at room temperature (RT). The resulting bands were visualized using enhanced chemi-luminescence (ECL) (Bio-Rad, USA). The following anti-bodies were used in this study: iNOS (1:2000, #ab15323, Abcam, USA); COX-2 (1:2000, #ab15191, Abcam, USA); IκBα (1:1000, #4812, Cell Signaling Technology, USA); p-IκBα (1:1000, #2859, Cell Signaling Technology, USA); p65 (1:2000, #8242, Cell Signaling Technology, USA); TREM2 (1:2000, #PA5-46980, Thermo Fisher Scientific, USA); Lamin B1 (1:5000, sc-374015, Santa Cruz Biotechnology, USA); β-actin (1:10,000, #3700, Cell Signaling Technology, USA); anti-rabbit IgG, HRP-linked antibody (1:2000, #7074, Cell Signaling Technology, USA); anti-mouse IgG, HRP-linked antibody (1:2000, #7076, Cell Signaling Technology, USA). The western blot bands were scanned, and the sum optical density of the bands

was quantitatively analyzed using the Kodak Digital Science 1D software (Eastman Kodak Company, USA). Firstly, we subtracted the background. Then, we selected the bands and quantified signal intensities. Data were exported for statistical analysis.

Enzyme-Linked Immunosorbent Assay

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to measure the protein expression levels of TNF-α (#MTA00B, R&D Systems, USA), IL-6 (#M6000B, R&D Systems, USA), IL-1β (#MLB00C, R&D Systems, USA), and PGE$_2$ (#ab133021, Abcam, USA). Briefly, the culture media were collected, and the analysis was performed following the manufacturer's instructions. The results were collected using 96-plate
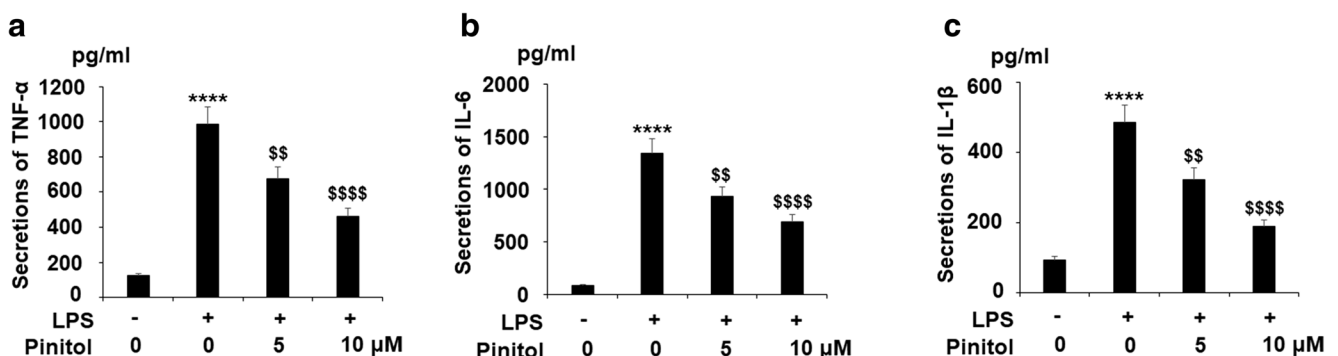

Fig. 2 Pinitol prevented LPS-induced secretion of pro-inflammatory cytokines. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** Secretions of TNF-α. **b** Secretions of IL-6. **c** Secretions of IL-1β (****$P < 0.0001$ vs. control group; $^{\$\$, \$\$}P < 0.01, 0.0001$ vs. LPS treatment group)
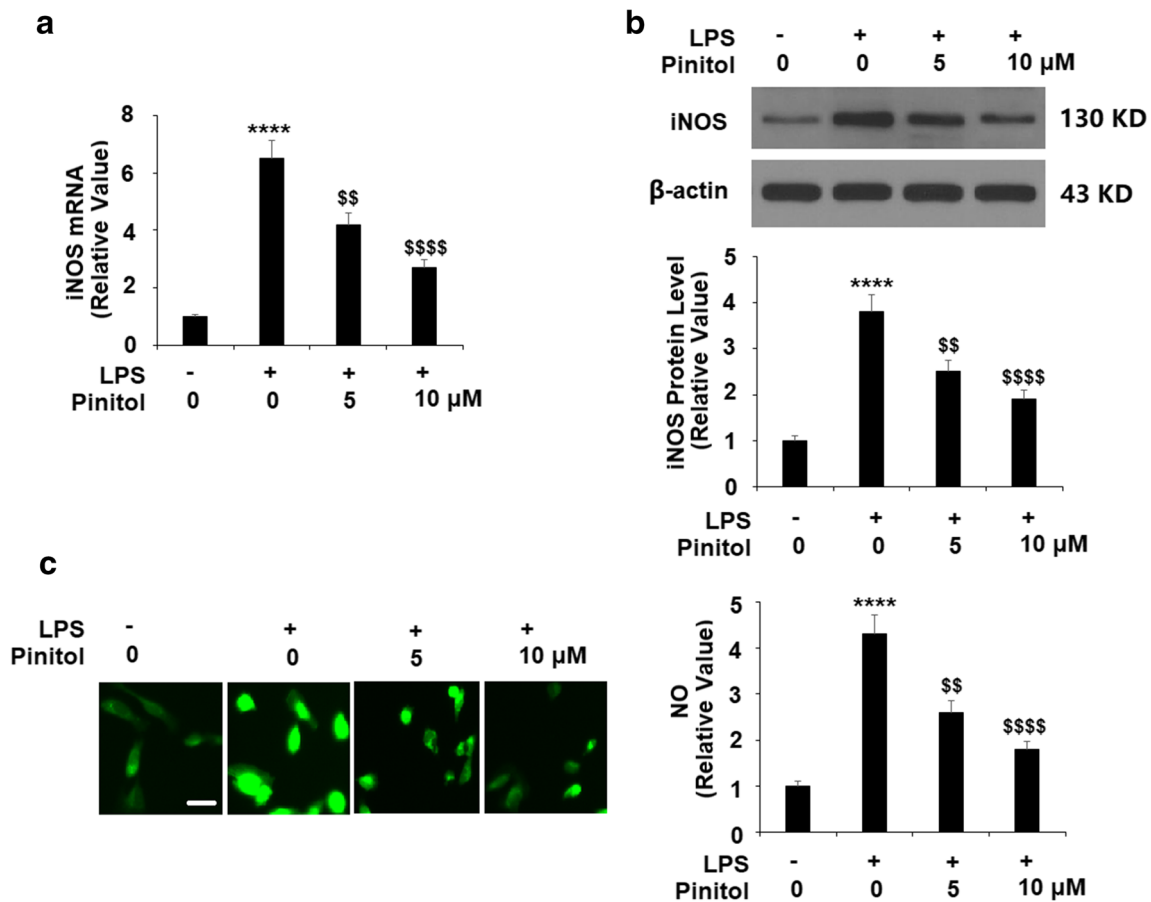
Fig. 3 Pinitol suppressed LPS-induced iNOS expression and NO production. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** mRNA of iNOS. **b** Protein of iNOS as measured by western blot analysis. **c** DAF-FM staining of NO; scale bar, 100 μm (****$P < 0.0001$ vs. control group; $^{\$\$}$, $^{\$\$}P < 0.01$, 0.0001 vs. LPS treatment group)

reader spectrometry. A standardized 4-PL curve was used to obtain absolute values.

Luciferase Activity

The transcriptional activity of NF-κB was determined by visualizing the 3x NF-κB bind sites containing luciferase vector. BV2 microglia were co-transfected with NF-κB promoter and a firefly promoter using Lipofectamine 2000 from Invitrogen (Carlsbad, CA). At 48 h after transfection, the cells underwent the indicated treatment, and

the lysates were collected. The dual-luciferase activity of NF-κB and renilla promoters was determined. The relative activity of NF-κB was calculated by normalizing to that of renilla luciferase.

Statistical Analysis

The data resulting from all experiments are presented as means ± standard error of measurement (S.E.M). Statistical analysis was performed using the software SPSS (Version 19.0). The one-way analysis of variance (ANOVA) method
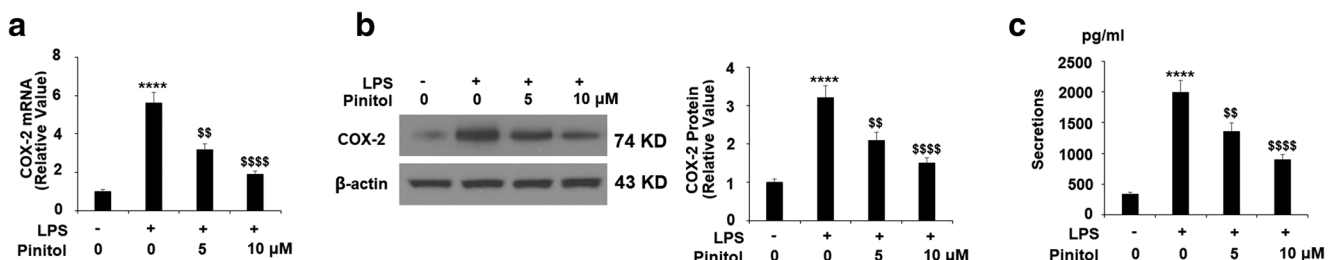

Fig. 4 Pinitol reduced LPS-induced expression of COX-2 and the production of PGE$_2$. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** mRNA of COX-2. **b** Protein of COX-2. **c** Secretions of PGE$_2$ (****$P < 0.0001$ vs. control group; $^{\$\$}$, $^{\$\$}P < 0.01$, 0.0001 vs. LPS treatment group)
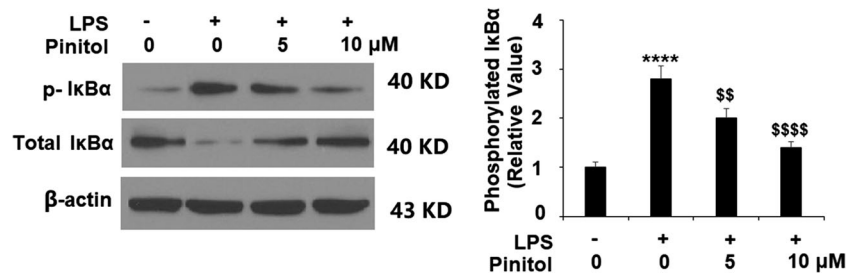
Fig. 5 Pinitol prevented LPS-induced phosphorylation and degradation of IκBα in BV2 microglia. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h.

Phosphorylated and total levels of IκBα (****$P < 0.0001$ vs. control group; $^{$$, $$}P < 0.01, 0.0001$ vs. LPS treatment group)

was used to determine statistical significance, followed by Turkey's post hoc test. A value of $P < 0.05$ was considered to be of statistical significance.

Results

Pinitol Reduced LPS-Induced Generation of ROS in BV2 Microglia

Microglia were pretreated with 5 and 10 μM pinitol for 3 h, followed by exposure to 1 μg/mL LPS for 24 h. To reveal the anti-oxidant effect of pinitol against LPS-induced oxidative stress, the generation of intracellular ROS was examined through dihydroethidium (DHE) staining. The results in Fig. 1 reveal that ROS levels gradually increased upon exposure to LPS by 3.5-fold. Conversely, the levels of ROS decreased to 2.4- and 1.4-fold when exposed to the two concentrations of pinitol. Thus, the results show that oxidative stress was impeded by pinitol through the inhibition of ROS.

Pinitol Prevented the Secretion of Pro-inflammatory Cytokines Induced by LPS

TNF-α, IL-1β, and IL-6 are important pro-inflammatory cytokines in the inflammatory response. Therefore, we investigated the impact of pinitol on the expression of these cytokines. The results in Fig. 2a showed that the secretions of TNF-α increased from 122.6 to 985.2 pg/mL with exposure to LPS. However, secretions of TNF-α decreased to 675.4 and 462.9 pg/mL after the addition of the two doses of pinitol. In Fig. 2b, we showed that treatment with LPS increased the secretions of IL-6 from 85.3 to 1344.1 pg/mL, while treatment with pinitol reduced it to 931.8 and 692.6 pg/mL. Similarly, the two doses of pinitol decreased the secretions of IL-1β induced by LPS from 485.3 to 322.7 and 189.7 pg/mL, as we showed in Fig. 2c.
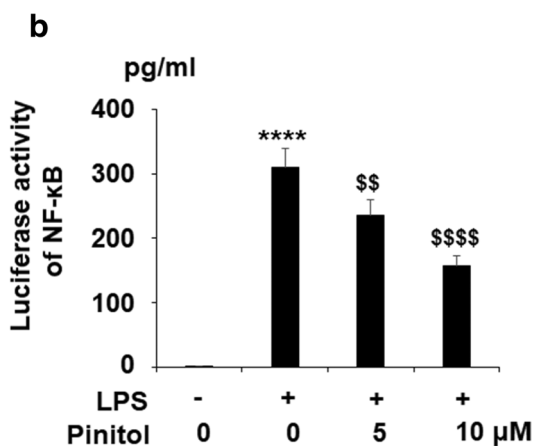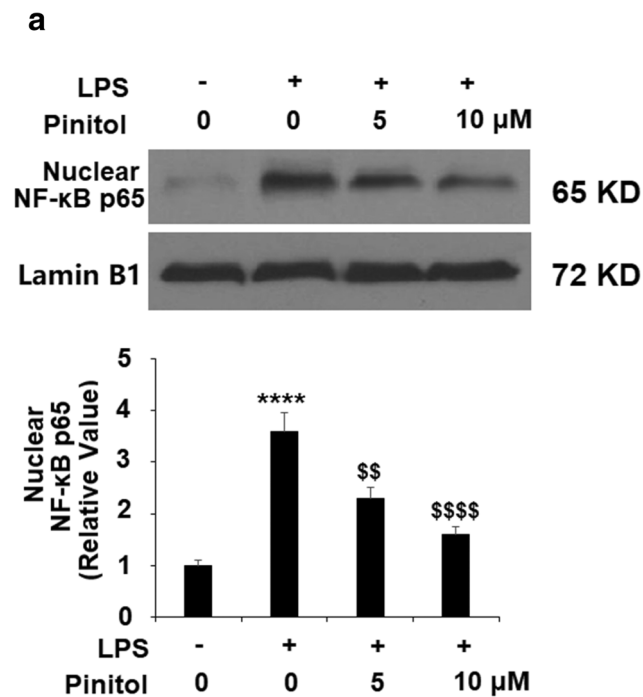

Fig. 6 Pinitol prevented LPS-induced activation of NF-κB. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** Nuclear translocation of NF-κB p65. **b** Luciferase activity of NF-κB (****$P < 0.0001$ vs. control group; $^{$$, $$}P < 0.01, 0.0001$ vs. LPS treatment group)
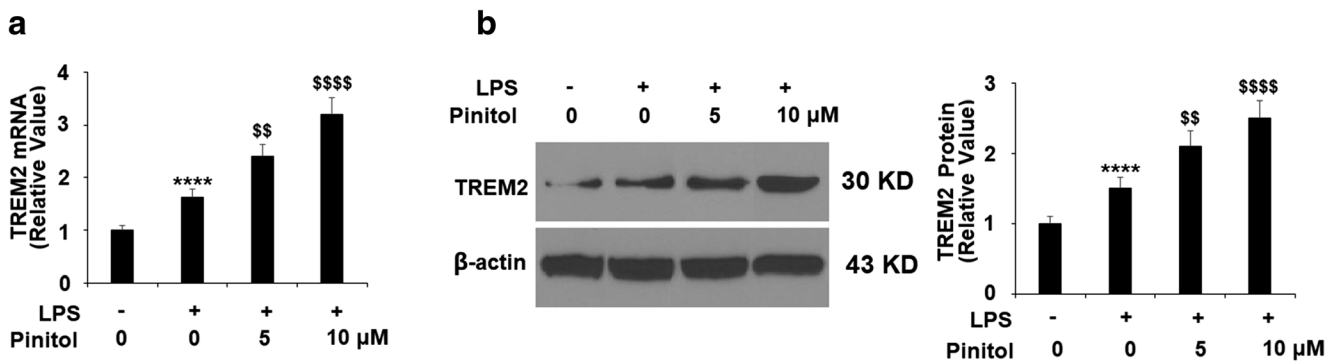
Fig. 7 Pinitol increased TREM2 in LPS-treated microglia. Cells were pretreated with 5 and 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** mRNA of TREM2. **b** Protein of TREM2 (****$P < 0.0001$ vs. control group; $^{\$\$, \$\$}P < 0.01$, 0.0001 vs. LPS treatment group)

Pinitol Suppressed iNOS Expression and NO Production Induced by LPS

iNOS is a critical regulatory enzyme in inflammation that acts through modulation of NO production. In Fig. 3a and b, we found that LPS stimulation increased iNOS at both the mRNA and protein levels, while the expression was decreased significantly by the two doses of pinitol. Notably, the DAF-FM staining results in Fig. 3c show that LPS treatment increased the production of NO to 4.3-fold, which was reduced to 2.6- and 1.8-fold by 5 and 10 μM pinitol, respectively. These results indicate that pinitol suppressed LPS-induced NO production via downregulation of iNOS.

Pinitol Reduced COX-2 Expression and PGE₂ Production Induced by LPS

COX-2 is another major inflammation-related enzyme involved in combining PGs, such as PGE₂. Results in Fig. 4a and b demonstrate that LPS significantly increased the expression of COX-2, which was attenuated by the pinitol dose-dependently. Consistently, in Fig. 4c, we showed that LPS treatment increased the secretion of PGE₂ from 337.8 to 1987.5 pg/mL, which was reduced to 1356.7 and 899.5 pg/mL by 5 and 10 μM of pinitol, respectively.

Pinitol Prevented LPS-Induced Activation of the IκBα/NF-κB Pathway in BV2 Microglia

It is well known that phosphorylation and degradation of IκBα is a trigger for NF-κB activation. Therefore, we studied the impact of pinitol on IκBα. Phosphorylated levels of IκBα increased to 2.8-fold by exposure to LPS alone, while it was reduced to 2.0- and 1.4-fold in the presence of the two concentrations of pinitol. Correspondingly, LPS treatment decreased the total level of IκBα by 45%, which was reduced by pinitol in a dose-dependent manner (Fig. 5).

Since the NF-κB signaling pathway plays a vital role in the induction of various pro-inflammatory cytokines and mediators, determining whether pinitol influences the activation of NF-κB is essential. The results in Fig. 6a showed that LPS treatment increased nuclear translocation of NF-κB p65 to 3.6-fold, while the levels were reduced by the two concentrations of pinitol to 2.3- and 1.6-fold. We further tested the transcriptional activity of NF-κB. The luciferase activity assay revealed that pinitol had a powerful effect against the LPS-induced increase in NF-κB activity (Fig. 6b).

The Anti-inflammatory Effects of Pinitol in Microglia Are Mediated by TREM2

TREM2 is a vital receptor of microglia. Interestingly, we found that pinitol treatment dose-dependently increased the expression of TREM2 at both the mRNA and protein levels, as we showed in Fig. 7a and b, suggesting the possible involvement of TREM2 in the effects of pinitol. To further verify the role of TREM2 in this process, the expression of TREM2 was knocked down by transfection with TREM2 siRNA for 12 h. This successful knockdown of TREM2 was revealed by western blot analysis (Fig. 8a). Notably, the results show that knockdown of TREM2 almost completely abolished the inhibitory effects of pinitol on NF-κB activation (Fig. 8b) and the secretion of TNF-α (Fig. 8c). This data suggests that TREM2 mediates the anti-inflammatory effects of pinitol in microglia.

Discussion

A 2012 report published by the World Health Organization (WHO) predicts that the number of patients with neurodegenerative diseases will reach over 70 million by 2030 and 106 million by 2050 (Word Health Organization 2012). Previous research has shown that such neurodegenerative diseases are associated with chronic neuroinflammation and primarily affect the elderly population (Eikelenboom et al. 2002; Marinova-Mutafchieva et al. 2009; Amor et al. 2010). Microglia, the primary cells in the brain, play a vital role in
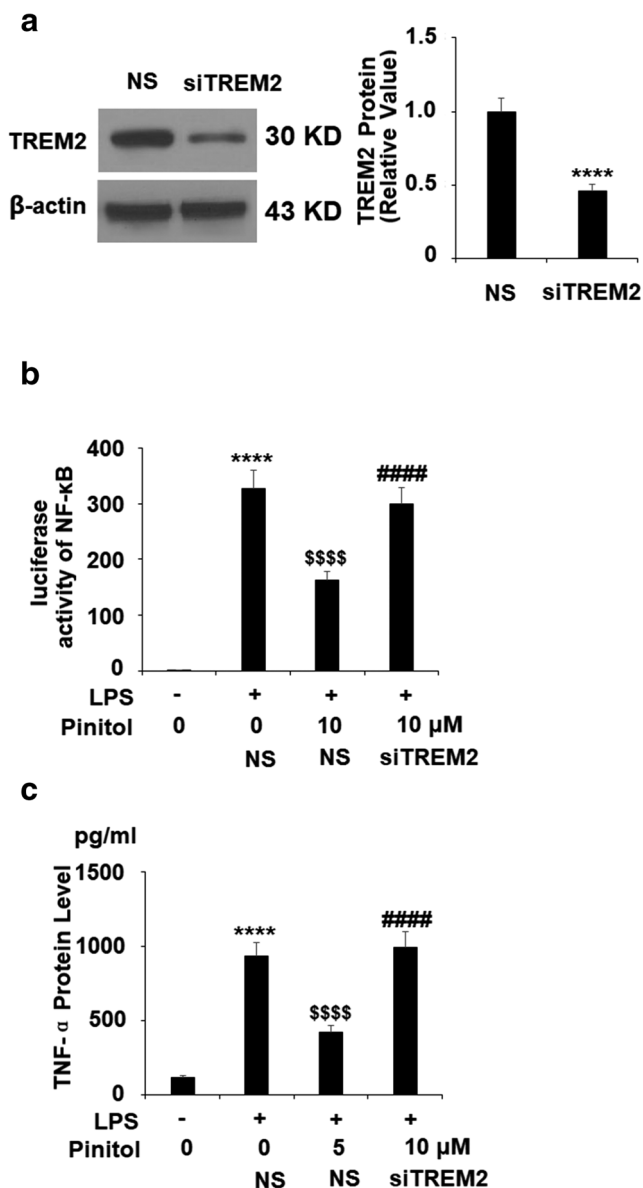
a

b

c

Fig. 8 Silencing of TREM2 abolished the protective effects of pinitol against LPS-induced inflammation in BV2 microglia. Cells were transfected with TREM2 siRNA for 12 h. Then, cells were pretreated with 10 μM pinitol for 3 h, followed by stimulation with 1 μg/mL LPS for 24 h. **a** Western blot analysis demonstrated successful silencing of TREM2. **b** Knockdown of TREM2 abolished the effects of pinitol in luciferase activity of NF-κB. **c** Knockdown of TREM2 abolished the inhibitory effects of pinitol in secretions of TNF-α (****$P < 0.0001$ vs. control group; $^{\$\$}P < 0.01$, 0.0001 vs. LPS treatment group; ####$P < 0.0001$ vs. LPS + 10 μM pinitol)

the progression and development of chronic inflammation in the CNS. Regulating over-activated microglia in neurodegenerative diseases could have a significant therapeutic impact. In this study, we demonstrated that pinitol has anti-neuroinflammatory properties in BV2 microglia stimulated with LPS. Our results show that pinitol reduces LPS-induced secretion of pro-inflammatory cytokines and mediators, suppresses the generation of ROS, and inhibits both

IκBα phosphorylation and NF-κB activation. Furthermore, we proved that TREM2 mediates these effects.

Microglia are significant contributors to oxidative stress in the CNS as they induce the release of ROS and other cytokines to modulate the inflammatory response (Loane and Kumar 2016). Low levels of ROS serve as signaling molecules to regulate the immune response against harmful pathogens. However, overproduction of ROS in chronic inflammation leads to neurotoxicity, which is a cause of neuronal apoptosis (Von et al. 2015). Exacerbation of oxidative stress and increased ROS in brain tissues greatly contributes to the pathological progression of neurodegenerative diseases. Our research found that the presence of pinitol ameliorates the oxidative changes induced by LPS, suggesting an essential role of pinitol in the oxidative balance in microglia.

The endotoxin hypothesis of neurodegeneration has been studied over the past few decades. An example of this hypothesis is that LPS concentrations are elevated in the blood and brain cells of patients with AD. Increased levels of these endotoxins have a direct impact on neurodegeneration (Brown 2019). LPS-induced aberrant expression and production of pro-inflammatory mediators produced by microglia results in neuroinflammation and promotes the progression of CNS disorders. The blockage of abnormal responses mediated by microglia, could potentially prevent the development of neurodegenerative diseases (Teeling and Perry 2009). Upregulation of TNF-α, IL-6, IL-1β, and PGE$_2$ are capable of initiating and amplifying the inflammatory response in brain cells (Lucas et al. 2006). Excessive generation of NO via over-activated microglia has been considered as another critical biomarker for neuroinflammation in neurodegenerative diseases. Our results demonstrate that pinitol strongly inhibits the production of TNF-α, IL-6, IL-1β, NO, and PGE$_2$, as well as the expression of COX-2 and iNOS. Our mechanistic investigation revealed that the protective effects of pinitol in inflammatory responses are dependent on the inhibition of IκBα/NF-κB activation. NF-κB is one of the most important signaling pathways regulating the expression of pro-inflammatory cytokines and mediators (Kaltschmidt and Kaltschmidt 2009). The protein p65, one of the NF-κB subunits, has well-documented significance in the expression of inflammatory mediators in microglia (Erica and Mollie 2019). It is known that the IκB inhibitors, such as IκBα, can prevent nuclear translocation of NF-κB p65 (Kopitar 2015). However, the phosphorylation and degradation of IκBα also trigger the transference of NF-κB p65 into the nucleus. Abnormal activation of NF-κB has been widely associated with the development of CNS disorders (Sivandzade et al. 2019). The inhibitory effects of pinitol on NF-κB activity have broadened our understanding of its beneficial effects against neuroinflammation.

Finally, we focused on TREM2, the microglial surface receptor. Previous studies have shown that TREM2 can mediate the functions of microglia in the CNS, such as clearance of

apoptotic neurons and secretion of pro-inflammatory cytokines (Takahashi et al. 2005; Hamerman et al. 2006). Our study has demonstrated that pinitol treatment increases the expression of TREM2 at both the mRNA and protein levels in BV2 microglia. On the other hand, silencing of TREM2 abolishes the effects of pinitol on NF-κB activity and secretions of TNF-α. These findings suggest that TREM2 is involved in the protective effects of pinitol against LPS-induced inflammation in microglia. TREM2 plays a critical role in transforming microglia from a homeostatic to a disease-associated state.

Additionally, TREM2 has been considered a principal regulator of microglial activity, including apoptosis, proliferation, phagocytosis, and inflammation, in various neurodegenerative diseases, such as AD and ischemia (Konishi and Kiyama 2018; Ulland and Colonna 2018). TREM2 has been proposed as a new therapeutic target for the treatment of these disorders. The involvement of TREM2 in the pharmacological functions of pinitol in microglial activity implicates a novel molecular mechanism.

Taken together, our findings suggest that pinitol has the potential to act as a therapeutic agent in neuroinflammation-related brain diseases by alleviating microglial activation.

Compliance with Ethical Standards

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