Molecular Neurobiology (2019) 56:2870–2880
https://doi.org/10.1007/s12035-018-1233-3

A Novel Synthetic Derivative of Phloroglucinol Inhibits Neuroinflammatory Responses Through Attenuating Kalirin Signaling Pathway in Murine BV2 Microglial Cells

Caixia Zang[1] · Hanyu Yang[1] · Lu Wang[1] · Yue Wang[1] · Xiuqi Bao[1] · Xiaoliang Wang[1] · Dan Zhang[1]

Received: 15 May 2018 / Accepted: 10 July 2018 / Published online: 31 July 2018

Abstract

Neuroinflammation has been implicated as an important factor in the neurodegenerative diseases, and multiple candidates with anti-inflammatory effects have been shown to be beneficial for the treatment of neurodegenerative diseases. Our previous study demonstrated that a novel synthetic phloroglucinol derivative from *Lysidice rhodostegia* roots (code name: Compound 21) exerted neuroprotective effect through suppressing neuroinflammation. The aim of this study was to reveal the underlying molecular mechanism. The results indicated that the anti-inflammatory effects of Compound 21 were mediated through suppression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and the production of reactive oxygen species (ROS). Further study showed that this suppression on NADPH oxidase was mediated by inhibiting the translocation and activity of its subunit Rac1. It is well established that Rac1 activation is regulated by a variety of guanine nucleotide exchange factors (GEFs), so we tried to find out whether GEFs were involved in the anti-inflammatory effects of Compound 21. The results showed that Compound 21 treatment down-regulated the expression and activity of GEF Kalirin, thus modulating the activity of Rac1 GTPase. Altogether, our data suggested that Compound 21 exerted the anti-neuroinflammatory effect through suppressing Kalirin signaling pathways, decreasing Rac1-NADPH oxidase activation and the subsequent pro-inflammatory cytokine production. The present study provided solid evidence to support Compound 21 as a potential candidate of neuroinflammatory inhibitor. Moreover, our findings have shed new light on the role of Rac1 and GEF Kalirin in neuroinflammation, which provides potential targets for neuroinflammation-related diseases, such as neurodegenerative diseases.

Keywords Neuroinflammation · Guanine nucleotide exchange factors · Kalirin · Rac1 GTPase · NADPH oxidase

Introduction

Neuroinflammation is extensively found in many central neural system diseases and usually reported to be the phenomena that preceding neuronal death [1, 2]. Neuroinflammation is characterized by activated glial cells and the production of pro-inflammatory mediators, such as reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), and prostaglandin E_2 (PGE_2) [3, 4], which damage surrounding neurons and eventually result in neuronal death. Accumulating evidence indicate that chronic neuroinflammation could remain unnoticed in the brain for years, inflicting continuous damage that could culminate later in life as Alzheimer's disease (AD) and Parkinson's disease (PD) [5, 6]. Thus, neuroinflammation has been considered to be one of the key points for the treatment of neurodegenerative diseases. Among all the cytokines that mediate neuroinflammation, ROS is considered to participate in inflammatory signaling pathways and exert direct neurotoxicity [7]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is considered to be an important source of ROS and a major contributor to neuroinflammation [8]. Therefore, regulation of NADPH oxidase activity and ROS release is of great importance to neuroinflammation. NADPH oxidase has both membrane subunits (gp91phox and p22phox) and cytosolic components (p47phox, p67phox, and Rac1). Translocations of cytosolic components to the membrane are essential for NADPH oxidase activation [9].

✉ Dan Zhang
danzhang@imm.ac.cn

[1] State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, 1 Xian Nong Tan Street, Beijing 100050, People's Republic of China

Given the participation of neuroinflammation in the progression of neuronal death, candidates with potential anti-inflammatory effects are highly appreciated for the treatment of neurodegenerative diseases. In our previous studies, a novel synthetic phloroglucinol derivative from *Lysidice rhodostegia* roots was found with strong anti-inflammatory effect. This compound is formulated as: (S)-3 hydroxy-2-((2,4,6-trimethoxybenzyl) amino) propanoic acid and the code name is Compound 21. We have reported that Compound 21 had protective effects in a PD mouse model and its anti-inflammatory effects contributed to its neuroprotective effect [10]. However, the exact mechanism of Compound 21 was still not elucidated. The purpose of this study was to explore the mechanism of the anti-neuroinflammatory effects of Compound 21.

Materials and Methods

Chemicals and Reagents

Compound 21 was synthesized by the Department of Pharmaceutical Chemistry, Institute of Materia Medica, Chinese Academy of Medical Science. It is a white powder with 99.9% purity.

Cell Cultures

BV2 cells (microglial cell) were grown in 90% DMEM supplemented with 10% fetal bovine serum and antibiotics. Cells were kept in a 5% CO_2 incubator in an atmosphere of 5% CO_2 at 37 °C.

Cell Viability Assay

BV2 cells were seeded at a density of 1×10^5 cells/mL in 96-well plates and incubated with LPS (1 μg/mL) and various concentrations of Compound 21 (0.1, 1, and 10 μM) for 24 h. BV2 cells were incubated with MTT solution (0.5 mg/mL) for 4 h at 37 °C, and cells were lysed with DMSO (150 μL) and shaken for 5 min. The absorbance at 595 nm was measured by a microplate reader.

Measurement of Inflammatory Cytokine Production

The production of inflammatory cytokines TNF-α and PGE_2 was measured by ELISA kits. BV2-enriched cultures were seeded at a density of 4×10^4 cells/well in a 24-plates. BV2 cells were pre-treated with Compound 21 (0.1, 1, and 10 μM) for 1 h, then incubated with LPS (1.0 μg/mL) for 6 h. After harvesting the supernatants of cells, the concentrations of TNF-α and PGE_2 were analyzed by TNF-α and PGE_2 ELISA kits.

Determination of Reactive Oxygen Species Production

The level of ROS was assayed by flow cytometry with fluorescent probe 2′7′-dichlorofluorescein diacetate (DCFH-DA). Cells of 2×10^6 were seeded 6-plates and pretreated with Compound 21 (0.1, 1, and 10 μM) for 30 min and exposed to LPS (1.0 μg/mL) with DMEM containing DCFH-DA (20 μM) for 3 h at 37 °C avoiding the light. After incubation, the BV2 cells were washed with PBS four times, and the DCFH-DA-loaded cells were examined by flow cytometry with excitation and emission wavelengths of 495 and 525 nm, respectively. It is known that the production intracellular ROS was proportional to the fluorescence intensity.

Rac1 Activity Assay

Cells were seeded in DMEM at a density of 3×10^6 cells in a 10-cm dish. After 24 h, cells were incubated with LPS (1.0 μg/mL) for 30 min, followed by treatment with Compound 21 (10 μM) for 30 min. Assay of Rac1 GTPase activity was determined using G-LISA Rac1 colorimetric-based kit (Cytoskeleton, Denver, CO, USA). In brief, cells were lysed and centrifuged to gain the soluble cell extract, and then, the total protein concentration was corrected. According to the manufacture's instruction, 50 μL of the extract volume was added to the assay plates and incubated on ice for 30 min. Active GTPase was detected by specific antibodies using a microplate reader (Bio-Tek mQuant).

Western Blot Analysis

BV2 cells were lysed using the RIPA buffer containing protease inhibitor and phosphatase inhibitor. Cytomembrane and cytoplasmic fractions were isolated by the membrane and cytosol protein extraction kit (Applygen Technologies, Beijing, China). Extracts were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were incubated with primary antibodies (COX-2, iNOS, Tiam1, Trio, ARHGEF, Rac1, and DOCK2, 1:1000, Abcam, MA, USA; p-IKKα, IKKα, p-IκB, and IκB, 1:1000, Santa Cruz Biotechnology, CA, USA; Kalirin and β-Tublin, 1:1000, Cell Signaling Technology, USA; β-actin 1:10,000, ABclonal Technology, Beijing, China) overnight at 4 °C and secondary antibody for 1 h. The blots were developed using ECL + Plus reagents (Yeasen, Shanghai, China) and band densities were analyzed by Gel-pro analyzer 3.

Co-immunoprecipitation

Cells were harvested and lysed in nondenaturing lysis buffer. In brief, 10-μL GEF antibodies (Tiam1, Kalirin, ARHGEF, DOCK2) and 10-μL agarose beads were added to 500 μL of

the corrected extract volume, and then were incubated overnight with end-over-end mixing gently at 4 °C. Immunoprecipitates were boiled for 5 min and then assayed by Western blot using anti-Rac1 antibody.

Rac1 Pull-Down Assay

BV2 cells were cultured in DMEM medium for 24 h and then incubated with 1 μg/mL LPS for 30 min prior to cell lysis. The Rac1 activation assay was carried out by Cell Biolabs's Rac1 Activation Assay Kit. In brief, the cell extracts were incubated with GST-PAK1 protein binding domain (GST-PBD) for 1 h at 4 °C with gentle end-over-end mixing. The GTP-Rac1 was detected by Western blot with an anti-Rac1 monoclonal antibody.

Immunofluorescent Staining

The BV2 cells were plated on cover slips in 24-well plates and fixed in 4% paraformaldehyde for 15 min at room temperature and then cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min. After being washed three times, 3% normal goat serum was used to block the cells for 2 h. The BV2 cells were incubated with primary antibodies (Rac1 1:100; Abcam, MA, USA; Kalirin 1:100, Cell Signaling Technology, USA) overnight at 4 °C, subsequently incubated with secondary antibodies (1:200; ABclonal Technology, Beijing, China) for 2 h avoiding the light. The cells were further stained with DAPI (2 μg/mL) and were observed and imaged using a Carl Zeiss microscope (Jena, Germany).

RNA Interference

Kalirin expression was knocked down in BV2 cells by Lipofectamine RNA iMAX Reagent. Specific siRNA for Kalirin (sense: GGCAGCUCCAAAUUCAUCUTT; antisense: AGAUGAAUUUGGAGCUGCCTT) and negative control siRNA and RNA iMAX Reagent were purchased from Invitrogen. The expression of Kalirin was analyzed by Western blot.

Rho GEF Exchange Assay

BV2 cells were seeded in 90% DMEM with 10% FBS at a density of 3×10^6 cells in a 10-cm dish. Cells were pretreated with Compound 21 (10 μM) for 30 min, following incubated with LPS (1.0 μg/mL) for 30 min. According to the manufactural instructions of Rho GEF exchange assay biochem kit (Cytoskeleton, Denver, CO, USA), the purified Kalirin was mixed with exchange reaction buffer and Rac1 GTPase for the kinetic readings ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 440$ nm) on a fluorescence plate reader.

Results

Compound 21 Suppressed LPS-Induced Inflammatory Responses in BV2 Cells

Microglial cell line BV2 cells were used to determine the inhibitory effect of Compound 21 on inflammation. MTT assay results excluded non-specific cytotoxicity of Compound 21 during the study (Figs. 1 and 2a). ELISA assay was first applied to measure the production of two major cytokines, TNF-α and PGE_2. ELISA results indicated that LPS trigged the production of TNF-α and PGE_2 in BV2 cells whereas the production was suppressed by Compound 21 (Fig. 2b, c). AS and COX-2 are major pro-inflammatory enzymes that produce the inflammatory cytokines. The expression of iNOS and COX-2 was increased after LPS stimulation, and Compound 21 treatment suppressed the elevated levels (Fig. 2d, e). It is well-known that NF-κB is a key regulator in the activation of chronic inflammatory response [11]. As shown in Fig. 2f–h, LPS treatment resulted in the upregulation of inhibitory κB kinase α (IKKα) phosphorylation and IκB phosphorylation and P65 translocation from cytoplasm into nucleus. Pretreatment with Compound 21 showed a beneficial effect on inhibiting IKKα and IκB phosphorylation, suggesting that Compound 21 is effective in suppressing the activation of NF-κB signaling pathway. All the results in Fig. 2 indicated the anti-inflammatory effect of Compound 21.

Compound 21 Treatment Inhibited ROS Release and Rac1-NADPH Oxidase Activation Stimulated by LPS

Flow cytometry was applied to examine the effect of Compound 21 on ROS release in LPS-treated BV2 cells. Different time points after LPS treatment (1, 2, 3, and 6 h) were selected to find the optimum time and 3 h was finally chosen for the further study (Fig. 3a). Compound 21 treatment suppressed ROS production in a concentration-dependent manner (Fig. 3b). NADPH oxidase is an important source for microglial ROS production (Gao et al. 2012). It has been reported that the translocation of its cytosolic components into the membrane is required for NADPH oxidase activation (Freeman et al. 1996; Mizrahi et al. 2006). Therefore, we sought to determine whether Compound 21 inhibited NADPH oxidase activation by measuring the translocation

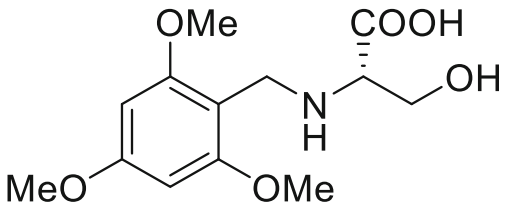

Fig. 1 The chemical structure of Compound 21

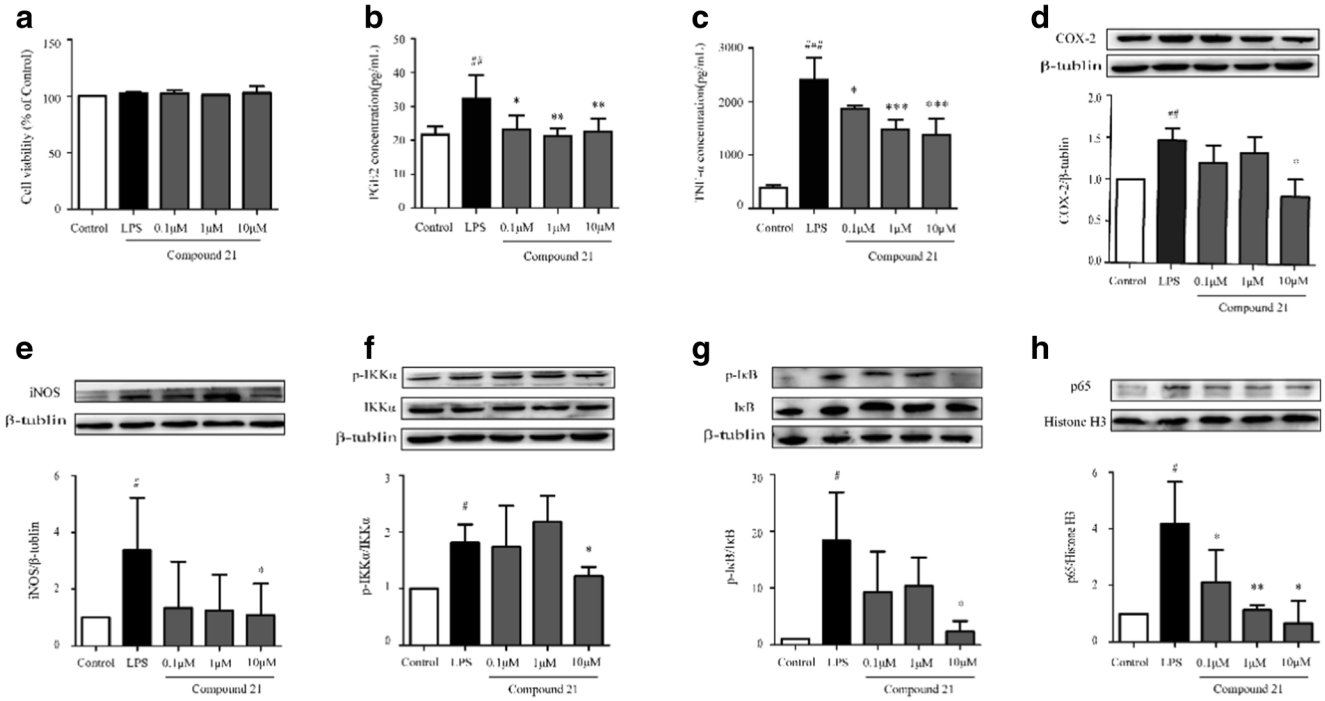

Fig. 2 Compound 21 suppressed LPS-induced neuroinflammation in BV2 cells. **a** Cell viability assayed by MTT. BV2 cells were stimulated with 1.0 μg/mL of LPS and different concentrations of Compound 21 (0.1, 1, and 10 μM) for 12 h. **b**, **c** The PGE_2 and TNF-α production in BV2 cells. BV2 cells were stimulated with LPS (1.0 μg/mL) for 6 h. **d**, **e** Expression of COX-2 and iNOS assayed by Western blot. BV2 cells were stimulated with 1.0 μg/mL of LPS and different concentration of Compound 21 for 12 h. **f**, **g** Phosphorylation of IKKα and IκBα level. **h** Nuclear translocation of p65. Data from three independent experiments are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. LPS group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. control group

of its cytosolic subunit Rac1. Western blot and immunofluorescence staining results showed an increase of Rac1 in the membrane 30 min after LPS treatment (Fig. 3c, d), and this increase was significantly blocked by treatment of Compound 21. These results indicated the inhibitory effect of Compound 21 on the activation of NADPH oxidase. Rac1 is a small GTPase with an inactive guanosine diphosphate (GDP)-bound state and an active guanosine triphosphate (GTP)-bound state. Rac1-GTP pull-down and Rac1-GTP enzyme-linked immunosorbent assays were used to evaluate Rac1 activity, and the results (Fig. 3e, f) showed that Compound 21 suppressed the Rac1 GTPase activity induced by LPS, thereby inhibiting neuroinflammation. Collectively, our data suggested that Compound 21 suppressed Rac1-NADPH oxidase activation and subsequent ROS release in BV2 cells.

Inhibition of Rac1 Caused a Decrease in Neuroinflammation

We then investigated the role of Rac1 in the regulation of neuroinflammation using Rac1 inhibitor NSC23766. NSC23766 targets the domain of Rac1-specific GEF Trio or Tiam1 to inhibit Rac1 activation (Gao et al. 2004). Rac1-GTP enzyme-linked immunosorbent assay and confocal results confirmed that NSC23766 (10 μM) treatment inhibited Rac1 activity (Fig. 4a, b). Moreover, NSC23766 significantly reduced inflammatory cytokine (TNF-α and PGE_2) production and inflammatory proteins (COX-2 and iNOS) expression induced by LPS (Fig. 4c–f). All the results revealed that Rac1 played a vital role in neuroinflammation and Rac1 inhibition could decrease the inflammatory reaction. Meanwhile, Compound 21 exerted comparable anti-inflammatory effect to NSC23766. Collectively, those data indicated that Rac1 GTPase is involved in inflammation and the effect of Compound 21 on Rac1 activity was similar to NSC23766.

Compound 21 Treatment Attenuated the Interaction Between Rac1 and Its Regulator GEF Kalirin

It is well established that the activity of Rac1 GTPase is regulated by GEFs. We then studied whether Compound 21 took part in the interaction between Rac1 and GEFs. We used Co-IP to authenticate the interaction between Rac1 and GEFs when LPS was added to BV2 cells, and then immunoprecipitated by different GEF antibodies. The results in Fig. 5a–d revealed that LPS stimulation led to the increased interaction between Rac1 and GEFs including Kalirin, Tiam1, and DOCK2 but not ARHGEF. The data (Fig. 5a) showed that Compound 21 significantly decreased the interaction between Rac1 and Kalirin. Confocal imaging results (Fig. 5e) also authenticated that the

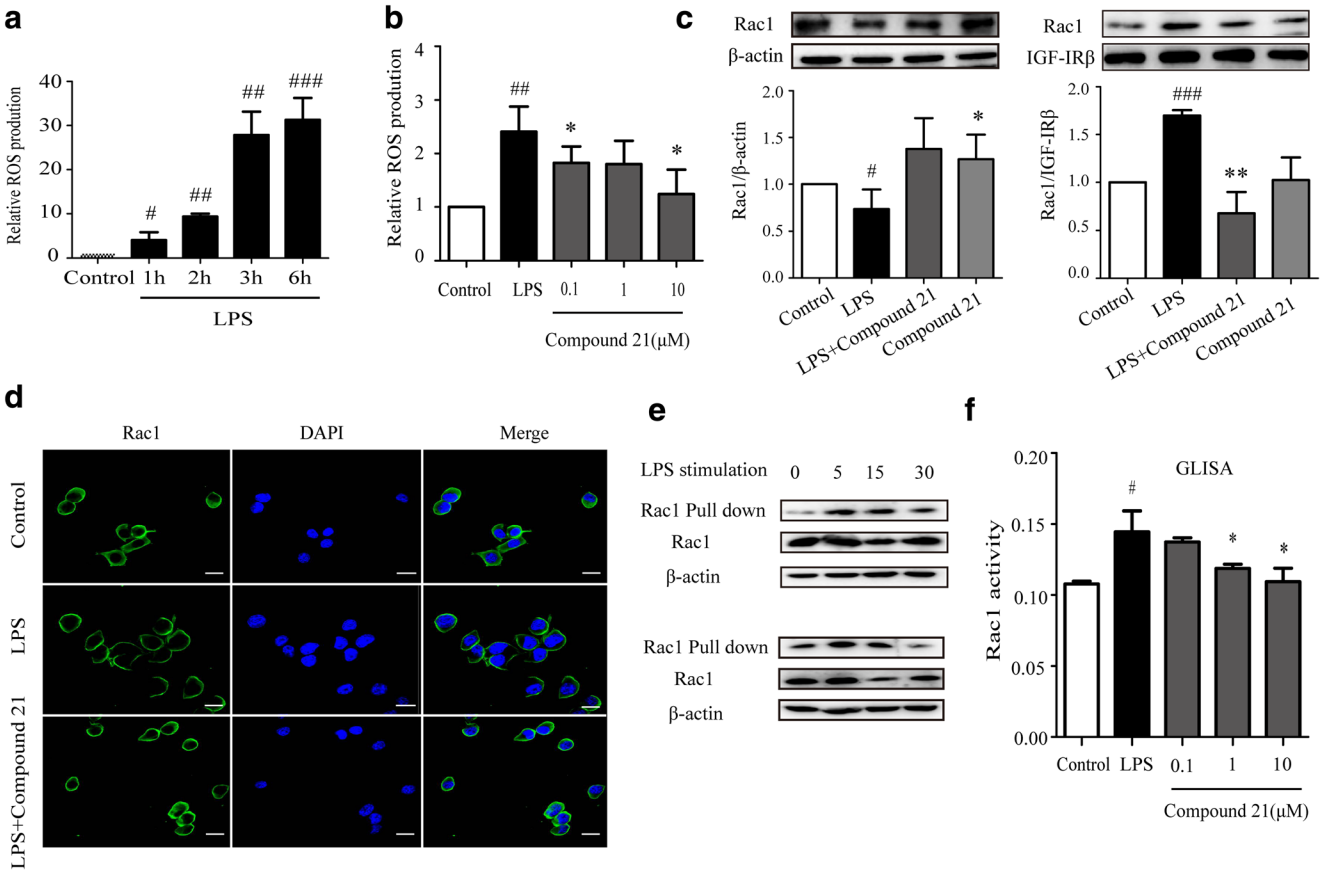

Fig. 3 Compound 21 treatment inhibited ROS production and Rac1-NADPH oxidase activation stimulated by LPS. **a**, **b** The production of ROS from BV2 cells. The BV2 cells were pretreated with Compound 21 (0.1, 1, and 10 μM) for 2 h, and then stimulated with 1.0 μg/mL LPS for 3 h, followed by incubation with 10 μM DCFH-DA for 30 min avoiding the light. The amount of ROS production was assayed by flow cytometry. **c** Translocation of Rac1 from cytoplasm to cytomembrane. Fractionated proteins were analyzed by Western blot assay. **d** Immunofluorescence staining with Rac1 (green) and DAPI (blue) in BV2 cells. Scale bar represents 20 μm. **e** Rac1 activity measured by Rac1 pull-down assay. The BV2 cells were treated with LPS for various time length (5, 15, and 30 min), and the 30 min was chosen. Pretreatment with Compound 21 for 2 h, and then stimulated by LPS for 30 min to measure Rac1 activation. **f** Rac1 activity measured by G-LISA assay. Data from three independent experiments are expressed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. control group

interaction between Rac1 and Kalirin was attenuated by Compound 21 treatment. All the data suggested that Compound 21 decreased the interaction between Rac1 and Kalirin, thereby attenuating neuroinflammation.

Kalirin Is the Key Factor in Neuroinflammation and Might Be the Target of Compound 21 on Microglia

We then investigated the role of Kalirin in neuroinflammation by knocking down endogenous Kalirin in BV2 cells with siRNA. The Kalirin expression was obviously decreased by Kalirin siRNA. Neuroinflammation reaction was also interfered by Kalirin siRNA as shown by the decreased production of inflammatory cytokines (TNF-α and PGE_2) and the decreased expression of COX-2 (Fig. 6a–c). The pull-down experiment showed that Kalirin interference led to decreased Rac1 activation stimulated by LPS (Fig. 6d), confirmed that Rac1 is regulated by Kalirin. Those data indicated that Kalirin is the key factor in neuroinflammation by regulating NADPH oxidase activation. In addition to the result that Compound 21 influenced the interaction between Rac1 and Kalirin, we further investigated whether Compound 21 had impact on Kalirin activity. We purified Kalirin protein with antibody by immunoprecipitation assay, and then defined the real-time exchange reaction of Rac1 by Rho GEF exchange assay biochem kit. The enhancement of fluorescent emission intensity in the presence of Rac1 GTPase and Kalirin reflected the respective Kalirin activity. Pretreatment with Compound 21 for 2 h significantly reduced the activity of Kalirin (Fig. 7a). Western blot and confocal imaging results revealed that Compound 21 treatment inhibited the overexpression of Kalirin (Fig. 7b, c). In conclusion, we speculated Kalirin as the important GEF involved in neuroinflammation, and Compound 21 suppressed the activity and expression of Kalirin to regulate the Rac1 activation and alleviated the neuroinflammation.

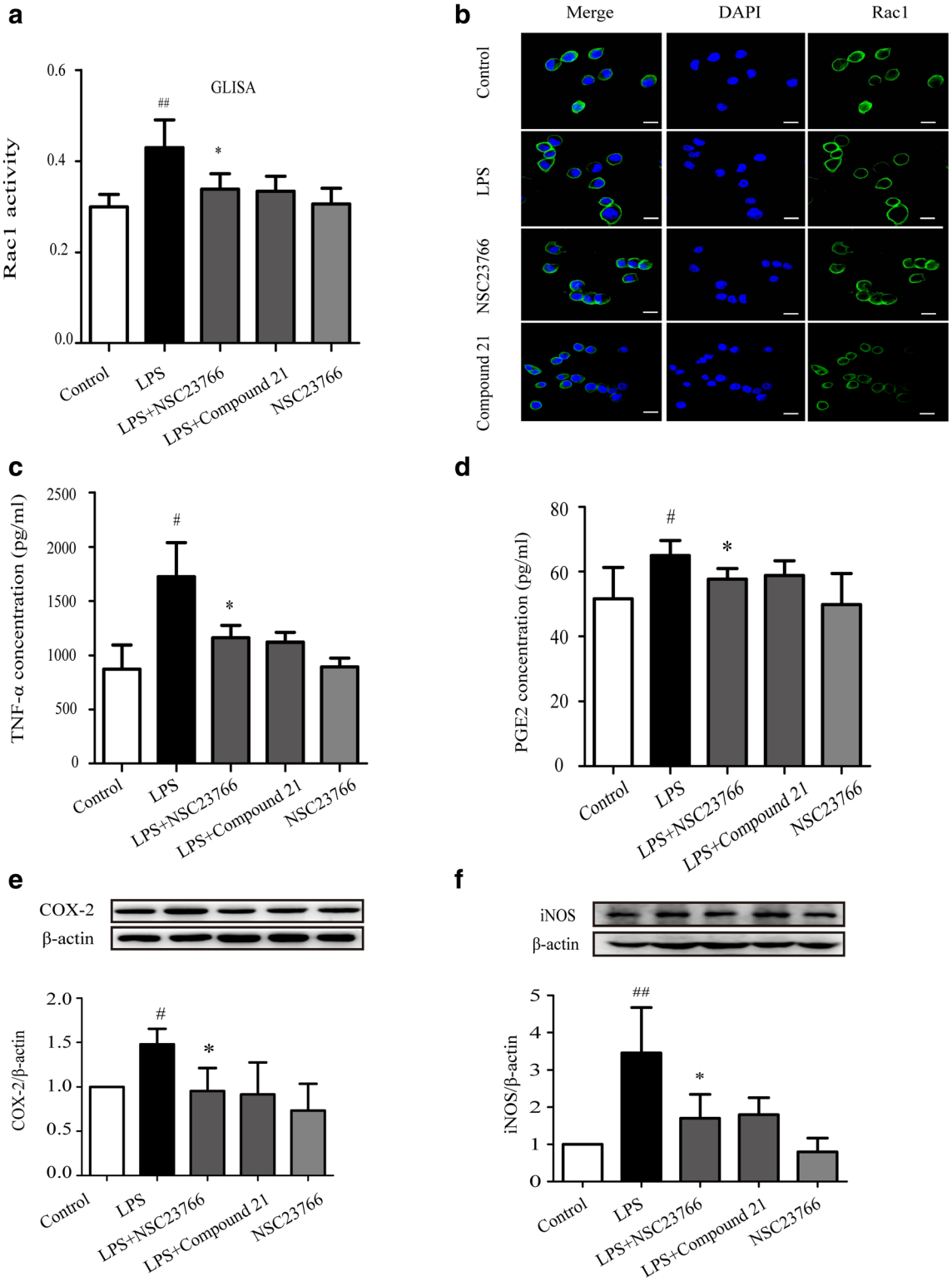

Fig. 4 Inhibition of Rac1 caused a decrease in neuroinflammation. **a** Rac1 activation assayed by GLISA assay. **b** Immunofluorescence staining with Rac1 (green) and DAPI (blue) in BV2 cells. **c**, **d** The TNF-α and PGE_2 production in BV2 cells. BV2 cells were pretreated with NSC23766 (10 μM) for 2 h following with LPS (1.0 μg/mL) stimulation for 6 h. **e**, **f** Expression of COX-2 and iNOS by Western blot assay. BV2 cells were pretreated with NSC23766(10 μM) or Compound 21 (10 μM) for 2 h, and then stimulated with 1.0 μg/mL of LPS for 12 h. Scale bar represents 20 μm. Data from three independent experiments are expressed as mean ± SD. $^{*}p < 0.05$ vs. LPS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with control group

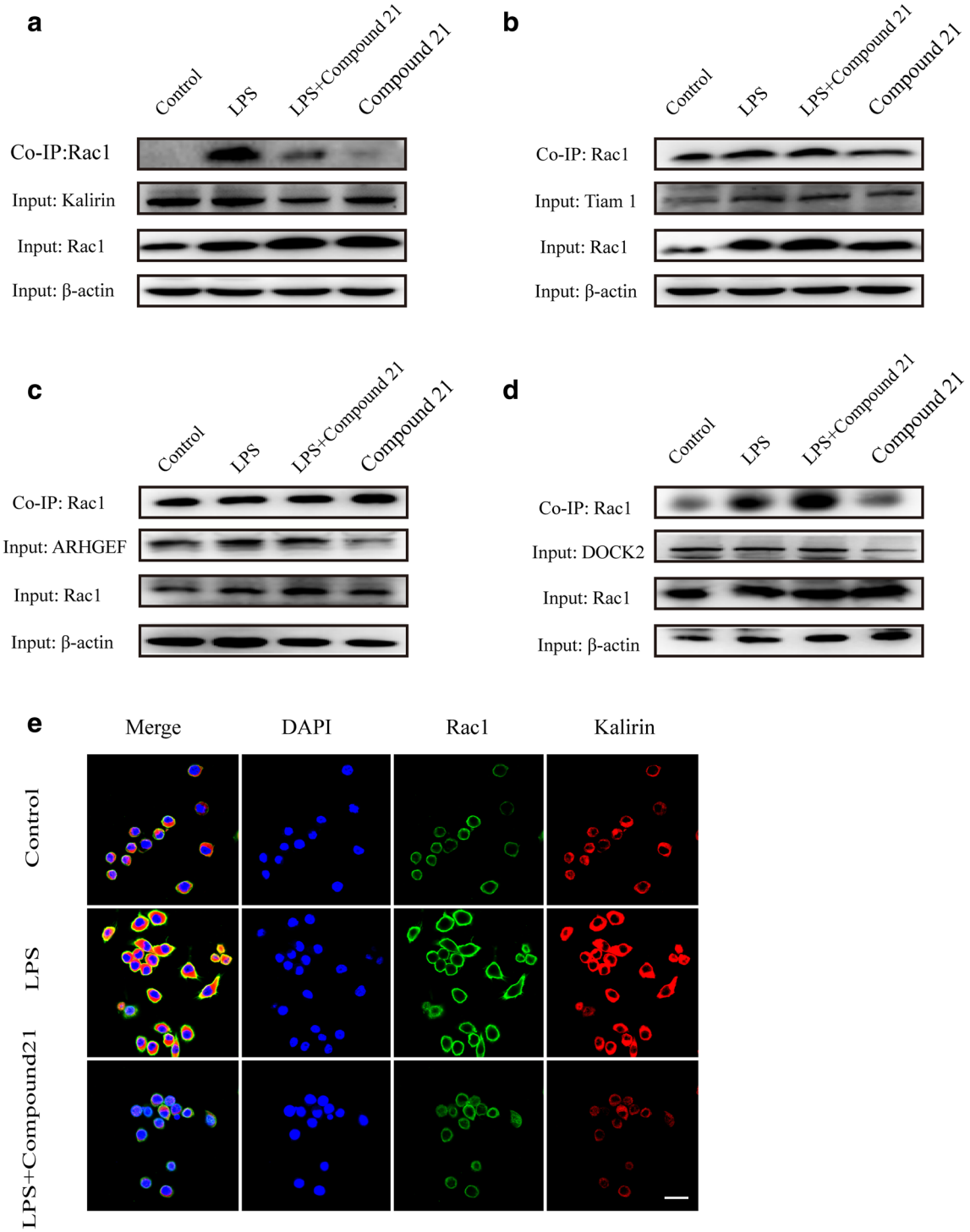

Fig. 5 Compound 21 treatment attenuated the interaction between Rac1 and its regulator GEF-Kalirin. **a**–**d** The effects of Compound 21 on the interaction between Rac1 and its regulators GEF (Kalirin, Tiam1, ARHGEF, DOCK2) shown by Co-IP. The BV2 cells were pretreated with Compound 21 (10 μM) for 2 h, and then stimulated with 1.0 μg/mL LPS for 30 min. **e** The interaction between Rac1 and Kalirin was shown by confocal imaging. Scale bar represents 20 μm

Discussion

Previous studies revealed that Compound 21 showed anti-inflammatory effects in an in vivo PD animal model [10], while the exact mechanism is still unknown. The findings in the study demonstrated that Compound 21 suppressed the Rac1-NADPH oxidase activation, the subsequent ROS and other inflammatory cytokines release, eventually inhibiting

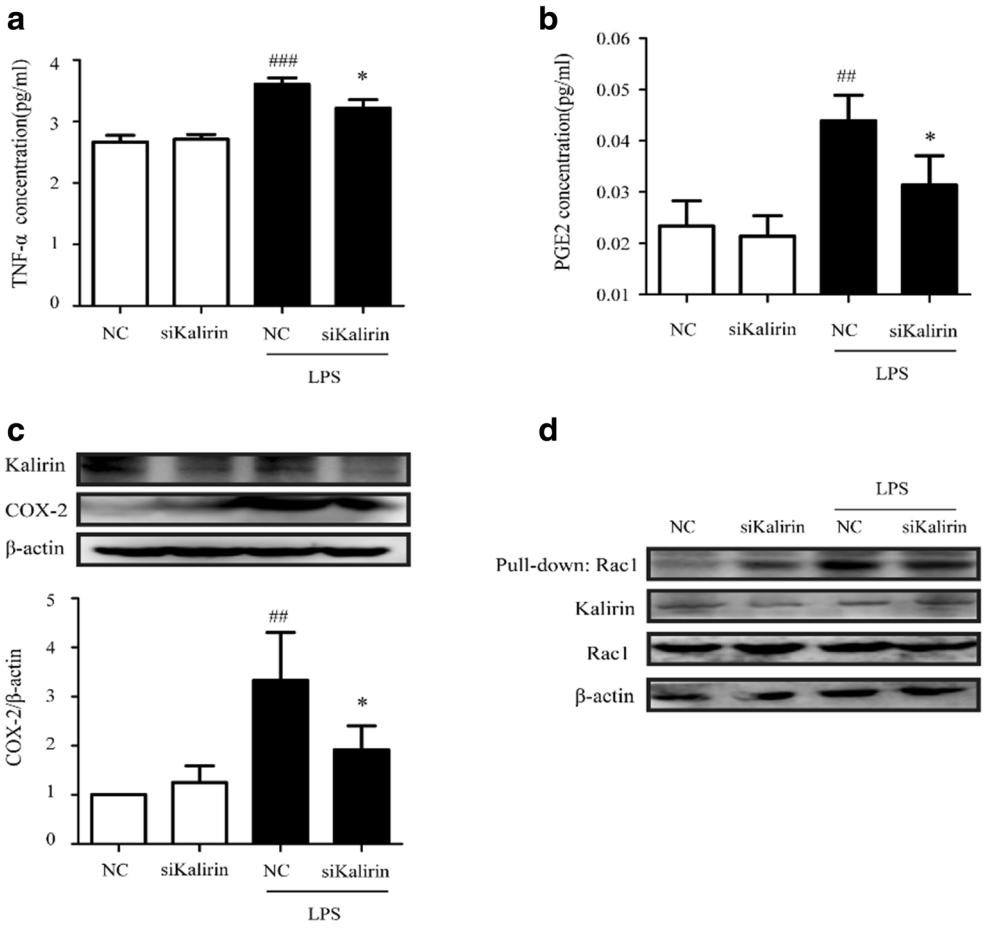

Fig. 6 Kalirin is involved in neuroinflammation in BV2 cells. **a**, **b** The PGE_2 and TNF-α production in BV2 cells. **c** Expression of COX-2 assayed by Western blot. **d** Rac1 activation by pull-down assay. The BV2 cells were pretreatment with Compound 21 (10 μM) for 2 h, and then stimulated by LPS for 30 min to measure Rac1 activation. Data from three independent experiments are expressed as mean ± SD. $^{*}p < 0.05$ vs. LPS group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.01$ vs. control group

the neuroinflammation. Further mechanistic study indicated that one GEF, Kalirin, mediated the anti-inflammatory effect of Compound 21. Our study revealed that Kalirin might be the target of Compound 21 on BV2 microglia.

Considering that neuroinflammation plays a vital role in neurodegenerative diseases [12, 13], neuroinflammation suppression might become an effective disease-modifying therapy and thereby open up a new strategy for the treatment of neurodegenerative diseases. It is important to highlight that there are plenty of natural compounds with anti-inflammatory activity, such as curcumin, punicalagin, and salvianolic acid B, which all showed nice neuroprotective effect in neurodegenerative disease models [14–16]. Based on this idea, we sought to find new treatment candidates for neurodegenerative diseases. Compound 21, a novel synthetic phloroglucinol derivative from *Lysidice rhodostegia* roots, was found from anti-inflammatory screening. In the in vivo study, Compound 21 has found to be a potential PD treatment candidate [10]. The purpose of our study was to explore the mechanism of the anti-neuroinflammatory effect of Compound 21. In the present study, we have found that Compound 21 reduced the expression of inflammatory proteins (COX-2 and iNOS) and the production of inflammatory cytokines (PGE_2 and TNF-α) in LPS-stimulated BV2 microglia. Moreover, Compound 21 treatment inhibited NF-κB signaling pathway. All the data confirmed the anti-inflammatory effect of Compound 21 in microglia.

ROS production is suggested to be upstream of many neuroinflammatory-related signal pathways [7]. NADPH oxidase, a prominent source of ROS, plays a vital role in the inflammatory response and neurotoxicity mediated by microglia cells. NADPH oxidase consists of membrane subunits (gp91phox and p22phox) and cytosolic subunits (p40phox, p47phox, p67phox, and Rac1), which translocate to cytomembranes when exposed to stimulation [17, 18]. There is overwhelming evidence that Rac1 is an important participant in the assembly and consequent activation of the NADPH oxidase [19, 20]. Rac1 induces a conformational change in p67phox, promoting interaction with gp91phox after Rac1-GTP binding to p67phox [21]. Of all subunits, Rac1 plays a coordinating role in the activation of NADPH oxidase [22]. The findings in the study revealed that Compound 21 treatment suppressed the NADPH oxidase activation by both reducing Rac1 translocation from cytosol to cytomembrane and attenuating Rac1 activity. The results suggested that Compound 21 modulated neuroinflammation by inhibiting Rac1-NADPH oxidase activation. To confirm the role of Rac1 in neuroinflammation, NSC23766, an inhibitor of Rac1 activation, was used. We

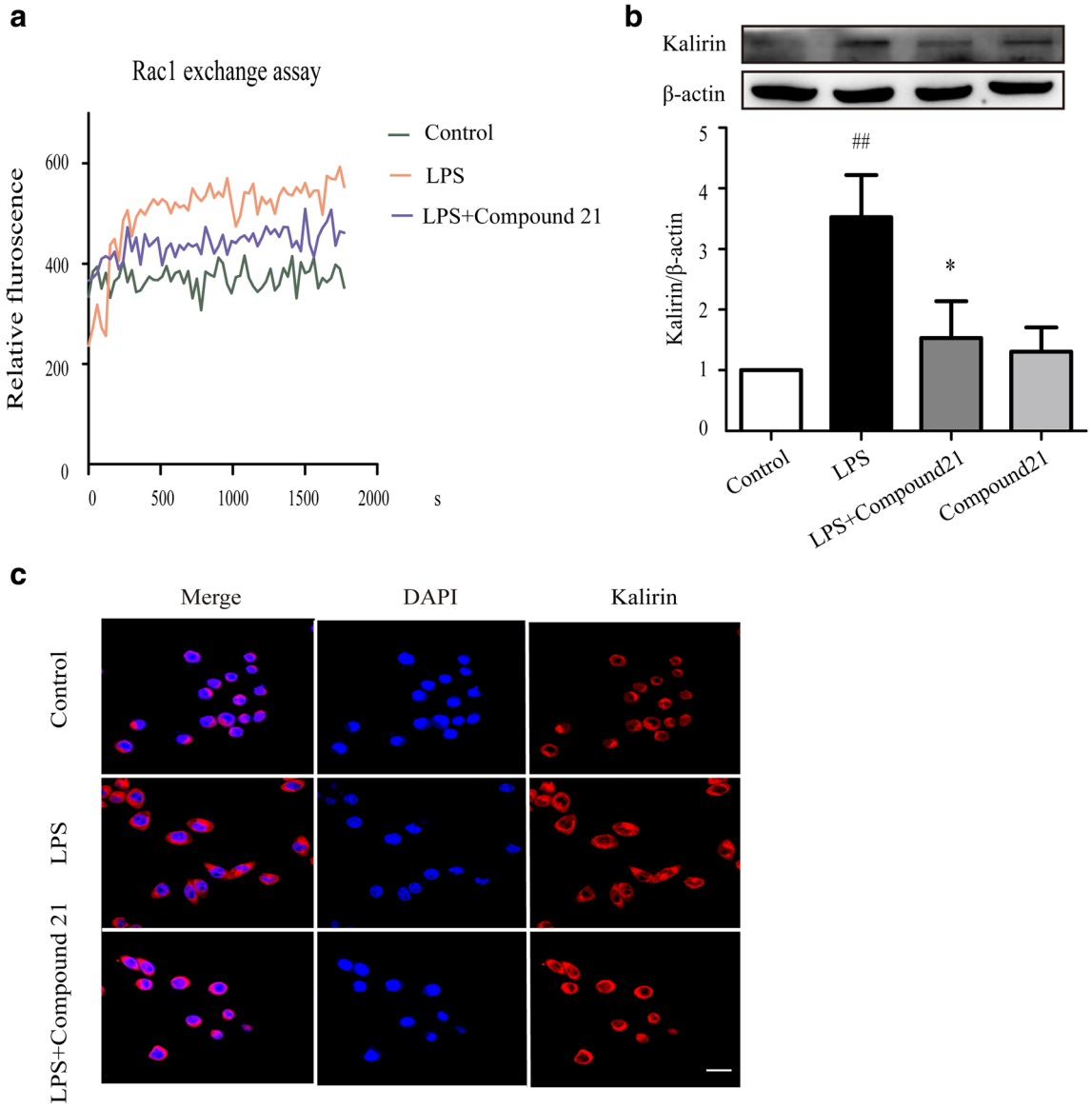

Fig. 7 Compound 21 inhibited the activity and expression of Kalirin. **a** The effect of Compound 21 on the activity of Kalirin by Rho GEF exchange assay. The BV2 cells were pretreated with Compound 21 (0.1, 1, and 10 μM) for 2 h, and then stimulated with 1.0 μg/mL LPS for 30 min. **b**, **c** The expression of Kalirin was shown by Western blot assay and immunofluorescence staining. BV2 cells were stimulated with 1.0 μg/mL of LPS and Compound 21 treatment for 12 h. Scale bar represents 20 μm. Data from three independent experiments are expressed as mean ± SD. $^{*}p < 0.05$ vs. LPS group, $^{\#\#}p < 0.01$ vs. control group

found that NSC23766 treatment inhibited the inflammatory response induced by LPS, and Compound 21 exerted comparable effect to NSC23766. The data suggested that Rac1 plays an important role in neuroinflammation and regulating Rac1 contributes to the anti-inflammatory effect of Compound 21.

It is well established that Rac1 activation is regulated by a variety of GEFs, and the extensively studied GEFs are Tiam1, Kalirin, Farp family (ARHGEF), and DOCK2. Co-IP assay was applied in the present study to evaluate the interactions between Rac1 and different GEFs when LPS was added, and results suggested that the interaction between Rac1 and Tiam1, Kalirin, and DOK2 was enhanced. Among all the GEFs tested, pretreatment with Compound 21 attenuated the interaction between Rac1 and Kalirin. We then further tested the role of Kalirin in neuroinflammation and the anti-inflammatory effect of Compound 21. We knocked down endogenous Kalirin in BV2 cells with sequence-specific siRNA and found that both the inflammatory level and Rac1 activation were decreased. The results indicated that Kalirin is involved in the activation of NADPH oxidase and the subsequent neuroinflammation. Further results revealed that Compound 21 treatment decreased the over-activation and over-expression of Kalirin induced by LPS. Collectively, the findings revealed that GEF family Kalirin might be the target of Compound 21 for its anti-inflammatory effects.

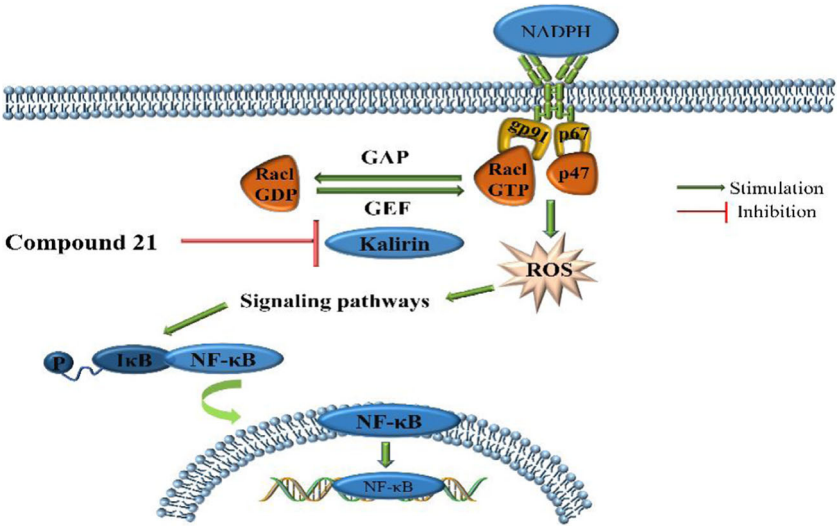

Fig. 8 Proposed mechanism for Compound 21 inhibits neuroinflammation in BV2 microglia. The GEF Kalirin regulates Rac1-NADPH oxidase activation, subsequent ROS production, and downstream signaling pathway. Compound 21 inhibits the Kalirin-mediated neuroinflammatory signaling pathway

Our study provided evidence supporting Compound 21 as a potential candidate of neuroinflammatory inhibitor and underlying molecular mechanism revealed that Compound 21 suppressed Kalirin signaling pathway and Rac1-NADPH oxidase activation (Fig. 8). The present study also suggested that targeting the specific enzymes involved in NADPH oxidase activation, such as Kalirin and Rac1 GTPase, may be beneficial for the treatment of inflammation-related neurodegenerative diseases.

Author's Contributions CXZ, HHY, LW, YW, and XQB performed the experiments. CXZ, XLW, and DZ designed the study, analyzed and interpreted data, and wrote the manuscript.

Funding This work was supported by National Natural Science Foundation of China (81773718, 81630097), and CAMS Innovation Fund for Medical Sciences (2016-I2M-3-011).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Manocha GD, Floden AM, Puig KL, Nagamoto-Combs K, Scherzer CR, Combs CK (2017) Defining the contribution of neuroinflammation to Parkinson's disease in humanized immune system mice. Mol Neurodegener 12:17. https://doi.org/10.1186/s13024-017-0158-z
2. Boza-Serrano A, Yang Y, Paulus A, Deierborg T (2018) Innate immune alterations are elicited in microglial cells before plaque deposition in the Alzheimer's disease mouse model 5xFAD. Sci Rep 8(1):1550. https://doi.org/10.1038/s41598-018-19699-y
3. Amor S1, Puentes F, Baker D, van der Valk P (2010) Inflammation in neurodegenerative diseases. Immunology 129(2):154–69. https://doi.org/10.1111/j.1365-2567.2009.03225.x
4. Fischer R , Maier O (2015) Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease: Role of TNF. Oxid Med Cell Longev. 2015: 610813
5. Kaur K, Gill JS, Bansal PK, Deshmukh R (2017) Neuroinflammation - a major cause for striatal dopaminergic degeneration in Parkinson's disease. J Neurol Sci 381(Supplement C):308–314. https://doi.org/10.1016/j.jns.2017.08.3251
6. Tiwari PC, Pal R (2017) The potential role of neuroinflammation and transcription factors in Parkinson disease. Dialogues Clin Neurosci 19(1):71–80
7. Patten DA, Germain M, Kelly MA, Slack RS (2010) Reactive oxygen species: Stuck in the middle of neurodegeneration. J Alzheim Dis: JAD 20(Suppl 2):S357–S367. https://doi.org/10.3233/JAD-2010-100498
8. Choi S-H, Aid S, Kim H-W, Jackson SH, Bosetti F (2012) Inhibition of NADPH oxidase promotes alternative and anti–inflammatory microglial activation during neuroinflammation. J Neurochem 120(2):292–301. https://doi.org/10.1111/j.1471-4159.2011.07572.x
9. Brandes RP, Weissmann N, Schröder K (2014) Nox family NADPH oxidases: Molecular mechanisms of activation. Free Radic Biol Med 76:208–226. https://doi.org/10.1016/j.freeradbiomed.2014.07.046
10. Wang Y-D, Bao X-Q, Xu S, Yu W-W, Cao S-N, Hu J-P, Li Y, Wang X-L et al (2016) A novel Parkinson's disease drug candidate with potent anti-neuroinflammatory effects through the Src signaling pathway. J Med Chem 59(19):9062–9079. https://doi.org/10.1021/acs.jmedchem.6b00976
11. Tak PP, Firestein GS (2001) NF-κB: A key role in inflammatory diseases. J Clin Investig 107(1):7–11
12. Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR, Lafaille JJ, Hempstead BL, Littman DR et al (2013) Microglia promote learning-dependent synapse formation through BDNF. Cell 155(7):1596–1609. https://doi.org/10.1016/j.cell.2013.11.030
13. Steardo L, Bronzuoli MR, Iacomino A, Esposito G, Steardo L, Scuderi C (2015) Does neuroinflammation turn on the flame in Alzheimer's disease? Focus on astrocytes. Front Neurosci 9:259. https://doi.org/10.3389/fnins.2015.00259
14. Yang F, Lim GP, Begum AN, Ubeda OJ, Simmons MR, Ambegaokar SS, Chen PP, Kayed R et al (2005) Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem 280(7):5892–5901. https://doi.org/10.1074/jbc.M404751200

15. Kim YE, Hwang CJ, Lee HP, Kim CS, Son DJ, Ham YW, Hellström M, Han S-B et al (2017) Inhibitory effect of punicalagin on lipopolysaccharide-induced neuroinflammation, oxidative stress and memory impairment via inhibition of nuclear factor-kappaB. Neuropharmacology 117:21–32. https://doi.org/10.1016/j.neuropharm.2017.01.025
16. Lee YW, Kim DH, Jeon SJ, Park SJ, Kim JM, Jung JM, Lee HE, Bae SG et al (2013) Neuroprotective effects of salvianolic acid B on an Aβ25–35 peptide-induced mouse model of Alzheimer's disease. Eur J Pharmacol 704(1–3):70–77. https://doi.org/10.1016/j.ejphar.2013.02.015
17. Niu X-L, Madamanchi NR, Vendrov AE, Tchivilev I, Rojas M, Madamanchi C, Brandes RP, Krause K-H et al (2010) Nox activator 1 (NoxA1): A potential target for modulation of vascular reactive oxygen species in atherosclerotic arteries: Niu, NoxA1 regulates VSMC NADPH oxidase. Circulation 121(4):549–559. https://doi.org/10.1161/CIRCULATIONAHA.109.908319
18. Ambasta RK, Kumar P, Griendling KK, Schmidt HH, Busse R, Brandes RP (2004) Direct interaction of the novel Nox proteins with p22phox is required for the formation of a functionally active NADPH oxidase. J Biol Chem 279(44):45935–45941. https://doi.org/10.1074/jbc.M406486200
19. Choi SS, Witek RP, Yang L, Omenetti A, Syn W-K, Moylan CA, Jung Y, Karaca GF et al (2010) Activation of Rac1 promotes hedgehog-mediated acquisition of the myofibroblastic phenotype in rat and human hepatic stellate cells. Hepatology (Baltimore, Md) 52(1):278–290. https://doi.org/10.1002/hep.23649
20. Hordijk PL (2006) Regulation of NADPH oxidases: The role of Rac proteins. Circ Res 98(4):453–462. https://doi.org/10.1161/01.RES.0000204727.46710.5e
21. Diekmann D, Abo A, Johnston C, Segal A, Hall A (1994) Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. Science 265(5171):531–533. https://doi.org/10.1126/science.8036496
22. Bai Y, Xiang X, Liang C, Shi L (2015) Regulating Rac in the nervous system: Molecular function and disease implication of Rac GEFs and GAPs. Biomed Res Int 2015:632450–632417. https://doi.org/10.1155/2015/632450