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Research Article
5-Nitro-2-(3-phenylpropylamino) Benzoic Acid Promotes Lipopolysaccharide-induced Inflammation via p38 MAPK Pathway in RAW264.7 Macrophages

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Abstract

Background and Objectives: 5-nitro-2-(3-phenylpropylamino) benzoic acid is a significant chlorine channel blocker and an agonist of G protein-coupled receptor 35 but its function in macrophage inflammatory response has not been reported yet. Therefore, this study aims to reveal the unrecognized role of 5-Nitro-2-(3-phenylpropylamino) benzoic acid in macrophage inflammation activation and its mechanism, which is helpful for further understanding of macrophage inflammation. **Materials and Methods:** For treatments, cells were pre-treated respectively with different drugs and then treated with Lipopolysaccharide for 4 hrs. Proteins were resolved by the SDS page and analyzed by immunoblotting using antibodies. The qRT-PCR was conducted with the SuperReal Pre Mix × Plus kit. One-Way ANOVA analysis of variance and t-test were using SPSS 13.0 statistical software package. **Results:** Silencing voltage-gated chloride channel 3 inhibited the expression of tumour necrosis factor-alpha in Lipopolysaccharide (100 ng mL^{-1})-induced murine macrophages RAW264.7. 5-Nitro-2-(3-phenylpropylamino) benzoic acid (100 μM) downregulates the Lipopolysaccharide-induced nuclear factor kappa-light-chain-enhancer of activated B cells phosphorylation level via inhibiting the chlorine channel. But G protein-coupled receptor 35 agonists up-regulate tumour necrosis factor-alpha in macrophages induced by Lipopolysaccharide. 5-Nitro-2-(3-phenylpropylamino) benzoic acid enhances LPS-induced macrophages inflammation by promoting the associated p38 mitogen-activated protein kinase after activating G protein-coupled receptor 35. **Conclusion:** Current work indicated that not a chlorine channel blocker but a G protein-coupled receptor 35 agonists 5-Nitro-2-(3-phenylpropylamino) benzoic acid acts to enhance the inflammatory response of macrophages, which suggested that G protein-coupled receptor 35 activations may promote inflammatory response and aggravate the clinical diseases including bacterial infection.

Key words: Inflammation response, Lipopolysaccharide (LPS), G protein-coupled receptor 35 (GPR35), mitogen-activated protein kinase (MAPK), chloride channel

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Macrophages are significant regulatory and effector immune cells in the body. They are at rest under normal conditions[1] and participate in numerous physiological and pathological processes when activated. They secrete specific cytokines to participate in inflammatory reactions and initiate specific immune responses finally[2-4]. Lipopolysaccharide (LPS) is a common inflammatory stimulus. LPS is recognized by Toll-like Receptor 4 (TLR-4), then promotes an intracellular signalling cascade, ultimately activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) signalling pathways leading to the secretion of proinflammatory cytokines, such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1 (IL-1)[5].

Current studies have found that chloride channels are involved in cellular immune responses and inflammatory responses. Studies have shown that chlorine channel blockers can inhibit the infection of Herpes Simplex Virus type 1 (HSV-1)[6] and inhibit microglial phagocytosis of *Escherichia coli*[7], suggesting that chlorine channels are involved in inflammatory responses. Initially, a voltage-gated chloride channel (ClC) was cloned by Jentsch *et al*.[8] from the electric organ of electric rays. Studies have shown that cytokines, such as TNF-α and IL-1, activate chloride currents that are dependent on ClC-3 expression[9]. Studies have shown that ClC-3 is a pro-inflammatory factor involved in the cytokines release of LPS-induced[10]. However, the current studies about chloride channels and inflammation mainly focus on vascular inflammation, the researches on macrophage inflammation need to be further explored.

G Protein-Coupled Receptors (GPCRs) widely expressed in cells play an important role in the inflammatory immune response. Studies have shown that the GPCRs signalling pathway interacts with the Toll-like receptor signalling pathway of innate immunity, enhancing the expression of inflammatory factors in human gingival epithelial cells[11]. G Protein-Coupled Receptor 35 (GPR35), a kind of GPCRs, has been found to promote the adhesion of white blood cells to vascular endothelial cells[12], suggesting that GPR35 plays a certain role in an inflammatory response. However, the function of GPR35 in inflammation has not been illuminated. In addition, the Mitogen-Activated Protein Kinase (MAPK) signalling pathway can be activated by GPCRs. MAPK is a group of serine-threonine protein kinases that can be activated by different extracellular stimuli. MAPK play a key role in many physiological and pathophysiological processes such as cell growth, differentiation, proliferation, apoptosis, stress adaptation and inflammation. The MAPK family consists of four members: p38 MAPK, extracellular regulated protein kinase 1/2 (ERK1/2), stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK) and ERK5/big mitogen-activated protein kinase 1 (BMK1). As an important member of the MAPK family, p38 MAPK also plays an important role in inflammation and stress response, which is considered a transfer station for numerous signal transduction pathways within cells[13]. Thus, p38 MAPK has become a target for the development of anti-inflammatory drugs.

5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) is a non-specific chlorine channel blocker[14] and an agonist of GPR35[15] but its function in macrophage inflammatory response has not been reported yet.

This study demonstrated that NPPB does not regulate LPS- induced macrophage inflammation via chloride channel but activates GPR35 to promote the p38 MAPK pathway mediating macrophage inflammation. Thus, the current study uncovered a previously unrecognized function of NPPB on the inflammatory response and found a new target GPR35 for activating inflammatory responses in macrophages. Current findings suggested that GPR35 activation may promote inflammatory response and aggravate clinical diseases including bacterial infection.

MATERIALS AND METHODS

Cell culture and reagents: The study was carried out at Jinan University in Guangzhou, China, in 2021. The mouse macrophage cell line RAW264.7 was purchased from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (No. 11995065, Gibco, Invitrogen, USA) complete medium containing 10% Foetal bovine serum (No. 10099-141, Gibco, Invitrogen, USA), 1% penicillin/streptomycin (No. 15070063, Gibco, Invitrogen, USA) in a 37°C incubator (NAPCO6500 CO_2 incubator, Thermo Fisher Scientific, USA) containing 5% CO_2 and 95% relative humidity. When the degree of cell fusion was about 80%, the following process was conducted. For LPS treatment, cells were treated with 100 ng mL^{-1} LPS (No. L3024, Sigma-Aldrich, USA) for 4 hrs. For NPPB and DIDS treatments, the cells were pre-treated respectively with 100 μM NPPB (No. 107254-86-4, Sigma-Aldrich, USA) and 100 μM DIDS (No. 67483-13-0, Sigma-Aldrich, USA) for 30 min then treated with 100 ng mL^{-1} LPS for 4 hrs. For GPR35 Agonist treatment, cells were pre-treated with 100 μM GPR35 Agonist (No. 123021-85-2, Sigma-Aldrich, USA) for 30 min then treated with LPS for 4 hrs. For MAPK treatment, cells were pre-treated with 20 μM SB203580 (No. 152121-47-6, Sigma-Aldrich, USA) for 1 hr and then treated with LPS for 4 hrs.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR): Total RNA was purified using Trizol (Invitrogen) according to the manufacturer's instructions, then quantified by OD 260/280 nm measurement. Followed the instructions of the SuperScriptTM III First-Strand Synthesis System RT-PCR kit (TaKaRa) for reverse transcription. The qRT-PCR was conducted with SuperReal Pre Mix × Plus (SYBR Green) kit (QIAGEN). All genes were normalized to GAPDH. Amplification primers (Thermo Fisher) were listed as follows: CLCN-1 sense (GCATTTGGAAGGCTGGTAGG), CLCN-1 antisense (TGTGGAGACTGTGTGGGAGAC), CLCN-2 sense (GGGAGTGG TGCTGAAAGAAT), CLCN-2 antisense (CCCAAAGAGGGAGAGG AACT), CLCN-3 sense (CCTGGCTGCTGATGTTATGA), CLCN-3 antisense (CTGAGGGCAAATCCCACTAA); CLCN-4 sense (GCCTG TTTGGTGGCATTTAG), CLCN-4 antisense (GGCAAGTGTTCA GCGTCAT), CLCN-5 sense (AATGAAGCCAAGCGGAGAG), CLCN-5 antisense (ACCAGAGCAGCGAAGAAAGA), CLCN-6 sense (GGCACAACCTGACAAATGAA), CLCN-6 antisense (ATCTG AGCAAACGGGTGAGT), CLCN-7 sense (GGCATCTCCCTGTC CTACCT), CLCN-7 antisense (GCAGTCATCAGCACCAACAT), TNF-α sense (CTTGTCTACTCCCAGGTTCTCTT), TNF-α antisense (GATAGCAAATCGGCTGACGG), IL-6 sense (GCTGGAGTCACAGA AGGAGTGGC), IL-6 antisense (GGCATAACGCACTAGGTTT GCCG), GAPDH sense (CTCGTGGAGTCTACTGGTGT), GAPDH antisense (GTCATCATACTTGGCAGGTT).

Antibodies and western blots: Cells were grown in a normal medium and then were treated with or without LPS, NPPB, DIDS, Tamoxifen, GPR35 agonists and SB203580. Cells were washed with cold PBS three times, then PBS was discarded and added lysate buffer (M-PER® Mammalian Protein Extraction Reagent, Pierce, USA) for 5 min, then cells were scraped and cell fragments were collected together with lysate into a 1.5 mL EP tube (operated on ice). The cell lysate was centrifuged at 12000 g min^{-1} for 10 min at 4°C and the supernatant was taken into a pre-cooled 1.5 mL EP tube. Protein concentrations were determined by BCA assay (Pierce, USA). Proteins were resolved by the SDS page and analyzed by immunoblotting using antibodies. The following primary antibodies were used: β-actin (Neomarker, USA), TNF-α (CST, USA), ClC-3 (Abcam, USA), NF-κB p65 (CST, USA), phosphorylated-NF-κB p65 (CST, USA), IκBα (CST, USA), phosphorylated-IκBα (CST, USA), p38 MAPK (CST, USA), phosphorylated-p38 MAPK (CST, USA), JNK (CST, USA), phosphorylated-JNK (CST, USA), ERK (Santa Cruz, USA), phosphorylated-ERK (Santa Cruz, USA), c-Jun (CST, USA), phosphorylated-c-Jun (CST, USA), GPR35 (Santa Cruz, USA). Images were taken on a Bio-Rad ChemiDoc XRS+System gel imager and grayscale scanning analysis was performed using Image Lab 4.0 (Bio-Rad) software.

Enzyme-linked immunosorbent assay (ELISA): The production of TNF-α and IL-6 was determined in cell culture supernatants using ELISA kits (RayBiotech, Norcross, America) according to the manufacturer's instructions.

RNA interference: RNA interference was carried out on RAW264.7 using Lipofectamine 2000 (Invitrogen) and diluted to 50 nmol L^{-1} when used. Cells were seeded the day before transfection and transfected at 30-50% confluence with the indicated 100 nmol L^{-1} ClC-3 siRNA oligonucleotides (GenePharma, China).

RNA-sequencing: The RNA-sequencing data was obtained from a dataset published elsewhere[16].

Statistical analysis: All data were expressed as the mean and standard error of the mean (SEM). One-way ANOVA analysis of variance and t-test were using SPSS 13.0 statistical software package and $p<0.05$ was considered statistically significant.

RESULTS

NPPB promotes inflammatory reaction in LPS-induced RAW 264.7 macrophages: To obtain inflammatory macrophages, RAW264.7 cells were activated by LPS. Whether LPS-induced inflammatory factors could be influenced by NPPB was investigated. The preliminary results suggested that mRNA expression of TNF-α and IL-6 were increased by NPPB with LPS treatment (Fig. 1a-b). Consistently, NPPB in combination with LPS enhanced TNF-α protein expression and NPPB alone did not induce TNF-α (Fig. 1c-d). NPPB increased LPS-induced TNF-α in a dose-dependent manner (Fig. 1e-f). Furthermore, the inflammatory cytokines were detected in cell culture supernatants, which were up-regulated upon NPPB treatment, compared to LPS treatment alone. The data show that NPPB inhibits LPS-induced secretion of inflammatory cytokines (Fig. 1g-h). These results indicate that NPPB promotes LPS-induced inflammatory reaction in RAW264.7 macrophages.

NPPB does not participate in the inflammatory response by inhibiting chloride channels: NPPB is widely used as a potent inhibitor of many different chloride channels. So whether NPPB participates in the inflammatory response by regulating chloride channels? Firstly, the LPS-induced RAW264.7 murine macrophages were treated with NPPB and another representative chloride channel blocker DIDS. Different chloride channel blockers had different effects on TNF-α expression in LPS-induced RAW264.7 cells, NPPB significantly increased TNF-α expression, while DIDS reduced the TNF-α expression (Fig. 2a-b).

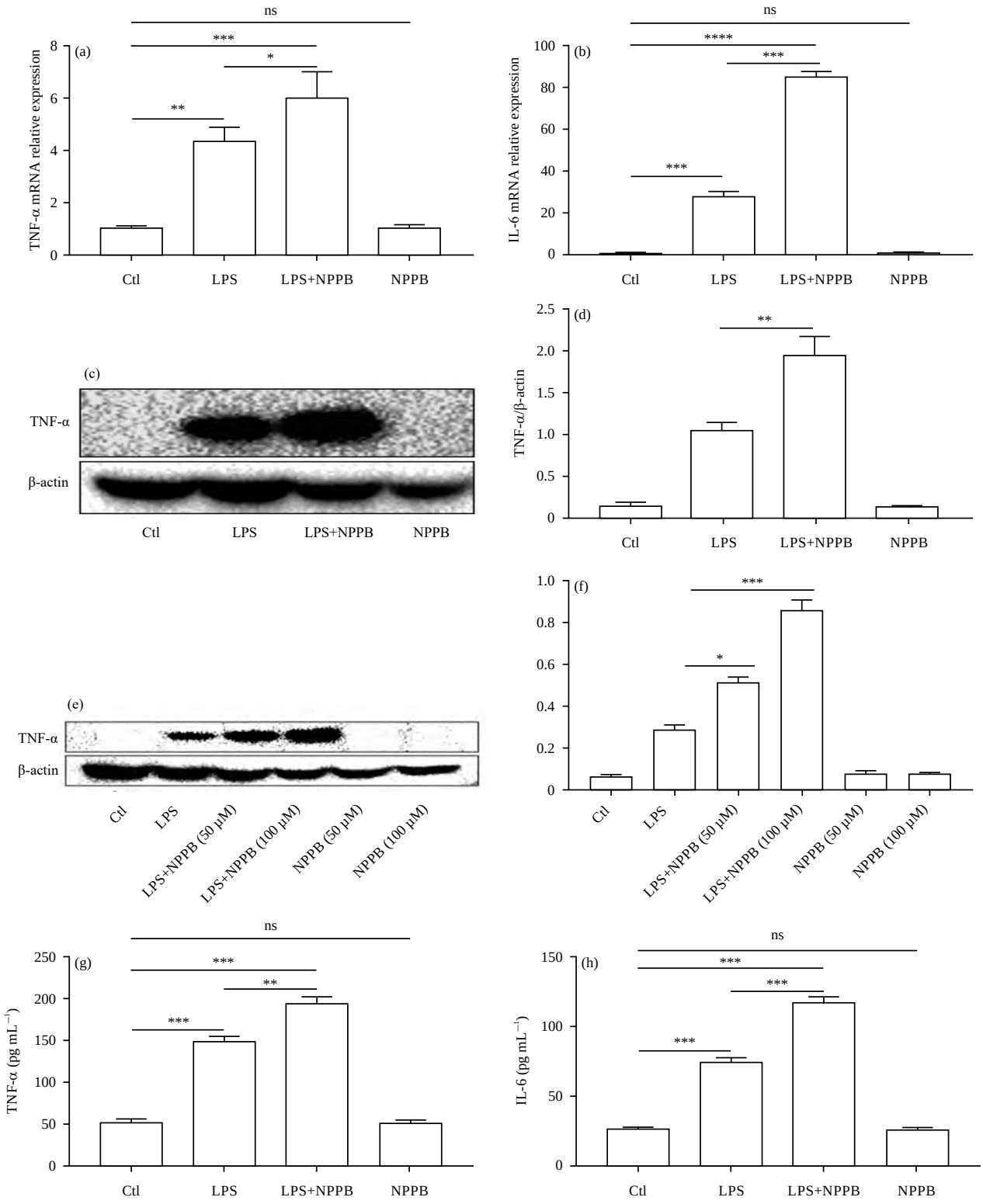

Fig. 1(a-h): NPPB up-regulates the expression of TNF-α in LPS-induced RAW264.7 macrophages, (a-b) TNF-α and IL-6 mRNA relative expression in each group (n = 4), (c-d) TNF-a protein expression and quantification in each group (n = 3), (e-f) TNF-α protein expression and quantification in each group treated with different concentrations of NPPB (n = 4) and (g-h) Absolute concentrations of TNF-α and IL-6 in each group (n = 5)

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

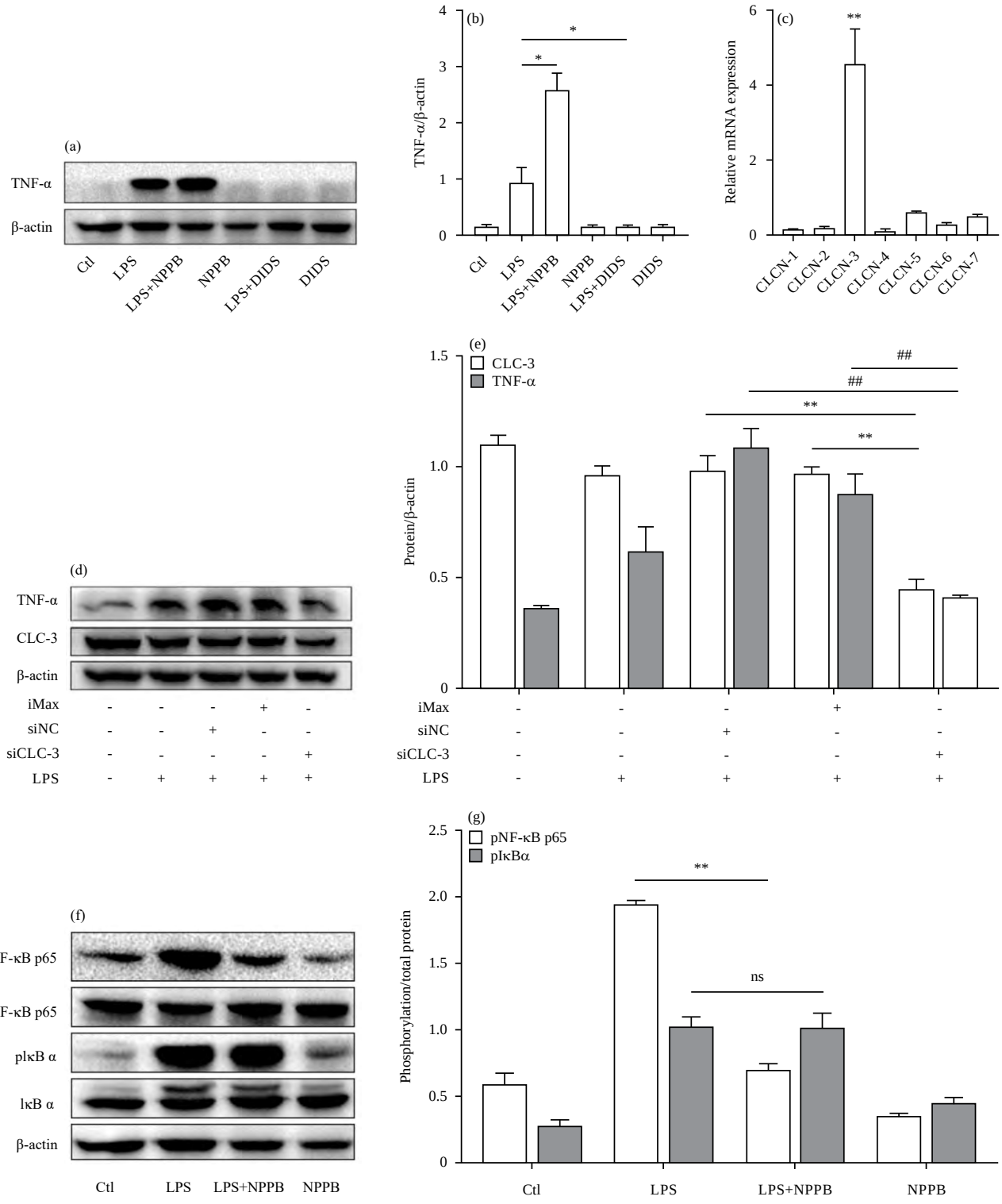

Fig. 2(a-g): Chloride channels are not the targets for NPPB to promote the inflammatory reaction, (a-b) TNF-α protein expression and quantification in each group treated with NPPB and the another chloride channel blocker DIDS (n = 4), (c) Relative mRNA expression level of ClC family members (n = 4), (d-e) TNF-a and CLC-3 protein expression and quantification in each group (n = 4) and (f-g) pNF-κB and pIκBa protein expression and quantification in each group (n = 4)

*p<0.05, **p<0.01, ##p<0.01

As the relative mRNA expression level of chloride channel protein, ClC-3 is significantly higher than other ClC family members (Fig. 2c), so the ClC-3 was focused. Treatment with ClC-3 siRNA significantly decreased the expression of ClC-3 protein. Compared with the LPS group, LPS-induced TNF-α expression was significantly reversed by the knockdown of ClC-3 (Fig. 2d-e). This result shows that ClC-3 inhibition reduces the expression of TNF-α in LPS-induced RAW264.7, which is contrary to the increased expression of LPS-induced TNF-α caused by the chloride channel blocker NPPB.

Previously a report suggests that ClC-3 regulates NF-κB inflammatory signalling pathway, which leads us to explore whether NF-κB is involved with TNF-α reduced by ClC-3 inhibition in LPS-induced macrophages. The results showed that the phosphorylations of p65 NF-κB and IκBα were significantly increased after LPS treatment but the phosphorylation of p65 NF-κB was reversed with NPPB pretreatment (Fig. 2f-g), suggesting that NPPB inhibits NF-κB signalling by inhibiting ClC-3. The results indicate that chloride channels are not the targets for NPPB to promote LPS-induced inflammatory reaction in RAW264.7.

NPPB promotes inflammatory reaction by activating GPR35: Nevertheless, studies have shown that NPPB is also an agonist for GPR35. Consistently, NPPB furtherly activated GPR35 and enhanced TNF-α expression in LPS-induced RAW264.7 (Fig. 3a-b). Then the effect of a known specific GPR35 agonist on TNF-α expression was investigated in LPS-induced RAW 264.7. The known specific GPR35 agonist had the same effect as NPPB, significantly increased the expression of TNF-α in LPS-induced RAW264.7 cells (Fig. 3c-d), suggesting that GPR35 activation upregulates LPS-induced inflammatory factors.

What is more, we reanalysed the RNA-sequencing data on sorted GPR35 negative and GPR35 positive macrophages from the colonic lamina propria. GPR35 positive macrophages showed higher TNF superfamily transcripts compared to GPR35 negative macrophages (Fig. 3e), which further supports current findings. Collectively, these findings indicate that NPPB up-regulates TNF-α by activating GPR35 in LPS-induced RAW264.7.

NPPB promotes inflammatory reaction by activating the p38-MAPK pathway: GPR35 has been reported to interact with extracellular signals to regulate p38 MAPK. To explore the mechanism of NPPB up-regulating LPS-induced inflammatory response in macrophages, the p38 MAPK signalling pathway was inhibited by SB203580. SB203580 reversed TNF-α expression increased by NPPB (Fig. 4a-b), which preliminarily confirms that p38 MAPK was involved in the inflammatory response promoted by NPPB.

To further confirm the role of the p38 MAPK pathway in the inflammatory response, the p38 MAPK protein expression was detected in RAW264.7 induced by LPS or LPS combined with NPPB. LPS induced phosphorylation of p38 MAPK, which was increased with NPPB treatment (Fig. 4c-d). This result shows that NPPB enhances LPS-induced inflammatory response by activating p38 MAPK. Moreover, MAPKs include ERK and JNK MAPK subfamilies. Thus, the other crucial proteins of the MAPK signalling pathway were detected after LPS treatment or LPS combined with NPPB treatment in RAW264.7. The phosphorylation of JNK and ERK showed no differences but the phosphorylation of c-Jun was inhibited with NPPB treatment (Fig. 4e-f). Here NPPB is proposed to promote LPS-induced TNF-α expression in macrophages by activating GPR35 to promote the p38 MAPK pathway.

DISCUSSION

Inflammation is an automatic defence triggered by stimuli. It can be triggered by various factors, such as biological, physical, chemical, pathological changes, foreign bodies and allergies. Macrophages produce inflammatory cytokines after polarization induced by LPS alone or associated with Th1 cytokines. In this study, the results show that NPPB can up-regulate inflammation of macrophages RAW264.7 induced by LPS at mRNA and protein levels. And the expression of TNF-α mRNA showed dose-dependent on the concentration of NPPB. In conclusion, NPPB can indeed promote macrophage inflammation.

Studies have shown that the chloride channel blockers DIDS downregulated ClC-3 and LPS-induced inflammatory cytokines *in vivo* and *in vitro*, via TLR4/NF-κB pathway[10]. It shows that ClC-3 plays an important role in inflammation[10]. However, the current study shows that different blockers have different effects on LPS-induced TNF-α expression in macrophage RAW264.7. NPPB, which has been reported as a blocker to classic chloride channel in HEK-293[17], significantly promoted TNF-α expression, while another representative chloride channel blocker DIDS[10] was able to reduce TNF-α expression. But it is noted that DIDS and silencing of ClC-3 both inhibited LPS-induced TNF-α expression in RAW264.7 cells, which is consistent with the previous study[10]. The activated IκBs are phosphorylated and separated from p65 NF-κB, which causes p65 NF-κB activation[18,19]. A previous report shows that ClC-3 deletion significantly reduces the activity of p65 NF-κB[9]. Interestingly, NPPB did down-regulate the phosphorylation level of NF-κB but finally up-regulated inflammation induced by LPS. Based on these results, ClC-3 chloride channel may not be the target for NPPB to up-regulate macrophage inflammatory response.

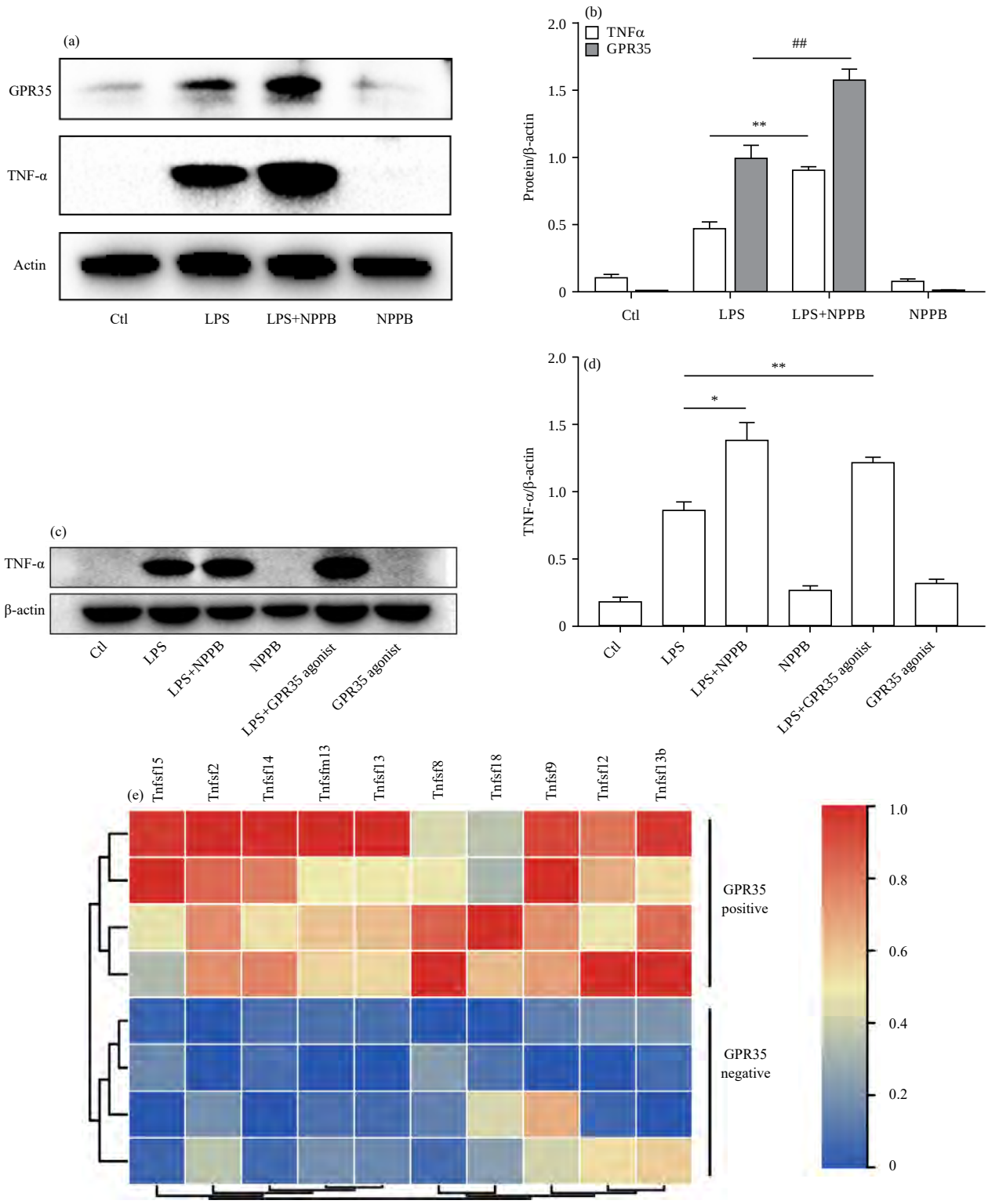

Fig. 3(a-e): GPR35 activated by NPPB up-regulates inflammatory response, (a-b) GPR35 and TNF-α protein expression and quantification in each group (n = 4), (c-d) TNF-α protein expression and quantification in each group treated with NPPB and another GPR35 agonist (n = 4), (e) Heatmap representation of TNF superfamily cytokine expression profiles from the RNA-sequencing of GPR35 positive and GPR35 negative colonic lamina propria macrophages

*p<0.05, **p<0.01, ##p<0.01

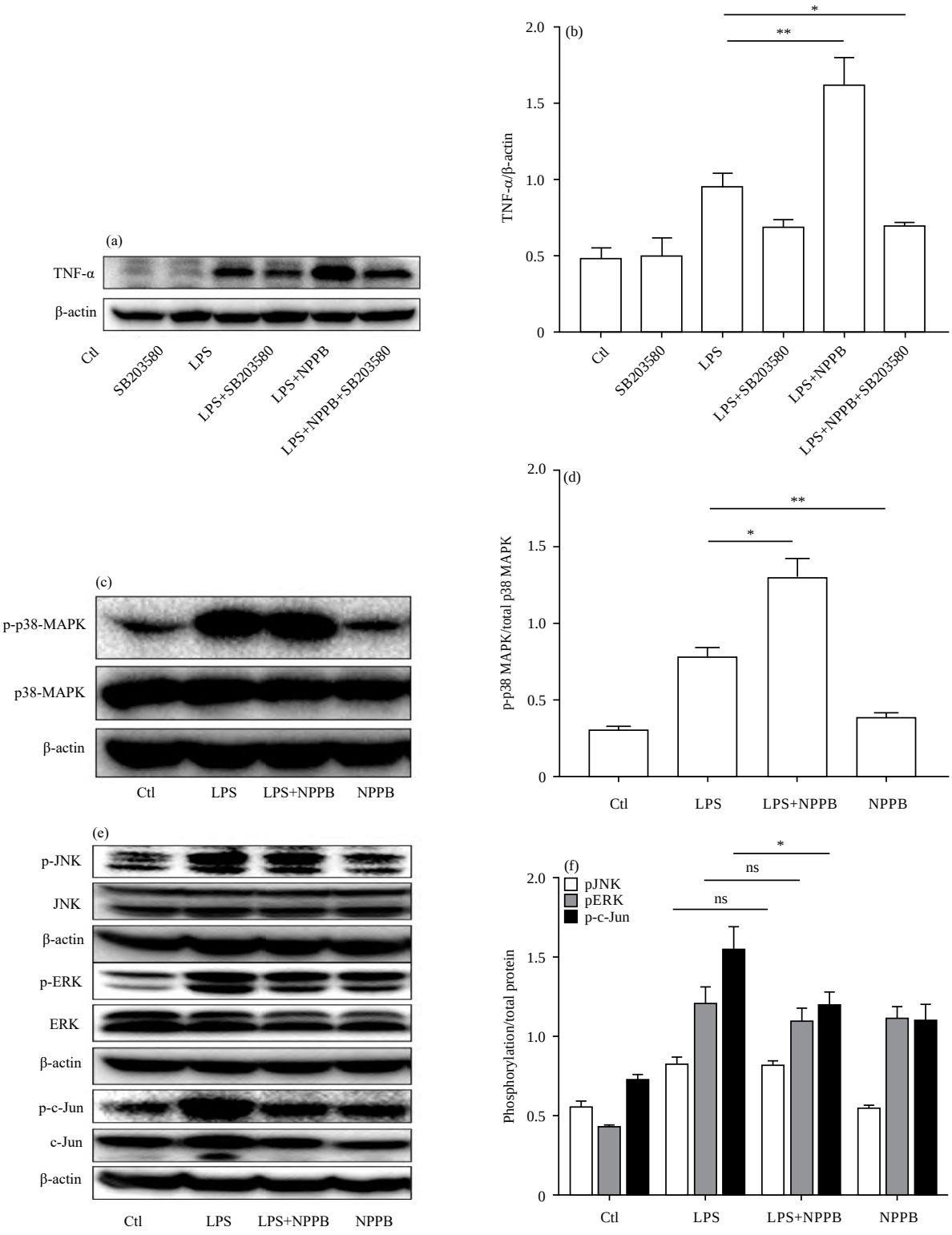

Fig. 4(a-f): NPPB up-regulates inflammatory response by activating p38-MAPK, (a-b) TNF-α protein expression and quantification in each group treated with NPPB and p38-MAPK inhibitor SB203580 (n = 4), (c-d) p-p38-MAPK protein expression and quantification in each group (n = 4), (e-f) p-JNK, p-ERK and p-c-Jun protein expression and quantification in each group (n = 4)

*p<0.05, **p<0.01

What is more, since NPPB activates the GPR35-Gi/o and GPR35-G16 pathway in human embryonic kidney 293 (HEK293) cells[15], it was explored whether NPPB act as a GPR35 agonist to promote macrophage to release inflammatory factors via the MAPK signalling pathway. In the current study, NPPB promotes macrophages inflammation, consistent with an already known GPR35 agonist. From another aspect, RNA-sequencing data showed that the expression of pro-inflammatory genes in GPR35 negative macrophages was lower than that in GPR35 positive macrophages[16]. These results led us to further explore the mechanism of inflammation promoted by GPR35. Several studies support that the MAPK pathway is one of the signalling pathways activated by GPCR agonists[20-22] and it is proved that phosphorylating ERK1/2 activates GPR35[21]. Furthermore, the current study found that NPPB act as a GPR35 agonist to enhance the inflammatory response of macrophages by activating p38 MAPK. At the same time, other key proteins of the MAPK pathway were detected, neither the phosphorylation level of JNK nor the phosphorylation level of ERK showed obvious differences but the phosphorylation level of C-Jun was inhibited after LPS plus NPPB treatment, compared with LPS treatment. It is suggested that NPPB promotes the expression of TNF-α in macrophages induced by LPS, via promoting the p38 MAPK inflammatory pathway by activating GPR35.

Interestingly, it is reported that activation of MAPK signalling pathways can inhibit the K^+ channels[22]. Consistent with the previous study[16], the current study demonstrated that the K^+ channel blocker phosphatidic acid[23] also activates GPR35 and promotes TNF-α expression (data not shown), which is worth researching further.

CONCLUSION

Current study revealed the unrecognized role of NPPB in macrophage inflammation activation and its mechanism, which is helpful for further understanding of macrophage inflammation and excavates a new target GPR35. It should be noticed that GPR35 activation may promote inflammatory response and aggravate clinical diseases including bacterial infection. Last but not least, the mechanism by which GPR35 activation promotes the inflammatory response through the MAPK signalling pathway needs further exploration.

SIGNIFICANCE STATEMENT

This study discovered NPPB promotes inflammatory reaction by activating GPR35 rather than inhibiting ClC-3. GPR35 activation may aggravate clinical diseases including bacterial infection, which may guide reasonable drug use. For electrophysiological researchers, it is sometimes necessary to consider whether the ion channel inhibitors chosen may affect G protein-coupled receptors and inflammatory responses. This study will help the researchers to uncover the critical relationship between inflammation and ion channels or G protein-coupled receptors, which is beneficial to further exploring new targets of inflammatory response.

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