Expression of TNF-α and IL-β can be suppressed via the PPAR-γ/mTOR signaling pathway in BV-2 microglia: A potential anti-inflammation mechanism

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Abstract. Currently, microglia are considered as crucial factors in suppressing inflammatory reactions, but the specific molecular mechanism remains unknown. To elucidate whether peroxisome proliferator-activated receptor-γ (PPAR-γ) can inhibit neuroinflammatory cytokine expression via the mTOR signal pathway, the BV-2 cell line was incubated with lipopolysaccharide (10 mM/ml) to induce an inflammatory injury. PPAR-γ was activated by rosiglitazone, and was inhibited by GW9662. The mTOR signal pathway was activated by phosphatidic acid (P.A.), while it was inhibited by rapamycin. Western blotting and reverse transcription-quantitative PCR were used to evaluate the expression levels of PPAR-γ/mTOR signal pathway related proteins and neuroinflammatory cytokines, including NF-κB, tumor necrosis factor (TNF)-α and interleukin (IL)-1β. When treated with P.A., the expression levels of phosphorylated (p)mTOR and p-ribosomal protein S6 kinase (pS6K) were significantly increased and the expression levels of TNF-α and IL-1β were significantly lower. However, the expression of PPAR-γ was similar in P.A. treated cells and cells treated with rapamycin. When PPAR-γ was activated, pmTOR and pS6K protein expression levels were significantly decreased, and the mRNA expression levels of TNF-α and IL-1β were significantly reduced, but this inhibition could be alleviated by administrating GW9662. Collectively, it was indicated that the mTOR signal pathway may be located downstream of PPAR-γ. Furthermore, neuroinflammatory reactions could be inhibited via the activation of PPAR-γ by suppressing the mTOR signal pathway in microglia.

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Abbreviations: ICH, intracerebral hemorrhage; PPAR-γ, peroxisome proliferator-activated receptor-γ

Key words: microglia, mTOR, neuroinflammation, PPAR-γ, toll-like receptor 4

Introduction

Intracerebral hemorrhage (ICH), which accounts for 10-15% of all strokes, is associated with a high rate of mortality and disability (1,2). In addition to the mass effect of hematoma, which causes primary brain injury and potential intracranial hypertension, secondary inflammation can affect the outcome of patients with ICH (3,4). Thus, inhibition of inflammation after ICH is important.

Microglia are considered crucial in the suppression of inflammatory reactions after ICH, although only M2 microglia have been shown to exert anti-inflammatory effects (5,6). It was previously reported that M2 microglia can not only suppress inflammation after ICH, but also inhibit neuroinflammation in patients with Alzheimer's disease (AD) (7) and ischemic stroke (8). Moreover, activation of the peroxisome proliferator-activated receptor γ (PPAR-γ) signaling pathway is speculated to be the mechanism underlying these effects. When the PPAR-γ signaling pathway is activated, more resting state microglia are transformed to the M2 type (4). However, the molecular mechanism via which M2 microglia exert their anti-inflammatory effects is not fully understood.

The mTOR signal pathway has been revealed to have a central role in cell metabolism, proliferation, differentiation and development (9). In our previous *in vivo* and *in vitro* studies, it was demonstrated that the mTOR signaling pathway was crucial for protection against ischemic injury, but could also exacerbate harmful inflammatory reactions in ischemia-reperfusion injury (10-12). Furthermore, in ICH studies, suppression of mTOR inhibits the expression of inflammatory factors (13), although the underlying mechanism remains to be elucidated. Thus, the role of mTOR in neuroinflammation remains controversial.

Currently, the downstream factors of the PPAR-γ signaling pathway that suppress inflammation in microglia remain

unknown. Although it has been reported that mTOR can regulate the expression of inflammatory factors in the central nervous system (14,15), few studies have elucidated the role of the interaction between PPAR-γ and mTOR in neuroinflammatory processes. It was hypothesized that PPAR-γ may inhibit certain inflammatory factors, including interleukin (IL)-1β, NF-kB and tumor necrosis factor (TNF)-α, via the mTOR signaling pathway, and thus the aim of the present *in vitro* study was to examine whether the activation of PPAR-γ can inhibit the expression of neuroinflammatory cytokines via mTOR signaling.

Materials and methods

Cell line and cell culture. BV-2 cells, an immortalized murine microglial cell line, were commercially obtained from the American Type Culture Collection for use in the present study; BV-2 cells were cultured according to manufacturer's instructions. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.; cat. no. C11995500CP) supplemented with 10% (v/v) FBS (Biological Industries; cat. no. 04-001-1ACS) and 1% penicillin/streptomycin (Genom Biotech Pvt; cat. no. GNM15140). All cells were maintained in a humidified incubator with 95% air and 5% CO_2 at 37˚C. Cells were seeded in 6-well culture plates at a density of 500,000 cells per well.

Study design and treatments. To elucidate the relationship between mTOR and the expression levels of inflammatory factors in microglia, including TNF-α, IL-1β and NF-κB, phosphatidic acid (P.A.; Sigma-Aldrich; Merck KGaA; 100 nM/well) and rapamycin (EMD Millipore; 100 nM/well) (16) were used to activate or inhibit mTOR signal pathway for 48 h with 95% air and 5% CO_2 at 37˚C, respectively. A flow chart of the current study design is presented in Fig. 1A.

Then, to examine whether mTOR could be regulated by the PPAR-γ signaling pathway, rosiglitazone (a PPAR-γ activator; Sigma-Aldrich; Merck KGaA; 50 mg/ml) and GW9662 (a PPAR-γ inhibitor; Sigma-Aldrich; Merck KGaA; 10 mg/ml) (17) were selected to interfere with the activation of PPAR-γ (Fig. 1B). Cells were incubated with lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA; 10 mM/ml) for 24 h with 95% air and 5% CO_2 at 37˚C to induce an inflammatory injury as previously described (18).

Protein preparation and western blot analysis. Protein expression levels of PPAR-γ, mTOR, phosphorylated (p)mTOR, ribosomal protein S6 kinase (S6K), pS6K, Toll-like receptor (TLR) 4, NF-κB, TNF-α and IL-1β were determined using western blotting as previously described (11). In brief, Cell pellets were homogenized in 100 μl lysis buffer (cat. no. p0013; Beyotime Institute of Biotechnology) supplemented with 1 mM phenylmethylsulphonyl fluoride (Sigma-Aldrich; Merck KGaA). Then, prepared protein (50 μg) in each lane was subjected to SDS-PAGE using 4-15% Ready Gel (cat. no. L050505A2; Bio-Rad Laboratories, Inc.) under 200 V for 45 min. Protein bands were transferred from the gel to PVDF (EMD Millipore) membranes under 100 V for 2 h. After blocking with with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 at 4˚C overnight, the membrane was incubated with primary antibodies overnight at 4˚C, followed by Alexa Fluor 488 donkey anti-rabbit (cat. no. A-21206; 1:5,000; Invitrogen; Thermo Fisher Scientific, Inc.) or anti-rat IgG secondary antibody (cat. no. A-21210; 1:5,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature in a dark room. Then, membranes were scanned using Typhoon trio (Cytiva), visualized using an enhanced chemiluminescence kit (Cytiva) and the optical densities of all protein bands were analyzed using IMAGEQUANT 5.2 software (Cytiva). All samples were run on the same gel. Protein bands were rearranged solely to ease comparison in figures. The manufacturers, dilutions and cat. nos. of all primary antibodies used are listed in Table SI.

Reverse transcription-quantitative PCR (RT-qPCR). qPCR was performed using SYBR Green QPCR system (Qiagen, Inc.). The protocol of RT-qPCR was described in our previous study (12). Total RNA was extracted from cultured microglia cells using PrepEase RNA Spin kit (Affymetrix; Thermo Fisher Scientific, Inc.; cat. no. 78767) according to manufacturer's protocol. Isolated RNA (1 mg) was reversed transcribed into cDNA using Verso cDNA Synthesis kit (Thermo Fisher Scientific, Inc.; cat. no. AB-1453/B) according to manufacturer's protocol. RT-PCR analyses were performed as described previously (12). GAPDH was used as an internal standard. The primers used for qPCR are listed in Table SII. Expression of each gene transcript was calculated using the $2^{-\Delta\Delta Cq}$ method and normalize to GAPDH (12).

Statistical analysis. All experiments were independently performed ≥3 times. Continuous variables are presented as the mean ± standard deviations or as median (interquartile range). For group comparisons, the one-ANOVA was used for continuous variables with a normal distribution, and a one-ANOVA followed by Dunnett's post hoc test was used for continuous variables with skewed distributions. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 23.0 (SPSS, Inc.) and MedCalc statistical software (version 15.2.2; MedCalc Software bvba).

Results

Suppression of mTOR signaling reduces the expression of TNF-α and IL-1β. To examine whether inflammatory reactions are exacerbated or alleviated by mTOR, P.A. (a mTOR agonist) and rapamycin (a mTOR antagonist) were used to modulate the activation of mTOR, and then the expression levels of PPAR-γ, mTOR signaling-related proteins and inflammatory cytokines were evaluated. The expression levels of mTOR and S6K were similar between the groups, but the expression levels of pmTOR (P=0.039) and pS6K (P=0.0073) were increased by P.A. treatment compared with rapamycin, saline or control groups (Fig. 2A); this indicated that the mTOR signaling pathway was successfully activated. It was found that PPAR-γ expression was not significantly different between the groups (P=0.0839). In addition, the protein expression levels of TNF-α (P=0.028) and IL-1β (P=0.0082) were significantly lower in the rapamycin vs. P.A. group, but there was no difference in the expression of NF-κB among the P.A., rapamycin and saline groups. These results suggested that some neuroinflammatory

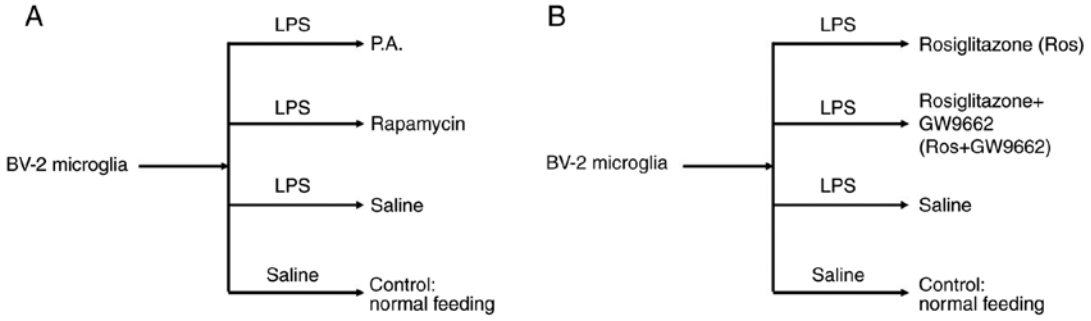

Figure 1. Schematic design and flow chart of the present study. (A) Effects of mTOR activation or inhibition on inflammations in BV-2. (B) Effects of PPAR-g activation on inflammations in BV-2. LPS, lipopolysaccharide; P.A., phosphatidic acid.

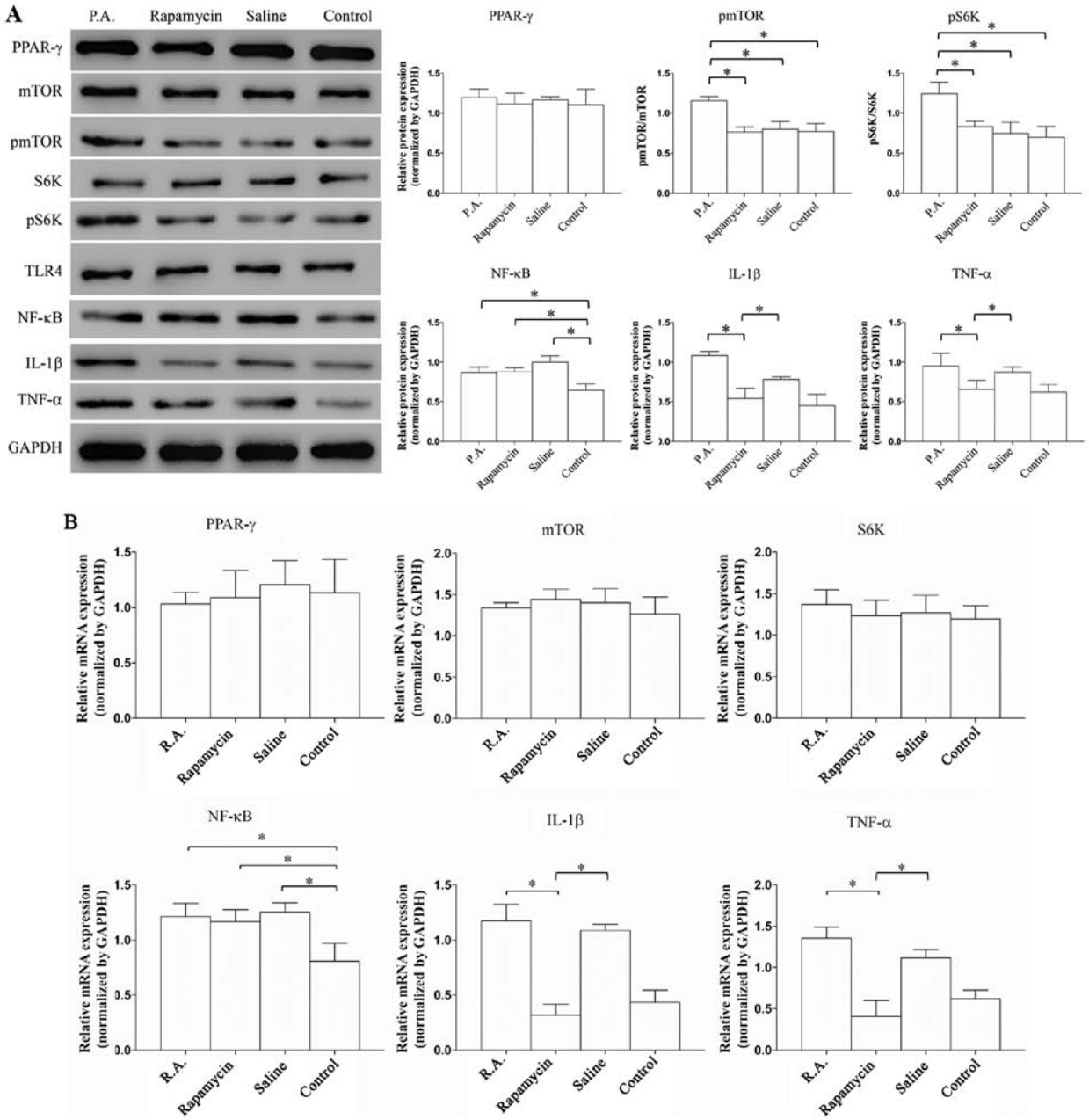

Figure 2. mTOR suppression is able to reduce the expression levels of TNF-α and IL-1β. Expression levels were detected by (A) western blot analysis and (B) reverse transcription-quantitative PCR. Expression levels of PPAR-γ, mTOR, S6K and TLR4 were similar within the four groups. The expression of NF-κB was significantly lower in the control group compared with the other groups. When administrated with P.A., the protein expression levels of pmTOR and pS6K were significantly increased. Neuroinflammatory factors, IL-1β and TNF-α, were significantly suppressed when treated by rapamycin. N=8-10 in ≥3 independent experiments. *P<0.05. TNF, tumor necrosis factor; IL, interleukin; P.A., phosphatidic acid; p, phosphorylated; TLR4, Toll-like receptor 4; S6K, ribosomal protein S6 kinase; PPAR-γ, proliferators-activated receptors-γ.

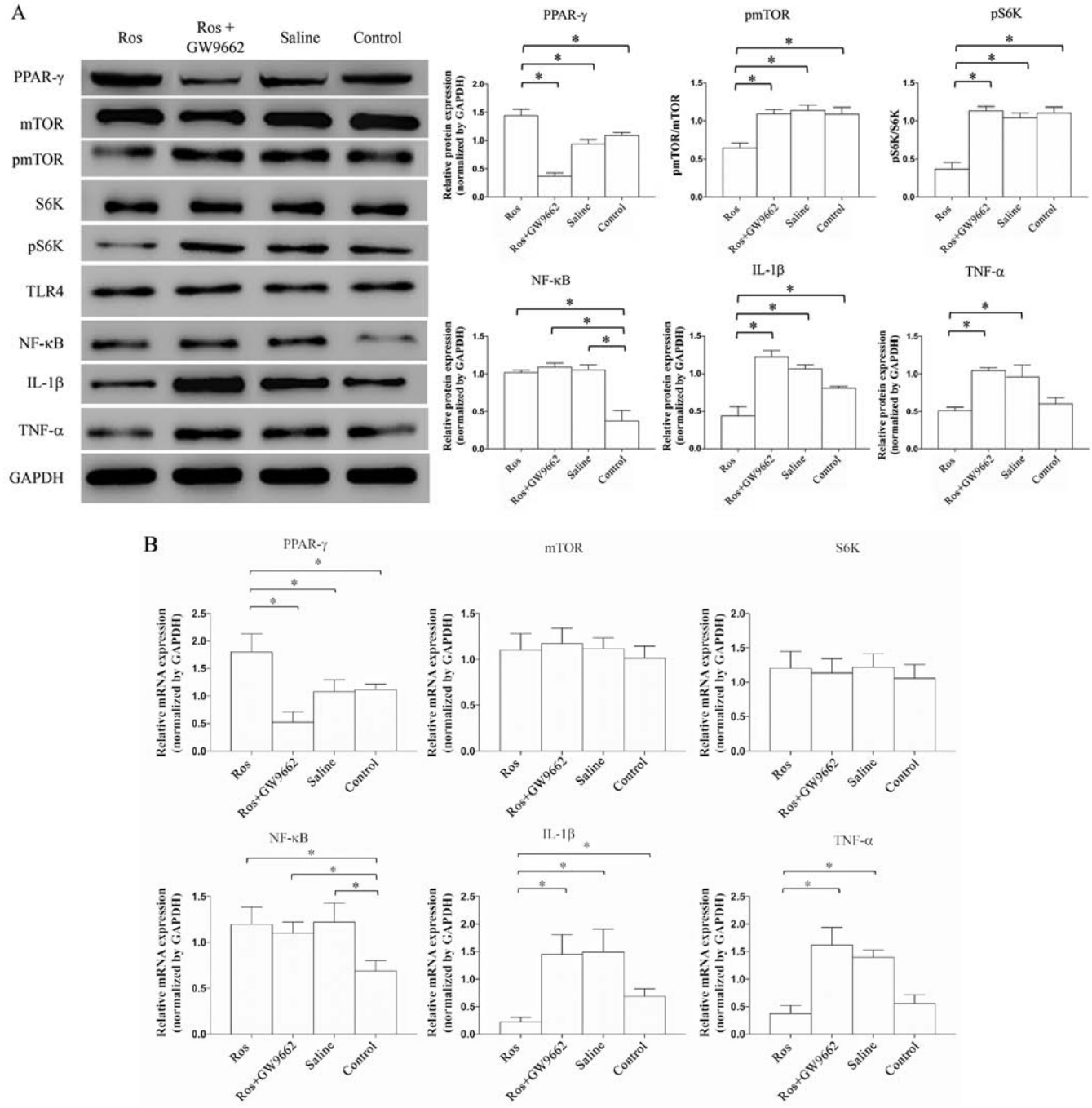

Figure 3. Activation of PPAR-γ can reduce the expression of TNF-α and IL-1β via inhibiting mTOR. Expression levels were detected by (A) western blot analysis and (B) reverse transcription-quantitative PCR. Expression levels of mTOR, S6K and TLR4 were similar. The expression of NF-κB was significantly lower in the control group compared with the other groups. When administrated with rosiglitazone, the expression levels of pmTOR and pS6K were significantly reduced. Moreover, the expression levels of IL-1β and TNF-α were significantly suppressed. N=8-10 in ≥3 independent experiments. *P<0.05. TNF, tumor necrosis factor; IL, interleukin; p, phosphorylated; TLR4, Toll-like receptor 4; S6K, ribosomal protein S6 kinase; PPAR-γ, proliferators-activated receptors-γ; Ros, rosiglitazone.

factors, including TNF-α and IL-1β, can be suppressed when the mTOR signaling pathway is inhibited.

RT-qPCR was used to detect the mRNA expression levels of related proteins and inflammatory factors. The mRNA expression levels of TNF-α (P=0.0097) and IL-1β (P=0.0053) were significantly decreased in the rapamycin and control groups, but the expression of NF-κB was similar among P.A, rapamycin and saline groups (Fig. 2B). The RT-qPCR results were in line with those of the western blot analysis.

Activation of PPAR-γ can prevent the expression of TNF-α and IL-1β via mTOR inhibition. After applying rosiglitazone, the expression of PPAR-γ was significantly increased (P=0.0047), while that of pmTOR (P=0.0041) and pS6K (P=0.0013) was significantly decreased compared with rosiglitazone+GW9662, saline or control groups; however, these effects were abrogated by GW9662 treatment (Fig. 3A). These findings indicated that the mTOR signaling pathway can be suppressed by the activation of PPAR-γ. Combined

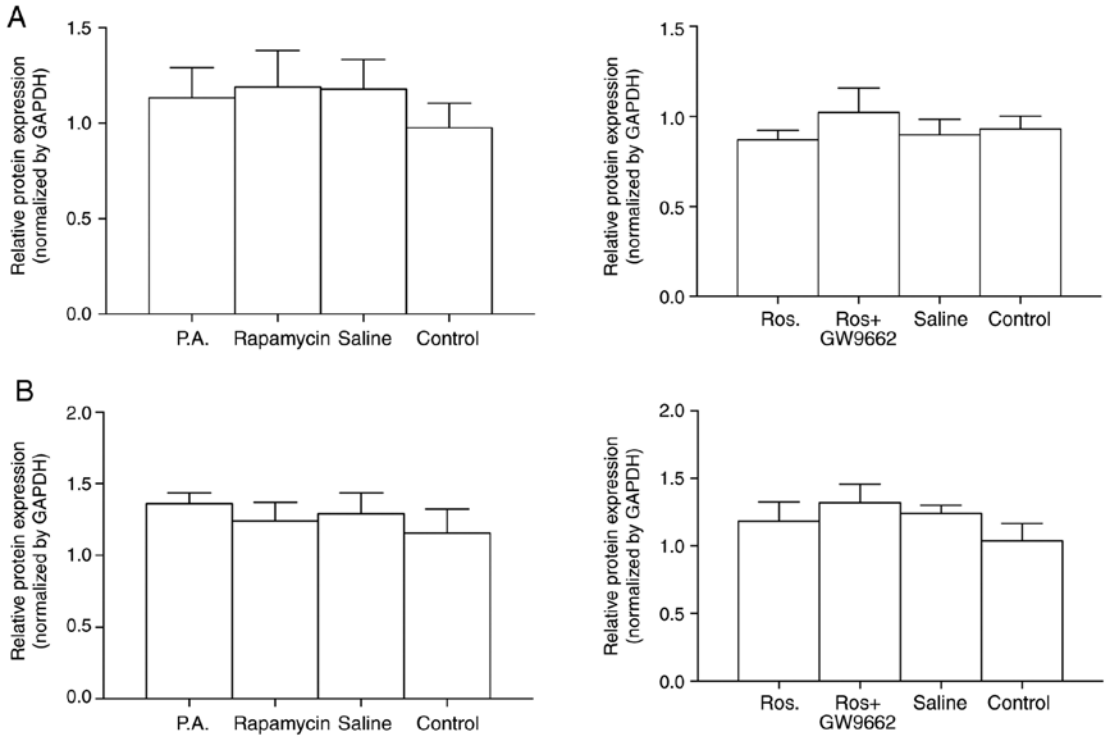

Figure 4. Expression of TLR4 in each group was similar. As detected by (A) western blotting and (B) reverse transcription-quantitative PCR, no significant differences in the expression of TLR4 were found. N=8-10 in ≥3 independent experiments. TLR4, Toll-like receptor 4; P.A., phosphatidic acid; Ros, rosiglitazone.

with the previous results demonstrating that the expression of PPAR-γ is stable regardless of whether mTOR is activated or inhibited (Fig. 2A), it was speculated that mTOR signaling likely occurs downstream of PPAR-γ. Moreover, as detected by western blotting, the protein expression levels of TNF-α and IL-1β were significantly reduced when rosiglitazone was administered (P=0.0086 and P=0.0073, respectively; Fig. 3A), and such anti-inflammatory ability was prevented by GW9662. However, the expression of NF-κB was not significantly different vs. the group without PPAR-γ activation.

Next, RT-qPCR was performed to evaluate the mRNA expression levels of related cytokines and proteins (Fig. 3B). The mRNA expression of PPAR-γ was significantly increased in the presence of the PPAR-γ agonist (P=0.0093), while the mRNA expression levels of mTOR and S6K were stable. In addition, the mRNA expression levels of TNF-α (P=0.028) and IL-1β (P=0.031) were significantly suppressed in the rosiglitazone group, and such inhibition was abrogated by treatment with a PPAR-γ antagonist (Fig. 3B). Furthermore, in either treatment, it was suggested that the expression of TLR4 was similar among the four groups (P=0.0831; Figs. 2A, 3A and 4). Collectively, the results suggested that the expression levels of neuroinflammatory cytokines can be suppressed via the PPAR-γ and mTOR signaling pathways.

Discussion

In this *in vitro* study, it was found that PPAR-γ activation inhibited the expression levels of TNF-α and IL-1β via the suppression of mTOR signaling in microglia, but NF-κB expression was not significantly affected. Therefore, the present study identified a possible anti-inflammation mechanism of microglia.

Since neuroinflammatory reactions have both protective and detrimental effects in the brain, these are considered a double-edged sword (19). Neuroinflammation is a crucial factor not only in the outcome of patients with AD (7), but also in acute neurological insults, including ICH (4) and traumatic brain injury (3). Microglia, which are the brain's resident innate immune cells, were identified in the present study as a key component of the neuroinflammatory response. Mechanistically, M2 microglia release cytokines to reduce inflammation, and migrate to damaged tissue to participate in regenerative processes (6). However, the mechanism via which PPAR-γ activation mediates the suppression of inflammatory cytokines in microglia remains unknown.

The specific function of mTOR in neurons is controversial. Previous studies have reported that mTOR activation may be harmful, and can exacerbate neuroinflammation after neuronal injury in rats (14,20); however, other studies suggested that mTOR has positive effects in ischemic rats (13,21). Along with these opposing findings, the present results suggested that mTOR may be regulated by different signaling pathways, proteins or mRNAs depending on the disease state.

In the present study, P.A. and rapamycin were used to test the aforementioned hypothesis that PPAR-γ may inhibit certain inflammatory factors, including IL-1β, NF-κB and TNF-α, via the mTOR signalling pathway. It was demonstrated that the expression of PPAR-γ was similar between the P.A. and rapamycin groups, which indicated that the mTOR signaling pathway was located downstream of PPAR-γ and may be involved in the regulation of inflammatory cytokines. When PPAR-γ was activated by rosiglitazone, the expression levels of mTOR and S6K were similar between the groups, but those of pmTOR and pS6K were significantly decreased; this

suggested that mTOR signaling can be inhibited by PPAR-γ activation, and such inhibition can be abrogated with GW9662. Moreover, the results indicated that PPAR-γ can inhibit the expression levels of IL-1β and TNF-α via the suppression of the mTOR signaling pathway.

It was previously shown that PPAR-γ activation could suppress the expression of NF-κB (22,23). However, in the present study, the expression of NF-κB was not significantly different between the treatment groups. A possible explanation for this finding is that the current study did not manipulate the expression of the TLR4. The present results suggested that the expression of TLR4 was similar among the four groups.

TLRs are responsible for detecting microbial pathogens and generating innate immune responses (24). TLR4 is a membrane receptor for various substances, including LPS and heme (23,24). It has also been reported that activation of TLR4 leads to the transformation of a larger proportion of resting state microglia into the M1 type, which promotes inflammation (25). In addition, harmful neuroinflammatory reactions can be exacerbated by the activation of TLR4 (26). Moreover, the expression of NF-κB can be positively regulated by TLR4, but negatively regulated by PPAR-γ (27,28). The present results suggested that inflammatory factors were inhibited by PPAR-γ via mTOR suppression; thus, the relationship between TLR4 and mTOR may be crucial in the regulation of neuroinflammation.

The interaction between TLR4 and mTOR has been investigated in a previous studies (24,25,28), especially in microglia, and it has been shown that dietary L-arginine attenuates intestinal mucosal disruption via the inhibition of the TLR4 and mTOR pathways (29). Furthermore, activation of mTOR plays an essential role in TLR4-triggered neutrophil and macrophage activation (30). A recent study also revealed that mTOR-dependent autophagy regulates gut inflammatory responses via the upstream TLR4/myeloid differentiation primary response 88/mitogen-activated protein kinase signaling and the downstream NF-κB pathway (31). The current findings suggested that PPAR-γ activation can suppress certain neuroinflammatory factors via mTOR. However, it is necessary to further elucidate the relationship between mTOR and TLR4 to increase the understanding of the anti-inflammation ability of microglia.

There were several limitations to the present study. First, an *in vivo* study is required to assess the *in vitro* results. Thus, future studies will modulate the expression of S6K (downstream of mTOR) to further validate the hypothesized molecular mechanism involving the interaction between mTOR and PPAR-γ. This will include determining whether PPAR-γ directly regulated or mediated mTOR, pNF-κB and p65 nuclear translocation. In addition, the relationship between mTOR and TLR4 requires further investigation.

In conclusion, the mTOR signaling pathway may be located downstream of PPAR-γ. Furthermore, neuroinflammatory reactions may be inhibited by the activation of PPAR-γ via the suppression of mTOR signaling in microglia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The study was designed by JLZ, JH and RX. The experiments were performed and data were collected by JLZ, CW, XX, YHD, BT, GC and QY, and the data were analyzed by ZYD, CW, YRS and JY. The manuscript was written by JLZ and RX, and was revised and approved by all authors. The manuscript was finally proofread and approved by JH and RX.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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