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Tiliroside, a dietary glycosidic flavonoid, inhibits TRAF-6/NF-κB/p38-mediated neuroinflammation in activated BV2 microglia

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ABSTRACT

Background: Tiliroside is a dietary glycosidic flavonoid which has shown *in vivo* anti-inflammatory activity. This study is aimed at evaluating the effect of tiliroside on neuroinflammation in BV2 microglia, and to identify its molecular targets of anti-neuroinflammatory action.
Methods: BV2 cells were stimulated with LPS + IFNγ in the presence or absence of tiliroside. TNFα, IL-6, nitrite and PGE_2 production was determined with ELISA, Griess assay and enzyme immunoassay, respectively. iNOS, COX-2, phospho-p65, phospho-IκBα, phospho-IKKα, phospho-p38, phospho-MK2, phosopho-MKK3/6 and TRAF-6 were determined by western blot analysis. NF-κB activity was also investigated using a reporter gene assay in HEK293 cells. LPS-induced microglia ROS production was tested using the DCFDA method, while HO-1 and Nrf2 activation was determined with western blot.
Results: Tiliroside significantly suppressed TNFα, IL-6, nitrite and PGE_2 production, as well as iNOS and COX-2 protein expression from LPS + IFNγ-activated BV2 microglia. Further mechanistic studies showed that tiliroside inhibited neuroinflammation by targeting important steps in the NF-κB and p38 signalling in LPS + IFNγ-activated BV2 cells. This compound also inhibited LPS-induced TRAF-6 protein expression in BV2 cells. Antioxidant activity of tiliroside in BV2 cells was demonstrated through attenuation of LPS + IFNγ-induced ROS production and activation of HO-1/Nrf2 antioxidant system.
Conclusions: Tiliroside inhibits neuroinflammation in BV2 microglia through a mechanism involving TRAF-6-mediated activation of NF-κB and p38 MAPK signalling pathways. These activities are possibly due, in part, to the antioxidant property of this compound.
General Significance: Tiliroside is a potential novel natural compound for inhibiting neuroinflammation in neurodegenerative disorders.

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1. Introduction

Neuroinflammatory responses in the microglia have been shown to cause neuronal damage in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis. Activated microglia is considered to be an important hallmark of brain inflammation, and plays a key role in regulating neuroinflammatory reactions [1]. Hyperactive microglia is known to release a variety of cytotoxic factors like reactive oxygen mediators, arachidonic acid derivatives and pro-inflammatory cytokines. Prostaglandin E2 (PGE_2) is an arachidonic acid derivative, the production of which is catalysed by cyclooxygenase enzymes in activated microglia. Cyclooxygenases are of two subtypes, cyclooxygenase-1 (COX-1) and cycloxygenase-2 (COX-2). COX-1 is constitutively expresses in most cell types, whereas expression of COX-2 is induced by various factors including inflammatory cytokines, and is mainly responsible for the production of PGE_2 [2]. In cultured rat brain microglia, bacterial lipopolysaccharide (LPS) has been shown to induce COX-2 expression, and this is prevented in the presence of inhibitors of NF-κB [3]. In addition to PGE_2, the cellular messenger nitric oxide (NO) has been widely implicated in neuroinflammation and neurodegenerative processes. Elevated levels of NO have been reported in both microglia and astroglia, which are activated during the neuroinflammatory response [4]. Significant body of evidence also suggests that the release of large amounts of NO from activated astrocytes and microglia, mediated by the inducible nitric oxide synthase (iNOS) enzyme, is important in the pathogenesis of neurodegenerative disorders. This is evident in the induction of neuronal death through both necrotic and apoptotic pathways [4]. Consequently, both COX-2-mediated PGE_2 and iNOS-

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mediated NO production in the microglia remain important targets in reducing neuroinflammation in the microglia.

During neuroinflammation, the transcription factor NF-κB regulates COX-2 and iNOS-mediated PGE_2 and NO production, respectively. In a resting state, the NF-κB dimer is associated with the inhibitory protein IκB which retains NF-κB in the cytosol. However, in response to inflammatory stimuli such as LPS and cytokines, IκB kinase (IKK) phosphorylates IκB, leading to its proteasomal degradation. This process releases active NF-κB, which is then translocated from the cytosol to the nucleus, to bind specific DNA promoter sequences and to induce gene transcription of pro-inflammatory genes [5].

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which are critical for regulation of the production of inflammation mediators. Increased activity of MAPKs in activated microglia and astrocytes make them potential targets for novel therapeutics [6]. The MAPKs have been shown to be critical regulators of pro-inflammatory cytokines in neurodegenerative process. Among these, the p38 MAPK has been shown to play a central role due to its importance in the production of the pro-inflammatory cytokines and other mediators. In response to stress, p38 activates the mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK-2) [7]. The activation and expression of MK-2 have been shown to be increased in microglial cells which were stimulated with LPS and gamma interferon [8]. Furthermore, the p38 MAPK-mediated regulation of inflammatory gene transcription and translation is dependent on MK-2 [9]. Consequently, inhibition of the p38 MAPK and MK-2 activation may have therapeutic uses in neuroinflammation.

Reactive oxygen species (ROS) are produced for the maintenance of many physiological functions and act as second messengers. However, accumulating evidence has suggested that the pathogenesis of neurodegenerative disorders, including AD, is related to excessive production of ROS and the resultant increased oxidative stress [10]. Increased ROS production has been shown to control the expression of several inflammatory mediators. Oxidative stress is also responsible for the expression of critical inflammatory target proteins such as COX-2, iNOS and the adhesion molecules induced by cytokines, infections and peptides [10–12]. Furthermore, microglia cells are known to release different inflammatory mediators in response to oxidative stress [10]. The nuclear factor erythroid 2 related factor 2 (Nrf2) is a critical regulator of endogenous inducible defence systems in the body. Under physiological conditions Nrf2 is mainly located in the cytoplasm. However, in response to oxidative stress, Nrf2 undergoes nuclear translocation and binds to specific DNA sites known as the antioxidant response elements (ARE), to initiate transcription of cytoprotective genes [13]. The relationship between Nrf2 and NF-κB is not well-understood. However, NF-κB has been shown to attenuate the transcription of genes that are under the control of Nrf2 [14]. Taken together, there appears to be a crosstalk between these transcription factors in neuroinflammation.

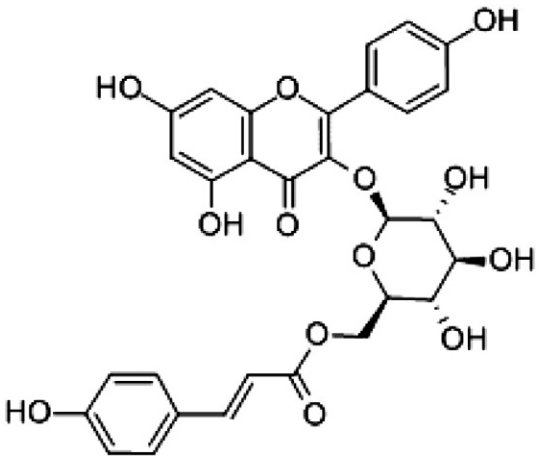

Fig. 1. Chemical structure of tiliroside.

Tiliroside (Fig. 1) is a glycosidic flavonoid found in several medicinal and dietary plants, such as linden, rose hip and strawberry [15]. This compound has been shown to exhibit anti-inflammatory, antioxidant, anticarcinogenic, and hepatoprotective activities [16]. In this study, we have investigated the effects of tiliroside on neuroinflammation following activation of BV2 microglia with a combination of lipopolysaccharide (LPS) and gamma interferon (IFNγ). We also evaluated whether tiliroside could activate the Nrf2/HO-1 antioxidant protective mechanism in BV2 microglia.

2. Materials and methods

2.1. Materials

Tiliroside (Sigma) was prepared in DMSO (Sigma) and stored in small aliquots at 20 °C. The following reagents were used: RPMI (Gibco), fetal bovine serum (Sigma), sodium pyruvate (Sigma), glutamine (Sigma), streptomycin/penicillin (Sigma), MEM-Eagles medium (Life Technologies). LPS (Sigma) derived from *Salmonella enterica* serotype typhimurium SL1181 and IFNγ (R&D Systems) derived from *E. coli*.

2.2. Cell culture

BV2 microglia cell line ICLC ATL03001 was purchased from Interlab Cell Line Collection (Banca Biologica e Cell Factory, Italy) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, streptomycin 40 μg/mL/penicillin 40 U/mL. Once confluent, cells were split 1:10 using trypsin/EDTA solution and cultured at 37 °C in 5% CO_2.

HEK293 cells were derived from human embryonic kidney, obtained from HPA Cultures (Salisbury, UK) and were cultured in MEM-Eagles medium. The medium was supplemented with 10%FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM L-glutamine, streptomycin 40 μg/mL/penicillin 40 U/mL. Once confluent, cells were split 1:10 using trypsin/EDTA solution and cultured at 37 °C in 5% CO_2.

2.3. Determination of cell viability

Viability of BV2 cells stimulated with LPS (100 ng/ml) + IFNγ (5 ng/ml) in the presence or absence of tiliroside (2–6 μM) was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates (2×10^5 cells/ml) and incubated for 48 h. Thereafter, cells were pre-treated with tiliroside (2–6 μM) for 30 min prior to stimulation with LPS (100 ng/ml) + IFNγ (5 ng/ml). Twenty-four hours after stimulation, culture medium was replaced with MTT solution (5 mg/ml) and incubated for 4 h at 37 °C in 5% CO_2. Thereafter 150 μl of MTT solution was replaced with DMSO and mixed thoroughly on a plate shaker and read at 540 nm.

2.4. Tumour necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) ELISA

BV2 cells were seeded in 96 well plates (2×10^5 cells /ml), cultured for 48 h. Thereafter, cells were pre-treated with tiliroside (2–6 μM) for 30 min prior to stimulation with LPS (100 ng/ml) + IFNγ (5 ng/ml). Twenty-four hours after stimulation, cell supernatants were collected and centrifuged. Concentrations of TNFα and IL-6 in cell supernatants were measured with a commercially available ELISA kit (BioLegend, UK), followed by measurements in a plate reader at a wavelength of 450 nm.

2.5. Nitrite assay

BV2 cells were seeded in 96 well plates (2×10^5 cells /ml), cultured for 48 h. Thereafter, cells were pre-treated with tiliroside (2–6 μM) for

30 min prior to stimulation with LPS (100 ng/ml) + IFNγ (5 ng/ml). Twenty-four hours after stimulation, cell supernatants were collected and centrifuged. Nitrite production was quantified using the Griess assay kit (Promega, Southampton, UK), followed by measurement at 540 nm. Nitrite concentrations were compared with sodium nitrite standard curve.

2.6. PGE_2 enzyme immunoassay (EIA)

BV2 cells were seeded in 6 well plates (2×10^5 cells /ml) and incubated for 48 h, followed by incubation with or without LPS (100 ng/ml) + IFNγ (5 ng/ml) for 24 h in presence or absence of tiliroside (2–6 μM) incubated for 30 min. Cell supernatants were collected and centrifuged at 1200 rpm for 5 min. PGE_2 accumulation was measured in supernatants with commercially available PGE_2 EIA kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.7. Preparation of cytoplasmic and nuclear extracts

Following stimulation, cell lysates were prepared by washing cells with PBS, followed by addition of lysis buffer and phenylmethylsulfonyl fluoride (PMSF), and centrifugation for 10 min. Nuclear extracts were prepared using EpiSeeker Nuclear Extraction Kit, according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, followed by addition of 20 μl of pre-extraction buffer and incubation on ice for 10 min. Thereafter, cells were centrifuged at 12,000 rpm for 1 min. Supernatants were discarded, and 10 μl of extraction buffer was added to the pellet and incubated on ice for 15 min, followed by centrifugation at 13,500 rpm for 15 min at 4 °C. The resulting nuclear extracts in the supernatants were collected.

2.8. Immunoblotting

Western blotting was performed to determine protein expressions in BV2 cells activated with LPS (100 ng/ml) + IFNγ (5 ng/ml) in the presence or absence of tiliroside (2–6 μM). Twenty-five micrograms of protein was subjected to sodium dodecyl sulfate–polyacrylamide (SDS) gel electrophoresis. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for 2 h. Membranes were then blocked at room temperature for 1 h and then incubated with primary antibodies overnight at 4 °C. Primary antibodies used in the experiments were rabbit anti-iNOS (Santa Cruz, 1:500), rabbit anti-COX-2 (Santa Cruz, 1:500), rabbit anti-phospho-IκBα (Santa Cruz, 1:250), rabbit anti-phospho-IKKα (Santa Cruz, 1:500), rabbit anti-phospho-p38 (Santa Cruz, 1:500), rabbit anti-phospho-MAPKAPK2 (Assay bio Tech, 1:1000), rabbit anti-TRAF-6 (Santa Cruz, 1:500), rabbit anti-HO-1 (Santa Cruz, 1:500) and rabbit anti-Nrf2 (Santa Cruz, 1:500). Blots were detected with Alexa Fluor® 680 goat anti-rabbit IgG (Life technologies, UK) using Licor Odyssey. Equal protein loading was assessed using rabbit anti-actin antibody (Sigma, 1:1000).

2.9. NF-κB dependent reporter gene assay

In order to determine the effect of tiliroside on the transactivation of NF-κB, a luciferase reporter gene assay was carried out. HEK293 cells were seeded out at a concentration of 4×10^5 cells/ml. Twenty-four hours later, cells were transfected with a pGL4.32[luc2P/NF-κB-RE/Hygro] Vector (Promega, UK), using TransIT®-LT1 transfection reagent (Mirus Bio LLC) and incubated for a further 16 h at 37 °C in 5% CO_2. After this period, culture medium was changed to Opti-MEM which contained 5% FBS, and cells incubated for a further 8 h. Thereafter, transfected HEK 293 cells were stimulated with TNFα (1 ng/ml) in the presence or absence of tiliroside (2–6 μM) for 6 h. NF-κB-mediated gene expression was measured with One-Glo luciferase assay kit (Promega, Southampton, UK) according to the manufacturer's instructions, using a Polar star Optima plate reader.

2.10. Measurement of cellular reactive oxygen species (ROS)

Intracellular ROS production was measured using DCFDA-cellular reactive oxygen species detection assay kit (Abcam, Cambridge, UK). BV2 microglia cells were seeded in 96 well plates at a density of 2×10^5 cells/ml. Once confluent, the cells were washed with PBS and stained with 20 μM DCFDA for 30 min at 37 °C. After incubation, the cells were washed and thereafter incubated for 30 min with or without tiliroside (2–6 μM) prior to stimulation with LPS (100 ng/ml) for 24 h. Fluorescence detection of ROS production was done using Polar star Optima plate reader.

2.11. Statistical analysis

All experiments were performed at least three times and in triplicates unless otherwise stated. Data are expressed as mean ± SEM. Statistical analysis was performed using one way ANOVA with post-hoc Student–Newman–Keuls test (multiple comparisons).

3. Results

3.1. Tiliroside reduced TNFα and IL-6 production in LPS + IFNγ-activated BV2 microglia

TNFα is one of the most significant molecules which mediate chronic neuroinflammation. We therefore tested whether tiliroside could prevent the production of this important cytokine from LPS + IFNγ-activated BV2 microglia. Our results show that tiliroside (4 and 6 μM) prevented TNFα production after stimulation with LPS + IFNγ for 24 h (Fig. 2a). Our results also show that tiliroside produced significant ($p < 0.05$) reduction in IL-6 production following activation of BV2 cells with LPS + IFNγ (Fig. 2b).

3.2. Tiliroside inhibited nitrite production through suppression of iNOS protein in LPS + IFNγ-activated BV2 microglia

Nitric oxide (NO) is an important mediator for regulating chronic inflammation in the central nervous system (CNS). Therefore we investigated the effects of tiliroside (2–6 μM) on nitrite production in BV2 microglia. Results show that there was marked increase in nitrite secretion when cells were stimulated with LPS + IFNγ alone, compared with unstimulated cells. Pre-treatment with tiliroside (2–6 μM) for 30 min prior to LPS + IFNγ stimulation significantly ($p < 0.05$) and dose-dependently inhibited nitrite production (Fig. 3a). Further investigations using immunoblotting showed that tiliroside (2–6 μM) produced significant ($p < 0.001$) suppression of iNOS protein expression (Fig. 3b). These results indicate that tiliroside regulated NO production in stimulated BV2 cells through inhibition of iNOS protein expression.

3.3. Tiliroside suppressed PGE_2 production by inhibiting COX-2 protein expression in LPS + IFNγ-activated BV2 microglia

Prostaglandin E2 (PGE_2) is an arachidonic acid derivative released by activated microglia through the enzymatic action of cyclooxygenase-2 (COX-2), a rate limiting enzyme [17]. We therefore evaluated the effects of tiliroside (2–6 μM) pre-treated for 30 min on PGE_2 production and COX-2 protein expression in LPS + IFNγ-activated BV2 microglia. As shown in Fig. 4a, stimulated BV2 cells produced detectable levels of PGE_2 compared to the untreated cells. Tiliroside (4 and 6 μM) significantly ($p < 0.01$) reduced PGE_2 production in a concentration-dependent manner. Also results in Fig. 4b show marked expression of COX-2 protein in LPS + IFNγ

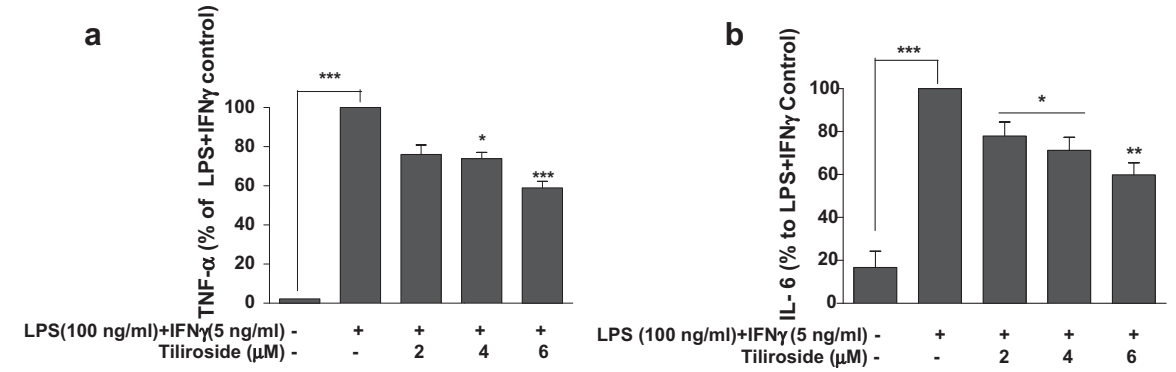

Fig. 2. Tiliroside reduced TNFα (a) and IL-6 (b) production in LPS + IFNγ-activated BV2 microglia. Cells were stimulated with LPS (100 ng/ml) + IFNγ (5 ng/ml) in the presence or absence of tiliroside (2–6 μM) for 24 h. At the end of the incubation period, supernatants were collected for ELISA measurements. Data are expressed as mean ± SEM for 3 independent experiments. Statistical analysis was performed using one way ANOVA with post-hoc Student–Newman–Keuls test (multiple comparisons; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison with LPS + IFNγ control.

stimulated cells. Pre-treatment with tiliroside (2–6 μM) significantly ($p < 0.01$) attenuated LPS + IFNγ-induced COX-2 protein expression. These results suggest that tiliroside inhibits LPS + IFNγ-induced PGE_2 production by inhibiting COX-2 protein expression in BV2 microglia.

3.4. Tiliroside inhibits neuroinflammation by targeting IKKα/IκB-NF-κB signalling pathway

NF-κB plays a central role in regulating the expression of pro-inflammatory genes like COX-2 which mediates PGE_2 production, and

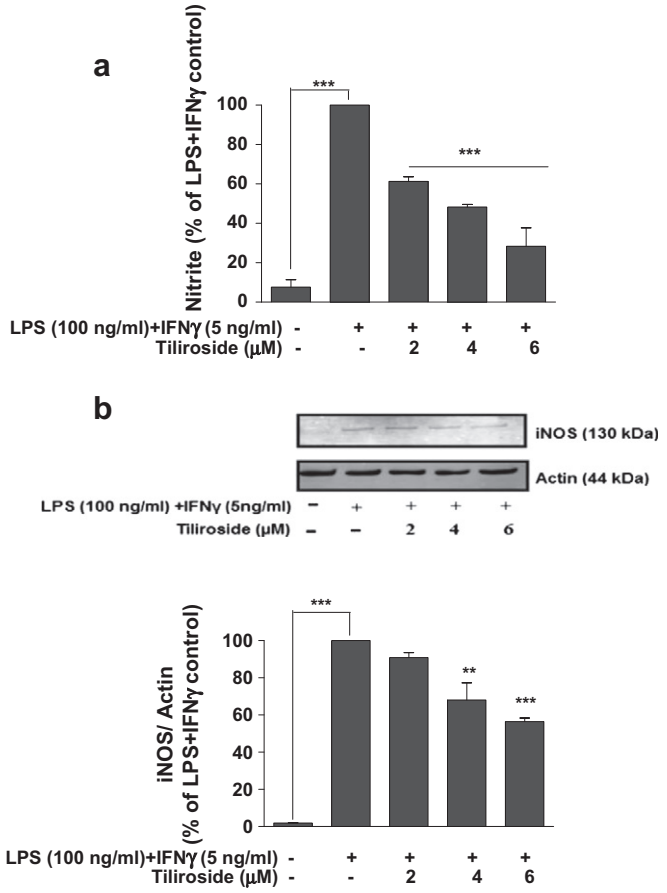

Fig. 3. Tiliroside inhibited nitric oxide (NO) release (a) and inducible nitric oxide (iNOS) protein expression (b) in BV2 cells stimulated with LPS + IFNγ. BV2 cells were stimulated with LPS (100 ng/ml) + IFNγ (5 ng/ml) in the presence or absence of tiliroside (2–6 μM) for 24 h. Data are expressed as mean ± SEM for 3 independent experiments. Statistical analysis was performed using one way ANOVA with post-hoc Student–Newman–Keuls test (multiple comparisons). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison with LPS + IFNγ control.

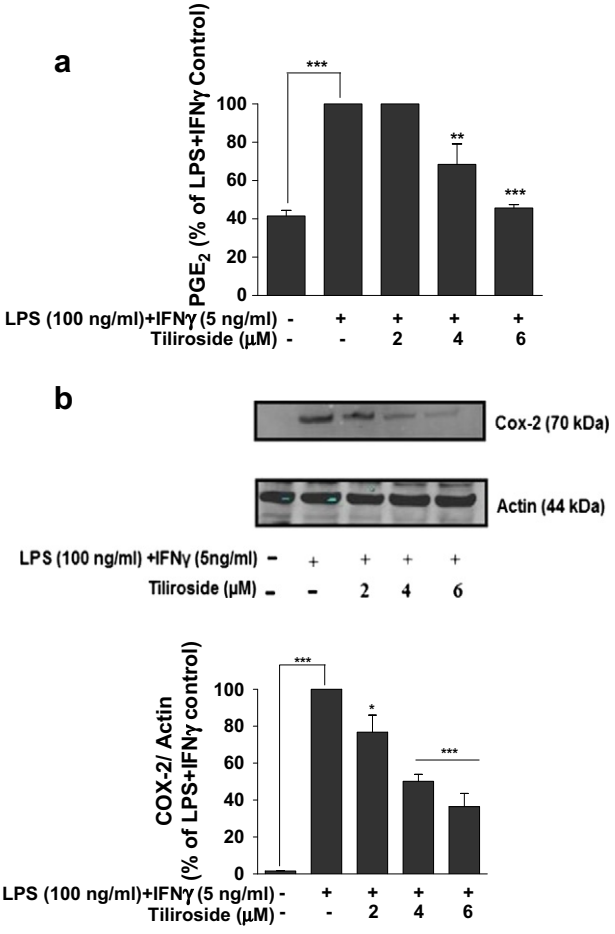

Fig. 4. Tiliroside attenuated PGE_2 production (a) and COX-2 protein expression (b) in LPS + IFNγ-activated BV2 microglia. Microglia were incubated in a medium containing 2–6 μM tiliroside for 30 min and then activated with LPS (100 ng/ml) + IFNγ (5 ng/ml) for 24 h. Tiliroside diminished PGE_2 production, as well as COX-2 protein expression in BV2 microglia. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, in comparison with LPS + IFNγ control.

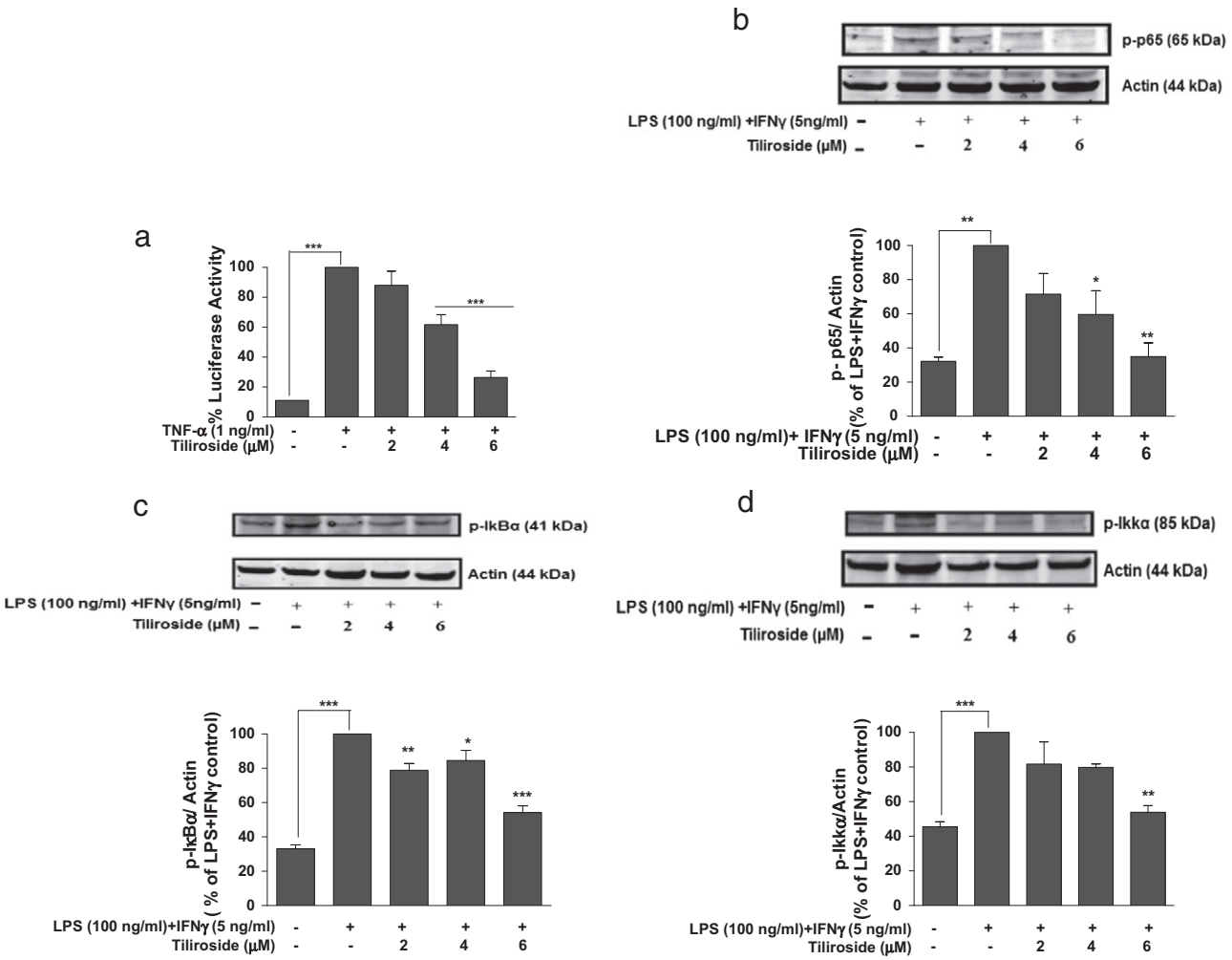

Fig. 5. Tiliroside inhibits NF-κB signalling pathway. TNFα-induced NF-κB-dependent gene expression in HEK 293 cells was inhibited by tiliroside (a). Transfected cells were incubated with different concentrations of tiliroside followed by stimulation with TNFα (1 ng/ml) for an additional 6 h. Luminescence was them measured. LPS + IFNγ-induced p65 subunit nuclear translocation was significantly inhibited by tiliroside in BV2 microglia cells (b). Tiliroside also attenuated phosphorylation of IκBα (c) and IKKα (d) in LPS + IFNγ stimulated BV-2 cells. BV2 cells were pre-treated for 30 min with tiliroside (2–6 μM) prior to stimulation with LPS + IFNγ, and then incubated for 1 h. At the end of the incubation, cytoplasmic or nuclear extracts were collected and western blot performed to measure phospho-IκBα, phospho-p65 and phospho-IKKα. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with LPS + IFNγ control.

iNOS which controls nitrite production in stimulated microglia [18]. To further determine whether tiliroside shows any general effect on NF-κB-mediated gene transcription, a luciferase reporter gene assay was used. HEK293 cells were transfected with a vector containing NF-κB regulated luciferase reporter construct, and the experiment revealed that tiliroside (4 and 6 μM) significantly ($p < 0.001$) inhibited NF-κB regulated luciferase reporter gene expression following stimulation with TNFα (1 ng/ml) (Fig. 5a).

Inactive NF-κB in cytoplasm exists as a complex with IκBα. Once this complex is phosphorylated by IKK, active NF-κB is translocated to the nucleus. Based on our observation that tiliroside produced an inhibition of NF-κB regulated reporter gene transcription, we examined whether the compound interfered with NF-κB signalling pathway in LPS + IFNγ-activated microglia. Firstly, we tested the effect of tiliroside on nuclear phosphorylated p65 subunit, and observed that at 4 and 6 μM of tiliroside, there was a significant ($p < 0.05$) inhibition of LPS LPS + IFNγ-induced nuclear translocation of the p65 subunit (Fig. 5b). Experiments to determine effects of the compound on upstream targets in the NF-κB signalling pathway following activation of microglia by LPS + IFNγ revealed significant ($p < 0.05$) inhibition of IκBα phosphorylation by tiliroside (2–6 μM) (Fig. 5c). This effect was further confirmed by significant ($p < 0.05$) inhibition of LPS + IFNγ-induced phosphorylation of IKKα by tiliroside (6 μM) (Fig. 5d). These results seem to suggest that tiliroside prevents neuroinflammation through mechanisms involving upstream targets in NF-κB signalling.

3.5. Tiliroside interferes with p38 signalling in BV2 microglia

Increasing evidence suggest that p38 mitogen activated protein (MAP) kinase regulates NF-κB-dependent transcription [19,20]. Also, LPS-stimulated and p38 MAPK mediated generation of NO, PGE_2 and TNFα has been observed in rat and human microglia [21–25]. Consequently, we investigated whether tiliroside interferes with p38 signalling in LPS + IFNγ-activated BV2 microglia. Firstly, we tested the effect of tiliroside on phosphorylation of p38 following activation of BV2 microglia with a combination of LPS and IFNγ. This was significantly ($p < 0.05$) inhibited by tiliroside (4 and 6 μM) (Fig. 6a). Further investigation on the downstream substrate of p38, MAPK-activated protein kinase 2 (MK2), showed an inhibition of its phosphorylation by tiliroside (4 and 6 μM), following activation of BV2 microglia with LPS + IFNγ (Fig. 6b). The activation of p38 is

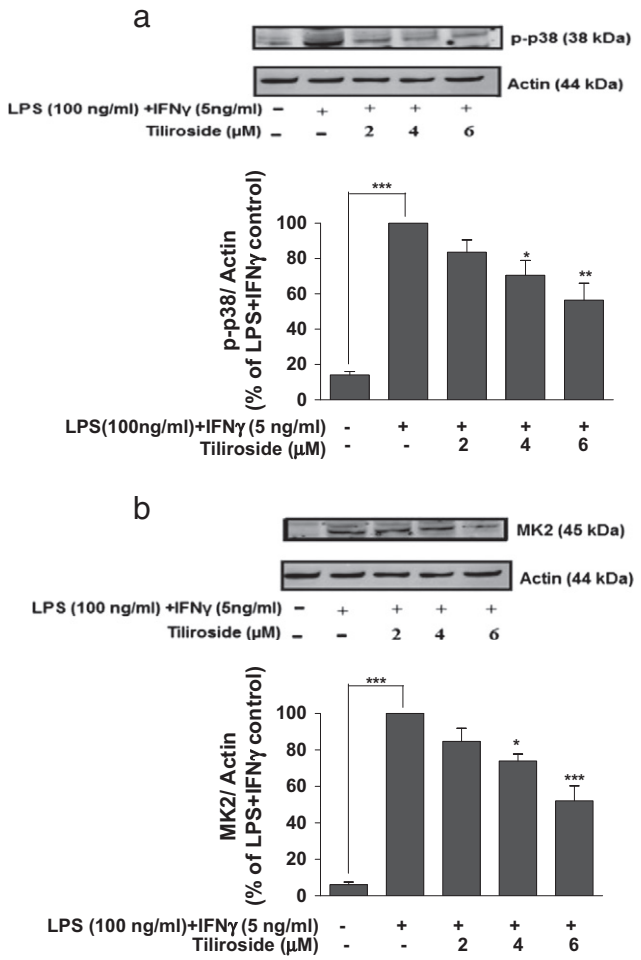

Fig. 6. Tiliroside inhibited LPS (100 ng/ml) + IFNγ (5 ng/ml)-induced phosphorylation of p38 (a) and MK2 (b) in BV2 microglia. Cells were pre-treated with tiliroside (2–6 μM) prior to activation with LPS + IFNγ. At the end of the incubation, cytoplasmic extracts were collected and western blot performed for phospho-p38 and phospho-MK2. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with LPS + IFNγ control.

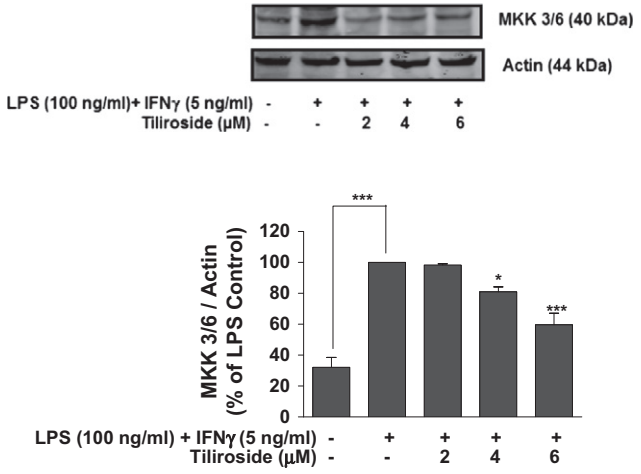

Fig. 7. Tiliroside inhibited LPS (100 ng/ml) + IFNγ (5 ng/ml)-induced MKK 3/6 phosphorylation in BV2 microglia. Cells were pre-treated with tiliroside (2–6 μM) in the presence or absence of LPS (100 ng/ml) + IFNγ (5 ng/ml) and incubated for 30 min. Western blot was performed for phospho-MKK 3/6. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with LPS control.

known to require phosphorylation by activated upstream MKK3/6, in response to diverse stimuli, including LPS and cytokines. In order to confirm whether tiliroside affects p38 signalling in activated BV2 microglia, we examined its effect on MKK3/6 phosphorylation. Our experiments showed that MKK3/6 are phosphorylated after 30 min of LPS + IFNγ exposure, and were significantly ($p < 0.01$) inhibited by tiliroside (6 μM) (Fig. 7).

3.6. Inhibitory actions of tiliroside on neuroinflammation is dependent on TRAF-6

LPS-induced TLR4-dependent activation of Toll adapter proteins (MyD88, IRAK-1 and TRAF-6) is one of the most important events upstream of the NF-κB inflammatory signalling pathway [26]. Here, we used western blot to show that TRAF-6 protein was maximally expressed within 10 min of LPS (100 ng/ml) + IFNγ (5 ng/ml) stimulation and its expression was inhibited by tiliroside (4 and 6 μM) (Fig. 8).

3.7. Tiliroside prevents the production of ROS and activates Nrf2/HO-1 antioxidant protective mechanisms in BV2 microglia

Chronic activation of microglia has been shown to produce ROS, which ultimately damage neurons. We therefore used the DCFDA assay to investigate the effect of tiliroside on cellular ROS, following LPS + IFNγ challenge. Our results show that at 6 μM, tiliroside produced significant ($p < 0.01$) reduction of ROS from BV2 cells, following activation by LPS (100 ng/ml) + IFNγ (5 ng/ml). At 2 and 4 μM, tiliroside did not produce significant reduction of ROS (Fig. 9a).

HO-1, which is transcriptionally controlled by Nrf-2, has been reported to be involved in the resolution of neuroinflammation [27]. Substances that activate the HO-1/Nrf-2 system have been suggested to have neuroprotective effect. We therefore investigated whether tiliroside could activate this antioxidant mechanism. Western blot experiments revealed that tiliroside (6 μM) produced significant increase in the expression of HO-1, while activation of nuclear Nrf-2 was achieved with 4 and 6 μM of the compound (Fig. 9b and c). These observations suggest that tiliroside prevents cellular ROS production and activates HO-1/Nrf2 antioxidant mechanism at 6 μM.

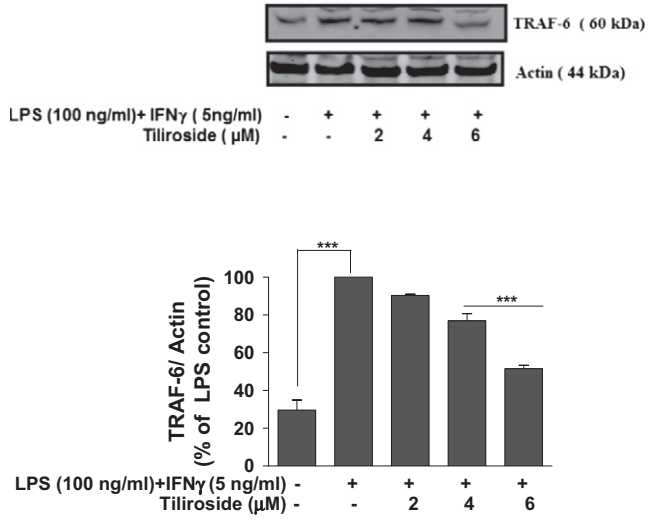

Fig. 8. Tiliroside inhibited LPS + IFNγ-induced activation of TRAF-6 in BV2 microglia. Cells were pre-treated with tiliroside (2–6 μM) in the presence or absence of LPS (100 ng/ml) + IFNγ (5 ng/ml) and incubated for 10 min. Western blot was performed for TRAF-6. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with LPS control.

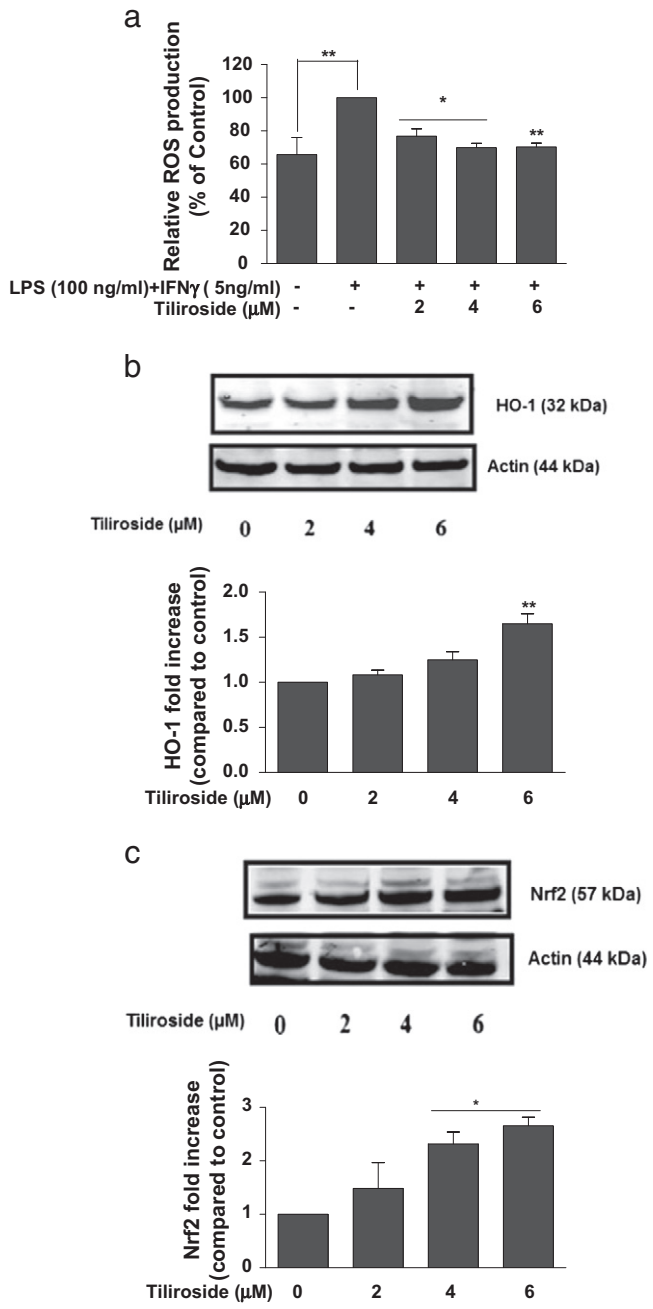

Fig. 9. Tiliroside suppressed LPS-induced ROS production in BV2 microglia. Cells were washed with PBS and stained with 20 μM DCFDA for 30 min at 37 °C, and then stimulated with LPS (100 ng/ml) + IFNγ (5 ng/ml) for 24 h in the presence or absence of tiliroside (2–6 μM). Fluorescence detection of ROS production was done using Polar star Optima plate reader (a). Tiliroside induced HO-1 (b) and Nrf2 (c) activity in BV2 microglia. BV2 cells were treated with the indicated concentrations of tiliroside for 24 h. Cytoplasmic or nuclear extracts were then subjected to western blot. All values are expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student–Newman–Keuls test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, in comparison with LPS control.

3.8. Tiliroside did not affect BV2 cell viability

In order to determine whether tiliroside affected the viability of BV2 cells, an MTT assay was carried out after incubating the cells with the compound for 24 h. Results showed that there was no significant difference in viability of cells treated with tiliroside (2–6 μM), when compared with control (untreated) cells (Fig. 10).

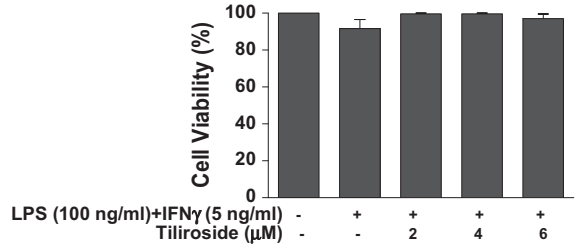

Fig. 10. Pre-treatment with tiliroside (2–6 μM) did not affect the viability of BV2 microglia stimulated with LPS + IFNγ. Cells were stimulated LPS (100 ng/ml) + IFNγ (5 ng/ml) in the presence or absence of tiliroside (2–6 μM) for 24 h. At the end of the incubation period, MTT assay was carried out on cells. All values are expressed as mean ± SEM for 3 independent experiments.

4. Discussion

Neuroinflammation is a critical component in the pathogenesis of neurodegenerative diseases. Sustained neuroinflammatory process may contribute to a set of events in the brain which eventually cause neuronal damage. Chronic activation of microglia cells has been observed in AD patients [28]. These microglia cells represent the first line of defence against invading pathogens or other types of brain tissue injury. Under pathological situations, such as neurodegenerative disease microglia become activated, migrate, and surround damaged or dead cells [29]. In this study, we have shown that tiliroside inhibits neuroinflammation in LPS + IFNγ-activated BV2 microglia.

TNFα and IL-6 have been shown to play a central role in initiating and regulating the cytokine cascade during an inflammatory response, and have been shown to be produced by activated microglia, along with other pro-inflammatory mediators and neurotoxic substances [29]. Consequently, we investigated whether tiliroside would prevent the production of these cytokines following LPS + IFNγ activation of BV2 microglia. Results demonstrate an inhibition of neuroinflammation by tiliroside, as observed by suppression of TNFα and IL-6 production. Earlier, tiliroside was reported to exhibit anti-inflammatory activity through the inhibition of phospholipase A2-induced paw oedema, TPA-induced ear inflammation, and leukocyte migration in mice [30]. Our results on the effect of tiliroside on TNFα in BV2 microglia seem to explain some of these *in vivo* data.

In the brain, nitric oxide is mainly produced by the microglia and its excessive production has been shown to be toxic to neurons [31]. Stimulation of glial cells has been proposed to lead to the generation of large quantities of nitric oxide, through the activation of iNOS. Nitric oxide is then released from astrocytes and microglia and target adjacent neurons [32]. In this study, we have demonstrated that tiliroside prevents nitric oxide production by inhibiting iNOS protein expression in activated BV2 microglia, suggesting another mechanism for inhibiting neuroinflammation. The cyclooxygenase (COX-2) enzyme pathway and subsequent generation of prostaglandins play a significant role in neuroinflammation. Therefore, in order to gain a broader understanding of the mechanisms of action of tiliroside in neuroinflammation, we explored its effect on COX-2 -mediated PGE_2 production. Interestingly, tiliroside suppressed PGE_2 production at 4 and 6 μM, while COX-2 protein expression was inhibited at all the concentrations investigated, showing that its activity is more pronounced at the level of COX-2 protein expression. All these observations show that tiliroside produces anti-neuroinflammatory actions in BV2 microglia.

In recent years there has been significant interest in the anti-neuroinflammatory and neuroprotective effects of flavonoids. Consequently, flavonoids have been explored for the reduction of the neuroinflammation-related neuronal damage and as potential preventive compounds for the progression of neurodegenerative diseases [33]. Some flavonoids which have been shown to inhibit neuroinflammation include quercetin [34–36], rutin [37], naringenin [38,39], and EGCG [40].

In the cytoplasm NF-κB is coupled to IκB to form an inactive complex. However, during inflammation, phosphorylation by active IKK leads to degradation of IκB by proteasomes, followed by NF-κB translocation into nucleus. Overwhelming evidence suggests that binding of translocated NF-κB to the promoter region of COX-2 expressing gene is required for the expression of COX-2 [41]. Also it is believed that NF-κB controls the transcription of different genes encoding neurotoxic inflammatory mediators. Consequently, we showed that tiliroside produced significant inhibition of general NF-κB-dependent gene expression, through suppression of TNFα-induced activation of the NF-κB-driven luciferase expression in HEK293 cells. Further experiments on specific effects of tiliroside on NF-κB signalling in BV2 microglia showed an inhibition of IκBα phosphorylation and activation of its upstream kinase, IKKα, by tiliroside following stimulation with LPS + IFNγ. Moreover, results revealed that tiliroside diminished LPS + IFNγ-mediated NF-κBp65 nuclear translocation in a concentration-dependent manner in BV2 microglia. Taken together, it is suggested that tiliroside inhibit neuroinflammation by targeting NF-κB signalling LPS + IFNγ-activated BV2 microglia.

MAP kinases play a crucial role in controlling signalling events that contribute to the production of neuroinflammatory mediators [42]. Reports have also linked the roles of ERK, p38 and JNK signalling pathways to COX-2 induction in different cell types [43]. Specifically, the p38 MAPK has been strongly linked to neuroinflammation. The p38 inhibitor SB203580 markedly inhibited iNOS protein expression in LPS induced BV2 microglia [44]. Of significance is the regulatory role of p38 MAPK in NF-κB transcriptional activity [19,20]. Interestingly, tiliroside attenuated the phosphorylation of p38 and its downstream substrate, MK2, in LPS + IFNγ-stimulated BV2 microglia. We confirmed the effect of tiliroside on p38 signalling by demonstrating its inhibitory action on MKK3/6 activation in LPS-stimulated BV2 microglia. MAPK kinase 3/6 (MKK3/6) is an upstream kinase of p38 and is phosphorylated by stimulation with LPS and cytokines. It is not clear from our studies if the action of tiliroside on NF-κB signalling is dependent on p38 activation.

Upon LPS stimulus, TLR4/MyD88 induces the expression of iNOS, COX-2 and the proinflammatory cytokines, through TNFα receptor-associated factor-6 (TRAF-6)-dependent activations of both NF-κB signalling pathway. Furthermore, LPS-induced activation of TRAF-6 is one of the most important events upstream of the IKKα/IκB-NF-κB inflammatory signalling pathway. In addition to NFκB activation, toll-like receptors can also initiate MAPK signalling cascades and activate multiple transcription factors, following LPS activation [45]. TRAF-6 can activate the MAPK kinase MKK3/6, which in turn activates p38. We showed that tiliroside suppressed TRAF-6 expression in LPS-stimulated BV2 microglia, suggesting that this compound probably targets TRAF-6, to inhibit IKKα/IκB-NF-κB and p38 signalling.

It is now recognised that ROS production by the microglia impact on neurons and modulate microglial activity [46]. The HO-1/Nrf2 antioxidant protective system, on the other hand, has been shown to exert anti-neuroinflammatory and neuroprotective effects. Our data demonstrate that LPS-induced ROS production in BV2 microglia was significantly downregulated by 6 μm tiliroside. At this concentration, the compound caused significant activation of HO-1 and Nrf-2 in BV2 microglia. We suggest that the antioxidant property of tiliroside in BV2 microglia probably contributes to its effect on neuroinflammation. Further studies will aim to establish the connection between its antioxidant and anti-neuroinflammatory effects.

The effects shown by tiliroside in this study are consistent with observations with other flavonoids. For example wogonin, a flavonoid found in *S. baicalensis*, has been shown to inhibit cytokines, NO, iNOS and NF-κB in C6 glia and BV2 microglia [47–49]. Baicalein is another flavonoid of *S. baicalensis* which inhibits neuroinflammation [50–52]. Quercetin, rutin and epigallocatechin gallate (EGCG) are popular flavonoids which have shown neuroinflammation inhibitory activity [34,37,40].

Taken together, we conclude that tiliroside inhibits neuroinflammation in BV2 microglia through a mechanism involving TRAF-6-mediated activation of NF-κB and p38 MAPK signalling pathways. These activities are possibly due, in part, to the antioxidant property of this compound. The outcome of this study is significant in the search for novel natural compounds for inhibiting neuroinflammation in neurodegenerative disorders.

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