RESEARCH ARTICLE

Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia

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Scope: In this study, the effects of punicalagin on neuroinflammation in LPS-activated microglia were investigated.
Methods and results: The ability of punicalagin to reduce the production of TNF-α, IL-6 and prostaglandin E_2 was measured in culture medium using enzyme immunoassay. TNF-α and IL-6 gene expression in mouse hippocampal slices was measured with PCR. cyclooxygenase-2 and microsomal prostaglandin E synthase 1 protein and mRNA were evaluated with Western blotting and PCR, respectively. Further experiments to investigate effects of punicalagin on protein expressions of inflammatory targets were also determined with Western blotting. Pretreatment of rat primary microglia with punicalagin (5–40 μM) prior to LPS (10 ng/mL) stimulation produced a significant ($p < 0.05$) inhibition of TNF-α, IL-6 and prostaglandin E_2 production. Punicalagin completely abolished TNF-α and IL-6 gene expression in LPS-stimulated hippocampal slices. Protein and mRNA expressions of cyclooxygenase-2 and microsomal prostaglandin E synthase 1 were also reduced by punicalagin pretreatment. Results show that punicalagin interferes with NF-κB signalling through attenuation of NF-κB-driven luciferase expression, as well as inhibition of IκB phosphorylation and nuclear translocation of p65 subunit in the microglia.
Conclusion: These results suggest that punicalagin inhibits neuroinflammation in LPS-activated microglia through interference with NF-κB signalling, suggesting its potential as a nutritional preventive strategy in neurodegenerative disorders.

Keywords:
Microglia / Neuroinflammation / NF-κB / Pomegranate / Punicalagin

1 Introduction

Neurodegenerative diseases, such as dementia, continue to be a major serious public health problem of an ageing population. Alzheimer's disease (AD) is the most prevalent type of dementia and accounts for about 60% of all cases [1].Considerable evidence suggests that neuroinflammation plays a role in AD and other neurodegenerative conditions [2]. In neuroinflammation, cytokines, chemokines, reactive oxygen species and reactive nitrogen species, and prostaglandin E_2 (PGE_2), play significant roles in the development and maintenance of inflammatory responses. These events are mediated through the microglia that are the resident immune macrophages in the CNS.

Evidence suggests that fruit and vegetable consumption is associated with reduced risk of chronic diseases, such as cardiovascular diseases, cancer and inflammatory disorders. Specifically, studies have suggested that diet and nutrition might be important modifiable risk factors for AD, and consumption of fruits and vegetables have been shown to reduce its risk [3]. Punicalagin (Fig. 1), a polyphenol found in the pomegranate fruit, *Punica granatum*, has been reported to exhibit anti-inflammatory activity. The compound was reported to exhibit in vivo anti-inflammatory effect through inhibition

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Abbreviations: AD, Alzheimer's disease; **IKK**, IκB kinase; **JNK**, c-Jun *N*-terminal kinase; **MAPK**, mitogen-activated protein kinase; **PGE2**, prostaglandin E_2; **TRAF6**, TNF receptor associated factor 6

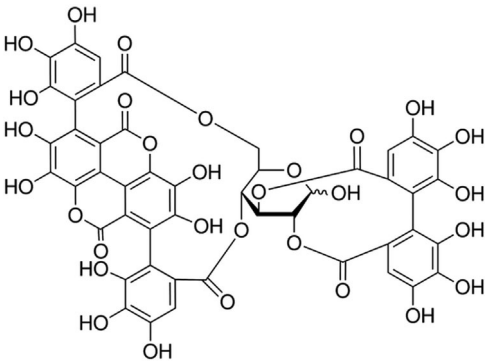

Figure 1. Punicalagin.

of paw oedema induced by carrageenan in rats [4]. In HT-29 human colon cancer cell line, punicalagin significantly reduced cyclooxygenase-2 (COX-2) protein expression and DNA binding of NF-κB [5]. Recently Xu et al. [6] reported that punicalagin inhibited LPS-induced inflammation in RAW264.7 macrophages. It has not been established whether punicalagin could inhibit neuroinflammation, a critical process in neurodegenerative disorders, such as AD. Consequently, we have investigated effects of punicalagin in LPS-induced neuroinflammation in activated microglia.

2 Materials and methods

Punicalagin was purchased from Sigma Chemicals and suspended in DMSO prior to experiments.

2.1 Cell culture

Primary mixed glial cell cultures were established from cerebral cortices of 1-day neonatal Sprague–Dawley rats as described in [7, 8]. Forebrains were minced, dissociated and collected by centrifugation (1000 × *g*, 10 min), resuspended in DMEM, containing 10% foetal calf serum and antibiotics (40 U/mL penicillin and 40 μg/mL streptomycin) and cultured in 5% CO_2 at 37°C. Floating microglia were harvested every week (between 2 and 7 wk) and reseeded into 75 cm^2 culture flask to give pure microglial cultures. The next day, cultures were washed to remove non-adherent cells, and fresh medium was added. HEK293 cells were obtained from the HPA Culture Collection (Salisbury, UK) and grown in MEM-Eagle's medium (Life Technologies, UK). Medium was supplemented with 10% foetal bovine serum (Sigma, UK), 2 mM L-glutamine, 1 mM sodium pyruvate and 40 units/mL penicillin/streptomycin (Sigma). Confluent monolayers were passaged routinely by trypsinisation. Cultures were grown at 37°C in 5% CO_2 until 80% confluence.

2.2 ATP assay for cell viability

Viability of primary microglia after treatment with punicalagin was determined by the ATP assay. ATP plays a central role in energy exchange in biological systems, and is present in all metabolically active cells. Thus, levels of ATP can be used to determine the functional integrity of cells. Cells (2×10^5/mL) were cultured for 48 h, and then incubated with or without LPS (10 ng/mL) in the absence or presence of punicalagin (5–40 μM) for 24 h. The concentration of ATP was measured through a sensitive luciferin-luciferase bioluminescent assay using a kit (Promega). After incubation, 100 μL of reconstituted substrate was added to the cells. Luminescence was then measured in Berthold Luminometer.

2.3 Determination of PGE_2 and cytokine (TNF-α and IL-6) production from activated microglia

Cultured primary microglia were incubated with or without LPS (10 ng/mL) in the absence or presence of punicalagin (5–40 μM) for 24 h. PGE_2 concentration was assessed in cell supernatants with a commercially available enzyme immunoassay (EIA) kit (Arbor Assays, Ann Arbor, MI, USA), followed by measurement at 450 nm. Concentrations of TNF-α 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. Experiments were performed at least three times and in triplicate.

2.4 Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described previously [9]. In brief, slice cultures were prepared from post-natal day P0–3 old C57BL/6 mice under sterile conditions. After decapitation, the brains were removed and the hippocampi from both hemispheres were acutely isolated in ice cold serum-free Hank's Balanced Salt Solution (HBSS), supplemented with 0.5% glucose and 15 mM HEPES. Isolated hippocampi were cut into 350–375 μM thick slices using a tissue chopper and were transferred to 0.4 μM culture plate inserts. Inserts containing hippocampi were placed in 6-well plates containing 1 mL of 0.5× minimum essential medium (MEM), containing 25% heat-inactivated horse serum, 25% Basal Medium Eagle (BME) without glutamate, 2 mM glutamax and 0.65% glucose. The slice cultures were kept at 35°C in a humidified atmosphere (5% CO_2) and the culture medium was refreshed the first day after preparation and every consecutive 2 days. After 6 days, slice cultures were pre-incubated for 30 min with punicalagin (5, 10, 20 and 40 μM); subsequently LPS (100 ng/mL) was added for further 4 h. We evaluated the effects of punicalagin on TNF-α and IL-6 gene expressions in LPS-stimulated mouse hippocampal slices using PCR.

2.5 Immunoblotting

Rat primary microglia were left untreated or treated with LPS (10 ng/mL) in the presence or absence of punicalagin (5-40 μM) for different periods. To obtain cytoplasmic extracts, cells were washed with PBS and lysed in 1.3× SDS-containing sample buffer without 1, 4-DTT or bromophenol blue containing 100 μM orthovanadate. Nuclear extracts were obtained using a commercially available nuclear extraction buffer (Abcam, UK). Protein contents were measured using the bicinchoninic acid method (Pierce), according to the manufacturer's instructions. For Western blotting, 40 μg of total protein from each sample was subjected to SDS-PAGE under reducing conditions. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked overnight at 4°C before incubation with primary antibodies. Primary antibodies used were goat anti-COX-2 (Santa Cruz; 1:500), rabbit anti-mPGES-1 (where mPGES-1 is microsomal prostaglandin E synthase 1; Agri Sera; 1:1000), anti-phospho-IκBα (Santa Cruz; 1:500), anti-p38 (Cell Signalling; 1:1000), anti-c-Jun *N*-terminal kinase (JNK) (Cell Signalling; 1:1000), anti-phospho-p38 (Cell Signalling; 1:1000), anti-phospho-JNK (Cell Signalling; 1:1000), anti-phospho-p42/44 (Cell Signalling; 1:1000), rabbit anti-phospho-p65 (Santa Cruz; 1:250), rabbit anti-TNF receptor associated factor 6 (TRAF-6) (1:1000) and rabbit anti-actin (Sigma; 1:5000). Primary antibodies were diluted in Tris-buffered saline (TBS), containing 0.1% Tween 20 (TBS-T) and 1% BSA. Membranes were incubated with the primary antibody overnight at 4°C, followed by incubation in secondary antibodies. All Western blot experiments were carried out at least three times.

2.6 Effect of punicalagin on COX-2 and mPGES-1 gene expressions

In order to further determine the effects of punicalagin on COX-2 and mPGES-1 expressions, we evaluated its effects on gene expression. Rat primary microglia were pre-incubated for 30 min with punicalagin (5–40 μM), subsequently LPS (10 ng/mL) was added for total 4 h. Effect of the compound on COX-2 and mPGES-1 gene expressions was determined using semi-quantitative PCR.

2.7 Semi-quantitative PCR

RNA preparation was done by using RNAspin mini RNA isolation kit (GE Healthcare, Freiburg, Germany) and for cDNA synthesis 1 μg of total RNA was reverse transcribed using M-MLV reverse transcriptase and random hexamers (Promega, Mannheim, Germany). The synthesised cDNA was the template for the real-time PCR amplification that was carried out by the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.), using iQ™ SYBR™ Green supermix (Bio-Rad Laboratories GmbH, Munich, Germany). Specific primers were designed by using Universal ProbeLibrary Assay Design Center (https://www.roche-applied-science.com/webapp/wcs/stores/servlet/CategoryDisplay?catalogId=10001&tab=Assay+Design+Center&identifier=Universal+Probe+Library&langId=-1#tab-3). Reaction conditions were 3 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 50°C and 45 s at 72°C and every cycle was followed by plate read. After that, 1 min at 95°C, 1 min at 55°C, followed by melt curve conditions of 65°C, 95°C with increment of 0.5°C for 5 s, followed by final plate read. GAPDH served as an internal control for sample normalisation and the comparative cycle threshold Ct method was used for data quantification. The following primer sequences were used in the present study. COX-2: Fwd 5′-GCCCAGCACTTCACGCATCAGT-3′; Reverse 5′-AAGTCCACCCCATGGCCCAGC-3′. mPGES-1: Fwd 5′-TGCAGCACGCTGCTGGTCAT-3′; Reverse 5′-GGCAAAGGCCTTCTTCCGCAG-3′. GAPDH: Fwd 5′-GTCGCCAGCCGAGCCACATC-3′; Reverse 5′-CCAGGCGCCCAATACGACCA-3′. TNF-α: Fwd 5′-CCTCCTGGCCAACGGCATGG -3′; Reverse 5′-AGCGCTGAGTTGGTCCCCCT-3′. IL-6: Fwd 5′-GCTGGTGACAACCACGGCCT-3′; Reverse 5′-CTCTCTGAAGGACTCTGGCTTTGT-3′; GAPDH: Fwd 5′-TGTCCGTCGTGGATCTGAC-3′; Reverse 5′-CCTGCTTCACCACCTTCTTG-3′.

2.8 NF-κB reporter gene assays

In order to determine the effect of punicalagin on NF-κB-mediated gene expression, a luciferase reporter gene assay was carried out as described earlier [10]. HEK293 cells were seeded out at a concentration of 4×10^5 cells/mL. Twenty-four hours later, cells were transfected with either a Cignal® NF-κB (SABiosciences) reporter, 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 media was changed to Opti-MEM and cells incubated for a further 8 h. Thereafter, transfected HEK293 cells were stimulated with TNF-α (1 ng/mL) in the presence or absence of punicalagin (5–40 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 Polarstar Optima Plate reader.

2.9 Statistical analysis

Values of all experiments were represented as mean ± SEM of at least three experiments. Values were compared using *t*-test (two groups) or one-way ANOVA with post hoc Student Newman–Keuls test (multiple comparisons). The level of significance was set at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

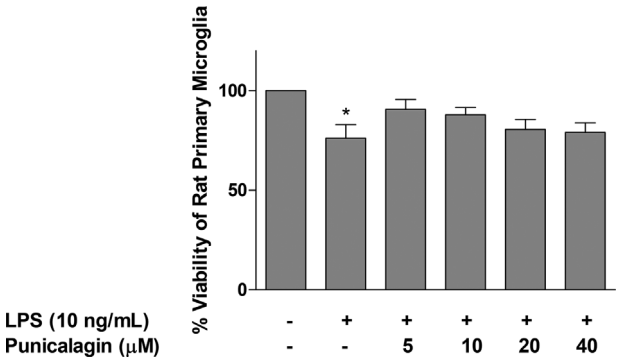

Figure 2. Pre-treatment with punicalagin did not affect the viability of rat primary microglia stimulated with LPS. Cells were stimulated with LPS (10 ng/mL) in the presence or absence of punicalagin (5–40 μM) for 24 h. At the end of the incubation period, ATP assay was carried out on cells. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test. $*p < 0.05$ in comparison with unstimulated control cells.

3 Results

3.1 Punicalagin did not affect the viability of microglia cells

In order to determine the toxicity of punicalagin to microglia, cell viability was measured after treating the cells with the compound for 24 h. Results showed that treatment with punicalagin (5–40 μM) prior to LPS stimulation did not have significant effect on the viability of primary microglia (Fig. 2). This suggests that the observed effects of the compound were not due to cytotoxicity as a result of decreased live cells.

3.2 Punicalagin suppresses the production of the pro-inflammatory cytokines in LPS-activated rat primary microglia and mouse hippocampal slices

The pro-inflammatory cytokines (TNF-α and IL-6) are known to be important mediators of microglia inflammation. We have therefore measured their production in supernatants of LPS-activated microglia using ELISA. After 24 h of LPS (10 ng/mL) stimulation, levels of TNF-α and IL-6 were significantly increased in culture supernatants (Fig. 3A). Pre-treatment with punicalagin (5–40 μM) resulted in significant and concentration-dependent reduction of TNF-α production into microglia cell supernatants. Results also showed significant suppression of IL-6 production by all concentrations of punicalagin tested. Using PCR, it was observed that stimulation of mouse hippocampal slice cultures with LPS (100 ng/mL) resulted in marked increase in the gene expression of both TNFα and IL-6. Interestingly, pre-treatment with punicalagin (5–40 μM) resulted in almost complete abolition of LPS-induced gene expression (Fig. 3B).

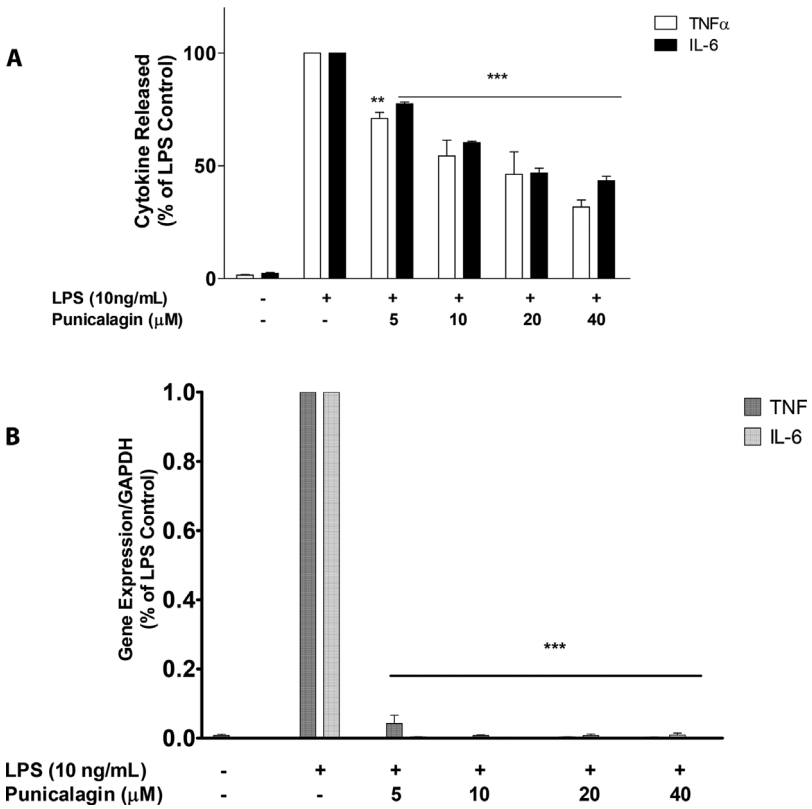

Figure 3. Punicalagin reduced TNF-α and IL-6 production in LPS-activated primary microglia. Cells were stimulated with LPS (10 ng/mL) in the presence or absence of punicalagin (5–40 μM) for 24 h. At the end of the incubation period, supernatants were collected for ELISA measurements (A). Punicalagin abolished TNF-α and IL-6 production from LPS-stimulated mouse hippocampal slices. Slice cultures were stimulated with LPS (100 ng/mL) in the presence or absence of punicalagin (5–40 μM) for 4 h. Thereafter TNF-α and IL-6 gene expressions were measured with PCR (B). All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test.$**p < 0.01$, $***p < 0.001$ in comparison with LPS control.

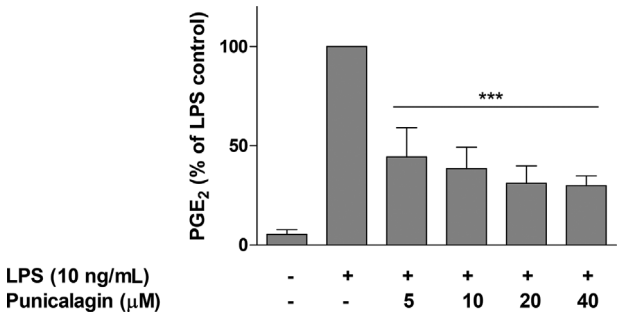

Figure 4. Punicalagin suppressed PGE_2 production in LPS-activated rat primary microglia. Microglia were incubated in a medium containing 5–40 μM punicalagin for 30 min and then activated by 10 ng/mL LPS for 24 h. Punicalagin diminished PGE_2 production in the microglia. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test. ***$p < 0.001$ in comparison with LPS control.

3.3 Punicalagin suppresses PGE_2 production by inhibiting COX-2 and mPGES-1 expressions in LPS-activated microglia

In the presence of LPS (10 ng/mL), there was a marked increase in PGE_2 production in supernatants of rat primary microglia, when compared to unstimulated cells. However, treatment with punicalagin (5–40 μM) prior to stimulation with LPS resulted in significant reduction in PGE_2 production, in comparison with LPS control (Fig. 4).

PGE_2 is synthesised during neuroinflammation through the enzymatic activity of COX-2. It is known that mPGES-1 is coupled to COX-2 in the biosynthesis of PGE_2. Consequently, we sought to determine whether the effect of punicalagin on PGE_2 was mediated through inhibition of the activities of these enzymes. Interestingly, experiments showed that punicalagin (5 μM) did not cause a significant reduction in COX-2 protein expression in microglia activated with LPS. However, significant reduction in expression was produced at 10 μM ($p < 0.05$) and at 20 and 40 μM ($p < 0.01$) of the compound (Fig. 5A). At mRNA level, punicalagin (5–40 μM) significantly

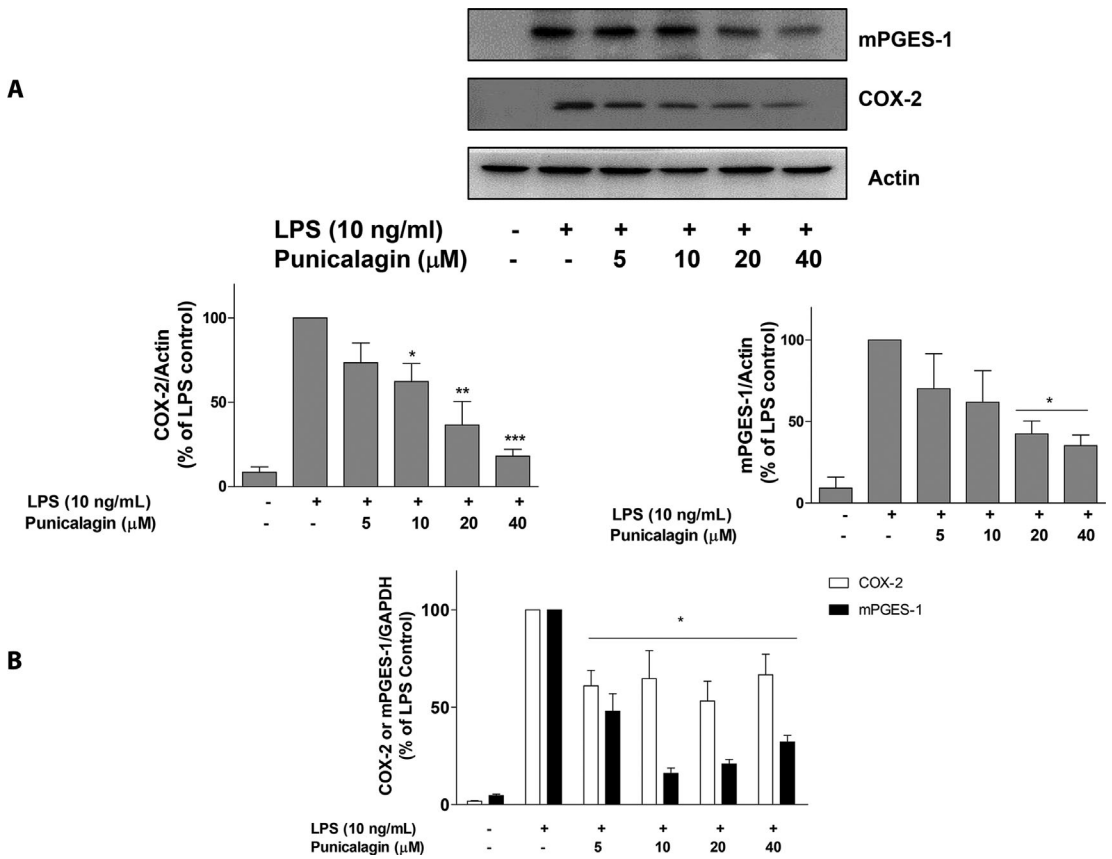

Figure 5. Punicalagin suppressed COX-2 and mPGES-1 protein expression (A), COX-2 and mPGES-1 mRNA expression (B) in LPS-activated rat primary microglia. Microglia were incubated in a medium containing 5–40 μM punicalagin for 30 min and then activated by 10 ng/mL LPS for 24 h (protein) and 4 h (mRNA). All values are expressed as mean ± SEM for three 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.

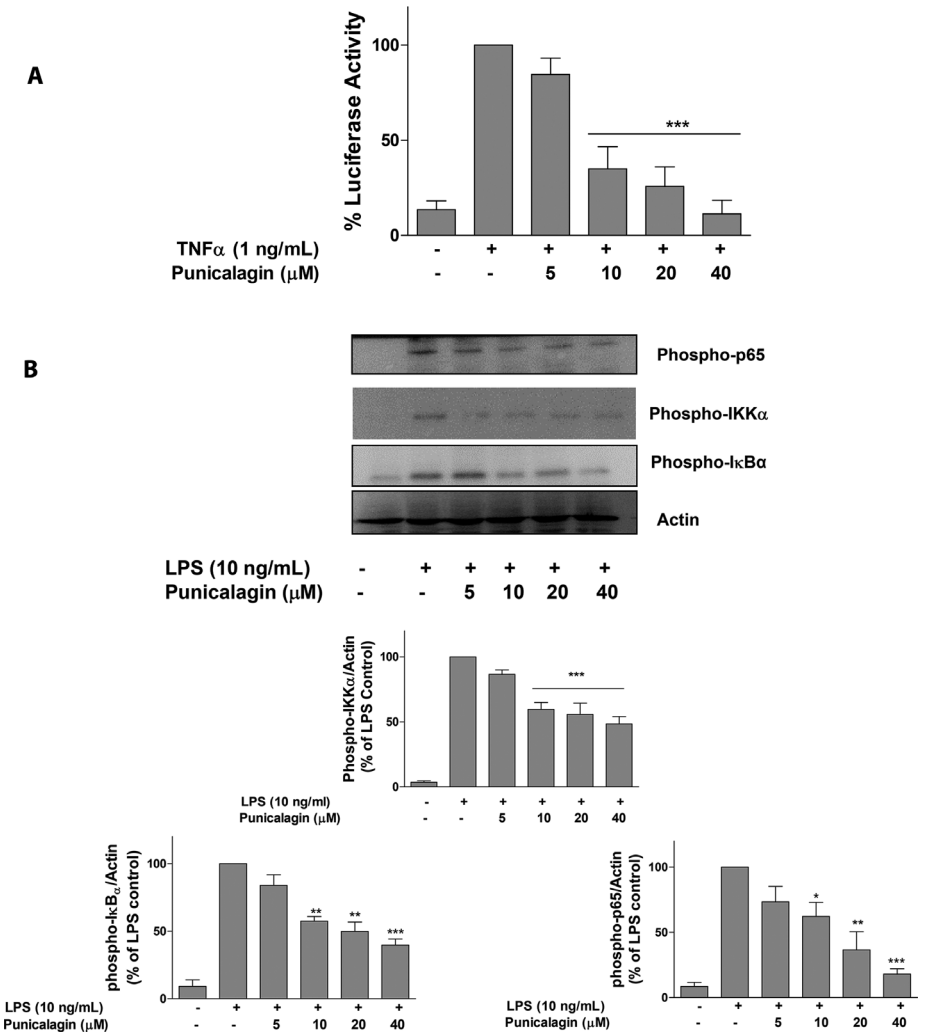

Figure 6. (A) Punicalagin inhibited NF-κB-mediated gene expression. HEK293 cells were transfected with a plasmid construct carrying a luciferase reporter gene controlled by NF-κB, and stimulated with TNF-α (1 ng/mL) in the presence or absence of punicalagin (5–40 μM). NF-κB-mediated gene expression was measured with ONE-Glo luciferase assay kit and luminescence measured. All values are expressed as mean ± SEM for three independent experiments performed in triplicates. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test. ***$p < 0.001$ in comparison with TNF-α control. (B) Punicalagin inhibited LPS-induced IKKα, IκBα and p65 phosphorylation in rat primary microglia. Cells were stimulated with LPS (10 ng/mL) in the presence or absence of punicalagin (5–40 μM) for either 10 (IKKα), 15 (IκBα) or 180 min (p65). At the end of incubation period, phosho-IKKα, phospho-IκBα and phospho-p65 protein expression were determined using Western blot with specific anti-phospho-IKKα, anti-phospho-IκBα and anti-phospho-p65 antibodies. All values are expressed as mean ± SEM for three 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.

($p < 0.05$) reduced COX-2 expression. This effect of punicalagin was not dependent on concentration (Fig. 5B).

To further understand the mechanisms involved in the suppression of PGE_2 production by punicalagin, we investigated its effect on protein and mRNA expressions of mPGES-1. Significant reduction ($p < 0.05$) in mPGES-1 protein expression in LPS-activated microglia by punicalagin was observed at 20 and 40 μM, while mRNA expression of this enzyme was significantly reduced ($p < 0.05$) at 5, 10, 20 and 40 μM (Fig. 5A and B).

3.4 Punicalagin inhibits neuroinflammation by interfering with NF-κB signalling pathway in LPS-activated microglia

Considering the role of NF-κB in neuroinflammation, we aimed to elucidate the effect of punicalagin on the activity of this transcription factor. First, we employed a reporter gene assay to determine the effect of punicalagin on the ability of NF-κB to control inflammatory gene transcription. To achieve this, we transfected HEK293 cells with a plasmid construct carrying a luciferase reporter gene controlled by NF-κB. We observed that stimulation of transfected cells with TNF-α (1 ng/mL) resulted in activation of the NF-κB-driven luciferase expression (Fig. 6A). This phenomenon was unaffected by 5 μM of punicalagin. However, pre-treatment with punicalagin (10–40 μM) resulted in significant ($p < 0.001$) and concentration-dependent inhibition of NF-κB-driven luciferase expression, demonstrating that punicalagin suppresses NF-κB-dependent gene expression in general.

One of the initial responses of the microglia to LPS stimulation is phosphorylation and degradation of IκB. Therefore, in order to establish the specific effects of punicalagin on NF-κB-mediated inflammatory signalling pathway in the microglia, we first investigated its effect on IκB kinase (IKK) and IκB phosphorylation, following stimulation of rat primary microglia with LPS (10 ng/mL). Using Western blot, we observed that LPS treatment resulted in phosphorylation of IKK and IκB within 10 and 15 min, respectively. These

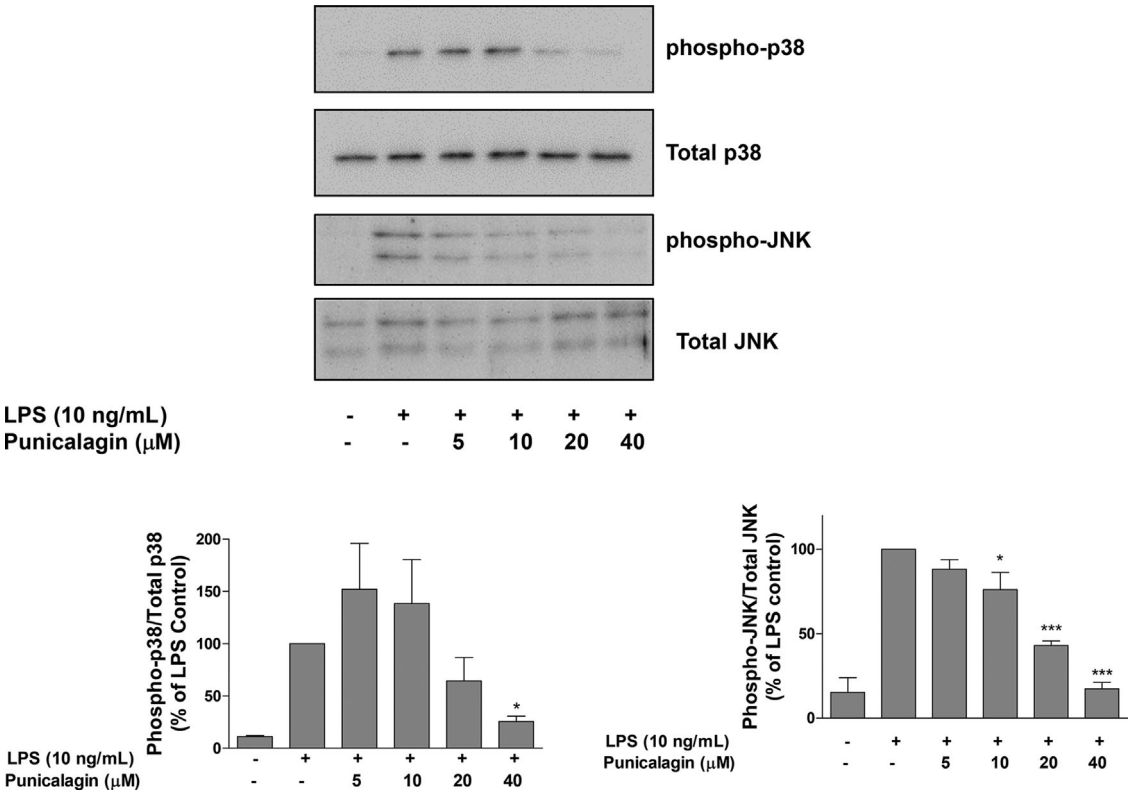

Figure 7. Punicalagin inhibited LPS-induced p38 and JNK MAPK phosphorylation in rat primary microglia. Cells were stimulated with LPS (10 ng/mL) in the presence or absence of punicalagin (5–40 μM) for 30 min. At the end of incubation period, phospho-p38 and phospho-JNK protein expressions were determined using Western blot with specific anti-phospho-p38 and anti-phospho-JNK antibodies. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test. $*p < 0.05$, $***p < 0.001$ in comparison with LPS control.

were significantly inhibited by pre-treatment with 10, 20 and 40 μM of punicalagin (Fig. 6B).

Once liberated from IκB, NF-κB is translocated into the nucleus in order to initiate inflammatory gene transcription. In order to confirm the activity observed on IκB phosphorylation, we next investigated the effect of punicalagin on p65 subunit nuclear translocation using Western blot. Figure 6B shows an increase in nuclear phospho-p65 following LPS stimulation, when compared with unstimulated cells. Nuclear translocation of p65 subunit was significantly inhibited by punicalagin (10–40 μM).

3.5 Inhibition of p38 and JNK phosphorylation contributes to the anti-inflammatory actions of punicalagin in LPS-activated microglia

Based on the reported roles of the mitogen-activated protein kinases (MAPKs) in neuroinflammation [11, 12], we sought to determine possible effects of punicalagin on the activities of p38, JNK and p42/44 MAPKs in LPS-activated rat primary microglia. Our results show that punicalagin produces modest effects on the MAPKs. Stimulation with LPS (10 ng/mL) resulted in marked phosphorylation of p38. This was significantly reduced ($p < 0.05$) by 40 μM of punicalagin, but not with the lower concentrations (Fig. 7). Interestingly, punicalagin (5–40 μM) produced a concentration-dependent and significant inhibition of JNK phosphorylation induced by LPS (10 ng/mL) stimulation (Fig. 7). Punicalagin did not have any effect on LPS-induced phosphorylation of p42/44 MAPK (data not shown).

3.6 Antineuroinflammatory effects of punicalagin are mediated through TRAF-6

LPS-induced Toll-like receptor 4 (TLR-4) dependent activation of TRAF-6 is one of the most important events upstream of the NF-κB inflammatory signalling pathway. Consequently, we investigated the effects of punicalagin on TRAF-6 protein expression in LPS-activated primary microglia. Figure 8 shows that punicalagin (10, 20 and 40 μM) inhibited TRAF-6 expression following LPS stimulation.

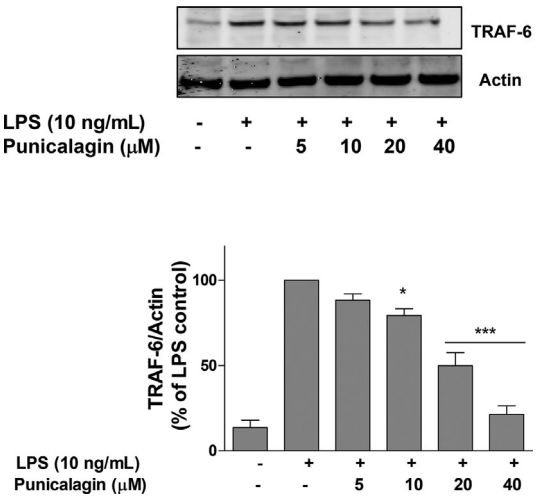

Figure 8. Punicalagin inhibited LPS-induced TRAF-6 expression in rat primary microglia. Cells were stimulated with LPS (10 ng/mL) in the presence or absence of punicalagin (5–40 μM) for 10 min. At the end of incubation period, TRAF-6 protein expression was determined using Western blot with specific anti-TRAF-6 antibody. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman–Keuls test. $*p < 0.05$, $***p < 0.001$ in comparison with LPS control.

4 Discussion

Accumulating evidence suggests that diets containing fruits, vegetables and natural products can play an important role in delaying the onset or slowing the progression of neurodegenerative diseases, such as AD [3]. Specifically, polyphenols have been shown to exhibit neuroprotective effects through their ability to suppress neuroinflammation and activate antioxidant mechanisms. In this study, we have demonstrated that a polyphenol found in pomegranate fruit, punicalagin, inhibits neuroinflammation in LPS-activated rat primary microglia by suppressing the production of pro-inflammatory cytokines (TNF-α and IL-6), and PGE_2 after 24 h of stimulation with LPS. This compound has also shown strong inhibition of TNF-α and IL-6 gene expressions in LPS-stimulated mouse hippocampal slices. These observations are consistent with results obtained by Xu et al. in RAW 264.7 macrophages [6]. They showed that punicalagin (25–100 μM) suppressed NO, PGE_2, TNF-α, IL-6 and IL-1β production from LPS-stimulated RAW 264.7 cells. Similarly, Winand and Schneider [13] reported inhibitory effects of punicalagin on TNF-α and NO production in LPS-stimulated RAW 264.7 cells, as well as IL-6 production in LPS-stimulated 3T3-L1 adipocytes.

The COX-2 enzyme pathway and subsequent generation of prostaglandins play a significant role in neuroinflammation. mPGES-1 is the terminal enzyme for the biosynthesis of PGE_2 during inflammation, and is functionally coupled with COX-2. This enzyme is markedly induced by pro-inflammatory stimuli and is down-regulated by anti-inflammatory glucocorticoids [14]. It has been shown that mPGES-1 inhibitors produced inhibition of PGE_2 production [15]. In order to gain a better understanding of the mechanisms of action of punicalagin in neuroinflammation, we investigated its effect on COX-2 and mPGES-1-mediated PGE_2 production. Our data show that punicalagin inhibited COX-2 and mPGES-1 after 24 h of LPS stimulation, suggesting that punicalagin acts to reduce PGE_2 production by interfering with both COX-2 and mPGES-1 enzymatic activities in LPS-activated microglia.

The transcription factor NF-κB plays a crucial role in neuroinflammation. In resting cells, NF-κB is sequestered in the cytoplasm by the inhibitory IκB protein. When activated by a variety of stimuli that includes LPS, IκB is phosphorylated by IKK. Phosphorylated IκB then undergoes ubiquitinisation and degradation [16]. Dissociation and degradation of IκB activates the translocation of NF-κB subunit from the cytosol to the nucleus. The translocated subunit thereafter facilitates the transcription of several pro-inflammatory genes, including those encoding pro-inflammatory cytokines, and COX-2. Furthermore, microglial NF-κB activation has been linked to brain damage through production of pro-inflammatory molecules [17–20], suggesting a role in neurodegenerative conditions. We therefore determined if punicalagin suppresses neuroinflammation through interference with this transcription factor. To investigate this, first, we employed a reporter gene assay to determine the effect of punicalagin on the ability of NF-κB to control inflammatory gene transcription. We observed that punicalagin significantly inhibited NF-κB-driven luciferase expression in HEK293 cells, suggesting that this compound is able to attenuate NF-κB-mediated gene expression in general. To confirm specific effects of punicalagin on NF-κB activity in the microglia, we used Western blot to investigate its effect on protein expression of phosphorylated IKKα, IκBα and nuclear phosphorylated p65. Results show that punicalagin significantly inhibited LPS-induced NF-κB signalling in microglia by suppressing the phosphorylation of IKKα, IκBα and nuclear p65 subunit within 10, 15 and 180 min of LPS stimulation. We therefore propose that punicalagin prevents neuroinflammation in LPS-activated microglia through NF-κB pathway.

Our studies have shown that punicalagin produced a modest suppressive action on the phosphorylation of p38 and JNK MAPKs following LPS activation. Interestingly, p42/44 MAPK phosphorylation was not affected by the compound. Studies have shown that treatment with LPS in primary astrocytes triggered the synthesis of inflammatory cytokines, through MAPKs signalling pathways [21]. Of particular interest is the role of p38, which has been shown to be acritical mediator of LPS-induced inflammation [22]. It appears that the effects of punicalagin on neuroinflammation are mediated mainly through targeting NF-κB signalling, while MAPK-mediated actions are minimal.

Studies have shown that the TLR-4-mediated TRAF-6/IKK/NF-κB pathway has been well established as a signalling pathway responsible for inflammatory responses

[23]. In addition to NF-κB activation, TLR-4 can also initiate MAPK signalling [24]. Based on the observation that punicalagin inhibited both NF-κB signalling and activation of p38 and JNK MAPKs in LPS-activated microglia, we attempted to establish a mechanism of these actions. Interestingly, punicalagin inhibited TRAF-6 protein expression, suggesting that this compound may inhibit the IKK/IκB/NF-κB signalling pathway, as well as p38 and JNK MAPK via selective inhibition of TRAF-6.

Taken together, this is the first time that punicalagin is shown to inhibit TRAF-6-mediated neuroinflammation. Future studies will focus on in vivo experiments to explore the therapeutic potentials of punicalagin in models of AD, Parkinson's disease and other neuroinflammation-related diseases.

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