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Original Article

The Anti-inflammatory Mechanism of Pu-erh Tea via Suppression the Activation of NF-κB/HIF-1α in LPS-stimulated RAW264.7 Cells

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Pu-erh tea, a popular and traditional Chinese tea, possesses various health-promoting effects, including inhibiting tumor cell progression and preventing type II diabetes and neurodegenerative disorders. However, the precise anti-inflammatory mechanisms are not well understood. In present study, we elucidated the anti-inflammatory mechanism of Pu-erh tea in lipopolysaccharide (LPS)-activated RAW264.7 cells. We explored the effects of Pu-erh tea on the levels of inflammatory-related genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and prostaglandin E_2 (PGE_2) in LPS-activated RAW264.7 cells. Moreover, we investigated its regulatory effects on nuclear factor-kappa B (NF)-κB and hypoxia-inducible-factor (HIF)-1α activation. The findings of this study demonstrated that Pu-erh tea inhibited the LPS-increased inflammatory cytokines and PGE_2 release, as well as COX-2 and iNOS expression. Moreover, we confirmed that the anti-inflammatory mechanism of Pu-erh tea occurs via the inhibition of NF-κB and HIF-1α activation. Conclusively, these findings provide experimental evidence that Pu-erh tea may be useful candidate in the treatment of inflammatory-related diseases.

Key Words Pu-erh tea, Inflammation, Nuclear factor-kappa B, Hypoxia-inducible-factor-1α

INTRODUCTION

Inflammation is a crucial pathological process that acts in response to tissue injury and biological stimuli (Duffield, 2003). Although the inflammatory reaction is considered a necessary response in the host defense process against pathogens, prolonged and excessive inflammatory response can lead to several chronic diseases. Activated macrophages play key roles in the inflammatory process, initiating and regulating immune responses by releasing variety of inflammatory mediators (McCormick et al., 2000; Liu et al., 2014). During an inflammatory response, macrophages release interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and other inflammatory mediators, leading to tissue damage. Additionally, IL-1β and TNF-α levels are increased in patients with inflammatory disorders, such as Crohn's disease and rheumatoid arthritis (Trefzer et al., 2003). Prostaglandin E_2 (PGE_2), induced by cyclooxygenase-2 (COX-2) at inflammatory sites, contributes to the swelling associated with inflammation and pain (Eisenach et al., 2010). Therefore, the down-regulation of inflammatory mediators is useful treatment strategies in inflammation-related disorders.

Nuclear factor-kappa B (NF-κB) conducts a key role in regulating inflammatory proteins during inflammatory conditions (Gadaleta et al., 2011). In response to inflammatory

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stimulants, such as infection, virus, and cytokine, the complex of IκB kinase is phosphorylated and the IκB is degraded. This in turn, activates NF-κB and translocate to the nucleus, leading to increased transcription of inflammatory mediators, including cytokines and COX-2 (Lee et al., 2020). Studies have shown that NF-κB activity is increased in patients with inflammatory diseases (Wong et al., 2001). Hypoxia-inducible-factor (HIF)-1α is a transcription factor that links inflammatory pathways (Giatromanolaki et al., 2003). In HIF-1α overexpressing mice, NF-κB activity and inflammatory genes expression were sequentially elevated. Clinical studies have demonstrated the overexpression of HIF-1α in inflammatory tissue from patients (Scortegagna et al., 2008). Therefore, modulating activation of NF-κB and HIF-1α may be helpful in development of a therapeutic strategy against inflammatory diseases.

Tea is one of the most popular beverages consumed worldwide. Pu-erh tea, a fermented Chinese tea, has a broad range of health-improving effects, including inhibiting tumor cell progression and preventing type II diabetes and neurodegenerative disorders (Du et al., 2012; Lin and Lin-Shiau, 2006). Although Pu-erh tea have long been used for effective diseases treatment, its accurate anti-inflammatory mechanisms are still not well elucidated. Therefore, we explored whether Pu-erh tea can attenuate the expression of inflammatory mediators, and the activation of HIF-1α and NF-κB in lipopolysaccharide (LPS)-induced RAW264.7 cells.

MATERIALS AND METHODS

Materials

LPS, avidin peroxidase (AP), Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazoliumbromide (MTT) and other reagent were supplied by Sigma (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) kits, Dulbecco's Modified Eagle's Medium (DMEM), bicinchoninic acid (BCA) and fetal bovine serum (FBS) were procured from Thermo Fisher Scientific Inc. (Rockford, IL, USA). The ELSIA assay kits for mouse IL-1β, IL-6 and TNF-α were procured from BD Biosciences (San Jose, CA, USA). Specific antibodies for NF-κB and HIF-1α were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of Pu-erh tea

The dried of Pu-erh tea (200 g) was pulverized into a powder and decocted in distilled water three times, and then concentrated and dried under vacuum rotary evaporator. The extract (yield, 13.8%) was filtered, freeze drying prior to being maintained at 4℃. The Pu-erh tea extract (PTE) were dissolved in PBS and filtered through a 0.22 μm syringe filter (GVS ABLUO, Fisher scientific, USA).

RAW264.7 Cell culture

Cells were maintained in DMEM containing 10% FBS supplemented with streptomycin (100 μg/mL) and penicillin (100 IU/mL) in 5% CO_2 atmosphere.

MTT assay

To evaluate the cytotoxicity by PTE, the MTT assay was conducted. Cells were treated with different concentrations of PTE (0.01, 01, and 1 mg/mL), and 50 μL of MTT solution was added. Then, the crystallized formazan was dissolved and the absorbance of plate was detected at 540 nm using a microplate reader (Molecular Devices, USA).

Cytokine assay

The content of IL-1β, IL-6 and TNF-α derived from cells were assayed by using a modification of an ELISA. Briefly, plates (96-well) were coated with IL-1β, IL-6 and TNF-α monoclonal Abs and then incubated overnight at 4℃. After washes, standard solution of IL-1β, IL-6 and TNF-α and sample were incubated. After washing with PBST, the plate was exposed to biotinylated IL-1β, IL-6 and TNF-α Abs followed by incubation for 2 h. After washing, AP and ABTS was sequentially exposed. The optical density was measured by a microplate reader at 405 nm.

PGE_2 assay

Cells (3×10^5 cells/well) were treated h with various dose of PTE (0.01, 0.1, and 1 mg/mL) and incubated with LPS (1 μg/mL) for 24 h. The PGE_2 content secreted from cell was measured using PGE_2 colorimetric assay kits. Briefly, the cell culture supernatant or PGE_2 standards were added to 96-well coated plates for 2 h. After the wells were washed,

50 μL of PGE_2 conjugate Ab were added. Washing with washing buffer and incubated with 100 μL of substrate solution for 30 minutes and treated with 50 μL of stop solution. Color development was measured at 405 nm using a microplate ELISA reader.

Measurement of nitric oxide (NO) concentration

Cells (3×10^5 cells/well) were treated with different concentrations of PTE for 1 h, and then incubated with LPS (1 μg/mL) for 24 h. Content of NO in cell culture supernatant was determined using Griess reagent. The concentration of NO was measured by quantitation of nitrite levels. The optical density was determined at 540 nm.

Isolation of nuclear fractions and western blot analysis

Cells (5×10^6 cells/mL) were seeded and treated with various concentrations of PTE for 1 h and LPS treated for 2 h. The cells were washed with PBS, collected, and isolated with the NE-PER Nuclear Extraction Reagent following the manufacturer's instruction. After protein quantification using BCA kit, the lysed protein was separated using gel electrophoresis, and electroblotted to membrane in transfer buffer. The membrane was then blocked with non-fat milk (5%), washed and blotted with primary Abs. After washing, membrane was reacted with horseradish-peroxidase-conjugated secondary Abs. After washing with PBST, the protein bands were detected by an ECL detection system.

Luciferase reporter gene assay

Cells were seeded and transfected with NF-κB-luc DNA and the medium was refreshed. The transfected cells were treated with different concentrations of PTE and treated with LPS for 2 h. Luciferase activity was evaluated using a Dual-Glo detection system following the manufacturer's protocols (Promega, WI, USA).

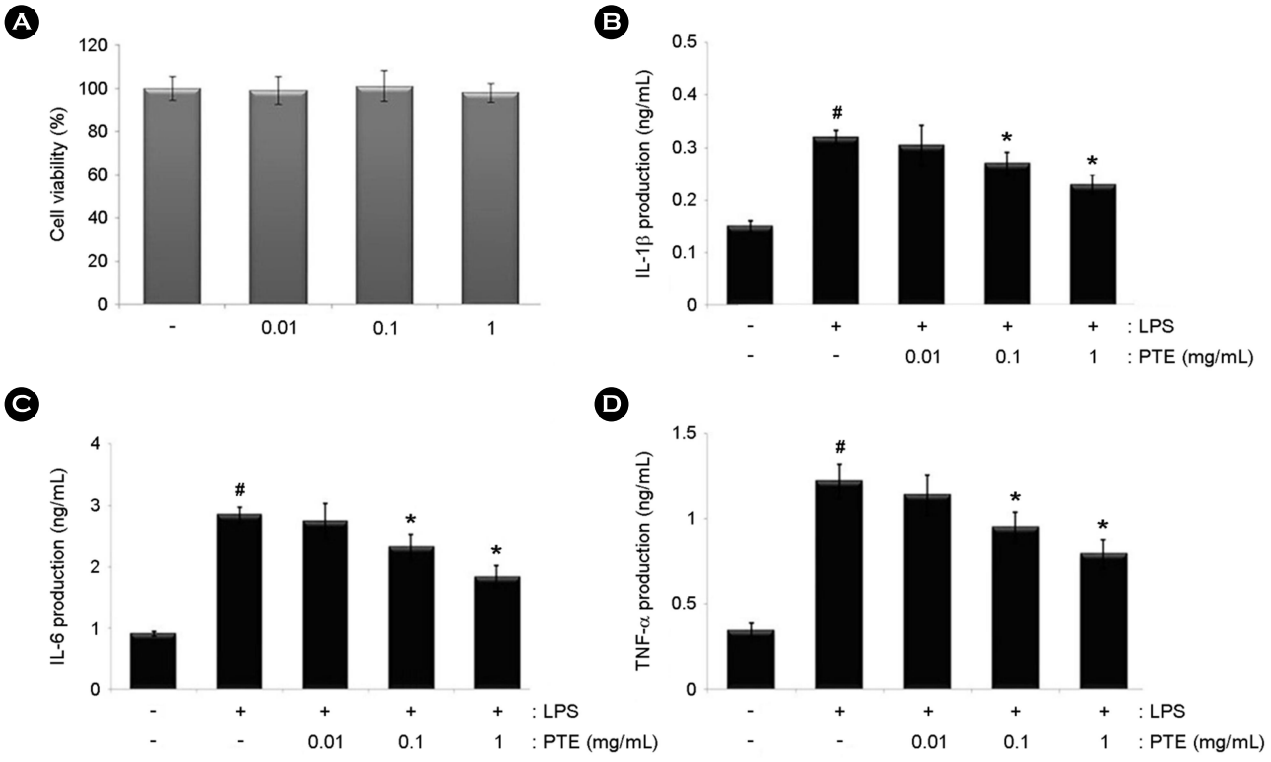

Fig. 1. Effects of PTE on cytotoxicity and secretion of inflammatory cytokines in activated macrophages. Cells (3×10^5 cells/mL) were treated for 1 h with various dose of PTE (0.01, 0.1, and 1 mg/mL), and incubated with LPS (1 μg/mL) for 24 h. (A) Cell viability was assessed using MTT assay. (B, C, and D) The levels of IL-1β, IL-6 and TNF-α in cell culture supernatant were analyzed using ELISA. Data are shown as mean ± S.D. of independent experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

Statistical analysis

Data were expressed as mean ± standard deviation (S.D). The statistical analyses were conducted using an independent *t*-test and ANOVA analysis. $P < 0.05$ was considered significant for the differences.

RESULTS

Regulatory effects of PTE on cytotoxicity and secretion of inflammatory cytokines in LPS-activated RAW264.7 cells

To identify the cytotoxicity of PTE with different concentrations of PTE (0.01, 0.1 and 1 mg/mL) using the MTT assay. As shown in Fig. 1A, the result revealed that PTE did not display cytotoxicity against macrophage cells. Next, we investigated the effects of PTE on IL-1β, IL-6 and TNF-α secretion from LPS-activated RAW264.7 cells. The cells were incubated with PTE (0.01, 0.1, and 1 mg/mL) prior to LPS treatment. As shown in Fig. 1B, C and D, LPS alone enhanced IL-1β, IL-6 and TNF-α secretion compared to that in the control. Whereas PTE markedly suppressed LPS-induced IL-1β, IL-6 and TNF-α release in a concentration-dependent manner. The inhibition rates of IL-1β, IL-6 and TNF-α secretion by PTE (1 mg/mL) were approximately 29.1% ($P < 0.05$), 35.2 ($P < 0.05$) and 33.8% ($P < 0.05$), respectively.

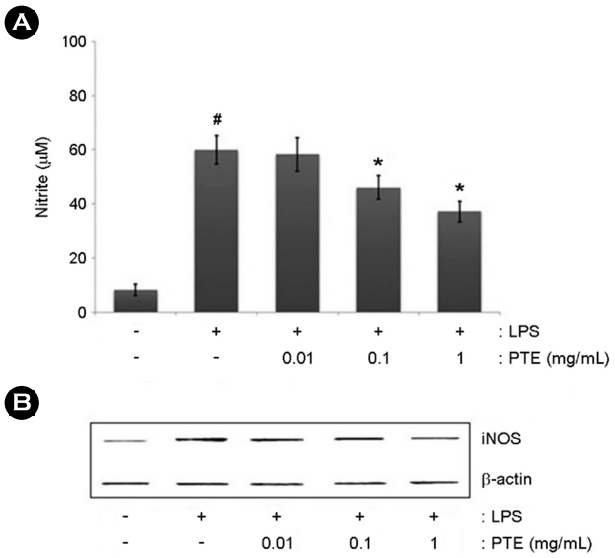

Fig. 2. Effects of PTE on NO and iNOS levels in activated macrophages. Cells were treated with PTE (0.01, 0.1, and 1 mg /mL) followed by LPS for 24 h. (A) NO production in cell culture supernatant was determined using the Griess kit. (B) The protein extract was assayed via western blot analysis for iNOS. Data are shown as mean ± S.D. of independent experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

Regulatory effects of PTE on the NO and iNOS levels in LPS- activated RAW264.7 cells

Because an excessive NO is associated with inflammatory processes (Kim et al., 2008), we determine the inhibitory effects of PTE on NO release. As shown in Fig. 2A, PTE induced a decrease in NO release in a concentration-dependent manner, with inhibition rates of NO levels by PTE (1 mg/mL) being determined as 37.7% ($P < 0.05$). Additionally, western blotting was conducted to determine the attenuative effect of PTE on the iNOS expression enhanced by LPS. The results showed that LPS enhanced an iNOS expression, whereas PTE significantly attenuated the iNOS expression in activated macrophages (Fig. 2B).

Regulatory effects of PTE on COX-2 expression and PGE_2 production in LPS-activated RAW264.7 cells

Enhanced COX-2 levels are associated with inflammatory processes (Ahn et al., 2005). Western blotting was performed to evaluate the effects of PTE on COX-2 expression induced by LPS. The cells were incubated with different concentrations of PTE (0.01, 0.1 and 1 mg/mL) and treated with LPS. We found that LPS markedly induced the expression of COX-2, however PTE attenuated increased COX-2 levels in a concentration-dependent manner (Fig. 3A).

COX-2 induces the PGE_2 biosynthesis, which contributes to swelling and pain associated with inflammation. Thus, we evaluated the inhibitory effect of PTE on PGE_2 production in LPS-activated RAW264.7 cells. Our results showed that PGE_2 levels were markedly up-regulated in response to LPS, whereas, this increase was significantly alleviated by PTE in a concentration-dependent manner (Fig. 3B). The inhibition rates of PGE_2 secretion by PTE (1 mg/mL) was approximately 39.7% ($P < 0.05$).

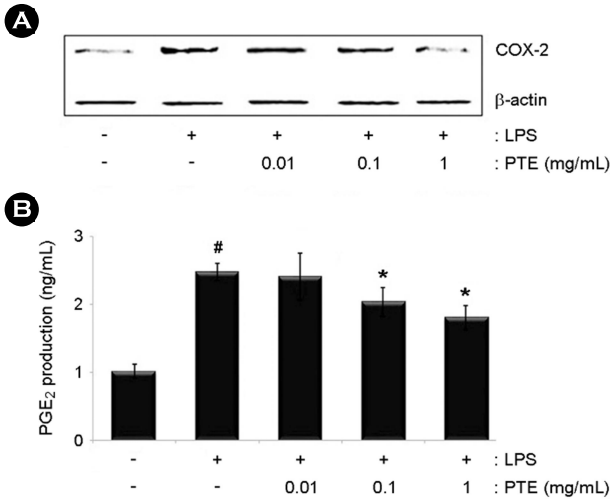

Fig. 3. Effects of PTE on COX-2 and PGE_2 levels in activated macrophages. (A) Cells (1×10^6 cells/mL) were treated with various dose of PTE (0.01, 0.1, and 1 mg/mL) and stimulated with LPS for 24 h. The protein extracts were tested via western blotting for COX-2. (B) Cells were treated h with various dose of PTE (0.01, 0.1, and 1 mg/mL) and incubated for 24 h with LPS (1 μg/mL). The levels of PGE_2 secretion in cell culture supernatant was evaluated with colorimetric assay kit. Data are shown as mean ± S.D. of independent experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

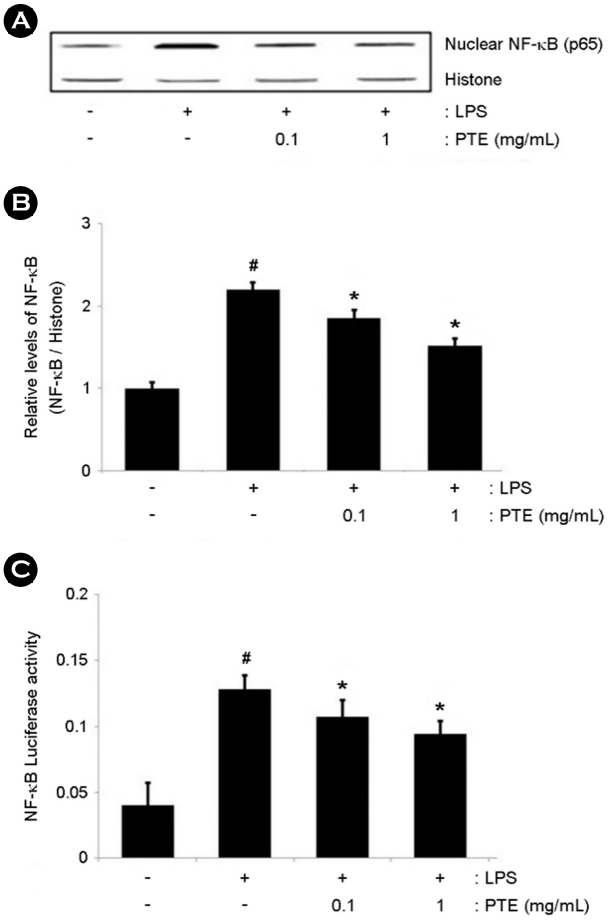

Fig. 4. Effect of PTE on NF-κB activation in activated macrophages. (A) Cells (3×10^6 cells/mL) were treated with different dose of PTE (0.1, and 1 mg/mL) and incubated with LPS for 2 h. Nuclear extracts were isolated by Nuclear Extraction kit and detected for NF-κB via western blot analysis. (B) NF-κB relative levels are shown. (C) The activity was detected by a luciferase detection system following the manufacturer's instruction. Data are shown as mean ± S.D. of independent experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

Regulatory effects of PTE on NF-κB activation in LPS-activated RA W264.7 cells

Because alleviation of NF-κB activity was considered as an anti-inflammatory target (Lappas et al., 2002), we hypothesis that the anti-inflammatory mechanism of PTE may be attributed to the blocking of NF-κB activation. Given that NF-κB activation requires the translocation of NF-κB into the nucleus, we examined the effects of PTE on the nuclear of NF-κB by western blot analysis. As shown in Fig. 4A, the expression levels of NF-κB in the nuclear fraction strongly enhanced in LPS-treated cells, but PTE decreased the elevated nuclear levels of NF-κB. The relative level of NF-κB is shown in Fig. 4B. Additionally, to examine the regulatory effect of PTE on NF-κB promoter activity, a luciferase activity assay was conducted. Our results confirmed that PTE attenuated NF-κB- luciferase activity enhanced by in LPS-stumualted RAW264.7 cells (Fig. 4C).

Effect of PTE on HIF-1α activation in LPS- stimulated RAW264.7 cells

HIF-1α excessive is related with inflammatory reaction by causing an enhanced in inflammatory mediators (Braverman et al., 2016). Therefore, to further examine the anti-inflammatory mechanism of PTE on inflammatory progress, we determined whether PTE suppress the HIF-1α activation in LPS-activated cells. We observed that that LPS enhanced the HIF-1α expression, however, the increase in HIF-1α activation was down-regulated by PTE (Fig. 5A). The HIF-1α relative level is represented (Fig. 5B).

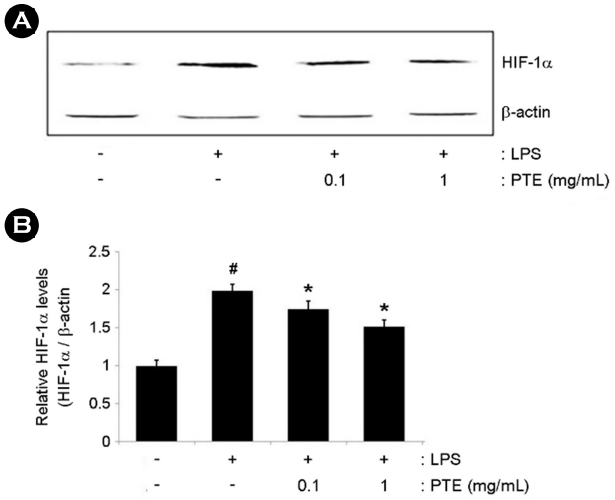

Fig. 5. Effect of PTE on HIF-1 α activation in activated macrophages. (A) Cells (3×10^6 cells/mL) were treated with different dose of PTE (0.1, and 1 mg/mL) and treated with LPS. The protein extract was tested via western blot analysis for HIF-1α. (B) HIF-1α relative levels are shown. Data are shown as mean ± S.D. of independent experiments ($^{\#}P < 0.05$ vs. control, $^{*}P < 0.05$ vs. LPS alone).

DISCUSSION

Tea is consumed worldwide and clinical studies have shown its health benefits. Pu-erh tea, a popular tea, has many health-improving properties including anti-oxidant, anti-obesity and anti-cancer properties. Although Pu-erh tea has long been used for the beneficial effective of diseases treatment, its accurate anti-inflammatory mechanisms are still not well examined. Our findings demonstrated that PTE attenuated the enhanced levels of TNF-α, IL-1β, IL-6, NO and PGE_2 secretion as well as the COX-2 and iNOS expression. Moreover, we indicated that the anti-inflammatory mechanism of PTE is contributed via suppressing the activation of NF-κB and HIF-1α in activated macrophages. This suggests that a mechanism by which PTE alleviate the inflammatory reaction.

Inflammation is biological response of the immune system that may be induced by a several of factors such as injury, infectious agent, and toxic compounds (Li et al., 2019). Macrophages actively contribute to inflammatory progress by increasing inflammatory mediators (Gordon and Taylor, 2005). Inflammatory cytokines contribute to the progression of the distributive inflammatory process (Kim et al., 2008). Excessive cytokines induce immune cell proliferation, pain and fever, resulting in inflammatory diseases. It has also been reported that IL-1β, IL-6 and TNF-α production is enhanced in patients of inflammatory diseases (Park et al., 2009). Thus, we explore whether the anti-inflammatory effects of PTE were mediated through the suppression of inflammatory cytokines. Our results showed that PTE significantly attenuated LPS-induced IL-1β, IL-6 and TNF-α production in LPS-stimulated cells. The inhibition rates of IL-1β, IL-6 and TNF-α by PTE (1 mg/mL were approximately 29.1% ($P < 0.05$), 35.2 ($P < 0.05$) and 33.8% ($P < 0.05$). By virtue of its ability to suppress the increased inflammatory cytokines, PET inhibited the immune hyperactivity and ensuing inflammatory response.

PGE_2, generated by COX-2 at inflammatory sites, contributes to the pain associated with inflammation. Additionally, excessive NO is associated with inflammatory processes. Studies indicated that blocking of inflammatory mediators is a beneficial strategy for treatment of inflammatory disorder. Subsequently, we observed that PTE suppressed the LPS-induced inflammatory mediators including COX-2, PGE_2 and NO. Based on these finding, we hypothesis that the anti-inflammatory effect of PTE may be involved in the attenuation of inflammation-related genes expression.

Accumulating evidence revealed that the NF-κB and HIF-1α (transcription factors) play a crucial role by inhibition the inflammatory gene transcription (Lee et al., 2020; Singh et al., 2012). It has been reported that the attenuation of NF-κB activation decreased an influx of inflammatory cells (Kelleher et al., 2007). Furthermore, HIF-1α links inflammatory pathways (Giatromanolaki et al., 2003). It has also been was been reported that HIF-1α overexpressing mice have an increase NF-κB activity and inflammatory genes expression. Clinical studies have revealed the overexpression of HIF-1α in inflammation tissue from patients (Scortegagna et al., 2008). Activation of HIF-1α promotes translocation of NF-κB subunits to the nucleus, resulting in elevation of the inflammatory cytokine expression in LPS-stimulated macrophages (Görlach and Bonello, 2008). Ginsenoside Rg3 was reported as having an anti-inflammatory effect by down-regulating the HIF-1α activation in activated-mast cells (Han et al., 2021). Porana sinensis Hemsl, traditional

Chinese medicine, inhibited the release of inflammatory cytokines and HIF-1α activation in LPS-induced RAW264.7 cell (Hu et al., 2022). Therefore, modulating NF-κB and HIF-1α activation may be helpful in developing a therapeutic strategy against inflammatory diseases. Our results herein indicated that PTE reduced NF-κB translocation into the nucleus as well as HIF-1α activation. We hypothesized that anti-inflammatory mechanism of PTE might derive from the suppression of NF-κB and HIF-1α activity in activated macrophages.

In conclusion, this study indicated that the anti-inflammatory activities of PTE may be attributed to the suppression of the production of TNF-α, IL-1β, IL-6, NO and PGE_2 as well as the expression of COX-2 and iNOS. Additionally, we found that the anti-inflammatory mechanism of PTE is caused by the alleviation of NF-κB and HIF-1α activation in activated macrophages. Collectively, our results conclusively suggested that PTE may be a promising agent for anti-inflammatory therapy.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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