




Article

Involvement of Antioxidant Defenses and NF- κ B/ERK Signaling in Anti-Inflammatory Effects of Pterostilbene, a Natural Analogue of Resveratrol

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Abstract: Pterostilbene (PTE), a natural stilbenoid occurring in grapes and berries, is recognized as a dimethylated analogue of resveratrol. This compound shows numerous notable pharmacological activities, including antiaging, anticancer, antidiabetes, antioxidant, and neuroprotection. This study investigates the anti-inflammatory properties of PTE in macrophage cells (RAW 264.7) against the lipoteichoic acid (LTA) stimulation. The expression of inflammatory tumor necrosis factor (TNF- α), interleukin-1 β (IL-1 β), and inducible nitric oxide synthase (iNOS) and the content of nitric oxide (NO) were detected in LTA-induced cells. In addition, a Western blot assay was used to detect mitogen-activated protein kinases: extracellular signal-regulated kinase (ERK)1/2, p38 MAPK, and c-Jun N-terminal kinase (JNK). The phosphorylation of I κ B and p65 and translocation of nuclear factor kappa B (NF- κ B) were assessed by Western blot and immuno-fluorescence staining. The results showed that PTE significantly attenuated NO production and TNF- α , IL-1 β , and iNOS expression in LTA stimulated cells. Among the activation of ERK, JNK, and p38 in cells treated with LTA, PTE at higher concentration had only inhibited ERK activation. However, PTE blocked I κ B phosphorylation, phosphorylation and nuclear translocation of p65NF- κ B. Fascinatingly, PTE enhanced antioxidant defense molecules as verified by the enhanced heme oxygenase-1 (HO-1) expression, catalase (CAT) antioxidant enzyme, and non-enzymatic antioxidant, and reduced glutathione (GSH) in LTA-induced RAW 264.7 cells. These results suggest that PTE exerts an anti-inflammatory property via attenuating NF- κ B/ERK signaling pathways as well as enriching antioxidant defense mechanisms.

Keywords: pterostilbene; cytokines; MAPK/NF- κ B; catalase; HO-1; reduced glutathione

1. Introduction

Inflammation is a major influence on the host defense against pathogenic encounters and the repair of normal tissue construction [1]. The main pro-inflammatory cells, macrophages, protect the body from external invaders by producing pro-inflammatory mediators and cytokines such as nitric oxide (NO), inducible nitric oxide synthase (iNOS),

tumor necrosis factor- α (TNF- α), and IL-1 β [2]. These mediators and cytokines induce cell and tissue damage, which are involved in chronic inflammatory diseases such as hepatitis, rheumatoid arthritis, and atherosclerosis [3,4]. Lipoteichoic acid (LTA), an outer membrane component of Gram-positive bacteria, is one of the critical players in the pathogenesis of sepsis [5]. LTA stimulates inflammatory reactions in the lung [6]. Thus, investigating the mechanisms that control LTA-mediated cell activation is crucial for diagnosis and treatment of lung inflammatory diseases. LTA induces the discharge of various cytokines such as IL-1 β , IL-6, and TNF- α [7]. A previous study showed that LTA induced TNF- α and IL-6 expressions via stimulating phosphorylation of ERK1/2 in macrophages; it also triggered translocation of nuclear factor (NF)- κ B from the cytoplasm to nuclei and its transactivation activity [8].

Heme oxygenase-1 (HO-1) is an inducible rate-limiting enzyme catalyzing cellular free heme to free iron, carbon monoxide (CO), and biliverdin/bilirubin in mammalian cells. The anti-inflammatory role of HO-1 is facilitated via the suppression of several pro-inflammatory mediators and cytokines [9]. Several studies established that HO-1 and its products, such as CO, inhibit the protein expression of iNOS, reducing NO production [10]. The expression of HO-1 is associated with several signaling pathways, such as mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor-E2-related factor 2 (Nrf2) [11]. MAPKs contribute essentially in immune defense, stress response, cellular proliferation, and apoptosis [12]. MAPK's activation controls the expression of a number of genes including HO-1 [13]. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and HO-1 play a key role in the defense against oxidative stress. Therefore, studies have proposed that antioxidant treatment may have defensive effects against oxidative damage and/or inflammatory diseases via stimulating the cellular antioxidant defense system.

In recent decades, the consumption of natural products for the treatment of inflammatory diseases is increasing as a novel approach in traditional medicine. Pterostilbene (PTE), a natural component of blueberry and an analog of resveratrol, has found higher bioavailability and lipophilic nature [14]. Remsberg et al. [15] studied the suppressive effects of PTE on inflammation, oxidative stress, and apoptosis in mammalian cells. Under hyperosmotic stress, PTE reduced the reactive oxygen species (ROS) production and expression of inflammatory mediators, including IL-1 β , IL-6, and TNF- α [16]. Oxidative stress induced by low-density lipoprotein in vascular endothelial cells was also found to be inhibited by PTE [17]. In addition, Chiou et al. [18] and Bhakkiyalakshmi et al. [19] reported the anti-hyperlipidemic and carcinogenic properties of PTE. A recent study established that a low dose of PTE (10 μ M) has excellent antitumor effect, suggesting safe and active therapeutic application of PTE [20]. Another recent animal study found PTE is an effective anti-inflammatory and antioxidant to prevent the damage of mycotoxins [21]. These findings motivated us to assess if PTE protects cells against LTA-induced inflammatory damage using RAW 264.7 macrophages as a model system. In addition, we also investigated if this anti-inflammatory effect is associated with NF- κ B/MAPK, HO-1 and antioxidant enzyme activity.

2. Materials and Methods

2.1. Materials

Pterostilbene (PTE, >98%, Figure 1A) was purchased from ChemFaces Biochem, Wuhan, Hubei, China. Potassium ferricyanide, ferric chloride, and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Anti-iNOS polyclonal antibody (pAb) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The antibodies against TNF- α , phospho-p38 MAPK Thr180/Tyr182, phospho-c-JNK (Thr183/Tyr185), phospho-p44/p42 ERK (Thr202/Tyr204), phospho-I κ B α Ser32/36, phospho-NF- κ B p65 (Ser536) pAbs were all purchased from Cell Signaling (Beverly, MA, USA). Anti-IL-1 β pAb was purchased from BioVision (Milpitas, CA, USA). Anti-HO-1 pAb was purchased from Enzo (Farmingdale, New York, NY, USA). NeoMarkers, Fremont, CA, USA, supplied

antibody against α -tubulin. PTE was dissolved in 0.1% DMSO. Sigma (St. Louis, MO, USA) supplied all other chemicals and reagents used in this study.

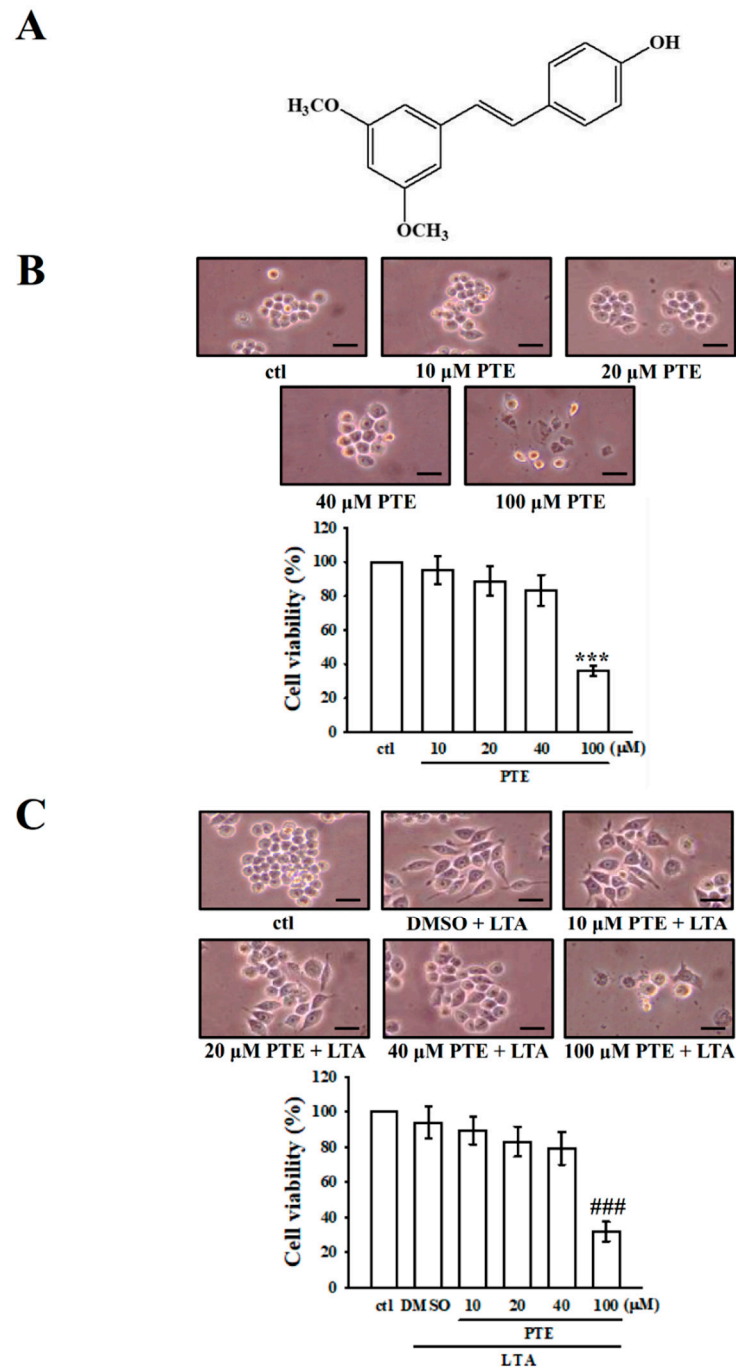


Figure 1. (A) The chemical structure of pterostilbene (PTE). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay and morphology of (B) PTE (10–100 μM) alone and (C) PTE pretreated lipoteichoic acid (LTA)-induced RAW 264.7 cells as described in the method section. Scale bar = 25 μm . Data offered are the means \pm standard error (S.E.M.) ($n = 4$); *** $p < 0.001$ vs. control cells; ### $p < 0.001$ vs. LTA induced cells.

2.2. MTT Assay and Morphology of RAW Cells

RAW 264.7 cells obtained from American Type Culture Collection (ATCC, Manassas, VA, U.S., TIB-71) were cultivated in DMEM medium at 37 $^{\circ}\text{C}$ under 5% CO_2 and 95% air. Cells at a density of 1×10^5 cells/well were pretreated with PTE (10–100 μM) for

30 min, followed by stimulation with LTA (5 µg/mL) for 24 h. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done by adding a 5 mg/mL of MTT working solution; culture medium was collected after 4 h incubation with this solution. A 300 µL of DMSO was added to dissolve the formed crystals. The formula of absorbance of treated-cells/absorbance of control cells × 100% was used to measure the cell viability index.

2.3. Quantification of NO Production

PTE at 20–40 µM was added to cells with or without LTA (5 µg/mL) for 24 h to measure the content of NO. Concisely, a 100-µL culture suspension was mixed with the same volume of 100 µL Griess reagent and incubated for 10 min. The optical density was measured at 550 nm by using a MRX absorbance reader. Sodium nitrite was used to derive reference standard curve to measure the NO production.

2.4. Western Blotting Study

Total protein was extracted from 6×10^5 cells. Equal amounts (50 µg) of the extracted proteins were run on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk for 40 min, the membrane was probed with various primary antibodies of target proteins for 2 h and subsequently incubated with HRP-conjugated donkey antirabbit IgG or sheep antimouse IgG for 1 h at room temperature. The intensity of protein bands was quantified by using the Biolight Windows Application, V2000.01 (Bio-Profil, Vilber Lourmat, France).

2.5. Confocal Microscopy Assay

Cells (5×10^4 per well) cultivated on cover slips were pretreated with PTE (40 µM) for 30 min, stimulated by LTA (5 µg/mL) for 1 h and subsequently fixed with 4% paraformaldehyde (10 min, 37 °C). Coverslips were double-washed by PBS, incubated with 0.1% Triton X-100 for 10 min, and then blocked with 5% BSA for 1 h. Further, coverslips were consecutively incubated with primary p65 antibody (4 °C, overnight) and secondary goat antirabbit IgG antibody (1 h, room temperature). Nuclei were stained with 4,6-diamidino-2 phenylindole (DAPI). A Leica TCS SP5 confocal spectral microscope imaging system (Mannheim, Germany) captured the images of nuclear translocation of p65.

2.6. Native Polyacrylamide Gel Electrophoresis (NATIVE-PAGE)

Native PAGE was performed to detect the antioxidant enzyme activity of CAT according to the method described by Woodbury et al. [22]. To this, the buffers and samples did not heat and avoid adding the SDS. The PAGE was run based on the equal amounts of 50 µg protein in an 8% gel.

2.7. Statistical Analyses

The data were expressed as mean ± standard error (S.E.M). One-way analysis of variance (ANOVA) was applied to determine the statistical difference among the groups. Statistical differences were observed as significant if the *p* value of the Student–Newman–Keuls test was *p* < 0.05.

3. Results

3.1. PTE on Viability and Morphology in RAW 264.7 Cells

Cell morphology and viability were examined to assess the toxicity of PTE in LPS-induced RAW 264.7 cells. Administration of RAW cells to 10, 20, and 40 µM PTE for 24 h did not affect cell viability (Figure 1B). When exposed to 100 µM, the viability of RAW cells was significantly affected. Similarly, exposure of cells to PTE (10–40 µM) for 30 min and then stimulated with LTA (5 µg/mL) for 24 h did not alter viability as well as cell morphology (Figure 1B,C). Therefore, the suitable concentrations of 20 and 40 µM PTE were used for the consequent analysis to omit the probability that the suppressive

result of PTE on LTA-induced inflammation was an effect of cytotoxicity triggered by cell viability reduction.

3.2. PTE Inhibited LTA-Induced NO Production by Tempering iNOS in RAW Cells

Nitric oxide, a pro-inflammatory mediator, can induce systemic inflammatory events [23]. The effect of PTE on NO production in LPS-stimulated RAW264.7 macrophages was determined in the culture medium using the Griess reaction. Figure 2A shows that PTE treatment significantly suppressed LTA-induced production of NO. Production of NO synthesis can be regulated by a rate-limiting enzyme inducible nitric oxide synthase (iNOS) [23]. The expression of iNOS was detected to examine if PTE inhibits NO production via the modulation of iNOS expression. As shown in Figure 2B,C, PTE inhibits LTA-induced protein expression of iNOS in RAW264.7 cells, inferring that PTE inhibited LTA-induced NO production through inhibiting the activity of iNOS.

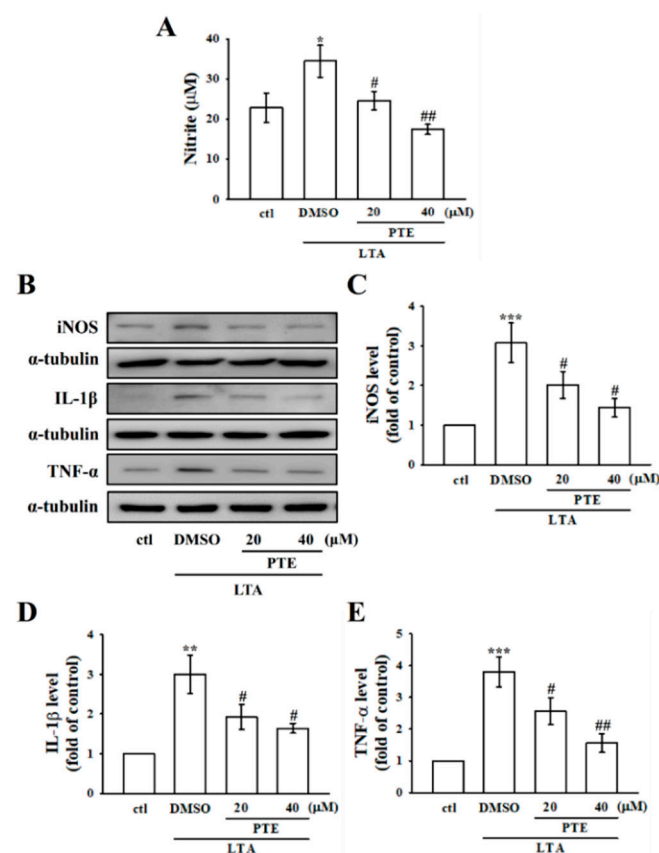


Figure 2. Effects of PTE on inflammatory mediators and cytokines (A–E). Cells were treated with 0.1% dimethyl sulfoxide (DMSO) or PTE (20 and 40 µM) for 30 min, and then stimulated by LTA (5 µg/mL) for 24 h. PTE reduces NO production (A), and protein expression of iNOS (C), IL-1β (D), and TNF-α (E) in LTA-induced RAW cells. Data expressed are the means ± S.E.M. ($n = 4$); * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control cells; # $p < 0.05$, and ## $p < 0.01$ vs. PTE pretreated cells.

3.3. PTE Inhibited LTA-Induced Inflammatory IL-1β and TNF-α Expression

As numerous inflammatory events are controlling by proinflammatory cytokines and they play a vital role in directing immune responses, the protein expression of pro-inflammatory cytokines including IL-1β and TNF-α was detected to govern whether PTE controlled their expression. As shown in Figure 2B,D,E, PTE inhibited LTA-induced IL-1β and TNF-α protein expression in RAW 264.7 macrophages.

3.4. PTE Inhibits ERK, but Not JNK and p38 MAPK Phosphorylation

Considering the fact that mitogen-activated protein kinases (MAPKs), including JNK, ERK, and p38, involve in inflammation-related signaling pathways, we studied the influence of PTE on the LTA-induced MAPK pathway. Figure 3 shows the induced expression of phosphorylated ERK1/2, JNK1/2 and p38 MAPK in RAW cells after exposure to LTA. Moreover, PTE had an inhibitory effect only on ERK activation when cells were pretreated at a higher concentration of PTE (40 μ M), but not on JNK and p38. These results indicated that PTE exposes its anti-inflammatory effects in LTA-induced RAW 264.7 cells via ERK signaling pathways.

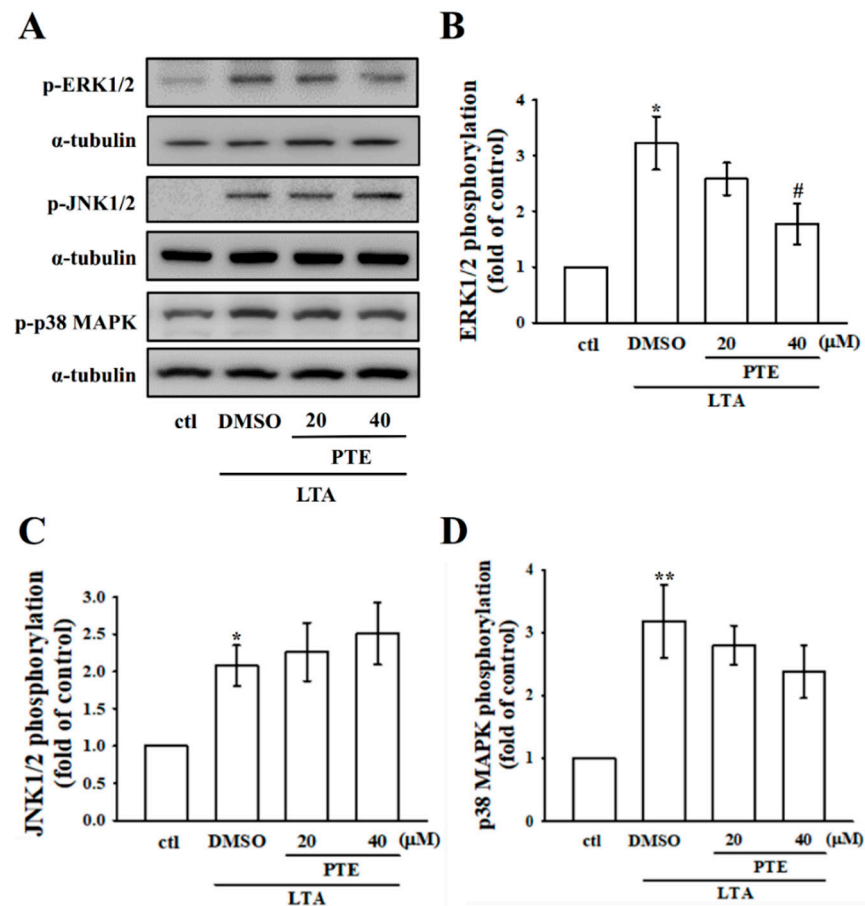


Figure 3. Effects of PTE on LTA-induced mitogen-activated protein kinases (MAPKs) phosphorylation in RAW cells (A–D). Cells were treated with 0.1% DMSO or PTE (20 and 40 μ M) for 30 min, followed by LTA (5 μ g/mL) for 1 h. PTE diminished the LTA-induced phosphorylation of ERK (B), but it did not have an effect on JNK (C), and p38 MAPK (D) phosphorylation. Data expressed are the means \pm S.E.M. ($n = 4$); * $p < 0.05$, and ** $p < 0.01$ vs. control cells; # $p < 0.05$ vs. PTE pretreated cells.

3.5. PTE Inhibited LTA-Induced NF- κ B Signaling Pathway

Nuclear factor-kappa B (NF- κ B) plays a vital role in controlling pro-inflammatory cytokines; it translocated to the nucleus upon activation to bind target DNA, which in turn controlled the expression of several inflammatory cytokines [24]. Consequently, we studied if PTE has an inhibitory effect on NF- κ B signaling pathways via phosphorylation of I κ B α and phosphorylation and nuclear translocation of p65 in LTA-induced macrophages by Western blotting and confocal assay, respectively. Figure 4 showed that PTE significantly diminished LTA-induced I κ B α (Figure 4A) and p65 (Figure 4B) phosphorylation, and inhibited nuclear translocation of p65 (Figure 4C), which represents that PTE inhibited LTA-induced inflammatory actions by hindering the NF- κ B signaling pathway.

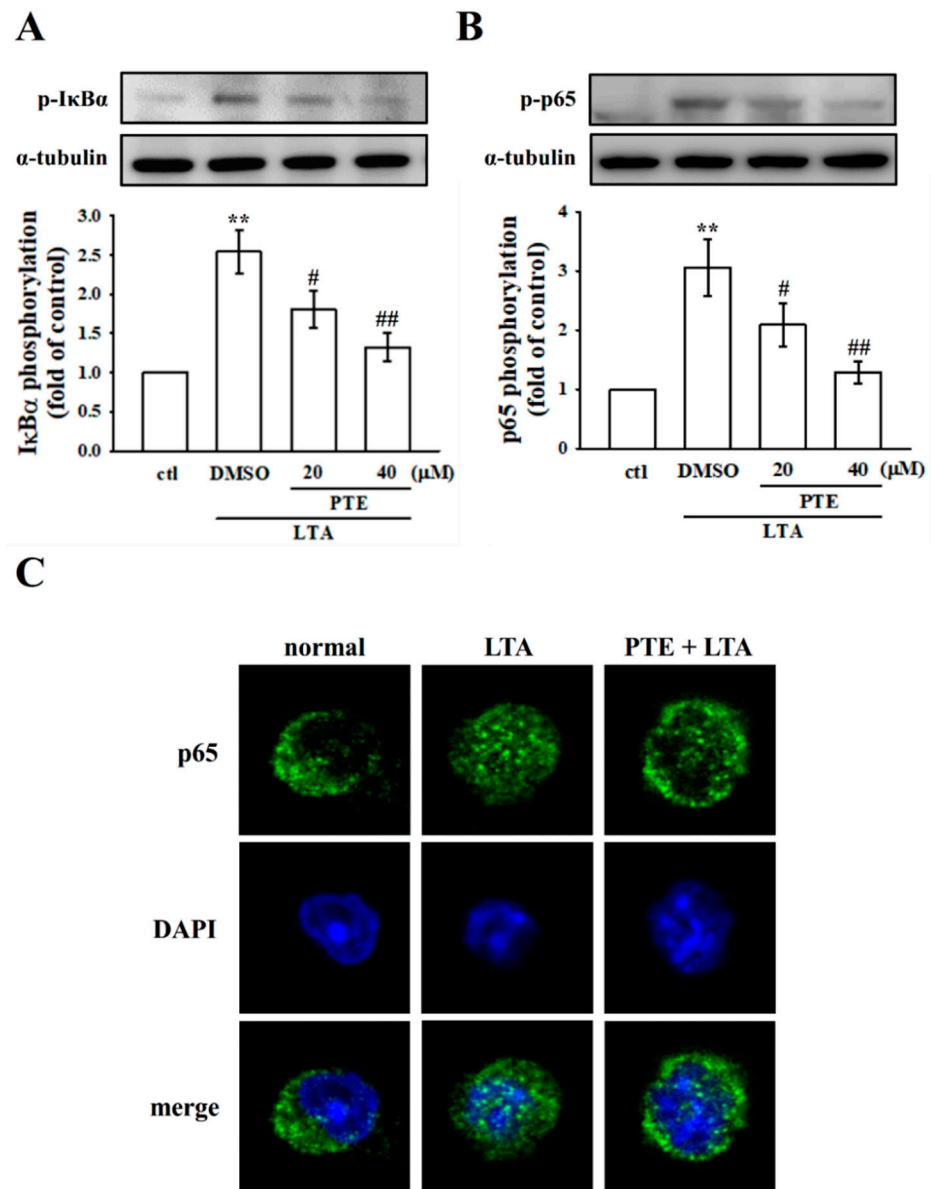


Figure 4. PTE regulates NF- κ B signaling pathway induced by LTA in RAW cells. Cells were treated with PTE (20 and 40 μ M) and LTA (5 μ g/mL) for 1 h. Western blotting assay was done to detect the phosphorylation of (A) I κ B α and (B) p65 in LTA-induced RAW cells. (C) PTE inhibited LTA-induced p65 nuclear translocation. ** $p < 0.01$ vs. control cells; # $p < 0.05$, and ## $p < 0.01$ vs. PTE pretreated cells ($n = 4$).

3.6. Effects of PTE on Antioxidant Defenses

The exploration of the expression of HO-1 showed a moderate decrease after LTA stimulation in RAW cells. Moreover, a concentration-dependent increase in HO-1 was detected in LTA-stimulated cells that had been pretreated with PTE ($p < 0.05$, Figure 5A). In addition, the activity of antioxidant enzyme catalase was found to increase in RAW cells when they were pretreated with PTE (Figure 5B). The trend concerning an increase in the activity of catalase (CAT) in cells pretreated with PTE was not concentration-dependent, as both 20 and 40 μ M PTE have similar effects on CAT activity. Moreover, LTA stimulation expressively reduced the content of GSH in RAW cells ($p < 0.01$), but this decrease was significantly raised in PTE-pretreated cells ($p < 0.05$, Figure 5C).

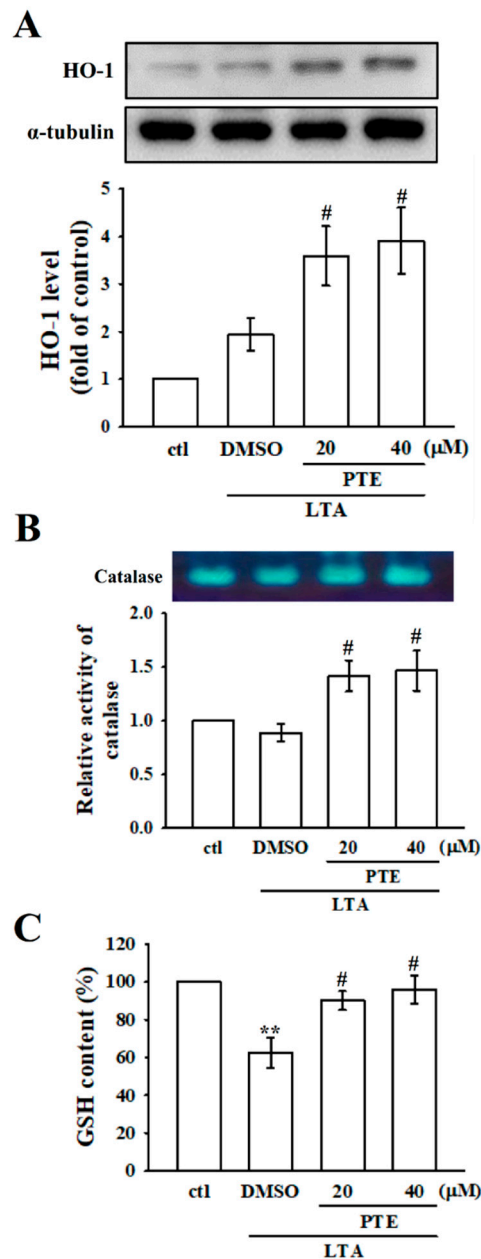


Figure 5. PTE enhances antioxidant defense molecules. Cells were treated with 0.1% DMSO or PTE (20 and 40 μ M) for 30 min followed by LTA (5 μ g/mL) for 24 h. PTE increases HO-1 expression (A), catalase (CAT) activity (B) and glutathione (GSH) content (C) in LTA-induced RAW cells. The figures are representative examples of three independent experiments. # $p < 0.05$ vs. LTA induced cells; ** $p < 0.01$ vs. control cells ($n = 4$).

4. Discussion

Pterostilbene, a natural compound in blueberries, is a potent derivative of the natural antioxidant resveratrol [25]. Several lines of evidence show that PTE has beneficial effects for cancer prevention and therapy, improving insulin sensitivity, adjusting blood glycemia and lipid levels, and conquering cardiovascular diseases, inflammation diseases, and aging [26]. Our recent study demonstrated that PTE has potent antiplatelet effects and suggested its possible therapeutic uses in thromboembolic disorders [27]. In this current study, we examined the anti-inflammatory effects of PTE in LTA-stimulated RAW264.7 cells. PTE showed anti-inflammatory effects via inhibiting the production of NO and its related iNOS enzyme expression. It expressively inhibited the protein expression of IL-1 β

and TNF- α . Additionally, it showed that PTE reserved LTA-induced inflammation chiefly by hindering the ERK-mediated NF- κ B pathway.

Nitric oxide (NO), a key cytotoxic agent, plays a role in the pathogenesis of numerous inflammatory diseases. The production of NO in activated macrophages via iNOS activation plays a major role in the pathology of several acute and chronic inflammatory conditions [28]. The activation of TNF- α , IL-6, and IL-1 β is associated with modulating inflammation and tumor progression [29]. Natural plant-derived products have been reported to inhibit the expression of iNOS [30]. Huang et al. [31] found that wogonin, a bioactive flavonoid, inhibits LTA-induced NO production and its gene expression in macrophages. In this study, PTE was found to significantly inhibit the production of NO in LTA-stimulated RAW 264.7 cells, without inducing cytotoxicity. The PTE's suppressive effect in LTA-induced NO production seems to involve the destruction of iNOS expression. Moreover, PTE expressively inhibited the LTA-induced production of TNF- α , and IL-1 β . This finding is consistent with a previous study showing that apigenin, a natural flavonoid compound, inhibited inflammatory diseases by inhibiting cytokines and TNF- α expression [32].

NF- κ B and MAPKs act as serious mediators of the release of inflammatory cytokines and control the expression of a variety of genes, such as TNF- α , IL-6, and iNOS [33]. Increasing evidence shows that the ERK signaling pathway contributes in regulating inflammatory events [34]. A study demonstrated that treatment with flavonoids, quercetin and luteolin blocked LTA-induced phosphorylation of ERK1/2 [35]. In this study, we found PTE treatment expressively attenuated LTA-induced ERK activation in RAW 264.7 cells. The phosphorylation of ERK1/2 is reported to be associated with NF- κ B activation [36], and the phosphorylation of I κ B α can be induced by ERK1/2-mediated I κ B kinase, activating the degradation of I κ B α and subsequently induced nuclear translocation of NF- κ B [8]. LTA increased the phosphorylation of I κ B and p65 and its translocation from the cytoplasm to nuclei, as this distinctive transcription factor stimulates by LTA [8]. LTA binds with TLR2, which in turn activates NF- κ B by protein kinases, and subsequently translocated to nuclei from the cytoplasm [37]. An in vitro study in microglia cells had shown that curcumin suppressed LTA-induced nuclear translocation and activation of NF- κ B [38]. Therefore, these results may suggest that PTE reduces the inflammatory actions in LTA-induced RAW cells via suppressing the activation of the ERK and NF- κ B pathways, as it stated above that ERK is associated with NF- κ B activation [36].

HO-1, an inducible stress protein, has antioxidant properties and plays an essential role in protection of oxidative stress in chronic disease [39]. In addition, the cellular protective role of HO-1 was reported via stopping a variety of inflammatory responses [40]. Various antioxidants can induce HO-1 to prevent oxidative damage and, therefore, compounds that can induce HO-1 expression may be favorable in the treatment of oxidative damage. Sun et al. [41] found quercetin stimulates HO-1 protein expression in LPS induced by 2 microglia cells. Similarly, we found PTE obviously raised HO-1 expression in LTA-induced cells. Moreover, the effect of PTE on antioxidant enzyme catalase (CAT) and glutathione was also established in LTA-induced cells. It shows that PTE elevates the activity CAT and the level of GSH. CAT enzyme is catalytically reacting on H₂O₂ to form H₂O [42]. A previous study revealed that curcumin, a natural anti-inflammatory compound, raises the activity of CAT to recover RAW cells from the LPS-induced ROS formation [43]. Glutathione (GSH), a cellular antioxidant found in various natural sources, contains L-cysteine, L-glutamic acid, and glycine [44]. Earlier investigations have shown that GSH inhibits oxidative stress-induced cell damage, and its decline contributes to the development of several diseases [45]. In addition, Guan et al. [46] found that liquiritin apioside, a main flavonoid component of licorice, had antioxidant properties by increasing the level of GSH against cigarette smoke-induced oxidative stress in lung epithelial cells. Here, the observed elevation of antioxidant defense molecules HO-1, CAT and GSH by PTE treatment indicates that PTE may at least be partially associated with the antioxidant mechanisms against LTA-induced inflammation in RAW cells.

5. Conclusions

The current study provides new insights into the potential antioxidative and anti-inflammatory mechanisms of PTE. PTE inhibited proinflammatory activation of macrophages by the NF- κ B/ERK signaling pathway via the potent attenuation of release of pro-inflammatory mediators (iNOS and NO) and cytokines (TNF- α and IL-1 β) by macrophages. In addition, PTE regulates LTA-induced inflammatory actions either by elevating the activity of CAT and GSH levels or by increasing the protein expression of HO-1. This study suggests that PTE exhibits antioxidant and anti-inflammatory effects in LTA-stimulated RAW 264.7 cells via the regulation of antioxidants as well as NF- κ B/ERK signaling pathways.

Author Contributions: T.J., C.-H.H. and J.-R.S. designed work and wrote the paper. M.-P.W., C.-W.H. and C.-H.H. carried out the experiments. C.-W.H., C.-L.C. and P.S.B. performed data analyses. M.M. provided interpretation and guidance on the manuscript. All authors have read and agreed to the published version of the manuscript.

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