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Cinnamaldehyde inhibits pro-inflammatory cytokines secretion from monocytes/macrophages through suppression of intracellular signaling

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Abstract

We investigated the *in vitro* anti-inflammatory effects of Cinnamaldehyde, a cytokine production inhibitor isolated from an essential oil produced from the leaves of *Cinnamomum osmophloeum* Kaneh, and its mechanism of action. Although Cinnamaldehyde has been reported to have contact sensitizing properties at high concentration (mM), we found that low concentration of Cinnamaldehyde (μM) inhibited the secretion of interleukin-1beta and tumor necrosis factor alpha within lipopolysaccharide (LPS) or lipoteichoic acid (LTA) stimulated murine J774A.1 macrophages. Cinnamaldehyde also suppressed the production of these cytokines from LPS stimulated human blood monocytes derived primary macrophages and human THP-1 monocytes. Furthermore, Cinnamaldehyde also inhibited the production of prointerleukin-1beta within LPS or LTA stimulated human THP-1 monocytes. Reactive oxygen species release from LPS stimulated J774A.1 macrophages was reduced by Cinnamaldehyde. The phosphorylation of extracellular signal-regulated kinase 1/2 and c-Jun N-terminal kinase 1/2 induced by LPS was also inhibited by Cinnamaldehyde; however, Cinnamaldehyde neither antagonize the binding of LPS to the cells nor alter the cell surface expression of toll-like receptor 4 and CD14. In addition, we also noted that Cinnamaldehyde appeared to elicit no cytotoxic effect upon J774A.1 macrophages under our experimental conditions, although Cinnamaldehyde reduced J774A.1 macrophages proliferation as analysed by MTT assay. Our current results have demonstrated the anti-oxidation and anti-inflammatory properties of Cinnamaldehyde that could provide the possibility for Cinnamaldehyde's future pharmaceutical application in the realm of immuno-modulation.

Keywords: Cinnamaldehyde; Lipopolysaccharide; Macrophage; Cytokine; Protein kinase

1. Introduction

In Asia area the cinnamon was popularly used in food and traditional herb medicine, moreover the cinnamaldehyde is a target component of cinnamon tree. The Cinnamaldehyde, a major and a bioactive compound isolated from the leaves of *Cinnamomum osmophloeum* kaneh

Abbreviations: LPS, lipopolysaccharide; LTA, lipoteichoic acid; poly-IC, polyinosinic-polycytidylic acid; TLR, toll-like receptor; TNF, tumor necrosis factor alpha; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

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(Cheng et al., 2004, 2006; Lee et al., 2005a). Cinnamaldehyde has been demonstrated to induce apoptosis by mitochondrial permeability transition in human promyelocytic leukemia HL-60 cells (Ka et al., 2003), and induced apoptosis in human hepatoma cells through activation of the proapoptotic Bcl-2 family proteins (Wu et al., 2005). Cinnamaldehyde also has been demonstrated to have anti-bacteria activities (Gill and Holley, 2004). Treatment of cultured mouse splenocytes with Cinnamaldehyde induced suppression of concanavalin A and lipopolysaccharide (LPS) induced proliferation of T cells in a dose-dependent manner, e.g., 1 μM of Cinnamaldehyde inhibited the LPS-induced proliferation by around 25% (Koh et al., 1998). Cinnamaldehyde has been reported to inhibit LPS induced DNA binding activity of NF-κB in addition to NF-κB transcriptional activity within cultured RAW 264.7 macrophages (Reddy et al., 2004). Studies have demonstrated that Cinnamaldehyde inhibited inducible nitric oxide synthase and nitric oxide production through inhibition of NF-κB activation within LPS stimulated RAW 264.7 macrophages in a dose-dependent manner with an IC_{50} value of 8 μM (Lee et al., 2002, 2005b). Furthermore, Cinnamaldehyde reduced interleukin-1beta (IL-1) induced cyclooxygenase-2 activity and consequently inhibits production of prostaglandin E2 in a dose-dependent manner within cultured rat cerebral microvascular endothelial cells (Guo et al., 2006). These results strongly suggest that Cinnamaldehyde can be used as an immuno-modulation agent. However, the effect of Cinnamaldehyde upon toll-like receptor (TLR) mediated pro-inflammatory cytokines production within cultured monocytes/macrophages would appear to be unclear at time of writing.

The innate immunological response is typically triggered by pathogen-associated molecular patterns that are shared by groups of different microbial pathogens, which are recognized by TLRs expressed on the cell surface of monocytes/macrophages (Medzhitov and Janeway, 1997). LPS, lipoteichoic acid (LTA) and polyinosinic-polycytidylic acid (polyIC) activates monocytes/macrophages by binding to TLR4, TLR2 and TLR3, respectively, followed by stimulating the production of pro-inflammatory cytokines within monocytes/macrophages, including tumor necrosis factor alpha (TNF), IL-1 and interleukin-6 (IL-6) (Takeda et al., 2003). The inflammatory bowel disease (IBD) is a systemic inflammatory disease primarily involving the gastrointestinal tract. TNF, IL-1 and IL-6 are key mediators in the pathogenesis of IBD (Ludwiczek et al., 2004). Recently, development of potential therapeutic approach to modulate inflammatory bowel disease has become ever more popular and, seemingly, important. These therapeutic approaches include inhibition of pro-inflammatory cytokines production (Osterman and Lichtenstein, 2007). MAP kinase inhibitors targeting various inflammatory cells and pathways also have become important modality in the treatment of patients with IBD (Baumgart and Sandborn, 2007). In this study, we demonstrated that Cinnamaldehyde could inhibit activation of MAP kinase and cytokines expression within TLRs activated monocytes/macrophages, suggesting that Cinnamaldehyde is a novel option for treatment of IBD. Some essential oils have been reported to inhibit cytokines expression from cells. For examples, Sugiol, a diterpene isolated from *Calocedrus formosana* bark inhibited LPS-induced TNF and IL-1 secretion from murine macrophages (Chao et al., 2005a). In addition, Terpinen-4-ol, the main component of the essential oil of *Melaleuca alternifolia* suppressed LPS-induced production of TNF, IL-1, IL-10 and PGE2 by human monocytes (Hart et al., 2000). However, the underlying molecular mechanisms for such capacity would, to the best of our knowledge, appear to have been less-frequently reported on.

The mitogen-activated protein kinases (MAPKs) consist of three subtypes including the extracellular signal-regulated kinases 1/2 (ERK1/2), stress-activated protein kinases (SAPKs)/c-Jun NH_2-terminal kinases 1/2 (JNK1/2) and p38 kinases (Yang et al., 2003). MAPKs play important roles in the regulation of cytokines gene expression, such regulation including TNF and IL-1 release within cultured human and murine macrophages (Hsu and Wen, 2002). In our previous study, we found that LPS stimulated IL-1 gene expression via the release of reactive oxygen species (ROS) and the activation of MAPKs within cultured human and murine macrophages (Hsu and Wen, 2002). In this current study, we isolated Cinnamaldehyde from essential oils produced from the leaves of *C. osmophloeum*, and demonstrated that Cinnamaldehyde inhibited ROS release and the activation of MAPKs as well as pro-inflammatory cytokines expression within TLRs activated cultured monocytes/macrophages. Our results provide support for the potential for the future pharmaceutical application of Cinnamaldehyde for immuno-modulation purposes.

2. Materials and methods

2.1. Materials

LPS (from *Escherichia coli* 0111:B4), LTA, polyIC, monoclonal anti-MAP kinase, activated (diphosphorylated ERK) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, monoclonal anti-actin antibody, PD98059 and SB203580 were purchased from Sigma Co. (St. Louis, MO). Mouse IL-1, IL-6 and TNF Enzyme-Linked Immunosorbent Assay (ELISA) Kits were purchased from R & D Systems, Inc. (Minneapolis, MN). Human IL-1 and TNF ELISA Kits were purchased from BioSource International, Inc. (Camarillo, CA). Anti-IL-1beta, polyclonal antibody, anti-rabbit IgG-HRP and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell cultures

Human primary monocytes were obtained from normal blood donor buffy coats (Taipei Blood Center, Taipei, Taiwan). Buffy coats cells were mixed with an equal volume of PBS, layered on Histopaque®-1077 (Sigma, St. Louis, MO, USA) and centrifuged at 400*g* for 30 min at 20 °C. The interface containing mononuclear cells were collected and washed twice with PBS. Thereafter, human primary monocytes (98% CD14+,

analyzed by flow cytometry) were isolated from mononuclear cells by the Monocyte Isolation kit II (Miltenyi Biotech, Auburn, CA, USA). Human primary monocytes were cultured in RPMI 1640 supplemented with 10% Fetal Calf Serum (FCS) (Hyclone, Logan, UT, USA). Human primary macrophages were obtained by culturing monocytes for 7 days in RPMI-1640 supplemented with 15% FCS at a density of $1.5 \times 10^5/cm^2$. During the cultured period, non-adherent cells were removed by washing with PBS. After 7 days of incubation, the adherent cells were used as human blood monocytes derived primary macrophages. Human THP-1 monocytes and murine J774A.1 macrophages were obtained from ATCC (Rockville, MD, USA) and propagated in RPMI-1640 medium supplemented with 10% heated-inactivated FCS and 2 mM L-glutamine (Life Technologies, Inc., MD), and cultured in a 37 °C, 5% CO_2 incubator.

2.3. Collection of essential oil from C. osmophloeum leaf

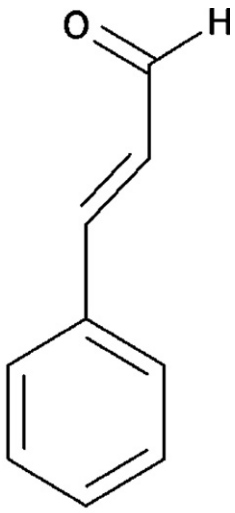
The leaves of 13-year-old *C. osmophloeum* were collected on August, 2003 from the Da-Pin-Ting of the Taiwan Sugar Farm located in Nantou County in central Taiwan. The species was identified by Mr. Yen-Ray Hsui of the Taiwan Forestry Research Institute, and the voucher specimens (reg. # WCCL-03-B014) were deposited at the laboratory of wood chemistry (School of Forestry and Resource Conservation, National Taiwan University). The fresh leaf oils of *C. osmophloeum* were obtained by using water distillation in a Clevenger-type apparatus for 6 h and their constituents determined by GC–MS (Cheng et al., 2004; Chao et al., 2005b). The sampling of essential oil was performed by a mass spectrometer, which was equipped with a PoLaris Q mass selective detector in the electron impact (EI) ionization mode (70 eV), and using a RTx-5 capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The oven temperature was held at 80 °C for 1 min to 200 °C held for 5 min at a rate of 4 °C/min. The injection temperature was 250 °C, detector temperature was 280 °C, and helium was used as a carrier gas at a split ratio of 10:1. Identification of the major components of *C. osmophloeum* leaf oil was confirmed by comparison with standards, by spiking, and on the basis of their mass spectral fragmentation using the Wiley GC–MS library. The quantity of compounds was obtained by integrating the peak area of the spectrograms.

2.4. Purification and identification of cinnamaldehyde

Leaf oils (1.5 g) of *C. osmophloeum* were purified by semi-preparative HPLC on a model L-7150 instrument (Hitachi, Japan) with a 250 × 10 mm i.d., 5 μm Luna Silica (absorption wavelength $\lambda = 254$ nm) (Phenomenex, American) column, ethyl acetate/hexane (15:85) mobile phase, 4 mL/min flow rate, and Hitachi L-7490 RI detector (Chao et al., 2005a). The pure Cinnamaldehyde compound (yield % = 85% and purity > 98%) was obtained ($t_R = 9.04$ min). The structure of the Cinnamaldehyde was identified by comparison of its physical and spectral data (Optical rotation, EIMS, ^{1}H NMR) with previous research values (Kakinuma et al., 1984; Hussain et al., 1986). The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance-500 MHz FT-NMR. The mass spectrum was obtained on a Finnigan MAT-95S mass spectrometer. Fig. 1 presents the structure of Cinnamaldehyde, its character being described as follows: Cinnamaldehyde, mild yellow oil; UV (EtOH), λ_{max}, nm (log ε): 285 nm. IR (KBr) $\nu_{max,}$ cm^{-1}: 2925, 2816, 2744, 1682, 1625, 1574, 1450, 1123, 748, 690. EIMS (70 eV), *m/z* (relative intensity) for C_9H_8O revealed 132, M^+ 132 (59), 131 (100), 103 (50), 77 (35), 51 (23). ^{1}H NMR (CD_3OD, 500 MHz) δ 6.67 (dd, 1H, $J = 7.7$ and 16 Hz, H-8), 7.45 (d, 1H, $J = 16$ Hz, H-7), 7.41 (dd, 1H, H-4), 7.40–7.41 (m, 2H, H-2 and H-6), 7.53–7.55 (m, 2H, H-3 and H-5), 9.67 (d, 1H, $J = 7.7$ Hz, H-9); ^{13}C NMR (CD_3OD, 500 MHz) δ 193.6 (C-9), 152.7 (C-7), 133.9 (C-1), 131.2 (C-4), 129.0 (C-2 and C-6), 128.5 (C-8), 128.4 (C-3 and C-5).

2.5. In vitro assay of H_2O_2 scavenger activity of cinnamaldehyde

In vitro H_2O_2 scavenger activity of cinnamaldehyde were performed by following process (Xu et al., 2004). One milliliter of Cinnamaldehyde was added one milliliter of 35% H_2O_2 to incubate 0 min and 1 h separately. The sample immediately using a normal-phase HPLC column (4.6 × 250 mm, Silica-60) attached to a L-4250 UV–Vis detector and an L-7100 pump (all from Hitachi, Tokyo, Japan) with a 20 μL sample injection loop. Two mobile phases, A (hexane) and B (EA), were used for all samples. All mobile phases were filtered (45 μm filter for organic solvent; Millipore) and degassed by sonication (Bransonic 52; Branson, Shelton, CT, USA). All solvents were HPLC grade. Each Cinnamaldehyde sample was re-suspended in ethanol at a concentration of 0.2 μL/mL, filtered and injected into the HPLC column with final volume of 10 μL. A linear gradient of 100% hexane to 0% hexane (0–100% mobile phase B) was used for 30 min running time at a flow rate of 1 mL/min. Sample was monitored by measuring UV absorption at 270 nm.



Cinnamaldehyde

Fig. 1. Chemical structure of Cinnamaldehyde from *C. osmophloeum*.

2.6. Flow cytometry analysis

Experiments for binding of LPS to cell surface, 2% paraformaldehyde fixed J774A.1 macrophages were incubated with Cinnamaldehyde (24 μM or 80 μM), anti-CD14 antibody (10 μg/mL) or isotype control antibody (10 μg/mL) (BD Biosciences, Mountain View, CA) for 30 min, followed by incubation with FITC-LPS for 30 min at 4 °C. After washing, cells were subjected to flow cytometry analysis on FACSCalibur using CellQuest software of Becton Dickinson Inc. (San Jose, CA). For cell surface expression experiments of TLR4 and CD14, J774A.1 macrophages were incubated with Cinnamaldehyde (24 μM or 80 μM) or LPS (1 μg/mL) for 30 min. Cells were fixed and cell surface expression of TLR4 and CD14 were measured by staining cells for 30 min with PE-conjugated anti-TLR4 antibody (IMGENEX Corporation, Carlsbad, CA) or PE-conjugated anti-CD14 antibody on ice, respectively. After washing, cells were subjected to flow cytometry analysis (Hua et al., 2007).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Biotinylated antibodies reagent (50 μL) was added to anti-mouse TNF, IL-1 and IL-6 precoated stripwell plates, respectively, with 50 μL supernatant concentrate from tested samples for various times and incubated at room temperature for 2 h. After washing the plate three times with washing buffer provided in kit components, 100 μL diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated and 100 μL premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL provided stop solution to each well to stop the reaction, the

absorbance of the plate was measured by MRX microplate reader, Dynex Tech. Inc., VA. at 450–550 nm wavelengths.

2.8. Western Blot

To investigate the inhibitory effect of Cinnamaldehyde on proIL-1 expression within LPS-stimulated J774A.1 cells, the cells were pretreated with Cinnamaldehyde for 30 min at 37 °C, followed by LPS (1 μg/mL) treatment for 6 h. The whole cell lysates were separated by 10% SDS–PAGE and electrotransferred to a polyvinylidene fluoride filter. Filters were incubated in blocking solution (5% non-fat milk in PBS with 0.1% tween 20). These blocking steps were performed at room temperature for 1 h. Filters were incubated with primary anti-IL-1 antibody for 2 h. After washing, filters were incubated with an HRP-conjugated secondary antibody directed against primary antibody. Filters were developed by an enhanced chemiluminescence Western Blot detection system.

2.9. Measurement of intracellular ROS production

Intracellular ROS stimulated by LPS was measured by detecting the fluorescent intensity of either 2′,7′-dichlorofluorescein diacetate (DCFH) or the improved analogue carboxyl-DCFH (CM-DCFH) (Molecular Probes, Inc., Eugene, OR) oxidized product, DCF (or CM-DCF). Briefly, 1×10^6 J774A.1 cells/mL grown in serum- and phenol red-free RPMI medium for 24 h, and then were preincubated with 2 μM CM-DCFH and Cinnamaldehyde (80 μM) or NAC (10 mM) at 37 °C for 30 min in the dark. To these were added fresh starvation medium containing LPS for additional incubation at the indicated times. The relative fluorescent intensity of fluorophore CM-DCF, which was formed by peroxide oxidation of the non-fluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a fluorometer, Cytofluor 2300 (Millipore Inc., Bedford, MA).

2.10. MTT assay for cell viability

The cytotoxicity of Cinnamaldehyde was assessed using the microculture tetrazolium (MTT) assay. After culturing on 96 well plates for 24 h, the cells were washed twice and incubated with 100 μL of 1 mg/mL of MTT for 2 h at 37 °C. The medium was discarded and 100 μL lysis buffer was then added. After 30 min incubation, the absorbance was measured at 570 nm using a microplate reader.

2.11. Statistical analysis

All values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffe's test.

3. Results

3.1. Cinnamaldehyde inhibits LPS-mediated pro-inflammatory cytokines production within murine J774A.1 macrophages

In order to investigate whether the Cinnamaldehyde isolated from the leaf essential oil from *C. osmophloeum* exhibits certain immuno-modulation activity within cultured J774A.1 macrophages, in particular here, J774A.1 macrophages were pre-treated with various concentrations of Cinnamaldehyde (0–80 μM) for a period of 30 min at 37 °C followed by challenge with LPS (ligand of TLR4) for 6 h. For non pre-treated control J774A.1 macrophages, LPS was noted to stimulate significant TNF secretion from J774A.1 macrophages to a level of about 66 ng/mL culture supernatants; within Cinnamaldehyde pre-treated cells, whereas LPS induced TNF secretion by J774A.1 macrophages to culture supernatants was concomitantly clearly reduced to around 40 ng/mL by 80 μM Cinnamaldehyde treatment (Fig. 2a). In addition, pre-incubated J774A.1 macrophages with PD98059, a pharmacological antagonist that inhibits the activation of MEK1 for 30 min, was noted to significantly reduce TNF secretion by J774A.1 macrophages to culture supernatants after 6 h LPS stimulation (Fig. 2a). Furthermore, in order to investigate the effect of Cinnamaldehyde on IL-6 production within LPS-stimulated J774A.1 macrophages, IL-6 production was monitored within J774A.1 macrophages stimulated by LPS in the presence or absence of Cinnamaldehyde for 6 h. The level of IL-6 within LPS stimulated J774A.1 macrophages was around 15 ng/mL (Fig. 2b). Upon treatment with Cinnamaldehyde (8, 24, 40 or 80 μM), the IL-6 production by J774A.1 macrophages was inhibited in a dosage-dependent fashion (11, 6, 6 and 6 ng/mL, respectively; Fig. 2b).

Following this, we tested the effect of Cinnamaldehyde upon LPS induced IL-1 expression by J774A.1 macrophages. Cells were pre-treated with Cinnamaldehyde for 30 min, followed by LPS challenge for 24 h. We found that IL-1 was secreted from LPS-stimulated cells into culture supernatant to a level of around 90 pg/mL, and correspondingly, the IL-1 concentration in supernatant dropped to levels of about 36, 32, 30 and 18 pg/mL by Cinnamaldehyde pre-treatment at a level of, respectively, 8, 24, 40 and 80 μM (Fig. 2c).

The molecular mechanism of the Cinnamaldehyde mediated down-regulation of IL-1 secretion was further investigated herein. Initially, we inquired as to whether the inhibitory effect of Cinnamaldehyde upon IL-1 secretion was regulated at the level of prointerleukin-1beta (proIL-1) (IL-1 precursor, 34 kD). LPS induced an approximate five-fold increase in proIL-1 expression compared to untreated controls (Fig. 2d). Such an LPS induced increased proIL-1 expression was reduced to approximately a three- and a 1.5-fold increase (compared to the control) by pretreatment with, respectively, 40 and 80 μM Cinnamaldehyde. In addition, inclusion of PD98059 reduced LPS induced proIL-1 expression to about the basal level.

3.2. Cinnamaldehyde inhibits LTA-, but not polyIC-mediated cytokines production

Studies have revealed that TLRs are the key molecules for recognizing pathogen-associated molecular patterns in order to elicit inflammatory responses (Takeda et al., 2003; Hsu and Wen, 2002). We investigated whether the inhibitory effect of Cinnamaldehyde is specific for TLR4-induced responses. Therefore, J774A.1 macrophages were stimulated with TLR2 or TLR3 ligand, and the effect of Cinnamaldehyde on cytokines production was examined. In addition to LPS, we used LTA and polyIC as ligands for TLR2 and TLR3, respectively (Takeda et al., 2003). We found that Cinnamaldehyde inhibited TNF (Fig. 3a)

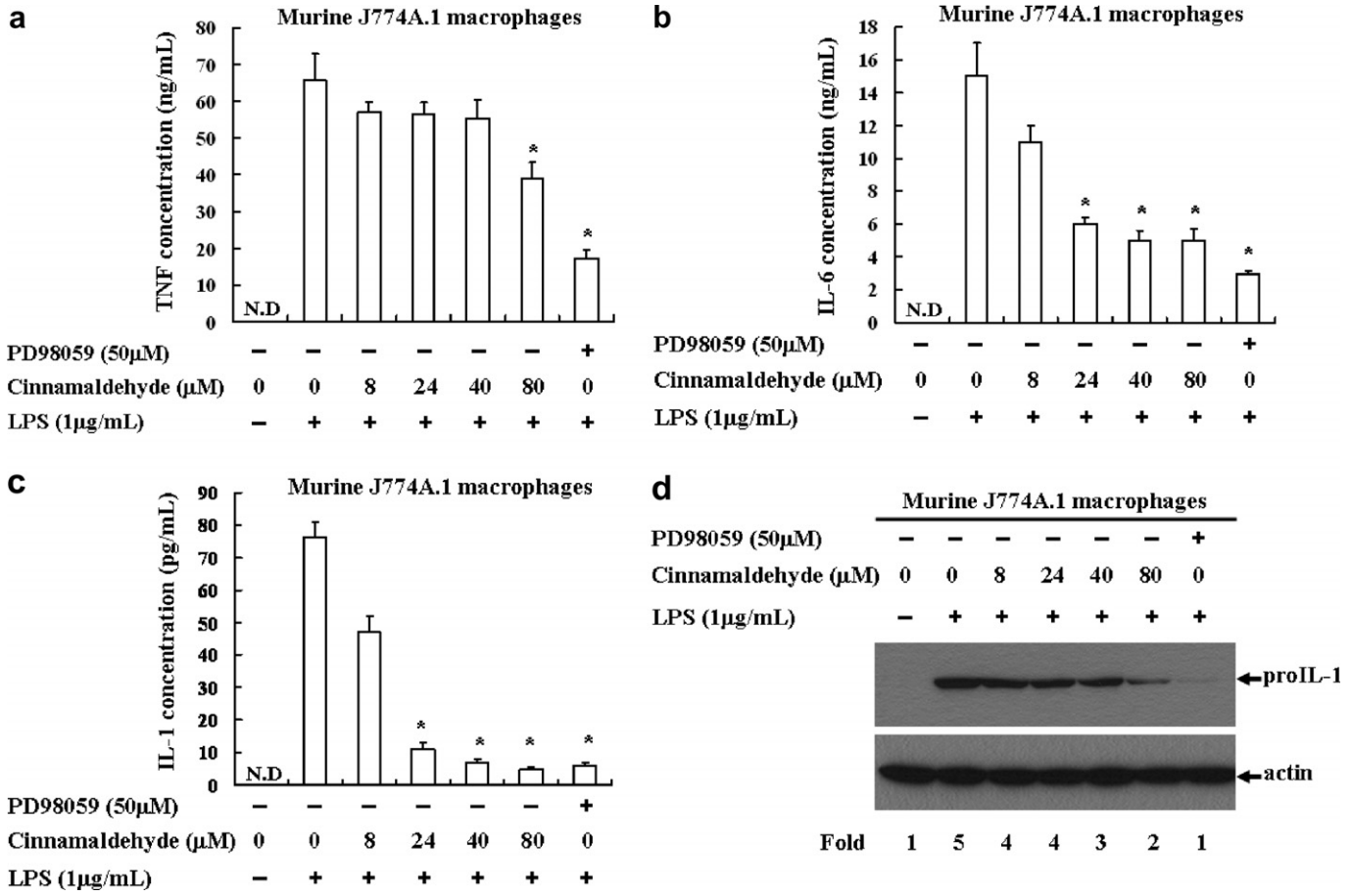


Fig. 2. Cinnamaldehyde inhibited cytokines production within J774A.1 macrophages. Cinnamaldehyde inhibited (a) TNF secretion, (b) IL-6 secretion, (c) IL-1 secretion and (d) proIL-1 expression within LPS-stimulated J774A.1 macrophages. Cells were pre-treated with various concentrations of Cinnamaldehyde or (50 μM) for 30 min prior to incubation with LPS (1 μg/mL) for 6 h (for TNF, IL-6 and proIL-1) or 24 h (for IL-1). TNF, IL-6 and IL-1 concentration in culture media were assayed by ELISA. The proIL-1 expression was assayed by Western blotting. ND means non-detectable; $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS alone.

and IL-1 (Fig. 3b) secretion from LTA stimulated J774A.1 macrophages in a dosage-dependent fashion; however, Cinnamaldehyde showed no significant inhibitory effect on TNF secretion within polyIC stimulated J774A.1 macrophages (Fig. 3c).

3.3. Cinnamaldehyde inhibits pro-inflammatory cytokines production within human blood monocytes-derived primary macrophages and human THP-1 monocytes

In order to test whether the inhibitory effect of Cinnamaldehyde on cytokines production is cell type specific or not, we used human blood monocytes derived primary macrophages and human THP-1 monocytes to test the hypothesis. The results indicated that Cinnamaldehyde was also effective within human blood monocytes derived primary macrophages and inhibited the secretion of LPS induced TNF and IL-1. In essence, secreted TNF levels of around 230 pg/mL were detected in the conditioned medium harvested from a J774A.1 macrophages culture at 6 h post stimulation for LPS stimulated cells, whereas LPS induced TNF secretion was reduced to around 110 pg/mL by 80 μM Cinnamaldehyde treatment (Fig. 4a). In addition, around 160 pg/mL IL-1 were secreted from 24 h LPS stimulated cells and LPS induced IL-1 secretion was reduced to around 70 pg/mL by 80 μg/mL Cinnamaldehyde treatment (Fig. 4b). Furthermore, Cinnamaldehyde also inhibited LPS induced TNF (Fig. 4c) and IL-1 (Fig. 4d) secretion within human THP-1 monocytes. LPS and LTA induced proIL-1 expression was also inhibited by Cinnamaldehyde within human THP-1 monocytes (Figs. 4e and 4f).

3.4. Effect of Cinnamaldehyde on LPS-binding to J774A.1 macrophages and on cell surface expression of TLR4/CD14

The results described above indicated that Cinnamaldehyde (80 μM) significantly inhibited cytokines expression; herein, we suggested that Cinnamaldehyde at this concentration may target an upstream event in LPS signaling or inhibit LPS binding to the cells. It is known that LPS binds to CD14/TLR4 complex on host cells such as monocytes and macrophages (Medzhitov and Janeway, 1997). We

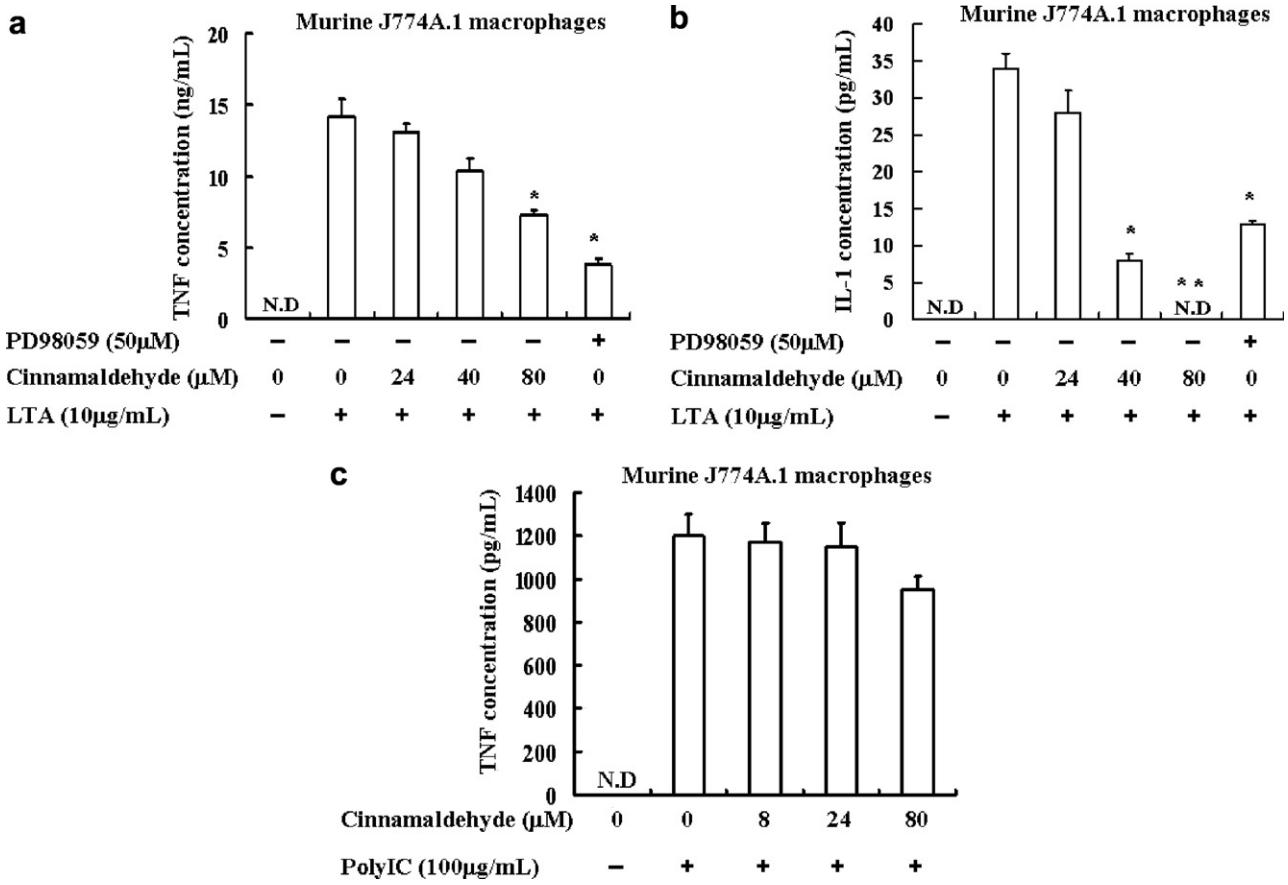


Fig. 3. Cinnamaldehyde inhibited LTA or polyIC mediated cytokines production within J774A.1 macrophages. Cinnamaldehyde inhibited LTA-induced TNF (a) and IL-1 (b) secretion within J774A.1 macrophages. Cells were pre-treated with various concentrations of Cinnamaldehyde or PD98059 (50 μM) for 30 min prior to incubation with LTA (10 μg/mL) for 6 h (for TNF) or 24 h (for IL-1). TNF and IL-1 concentration in culture media were assayed by ELISA. (c) J774A.1 macrophages were pretreated with various concentrations of Cinnamaldehyde for 30 min prior to incubation with polyIC (100 μg/mL) for 3 h. TNF secretion in culture media was assayed by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$ versus LTA alone.

conducted experiments to evaluate the effect of Cinnamaldehyde on LPS binding to the macrophages and on cell surface expression of TLR4 and CD14. J744A.1 macrophages were used in this experiment to use a neutralizing anti-murine CD14 monoclonal antibody as a positive control and use an isotype control antibody as a negative control in this experiment. The cells were incubated with FITC-conjugated LPS and the LPS binding was analyzed by flow cytometry. Pre-incubation of cells with anti-CD14 monoclonal antibody resulted in significantly inhibition of the binding of LPS to cells; however, the binding was not blocked by isotype control antibody. Thus, the binding of LPS to cells was CD14-dependent. In contrast to the anti-CD14 monoclonal antibody, Cinnamaldehyde did not block the binding of LPS to cells even at a concentration of 80 μM (Fig. 5a). Furthermore, we next analyzed surface expression of TLR4 and CD14, essential signaling and binding receptors for LPS upon Cinnamaldehyde treatment. When J774A.1 macrophages were stimulated with LPS for 30 min, expression of TLR4 and CD14 was partially decreased; however, the surface TLR4 and CD14 expression was not significantly changed by Cinnamaldehyde treatment (Fig. 5b). These results suggest that Cinnamaldehyde inhibits cytokines production without reducing LPS receptors expression and without antagonizing the binding of LPS to TLR4/CD14 complex.

3.5. Cinnamaldehyde inhibits LPS-induced phosphorylation of MAPKs and ROS release

From the results of our previous study, we note that MAPKs play important roles in the regulation of proIL-1/IL-1 expression within LPS-stimulated J774A.1 macrophages (Hsu and Wen, 2002; Chao et al., 2005b). Therefore, for this study, we investigated as to whether the LPS mediated activation of MAPKs was altered by Cinnamaldehyde pretreatment. As can be seen from Fig. 6a, LPS strongly induced the activation of ERK1/2, JNK1/2 and p38 within J774A.1 macrophages, yet by contrast, LPS-mediated activation of ERK1/2 and JNK1/2, but not p38, was significantly inhibited by Cinnamaldehyde. In essence, ERK1/2 showed 5, 5, 6, 7 and 3-fold increase in level of phosphorylation compared to that observed for control cells at respectively, 10, 20, 30, 60 and 120 min post stimulation for LPS

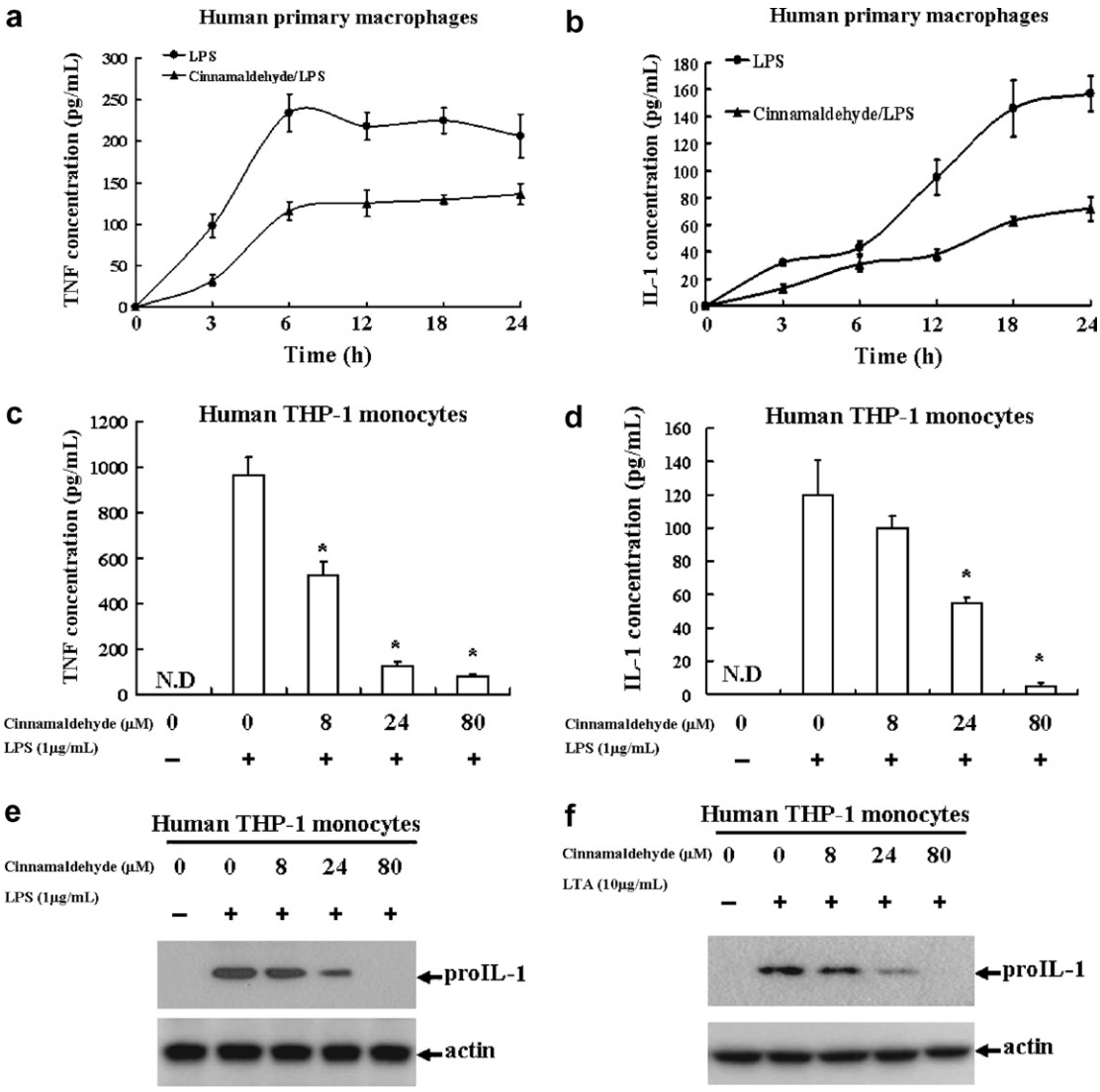


Fig. 4. Cinnamaldehyde inhibited LPS mediated cytokines production within human blood monocytes derived primary macrophages and human THP-1 monocytes. Cinnamaldehyde inhibited LPS-induced TNF (a) and IL-1 (b) secretion within human blood monocytes derived primary macrophages. Cells were pre-treated with Cinnamaldehyde for 30 min prior to incubation with LPS (1 μg/mL) for 0–24 h. TNF and IL-1 concentration in culture media were assayed by ELISA. The error bar for all experiments was mean ± SD ($n = 3$). Cinnamaldehyde inhibited LPS-induced TNF (c) and IL-1 (d) secretion within human THP-1 monocytes. Cells were pre-treated with various concentration of Cinnamaldehyde for 30 min prior to incubation with LPS (1 μg/mL) for 6 h (for TNF) or 24 h (for IL-1). TNF and IL-1 concentration in culture media were assayed by ELISA. $^{*}p < 0.05$; $^{**}p < 0.01$ versus LPS alone. Cinnamaldehyde inhibited LPS (e) or LTA (f) induced proIL-1 expression within THP-1 monocytes. Cells were pre-treated with various concentrations of Cinnamaldehyde for 30 min prior to incubation with LPS (1 μg/mL) or LTA (10 μg/mL) for 6 h. The proIL-1 expression was assayed by using Western blotting. The data are one of the representative experiments ($n = 3$).

stimulated cells. In the Cinnamaldehyde pre-incubated cells, LPS induced ERK1/2 phosphorylation reduced to 1, 1, 5, 3 and 2-fold compared to that observed for control cells at respectively, 10, 20, 30, 60 and 120 min post stimulation for LPS stimulated cells. In addition, LPS induced phosphorylated JNK1/2 increased to 2, 5, 4, 2 and 2-fold at respectively, 10, 20, 30, 60 and 120 min post stimulation compared to untreated control cells, whereas LPS did not induce JNK1/2 phosphorylation in Cinnamaldehyde pre-treated cells.

We therefore suggest that the inhibitory effect of Cinnamaldehyde upon LPS induced cytokines expression may result from the inhibition of the activation of MAPKs. In our previous study, we noted that ROS play important roles in LPS mediated phosphorylation of MAPKs and IL-1 gene expression (Hsu and Wen, 2002). In this study, we found that pre-treatment of cultured murine J774A.1 macrophages with Cinnamaldehyde effectively decreased LPS induced ROS (H_2O_2) release (Fig. 6b). Furthermore, based on the chemical structure of Cinnamaldehyde, we hypothesize that the aldehyde group of Cinnamaldehyde may be oxidized into acid group by H_2O_2 and H_2O_2 deoxidized into H_2O, simultaneously. In order to test our hypothesis, Cinnamaldehyde and H_2O_2 were mixed *in vitro* for 1 h, followed by detecting the possible structure changes of Cinnamaldehyde and H_2O_2 using HPLC. We

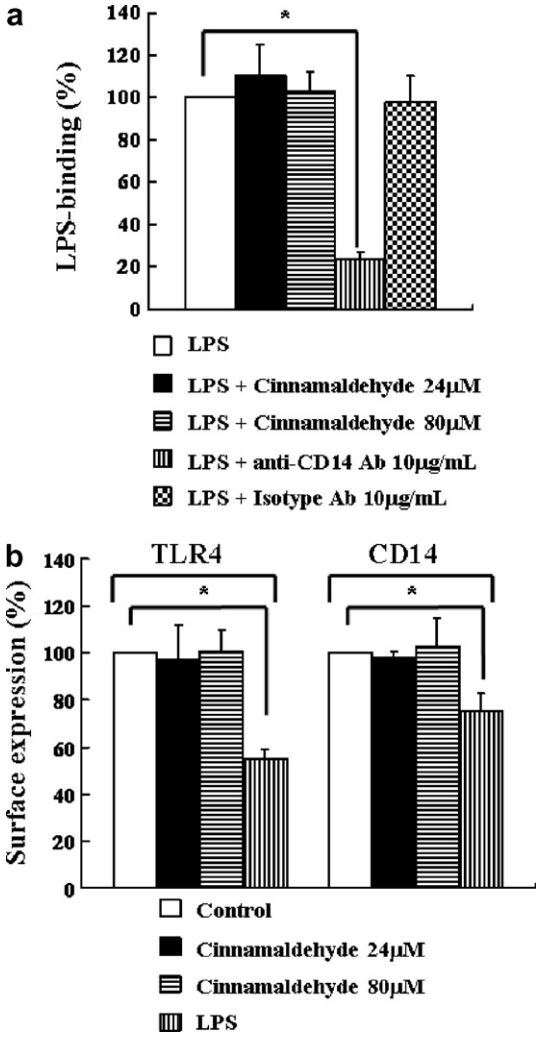


Fig. 5. Effect of Cinnamaldehyde on LPS-binding to J774A.1 macrophages and cell surface expression of TLR4 and CD14. (a) Cells were incubated with Cinnamaldehyde (24 μM or 80 μM), anti-mouse CD14 monoclonal antibody (10 μg/mL) or isotype control antibody (10 μg/mL) for 30 min at 4 °C and further incubated with FITC-conjugated LPS (1 μg/mL) for additional 30 min. The cells were analyzed by flow cytometry. (b) Cells were incubated with Cinnamaldehyde (24 μM or 80 μM) or LPS (1 μg/mL) for 30 min at 37 °C. After washing, cells were fixed with 2% paraformaldehyde, followed by stained with PE-conjugated TLR4 or CD14 antibody for 30 min, then analyzed by flow cytometry. $^{*}p < 0.05$; $^{**}p < 0.01$.

found that Cinnamaldehyde do not reactive with H_2O_2 directly *in vitro* (Fig. 6c). This result also confirm by GC–MS (data not shown). Our results indicate that the Cinnamaldehyde mediated inhibition of TNF and proIL-1/IL-1 expression, as well as the phosphorylation of MAP-Ks maybe, at least in part, due to its antioxidant activity (Hsu and Wen, 2002).

3.6. Effect of Cinnamaldehyde on J774A.1 macrophages viability

In this study, we found that during the 24 h period of J774A.1 macrophages cultured at 37 °C, the cell number of Cinnamaldehyde-treated group was increased, although to a lesser extent than was the case for untreated control cells (Fig. 7a), yet by contrast, Cinnamaldehyde reduced J774A.1 macrophages proliferation at a concentration above 40 μM as demonstrated by an MTT assay (Fig. 7b). These results indicate that Cinnamaldehyde does not exhibit any cytotoxic effect, but slight reduced proliferation within J774A.1 macrophages.

4. Discussion

C. osmophloeum is an endemic tree that grows in Taiwan's natural hardwood forests at middle elevations between 400 and 1500 m (Liu et al., 1988). It has been of interest to researchers because the chemical constituents of its leaf essential oil are similar to those of the famous *Cinnamomum cassia* bark oils (Chang et al., 2001; Hu et al., 1985). Cinnamon oil is commonly used in the food industry for its special aroma. If we could obtain the Cinnamaldehyde from distilling the *C. osmophloeum* leaves, there is no need to cut down the tree, which would be a sustainable utilization.

The innate immune system plays essential roles in host defense against microorganisms' infections. Immune responses to microbial components known as pathogen-associated molecular patterns are recognized and regulated by cellular receptors, including TLRs (Medzhitov and Janeway, 1997). Pathogen-associated molecular patterns are antigenic and able to activate monocytes/macrophages to secrete various inflammatory cytokines including: TNF, IL-1 and IL-6 (Takeda et al., 2003). While mediation of inflammation against pathogen infection by these inflammatory cytokines could be beneficial to the host; however, over-expression of these cytokines may cause inflammatory diseases, including inflammatory bowel disease (Ferrero-Miliani et al., 2006; Nakamura et al., 2006; Scheller et al., 2006). Cinnamaldehyde has been demonstrated to exhibit anti-tumor activities (Jeong et al., 2003; Ka et al., 2003; Wu et al., 2004), anti-bacteria activities (Gill and Holley, 2004), anti-proliferation activities (Koh et al., 1998) and anti LPS induced NF-κB transcriptional activities (Lee et al., 2002; Lee et al., 2005a,b; Reddy et al., 2004). In the present study, we demonstrated that Cinnamaldehyde inhibited TLR4 and TLR2 induced cytokines production. Our current results provided a potential medical application in modulating inflammatory diseases.

Cinnamaldehyde suppressed the LPS-induced production of TNF, IL-6 and IL-1 (Fig. 2). These data suggest that Cinnamaldehyde could show suppressive effects on the production of various types of inflammatory mediators, including those examined in this study. In addition, the inhibitory effects of Cinnamaldehyde on cytokine production were similar both within mouse and human macrophages as well as within human THP-1 monocytes; this suggests that differences in species do not greatly affect the efficacy of Cinnamaldehyde. The concentration of Cinnamaldehyde required for inhibition of LPS induced

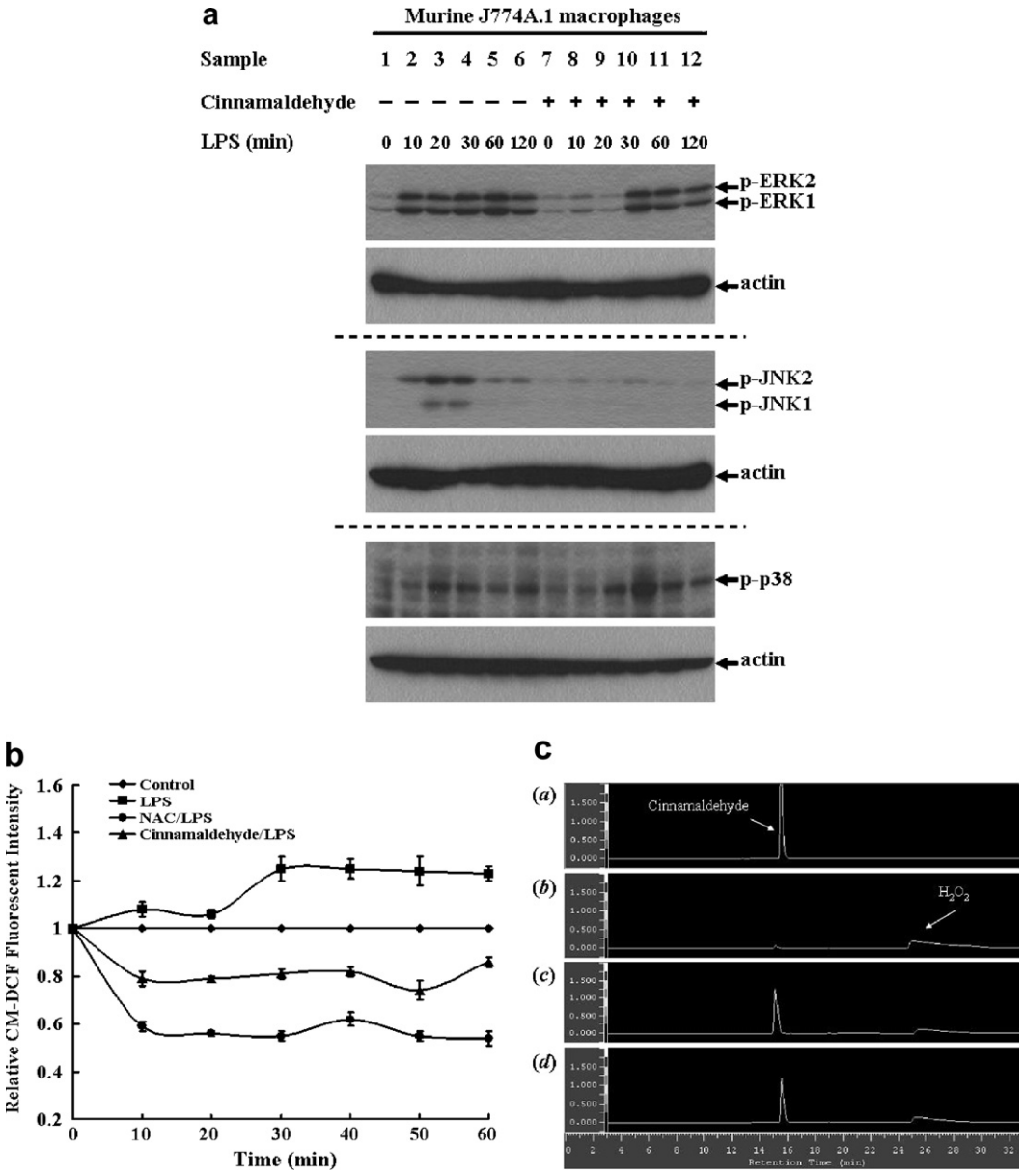


Fig. 6. Cinnamaldehyde inhibited MAPKs phosphorylation and ROS release within LPS stimulated J774A.1 macrophages. (a) Cells were pre-treated with Cinnamaldehyde (80 μM) for 30 min prior to incubation with LPS (1 μg/mL) for 0–120 min. The phosphorylation level of ERK1/2, JNK1/2, and p38 were analyzed by Western blotting with anti-diphosphorylated ERK1/2, anti-diphosphorylated JNK1/2 and anti-diphosphorylated p38 monoclonal antibody, respectively. The data are one of the representative experiments ($n = 3$). (b) Cells were pre-treated with CM-H2DCFDA (2 μM) for 30 min, followed by substitution with fresh medium. Cells were treated with Cinnamaldehyde (80 μM) or NAC (10 mM) for 30 min, respectively, prior to incubation with LPS (1 μg/mL) for 0–60 min. The relative fluorescence intensity of fluorophore CM-DCF was detected as described in Section 2. The data are expressed as means ± SE of three independent experiments. (c) Determine the interaction of Cinnamaldehyde and H_2O_2 by HPLC analysis. (a) Cinnamaldehyde only. (b) H_2O_2 only. (c) Cinnamaldehyde was incubated with H_2O_2 for 0 min. (d) Cinnamaldehyde was incubated with H_2O_2 for 1 h. The samples were analyzed on silica-60 column using a normal phase and a linear gradient of 100% hexane to 0% hexane (0–100% EA) at a flow rate of 1.0 mL/min for 30 min. Sample was monitored by measuring UV absorption at 270 nm.

cytokine production would appear to be quite compatible with its antibacterial activity against various bacteria (Burt, 2004). The anti-inflammatory properties of Cinnamaldehyde would appear to be similar to the anti-inflammatory properties of certain other essential oils deriving from certain other plants. For examples, the essential oil of *Bupleurum fruticescens* composed by two major components, alpha-pinene and beta-caryophyllene, against the carrageenin-induced hindpaw edema in adrenolectomized rats (Martin et al., 1993). Geranium essential oil dose-dependently suppressed leukocytes and neutrophils recruitment into the peritoneal cavity induced by intraperitoneal injection of casein in mice (Abe et al., 2004). In addition, the essential oil of Cordia verbenacea significantly decreased TNF production in carrageenan-injected rat paws (Passos et al., 2007). Interestingly, Cinnamaldehyde at a concentration of 24 μM, exhibited a significant inhibitory effect upon LPS induced IL-1 secretion within

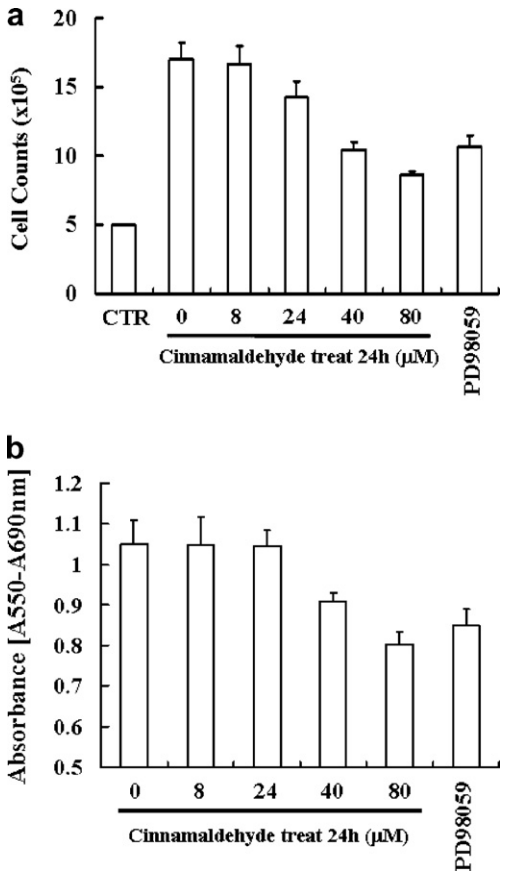


Fig. 7. Effects of Cinnamaldehyde on J774A.1 macrophages viability and proliferation. (a) Cells were treated with one of Cinnamaldehyde (0–80 μM), DMSO vehicle, or PD98059 (50 μM) for 24 h, followed by using trypan blue (0.4%) staining and counting. The error bar for all experiments was means ± SD ($n = 3$). (b) Cells were treated with one of Cinnamaldehyde (0–80 μM), DMSO vehicle or PD98059 (50 μM) for 24 h, followed by incubation with the MTT reagent and precipitate solubilized, and the absorbance (A550–A690) was measured by spectrophotometry. The bar graph with the mean absorbance values of three separate experiments is shown.

J774A.1 macrophages (Fig. 2c); however, concentration of Cinnamaldehyde for significant inhibiting LPS induced proIL-1 expression is 80 μM (Fig. 2d), indicating that Cinnamaldehyde elicited a more potent inhibitory effect upon IL-1 secretion than its inhibitory effect upon proIL-1 expression. These results indicate that activity of interleukin-1 converting enzyme (Cerretti et al., 1992; Hsu and Wen, 2002), the enzyme controlling IL-1 release, may be inhibited by Cinnamaldehyde. Our results suggest the possibility that Cinnamaldehyde may be able to be used to protect certain hosts from inflammatory diseases; although we acknowledge that the effect of Cinnamaldehyde upon LPS induced cytokine production *in vivo* needs to be further investigated.

The initial process in the activation of macrophages by LPS is the recognition of LPS by CD14 and TLR4 on the cell surface (Wright et al., 1990). Following LPS binding to CD14 and TLR4, TLR4 signaling pathways consist a MyD88-dependent and a MyD88-independent pathway. In common MyD88-dependent pathway, IL-1 receptor-associated kinase (IRAK) was recruited to TLR4 through interaction with MyD88. IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of downstream signaling pathways, including MAPKs and NF-κB (Takeda and Akira, 2004). In our previous study, we have demonstrated that ligation of LPS to TLR4 induced IL-1 gene expression via ROS-mediated MAPKs activation, including ERK1/2, JNK1/2 and p38 (Hsu and Wen, 2002). To determine whether Cinnamaldehyde inhibits cytokines production through interference LPS binding to CD14, the LPS binding assay were conducted. We found that Cinnamaldehyde did not block the CD14 mediated binding of LPS to macrophages (Fig. 5a) although it suppressed LPS induced cytokines production. We found that Cinnamaldehyde did not alter the cell surface expression of TLR4 and CD14 (Fig. 5a), which are critical for LPS mediated signal transduction. Taken together, Cinnamaldehyde is not an LPS antagonist but can inhibit an LPS induced signaling process that is elicited after binding of LPS to the receptor complex.

ROS plays important roles in LPS-induced activation and gene expression within macrophages (Chiang et al., 2006; Woo et al., 2004). In our previous study, we noted that *N*-acetylcysteine, a ROS scavenger, inhibited the LPS induced activation of MAPKs and proIL-1/IL-1 expression within LPS-stimulated J774A.1 macrophages, indicating that ROS acts upstream of MAPKs and regulates proIL-1/IL-1 expression (Hsu and Wen, 2002). Certain antioxidants, such as flavonoids and garlic oil derivatives, exhibited anti-inflammatory activities through control of cytokine induction during inflammation (Chang et al., 2005; Comalada et al., 2006). We have also demonstrated that Sugiol, a diterpene isolated from *C. formosana* Bark could effectively reduce ROS production and cytokines expression within LPS stimulated macrophages (Chao et al., 2005a). In this study, we are the first to report that Cinnamaldehyde inhibited LPS induced ROS release (Fig. 6b), yet by contrast, Cinnamaldehyde has been reported to induce ROS release within certain tumor cells (Ka et al., 2003 and Wu et al., 2004). We demonstrated that Cinnamaldehyde can not scavenge H_2O_2 directly by using HPLC and GC-MS methods, thus we suggest that Cinnamaldehyde-mediated reduction of H_2O_2 release through influence the H_2O_2 generation. The detailed molecular mechanism of Cinnamaldehyde-mediated reduction of H_2O_2 release needs further investigation.

In order to gain further insight into the mechanism of the Cinnamaldehyde mediated down-regulation of LPS induced cytokines expression, we examined the phosphorylation of intracellular signaling molecules involved in the LPS mediated signaling pathways. We previously have reported that LPS stimulates JNK1/2, ERK1/2 and p38 activation within J774A.1 macrophages are important for IL-1 expression (Hsu and Wen, 2002); herein we have demonstrated that pre-exposure of such cells to Cinnamaldehyde dramatically decreases the LPS mediated activation

of JNK1/2 and ERK1/2, but not p38. These results indicate that Cinnamaldehyde down-regulation of cytokines production results, principally, from the reduced activation of JNK1/2 and ERK1/2. Further, the anti-proliferation effect of Cinnamaldehyde may be able to be explained by its inhibitory effect upon LPS induced ERK1/2 activation, a mechanism which is related to cell proliferation and growth (Yang et al., 2003).

Because Cinnamaldehyde has been shown to regulate immune responses (Koh et al., 1998; Reddy et al., 2004; Lee et al., 2002, 2005a,b), some groups have investigated whether Cinnamaldehyde is a sensitizer for human or mice. Cinnamaldehyde has been reported as a potent skin sensitizer that causes allergic contact dermatitis in humans (Smith et al., 2000). Cinnamaldehyde also caused an up-regulation of the co-stimulatory molecule CD86, of intercellular adhesion molecule CD54 and of the HLA-DR antigen of immature dendritic cells (Tuschl and Kovac, 2001). Dermal treatment with Cinnamaldehyde resulted in an increase in the percentage of B cells in the auricular lymph nodes and expression of CD86 on B cells of mice, indicating that Cinnamaldehyde as a moderately potent sensitizer (Karrow et al., 2001). Instead, Cinnamaldehyde did not induce pro-inflammatory cytokines production in cell culture or in mice model (Tuschl and Kovac, 2001; Karrow et al., 2001) and mouse ear-swelling test did not identify Cinnamaldehyde as a contact allergen (Karrow et al., 2001). Cinnamaldehyde inhibited LPS-induced iNOS expression and NO production (Lee et al., 2005a,b) as well as LPS-induced cytokines production (this manuscript) at concentration of μM level; however, Cinnamaldehyde showed contact sensitizer properties at concentration of mM level, which is 1000-fold higher than that of inhibiting LPS-induced responses (Karrow et al., 2001). These data support the hypothesis that Cinnamaldehyde has the potential in medicinal application in the realm of immuno-modulation.

In summary, we have used cultured murine J774A.1 macrophages, human blood monocytes derived primary macrophages and human THP-1 monocytes to study, *in vitro*, the immuno-modulating effect of Cinnamaldehyde, a chemical compound derived from the leaves of *C. osmophloeum*. Cinnamaldehyde inhibited the production of pro-inflammatory cytokines production within LPS, LTA and polyIC stimulated macrophages and monocytes. We also found that Cinnamaldehyde-mediated inhibition of cytokines production maybe, at lest on part, due to reduction of ROS release as well as JNK1/2 and ERE1/2 activation within LPS stimulated J774A.1 macrophages. The antioxidant and anti-inflammatory properties of Cinnamaldehyde herald the possibility of its future pharmaceutical use for immuno-modulation purposes.

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