

Bromelain Inhibits Lipopolysaccharide-Induced Cytokine Production in Human THP-1 Monocytes via the Removal of CD14

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Bromelain has been reported to have anti-inflammatory and immunomodulatory effects. However, the anti-inflammatory mechanism of bromelain is unclear. Therefore, we investigated the effect of bromelain on cytokine production from lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMC) and monocytic leukemia THP-1 cells. The result showed that bromelain (50–100 µg/ml) significantly and reversibly reduced tumor necrosis factor (TNF)- α interleukin- (IL)-1 β and IL-6 from LPS-induced PBMC and THP-1 cells. This effect was correlated with reduced LPS-induced TNF- α mRNA and NF- κ B activity in THP-1 cells. In addition, bromelain dose-dependently inhibited LPS-induced prostaglandin E₂, thromboxane B₂ and COX-2 mRNA but not COX-1 mRNA. Importantly, bromelain degraded TNF- α and IL-1 β molecules, reduced the expression of surface marker CD14 but not Toll-like receptor 4 from THP-1 cells. Taken together, the results suggest that the suppression of signaling pathways by bromelain's proteolytic activity may contribute to the anti-inflammatory activity of bromelain.

Keywords Bromelain, Monocytes, NF- κ B, COX-2, TXB2, MAP Kinases, CD14.

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INTRODUCTION

Bromelain is a group of proteases extracted from the stem of pineapple. Bromelain is known for its anti-inflammatory effect (Seligman, 1962) and providing relief for adults with mild knee pain (Walker, 2002). Other pharmacological activities of bromelain have been reported, such as reduction of thrombogenesis, anti-hypertension, regulation of immune functions, anti-microbial infections and inhibition of cancer cell growth (Taussig and Batkin, 1988; Chandler and Mynott, 1998; Maurer, 2001). Bromelain can simultaneously enhance and inhibit immune cell responses *in vitro* and *in vivo* via a stimulatory action on accessory cells and a direct inhibitory action (Engwerda et al., 2001; Hou et al., 2006). In human macrophages/monocytes and mixed lymphocyte culture, bromelain induced a significant increase in interleukin (IL)-6, tumor necrosis factor alpha (TNF- α) and γ -interferon (IFN γ) (Barth et al., 2005; Rose et al., 2005). Bromelain also enhanced IFN- γ -mediated TNF- α production by murine macrophages. Bromelain's effect is independent of endotoxin receptor activation and is not caused by direct modulation of IFN- γ receptors. Instead, bromelain either enhances or acts synergistically with IFN- γ receptor-mediated signals (Engwerda et al., 2001). On the other hand, bromelain blocks activation of extracellular signal-regulated kinase (ERK) in Th0 cells stimulated via the T cell receptor, or stimulated with combined PMA and calcium ionophore. However, this inhibitory activity of bromelain is dependent on its proteolytic activity (Mynott et al., 1999). We also found that bromelain inhibited lipopolysaccharide (LPS)-activated mitogen-activated protein kinase (MAPK) in microglial cells (Hou et al., 2006).

LPS is an endotoxin from gram-negative bacteria, which provokes inflammatory and immunological responses. LPS binds to LPS-binding protein (LBP) in plasma and is delivered to the cell surface receptor CD14. Then LPS is transferred to the transmembrane signaling receptor toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS stimulation of human monocytes activates several intracellular signaling pathways that include the I κ B kinase (IKK)-nuclear factor (NF)- κ B pathway and three MAPK pathways: ERK, c-Jun N-terminal kinase (JNK) and p38 (Guha and Mackman, 2001). In human and rodent macrophages/monocytes, cytokine production and steady state mRNA levels were increased in response to LPS. Expression of IL-1, IL-6 and TNF- α has been shown to be dependent on the activation of the transcription factor, NF- κ B (Baldwin, 1996; Yoza et al., 1996). NF- κ B can function upstream of cyclooxygenase 2 (COX-2) to control transcription of this gene. Cyclopentenone prostaglandins inhibit NF- κ B activation but prostaglandin E₂ (PGE₂) promotes the transcriptional activity of NF- κ B (Poligone and Baldwin, 2001) and the COX-2 inhibitor can modulate NF- κ B (Shishodia et al., 2004).

Studies of proteolytic enzymes in rheumatic disorders have mostly been conducted on enzyme preparations consisting of combinations of bromelain,

papain, trypsin and chymotrypsin (Leipner et al., 2001; Dessor et al., 2001). Mechanism of this therapy on human remains unclear. Therefore, in this study, we examined the effect of bromelain on cytokine production from human peripheral blood mononuclear cells (PBMC) and human monocytic leukemia THP-1 cells with endotoxin stimulation. The effect of bromelain on signaling pathway and inflammatory factors was further investigated in THP-1 cells.

MATERIALS AND METHODS

Reagents Bromelain was kindly provided by HONG MAO BIOCHEMICALS (Rayong, Thailand). Endotoxin content was measured by *Limulus* Amebocyte Lysate Pyrochrome Kit (Cape Cod Inc, E. Falmouth, MA, USA). The following were purchased: LPS from *Escherichia coli* serotype 0111:B4 and phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO, USA); cell culture ingredients (Life Technologies, Grand Island, NY, USA); fetal bovine serum (FBS) from Biological Industries (Kibbutz Beit Haemek, Israel); rabbit anti-mouse β -Actin (Calbiochem, Temecula, CA, USA) and anti-phospho MAPKs (Promega, Madison, WI, USA); streptavidin-horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson, West Grove, PA, USA); FITC-conjugated mouse anti-CD14, R-phycoerythrin (R-PE)-conjugated mouse anti-human TLR4 monoclonal antibody (PharMingen, San Diego, CA); PGE₂, thromboxane B₂ (TXB₂), TNF- α , IL-1 β and IL-6 ELISA kits (R&D, Minneapolis, MN, USA); ActivELISA™ kit, NF- κ B p65 (IMGENEX, San Diego, CA, USA). M-PER solution was obtained from Pierce (Minneapolis, MN). E-64 was obtained from Peninsula Laboratories (Belmont, CA).

Proteolytic activity of bromelain Recombinant TNF- α and IL-1 β protein (5 μ g/ml each) were treated with bromelain (1–100 μ g/ml) for various incubation periods at room temperature. The reaction was stopped by adding 4x sample buffer and heated at 95°C in a dry bath for 15 min. The recombinant protein was then separated by electrophoresis on 15% polyacrylamide gel and stained by coomassie brilliant blue. ELISA assay was also used to confirm this effect.

Inhibition of proteolytic activity of bromelain Bromelain (casein digesting unit, 1666 CDU/mg) diluted in 3 μ M dithiothreitol (DTT) was incubated with 100 μ M E-64 and 60 mM sodium acetate (pH 5) for 10 min at 37°C. The inactivated bromelain was then dialyzed overnight in phosphate-buffered saline (PBS) at 4°C. The total inactivation of bromelain was achieved as assayed with the casein.

Preparation of PBMC Blood of healthy donors were obtained with informed consent form from Taichung Blood Center (Taichung, Taiwan). Human PBMC were then purified by Ficoll-paque gradient centrifugation. The cells were prepared in RPMI-140 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Cell culture THP-1 cell line was maintained in DMEM culture medium supplemented with 10% FBS and antibiotics at 37°C in a humidified incubator under 5% CO₂. Confluent cultures were passed by trypsinization. For experiments, THP-1 cells, 10⁶ cells/ml were differentiated by treatment with 200 nM PMA for 48 h and rested for 24 h. The cells were washed twice with warm DMEM, and then treated in serum-free medium. In all experiments, cells were treated with bromelain for the indicated times with and without LPS. Bromelain and LPS was dissolved in PBS.

Cell viability assay Cell viability was measured by production of blue formazan product from the colorless substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the mitochondrial hydrolases that are only present in living cells. The assay was measuring the absorbance at 540 nm using a microplate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA).

Enzyme-linked immunosorbent assay (ELISA) TNF- α , IL-1 β , IL-6, PGE₂ and TXB₂ were measured by R&D ELISA kits. The absorbance at 450 nm was determined using a microplate reader (spectraMAX 340).

Preparation of nuclear extract and NF- κ B p65 detection The cells were washed with cold PBS, harvested by centrifugation, and resuspended in hypotonic lysis buffer and incubated on ice for 15 min and then mixed with 0.75% Nonidet P-40 (NP-40) solution to disrupt the cells. The nuclear extract was collected by centrifuged at 14,000 rpm for 1 min and stored at -80°C. Protein concentration was determined by Bradford assay (Bio-Rad, Hemel, Hempstead, UK). p65 activity was detected by NF- κ B p65 ELISA kit (IMGENEX) according to the manufacture's instruction.

Isolation of RNA and RT-PCR After treatment of cells, total cellular RNA was extracted with a cold RNA extraction solution (Ultraspec RNA; Biotex Laboratory Inc., Houston, TX). The RT-PCR assays were performed with a Titan™ One Tube RT-PCR System kit (Boehringer, Mannheim, Germany). Briefly, 1 μ g of total RNA from each sample was added to 50 μ l of a reaction mixture containing 0.2 mM dNTP, 0.4 μ M each of sense and antisense specific primers, 5 mM DTT, 5 U RNase inhibitor, 1 μ l of AMV reverse transcriptase and Expand™ High Fidelity enzyme mix. The primer sequences were listed in Table 1. The β -actin (540-bp fragment) was served as a control for the RNA isolation and reverse-transcription. RT-PCR was conducted in a Perkin Elmer Cetus thermocycler (Norwalk, CT) with denaturation at 94°C for 45 s, annealing at 60°C (for COX-1), 55°C (for COX-2), or 60°C (for β -actin) for 45 s, and extension at 72°C for 2 min. The PCR product was electrophoresed in a 2.5(agarose gel and verified by predicted size. Quantification of the band density was performed by densitometric analysis (Digital Image Analysis System, PDI, Huntington Station, NY), and calculated as the optical density \times area of band.

Flow cytometry analysis THP-1 cells were washed with PBS and resuspended in RPMI 1640. The cells (1 \times 10⁶) were treated with different concentration

Table 1: The Primer Sequences and the Condition of PCR.

Gene	Primers	PCR parameters	Size (bp)	
TNF- α	5'-ATGAGCACTGAAAGCAGATC 3'-CAGACCCCGTCCAGATGAAAC	Denature Annealing Extension	94°C, 1 min 60°C, 1 min 72°C, 1 min	684
IL-1- β	5'-GCCCTAAACAGATGAAGTGC 3'-CCCTGAGTGTCTGTTTTTCG	(D) (A) (E)	94°C, 1 min 56°C, 45 s 72°C, 1 min	314
COX-1	5'-TGCATGTGGCTGTGGATGTCATC 3'-CACTAAGACAGACCCCGTCATCTC	(D) (A) (E)	94°C, 1 min 60°C, 45 s 72°C, 2 min	450
COX-2	5'-GAACATTGTGAACAACATCCC 3'-TTGGTGGCATAATCATCAGA	(D) (A) (E)	94°C, 1 min 55°C, 45 s 72°C, 2 min	615
β -actin	5'-GTGGGCCGCTCTAGGCACCAA 3'-CTCTTTGATGTCACGCACGATTC	(D) (A) (E)	96°C, 1 min 60°C, 45 s 72°C, 2 min	540

bromelain and incubated at 37°C for 40 min (without LPS) or 24 h (with LPS). After incubating, the cells were washed with PBS and resuspended in 100 μ l PBS containing 1% bovine serum albumin, incubated for 30 min with appropriate dilutions of either FITC- anti-CD14 or R-PE anti-TLR4 antibodies. The cells were washed and then analyzed on a FACScan cytofluorograph (Becton Dickinson, San Jose, CA). For each experiment, 10000 events were collected and analysed using the CELLQUEST program based on their forward and side light-scatter profiles. The isotype control histograms are not shown in order to simplify the figures unless otherwise indicated.

Statistical analysis All data were expressed as the mean \pm SEM. For single variable comparisons, Student's t-test was used. For multiple variable comparisons, data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. P values less than 0.05 were considered significant.

RESULTS

Effect of bromelain on cytokine production Human PBMC and THP-1 cells were treated with different concentrations of bromelain (1–100 μ g/ml) with and without LPS (1 μ g/ml) and then incubated for 24 h. At concentrations used in this study, none of the LPS or bromelain treatments caused toxicity to cells as judged by the MTT assay (data not shown). IL-1 β , IL-6 and TNF- α productions from culture supernatants of PBMC and THP-1 cells were slightly induced by bromelain partly because of endotoxin contamination (0.25 EU/100 μ g, as determined by Limulus Amebocyte Lysate Pyrochrome assay). However, bromelain dose-dependently reduced these cytokines elevation by LPS stimulation significantly as compared with that of the LPS control (Table 2). The

Table 2: Effect of Bromelain on Suppression of LPS-Induced Cytokine Production in Human PBMC and THP-1 Cells.^a

Concentration	LPS (1 µg/ml)	LPS+Bromelain (% of inhibition)		
		10	50	100 (µg/ml)
Human PBMC (2 x 10 ⁶)				
IL-1β ^b (pg/ml)	820 ± 40	830 ± 90 (0)	790 ± 40 (4)	610 ± 42* (26)
IL-6 (pg/ml)	1110 ± 35	760 ± 52 (31)	370 ± 20*(67)	192 ± 35* (83)
TNF-α (pg/ml)	760 ± 30	500 ± 20*(34)	350 ± 18*(64)	190 ± 22* (75)
Human THP-1 cells (1 x 10 ⁶)				
IL-1β ^b (pg/ml)	790 ± 10	400 ± 15*(42)	50 ± 4* (93)	10 ± 3* (98)
IL-6 (pg/ml)	400 ± 90	21 ± 9* (95)	15 ± 9* (96)	14 ± 5* (96)
TNF-α (pg/ml)	1100 ± 30	700 ± 30*(49)	230 ± 40*(100)	200 ± 20*(100)
PGE ₂ (pg/ml)	140 ± 10	116 ± 13*(48)	105 ± 15*(70)	65 ± 11*(100)
TXB ₂ (pg/ml)	118 ± 9	100 ± 10*(72)	75 ± 7*(100)	74 ± 5* (100)
COX-1 mRNA	120 ± 20	120 ± 10 (0)	130 ± 20 (0)	126 ± 15 (0)
COX-2 mRNA	185 ± 10	189 ± 10 (0)	152 ± 5* (39)	100 ± 5* (100)
NF-κB (pg/ml)	65 ± 7	-	50 ± 6* (65)	40 ± 5* (96)

^aCell cultures were stimulated with LPS in the presence or absence of bromelain and cultured for various time at 37°C in a humidified incubator under 5% CO₂. Cytokine, PGE₂ and TXB₂ from culture supernatants were determined by ELISA assay. NF-κB p65 level was detected by an ActivELISA™ kit (IMGENEX). Data represents the mean ± SE from at least four separate experiments. *, p < 0.05 as compared with the LPS group.

^bUnstimulated THP-1 control values for IL-1, IL-6 and TNF-α were 98 ± 30, 0, and 282 ± 21 pg/ml, respectively; PGE₂, TXB₂, and NF-κB levels were 90 ± 3, 86 ± 4, and 38 ± 4 pg/ml, respectively.

suppression of IL-6 and TNF-α productions and mRNA expressions by bromelain was more effective than that of the IL-1β (Table 2 and data not shown). The LPS-induced cytokine production in THP-1 cells was similarly suppressed by bromelain dose-dependently, either pretreated bromelain for 30 min and washed or cocultured with bromelain without wash. We reported the data with bromelain remained in the culture after treatment. The suppression of cytokine (93%–100%) was sustained for 24 h with the higher concentrations of bromelain (50–100 µg/ml). Interestingly, both IL-1β and TNF-α were degraded by 50–100 µg/ml of bromelain 38 to 52% and 28 to 49%, respectively as observed from 15% polyacrylamide gel electrophoresis and ELISA assay (data not shown).

Bromelain suppressed LPS-induced NF-κB, COX-2 and products Previous reports showed that agents decrease proinflammatory cytokine expression through the inhibition of NF-κB activation (Desser et al., 1993). Indeed, bromelain (50–100 µg/ml) inhibited the LPS-induced NF-κB activation by 23–39%. RT-PCR analyses were performed to assess the effect of bromelain on LPS-induced COX-1 and COX-2 mRNA steady-state levels. THP-1 cells were treated with various concentrations of bromelain (1–100 µg/ml) and LPS (1 µg/ml) for 4 h. The accumulation of COX-2 mRNA levels was inhibited 39%, 71%, and 100% by bromelain (50, 70, and 100 µg/ml, respectively), whereas COX-1

mRNA showed no change after such treatment. Treatment with 100 µg/ml bromelain abrogated the LPS-induced increase in COX-2 mRNA. Bromelain dose-dependently ($p < 0.05$) inhibited the LPS-induced PGE₂ and TXB₂ by 50–100 µg/ml bromelain in the 24 h culture (Table 2).

Bromelain suppressed LPS-induced MAPK activation LPS induces the activation of MAPKs, and subsequently activates the transcription factors with the attendant induction of COX-2 (Mestre et al., 2001). We found that bromelain dose-dependently suppressed LPS-activated phosphorylation of ERK1/2, JNK, and p38 MAPKs at the concentrations of 50–100 µg/ml (Figure 1). We then asked whether the effect was due to the proteolytic activity of bromelain. Bromelain was treated with a specific cysteine protease inhibitor, E-64 to inactivate its protease activity (Barrett et al., 1982). Inactivation of protease activity was confirmed by as assayed with the casein. E-64 treated bromelain reduced the inhibition on JNK from 50% to 26% and p38 from 37% to 8% (Figure 2).

Bromelain affected cell surface molecules We next address the question whether the proteolytic activity of bromelain exert on the cell surface molecules. The CD14 and TLR4 expressions on bromelain-treated THP-1 cells

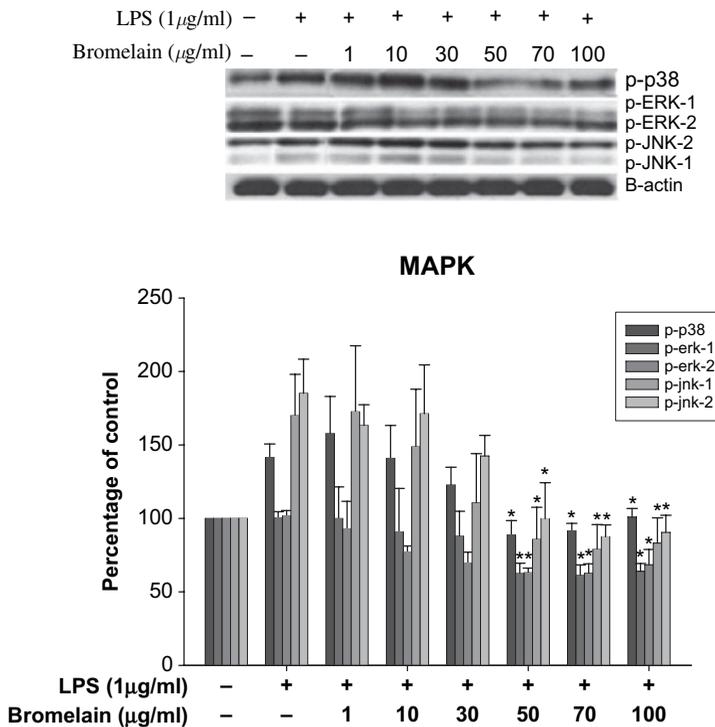


Figure 1: Bromelain inhibited LPS-induced phosphorylated-MAP kinases in human THP-1 cells. The Western blot is representative of four separate experiments. * $p < 0.05$ as compared with the LPS group.

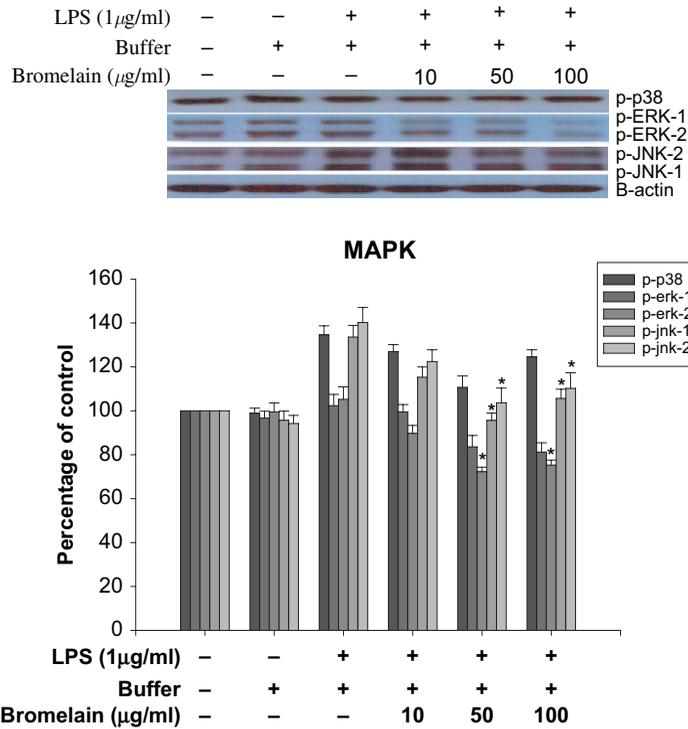


Figure 2: Non-protolytic bromelain inhibited LPS-induced phosphorlated-MAP kinases in THP-1 cells. Western blots showed that E-64 treated bromelain (10–100 μM) on the LPS-induced phospho-JNK and ERK MAPKs at 10 min. Values represent the mean from four separate experiments. **p* < 0.05 as compared with the LPS group.

were examined by flow cytometry. The result showed that bromelain dose-dependently reduced the surface marker CD14 expression but enhanced TLR4 expression of THP-1 cells (Figure 3). After treating THP-1 cells with LPS for 24 h, both CD14 and TLR4 staining were reduced from their medium control levels. However, CD14 but not TLR4 staining was dose-dependently reduced by bromelain (Figure 4). The TLR4 expression was reduced in LPS- and bromelain (100 μg/ml)-treated groups as compared with medium control.

DISCUSSION

The present study demonstrated that bromelain significantly reduced IL-1β, IL-6 and TNF-α from THP-1 cells after LPS-stimulation. The effect is associated with the inhibition of the LPS-induced NF-κB p65 activity and COX-2 mRNA. In addition, bromelain dose-dependently (10–100 μg/ml) inhibited the LPS-induced TXB₂ and PGE₂ levels. Bromelain suppressed LPS-activated MAPKs similar to our previous result (Hou et al., 2006). Interestingly,

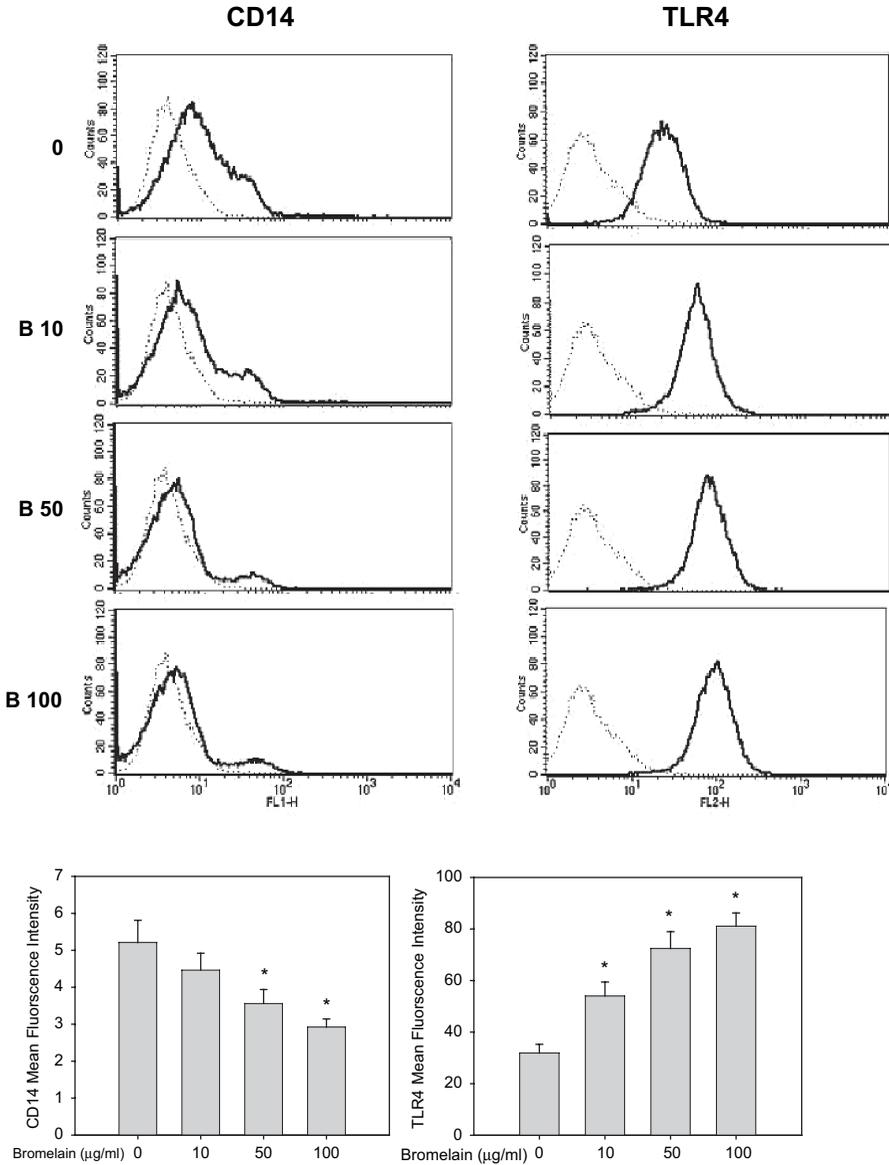


Figure 3: Flow cytometric analysis of bromelain-treated THP-1 cells. Cells were pretreated with bromelain for 40 min and then washed, and stained with FITC-conjugated mouse anti-CD14 or R-phycoerythrin-conjugated mouse anti-human TLR4 monoclonal antibody. The histograms are representative of four independent experiments. * $p < 0.05$ as compared with the LPS group (CD14) or control (TLR4).

non-proteolytic form of bromelain also affected LPS-activated MAPKs significantly by suppressing 26% of JNK and 8% of p38 MAPK. Importantly, we found that bromelain degraded TNF- α and IL-1 β molecules and reduced the leukocyte marker CD14 expression from THP-1 cells.

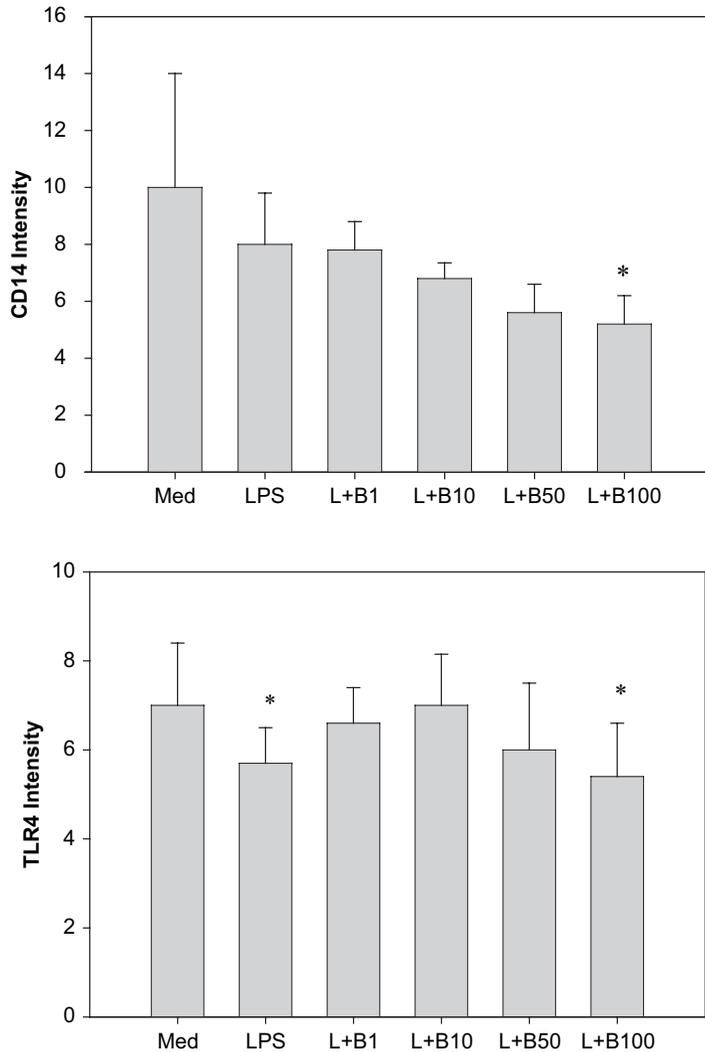


Figure 4: Flow cytometric analysis of LPS-stimulated and bromelain-treated THP-1 cells. Cells were pretreated with bromelain for 40 min and washed. Then cells were stimulated with LPS for 24 h and then washed and stained with FITC-conjugated mouse anti-CD14 or R-phycoerythrin-conjugated mouse anti-human TLR4 monoclonal antibody. The histograms are representative of four independent experiments. * $p < 0.05$ as compared with the medium control.

Bromelain inhibited the LPS-induced NF- κ B activities in THP-1 cells by 39%, which correlated with the reduced cytokine levels. IL-1, IL-6 and TNF- α production has been shown to be dependent on the activation of the transcription factor, NF- κ B (Yoza et al., 1996; Baldwin, 1996). COX-2 has both pro- and anti-inflammatory natures (Poligone and Baldwin, 2001) that COX-2 expression inhibits nuclear translocation of NF- κ B, and PGE₂ and its analogs

promote the inherent transcriptional activity of the p65/RelA subunit of NF- κ B. Bromelain suppressed cytokine production by LPS-stimulation of THP-1 cells was correlated with the reduced COX-2 mRNA, but not COX-1 mRNA. COX-2 plays a prominent role in the inflammation associated with adjuvant arthritis and COX-2 derived PGs unregulated COX-2 and IL-6 expression at inflammatory sites (Anderson et al., 1996). Because COX-1 expression dominates normal tissues whereas COX-2 mRNA is induced at the inflammatory site, COX-2 selective inhibitors are made for anti-inflammatory drugs without gastric toxicity (Seibert et al., 1997). More recently, selective COX-2 inhibitors (coxibs) were designed to inhibit the production of COX-2-dependent inflammatory prostanoids and to leave intact the cytoprotective COX-1 products such as PGE₂ and PGI₂. The coxibs exhibit efficacy in chronic inflammatory conditions, such as osteoarthritis and rheumatoid arthritis, and associate with a reduced incidence of GI toxicity. However, concerns regarding cardiovascular safety in high-risk patients have evolved. These concerns were driven initially by the concept that inhibition of COX-2-derived endothelial PGI₂ without concomitant inhibition of platelet thromboxane A₂ would result in increased cardiovascular risk (Meagher, 2003; Juni et al., 2004; Mattia and Coluzzi, 2005). Our result showed that bromelain acted as a COX-2 inhibitor but also inhibited LPS-induced PGE₂ and TXB₂ in THP-1 cells, therefore bromelain could have advantages over these concerns.

LPS binds TLR4 and its accessory protein MD2 is depended on the presence of LBP in plasma and cell surface receptor CD14 (Rabehi et al., 2001; Liu et al., 2002; Heagy et al., 2003). The LPS stimulation of human monocytes activates several intracellular signaling pathways and activates a variety of transcription factors that include NF- κ B (p50/p65) and AP-1 (c-Fos/c-Jun), which coordinate the induction of many genes encoding inflammatory mediators (Guha and Mackman, 2001; Heagy et al., 2003). We found that bromelain dose-dependently suppressed LPS-activated phosphorylation of JNK and p38 MAPKs. We also found that bromelain reduced the CD14 cell marker but enhanced the TLR-4 in THP-1 cells. This may explain its immune enhancing effect of increased cytokine in human macrophages/monocytes and modified mixed lymphocyte cultures which was partly due the endotoxin contamination in the preparation (Barth et al., 2005; Rose et al., 2005). However, in the presence of LPS, both CD14 and TLR-4 expressions were reduced from the control levels and bromelain (100 μ g/ml) further reduced CD14 expression from the LPS level. This result is different from a recent report that shows the LPS-stimulation induces CD14 and TLR4 expression in human monocytes from both surgical and control patients but not from septic patients (Tsujimoto et al., 2006). The discrepancy is not clear at present and needs further study with PBMC from normal healthy volunteers to clarify it. It is clear that membrane-associated proteins, CD14, CD11b/CD18, and TLR4, may serve as LPS recognition and/or

signaling receptors in human and murine monocytes/macrophages. It is known that at lower concentrations of LPS, the induction of proinflammatory cytokine genes requires the participation of both CD14 and TLR4, whereas at higher concentrations of LPS, the expression of a subset of LPS-inducible genes does not require CD14 molecule (Liu et al., 1994; Perera et al., 2001). When LPS-stimulation of naive monocytes is performed in medium with anti-CD14 antibody or without serum, there is marked lower of TNF release (Heagy et al., 2003). Therefore, reduction of CD14 expression in the surface of THP-1 by bromelain could inhibit cytokine production. The reduced CD14 would affect the binding of TLR4 with LPS, and in turn cause lower activation of MAPKs in LPS-stimulated THP-1 cells. In support of this hypothesis, we found that inhibition of p38 MAPK by bromelain in LPS-stimulated THP-1 cells was largely lost by the treatment of bromelain with E-64, a selective cysteine protease inhibitor. This is in agreement with the result of bromelain-inhibited T cells activation is dependent on its proteolytic activity (Mynott et al., 1999). The mechanism of nonproteolytic activities of bromelain is not clear and awaits further study. Bromelain affects a broad range of cell surface molecules from lymphocytes, monocytes, and granulocytes under physiologically relevant conditions. *In vitro* bromelain treatment of leukocytes in whole blood proteolytically altered 14 of 59 leukocyte markers including CD4, CD14, CD16, CD44 and CD45RA (Hale et al., 2002). It has been shown that proteolytic activity of bromelain is required for anti-inflammatory effects *in vivo*, although the precise mechanism has yet to be determined (Desser et al., 2001; Hale et al., 2005). Our results of reduced CD14 expression by bromelain were consistent with these findings. In addition, CD14 was largely restored in THP-1 cells either washed after incubating with the lower concentration of bromelain for 30 min or cocultured at 24 h and correlated with rebound cytokine production. Interestingly, we found that both IL-1 β and TNF- α protein were degraded by 50–100 μ g/ml of bromelain. This is due to the proteolytic activity of bromelain and this additional beneficial anti-inflammatory effect awaits further study *in vivo*.

In conclusion, our results provide mechanisms of bromelain, which reversibly modify inflammation under LPS stimulation. The anti-inflammatory effect of bromelain was in part by degrading cytokine molecules and reducing the expression of CD14 that leads to the reduced LPS-induced PGE₂, TXB₂, MAPKs, NF- κ B, COX-2 mRNA and cytokine.

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