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Inhibitory Action of Tsunokaori Tangor Peel on the Lipopolysaccharide-Induced Inflammatory Response in RAW 264.7 Macrophage Cells

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Abstract We evaluated the effects of extracts of Tsunokaori tangor peel on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E_2 (PGE_2) in RAW 264.7 cells. The ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) markedly inhibited the production of NO and PGE_2 in LPS-stimulated RAW 264.7 cells. Consistent with these findings, the expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins were down-regulated in a dose-dependent manner. Additionally, EA-TTP decreased the expression iNOS mRNA but not COX-2 mRNA. To determine the upstream signaling mechanism for the down-regulation of LPS-induced iNOS expression, we investigated the effect of EA-TTP on the degradation and re-synthesis of IκBα. EA-TTP dose-dependently delayed IκBα degradation and increased IκBα re-appearance following degradation, suggesting this as the mechanism by which EA-TTP suppressed iNOS gene expression. The EA-TTP also dose-dependently reduced the expression of the cellular stress-response protein heme oxygenase-1, and inhibited the LPS-induced sustained activation of extracellar signal-regulated kinase (ERK).

Keywords: Tsunokaori tangor peel, inducible nitric oxide synthase, cyclooxygenase-2, heme oxygenase-1, IκBα

Introduction

Chronic inflammation leads to destruction of normal tissue integrity and can influence a variety of pathologies, such as cancer and neurodegenerative diseases as well as inflammatory diseases (1, 2). Inflammation is associated with a large range of inflammatory mediators which initiate inflammatory responses, recruit and activate other cells to the site of inflammation and subsequently resolve the inflammation (3).

Nitric oxide (NO) regulates many biological functions (4), but excessive NO production has been implicated in the pathogenesis of inflammatory tissue injury and several other disease states (5, 6). The expression of inducible nitric oxide synthase (iNOS) results in high NO levels, profoundly influencing cell and tissue function, and causing cell damage (7). Prostaglandin E_2 (PGE_2) is the major metabolite produced by cyclooxygenase-2 (COX-2) at sites of inflammation where it contributes to edema, pain sensitization, and increased local blood flow (8). Therefore, the expression of iNOS and COX-2 must be tightly regulated to achieve the maximal benefits of NO and PGE_2 while avoiding toxicity.

Heme oxygenase-1 (HO-1) is a stress-response protein induced by a variety of stimuli associated with oxidative stress (9, 10). In macrophages, HO-1 expression is up-regulated by inflammatory mediators such as lipopolysaccharide (LPS) (11). HO-1 thus provides a relevant and sensitive index by which to assess alterations in the cellular inflammatory response.

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Nuclear factor kappa-B (NF-κB) is a transcription factor that plays a key role in inflammatory and immune responses, and is one of the critical transcription factors mediating iNOS, COX-2 and HO-1 expression (12). In nonstimulated cells, NF-κB is sequestered in the cytoplasm through interactions with a set of specific inhibitory proteins, the IκBs (13). In response to cell stimulation, IκBs are rapidly phosphorylated by IκB kinase (IKK) and phosphorylated IκBs are degraded by the 26S proteasome (14, 15). The degradation of the phospho-IκBs releases the NF-κB dimers and NF-κB is translocated to the nucleus where it activates transcription of specific target genes (16).

Citrus fruits are a dietary source of abundant antioxidants and have been used in traditional medicine in Korea, Japan, and China. The major flavonoids (*e.g.* hesperidin, naringin, and polymethoxylated flavones) of citrus fruits have been reported to possess anti-oxidant and anti-cancer activities (17, 18). In particular, the fruit peel has been shown to be more efficient than the corresponding juice-containing portions at eliminating free radical species (19) and essential oils extracted from citrus peels have been reported to have antibacterial and antifungal activities (20). Furthermore, naringin, quercetin, and nobiletin, charactieristic flavonoids of citrus peel, were shown to have an anti-inflammatory effect *in vivo* and *in vitro* through the inhibition of iNOS and/or COX-2 expression (21-23).

A commercial tangor cultivar, Tsunokaori tangor (Kiyomi tangor × Okitsu Wase Satsuma mandarin) is widely cultivated in Jeju Island, Korea. This hybrid fruit, which is very sweet with moderate acidity and a good aroma, is a popular dietary source of abundant antioxidants. However the peel is typically discarded, and there has been no comprehensive evaluation of its therapeutic benefits and commercial value.

The aim of this work was to evaluate the availability of Tsunokaori peel as an immunomodulating functional food. In this study, we examined the inhibitory effects of Tsunokaori tangor peel on the LPS-induced inflammatory response in murine macrophage RAW 264.7 cells. Furthermore, we investigated the associated molecular events by determining which signaling pathways were affected.

Materials and Methods

Plant materials and preparation of extracts Tsunokaori tangor fruits were collected from the Seogwipo area of Jeju Island, Korea. The dry powdered peels of ripe fruits were extracted three times with methanol (MeOH), and the combined extracts were concentrated to yield the MeOH extract. The MeOH extract was suspended in water and partitioned in the following order: n-hexane (hexane), ethyl acetate (EtOAc), n-butanol (BuOH), and water. The yields of the hexane, EtOAc, BuOH, and water fractions were 2.2, 5.3, 21.1, and 62.4% of the MeOH extract, respectively.

Chemicals Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco-BRL (Gaithersburg, MD, USA). The enzyme immunoassay (EIA) kit for PGE_2 was obtained from R&D Systems (Minneapolis, MN, USA). Monoclonal antibodies against iNOS, IκBa, phospho-IκBa, phospho-extracellular signal-regulated kinase (ERK) 1/2, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-COX-2 antibody was obtained from BD Transduction Laboratories (Lexington, KY, USA). Antibodies against phospho-c-jun N-terminal kinase (JNK), phospho-p38, p38, and ERK1/2 were obtained from Cell Signaling Technologies (Beverly, MA, USA). The iNOS, COX-2, and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea).

Cell culture The RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were grown at 37°C in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin sulfate (100 mg/mL) in a humidified atmosphere with 5% CO_2. The cells were incubated with the extract samples at various concentrations and stimulated with LPS (100 ng/mL) for 24 hr.

Assessment of cell viability To evaluate the cytotoxicity of the extracts on RAW 264.7 cells, the cultures were exposed to various concentrations of the test samples for 24 hr. Stock test solutions were prepared in 50% ethanol at 100 times the highest extract concentration tested and were serially diluted to the desired concentrations. For the control treatment, sister cultures were exposed to 0.5% ethanol, which had no effect on cell viability (data not shown). After each treatment, cell viability was assessed using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. In brief, MTT was added to each well at a final concentration of 0.5 mg/mL, the cells were incubated at 37°C for 3 hr, the medium was removed, and the formazan crystals produced in the wells were dissolved by the addition of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm using a Quant microplate spectrophotometer (Bio-TEK instruments Inc., Winooski, VT, USA). The concentration of each test sample that resulted in increased absorbance at 570 nm relative to the control in 50% of the cells (50% toxic concentration, TC_{50}) was determined from the dose-response curves. To validate the results of the MTT reduction assay, the activity of lactate dehydrogenase (LDH) released into the culture medium was also measured in some experiments according to a previously described method (24), and using the CytoTox®96 non-radioactive assay kit (Promega, Madison, WI, USA) which measures the conversion of tetrazolium salt into a red formazan product. The percentage of LDH released from the treated cells was calculated by comparing the observed amount of LDH released to the maximum amount released after the addition of 1% Triton X-100.

Determination of nitrite concentration The nitrite that accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction (25). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl], the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as a blank in all the experiments. The amount of nitrite in the samples was determined from a sodium nitrite standard curve. The concentration of each test sample that reduced the NO production by 50% with respect to the control (50% inhibition concentration, IC_{50}) was estimated. The selectivity index (SI) was determined as the ratio TC_{50}/(50% toxicity concentration) EC_{50}.

Determination of PGE_2 The PGE_2 concentration in the culture medium was quantified using a competitive enzyme immunoassay kit (R&D Systems) according to the manufacturer's instructions.

Western blot analysis Cellular proteins were extracted from the control and test sample-treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, and 0.5 mM Na orthovanadate) containing 5 μg/mL each of leupeptin and aprotinin, and the suspension was incubated for 60 min at 4°C. The cell debris was removed by microcentrifugation followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Samples containing equal amounts of proteins were separated by 8 or 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked with TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk for 1 hr at room temperature. The membranes were incubated overnight in primary antibodies diluted in TBST, then washed with TBST and incubated in horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature. The protein bands

were visualized using an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) RAW 264.7 cells were treated with 100 ng/mL LPS in the presence or absence of sample for 6 hr. Total RNA was purified from the cells using Tri reagent® (MRC, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μg of total RNA using the Improm-II™ reverse transcription kit (Promega). The synthetic gene-specific primer sets used for the PCR were: 1) iNOS forward 24-mer, 5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3' and reverse 24-mer, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'; 2) COX-2 forward 20-mer, 5'-CAC TAC ATC CTG ACC CAC TT-3' and reverse 20-mer, 5'-ATG CTC CTG CTT GAG TAT GT-3'; and 3) b-actin forward 22-mer, 5'-AGG CTG TGC TGT CCC TGT ATG C-3' and reverse 22-mer, 5'-ACC CAA GAA GGA AGG CTG GAA A-3'. The PCR consisted of 23 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 45 sec. The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and visualized by UV-induced fluorescence.

Statistical analysis The statistical analysis was performed using either the two-tailed Student's *t*-test or ANOVA for comparisons between two or multiple groups. Experimental results are expressed as the mean±SD.

Results and Discussion

Effects on cell viability We first evaluated the effects of the test samples on the viability of RAW 264.7 cells. The MeOH extract of the Tsunokaori tangor peel and its BuOH and water fractions did not affect cell viability at the concentrations used (31-1000 μg/mL), whereas the hexane fraction dramatically reduced cell viability in a concentration-dependent manner. The EtOAc fraction markedly reduced cell viability at higher concentrations (Fig. 1A). The calculated TC_{50} values of the hexane and EtOAc fractions for the RAW 264.7 cells were 82.1 and 477.8 μg/mL, respectively (Table 1).

Effects on LPS-induced NO production To determine the effects of the Tsunokaori tangor peel MeOH extract on LPS-induced NO production in RAW 264.7 cells, the cells were treated with LPS (100 ng/mL) in the presence or absence of the test samples for 24 hr. As shown in Fig. 1B, the MeOH extract and its hexane and EtOAc fractions showed a dose-dependent inhibitory effect on LPS-induced NO production. The calculated IC_{50} values of the MeOH

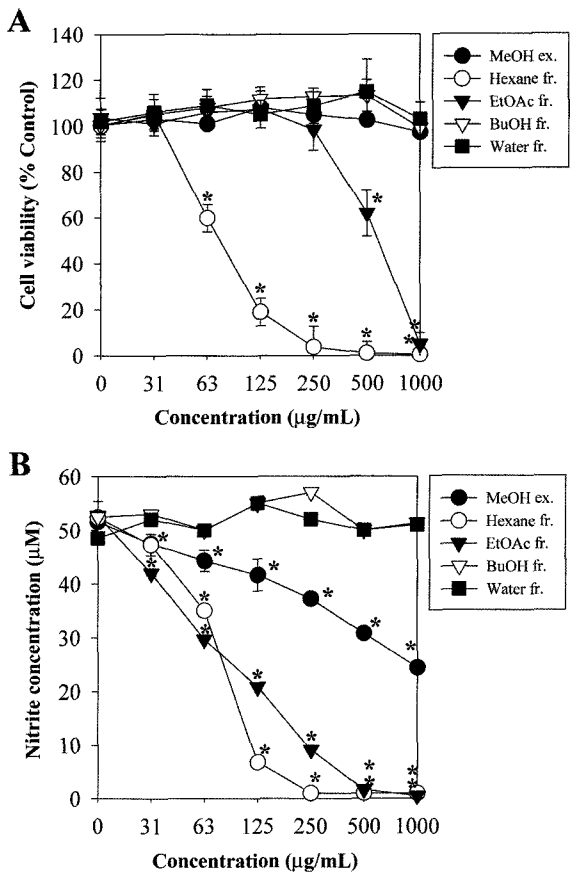

Fig. 1. Effects of the methanol extract of Tsunokaori tangor peel and its fractions on cell viability and LPS-induced NO production in RAW 264.7 cells. (A) Cells were cultured for 24 hr with the methanol extract of Tsunokaori tangor peel or its fractions at the concentrations shown. Data represent the mean±SD of triplicate experiments. *$p<0.05$ *vs.* cell viability in the absence of test samples. (B) Cells were incubated with LPS (100 ng/mL) with or without the methanol extract of Tsunokaori tangor peel or its fractions. Data represent the mean±SD of triplicate experiments. *$p<0.05$ vs. NO production in the absence of test samples.

Table 1. Cell toxicity of the methanol extract of Tsunokaori tangor peel and its fractions, and their effects on LPS-induced NO production in RAW 264.7 cells

	TC_{50}[1], μg/mL	IC_{50}[2], μg/mL	Selectivity Index[3]
MeOH extract	> 1000	756.7±3.8	> 1.32
Hexane fraction	82.1±1.6	77.8±1.4	1.06
EtOAc fraction	477.8±10.9	84.3±3.2	5.67
BuOH fraction	> 1000	> 1000	-
Water fraction	> 1000	> 1000	-

[1]TC_{50} is the concentration producing 50% toxicity in RAW 264.7 cells.
[2]IC_{50} is the concentration producing 50% inhibition of NO production in RAW 264.7 cells.
[3]Selectivity Index = TC_{50}/IC_{50}.

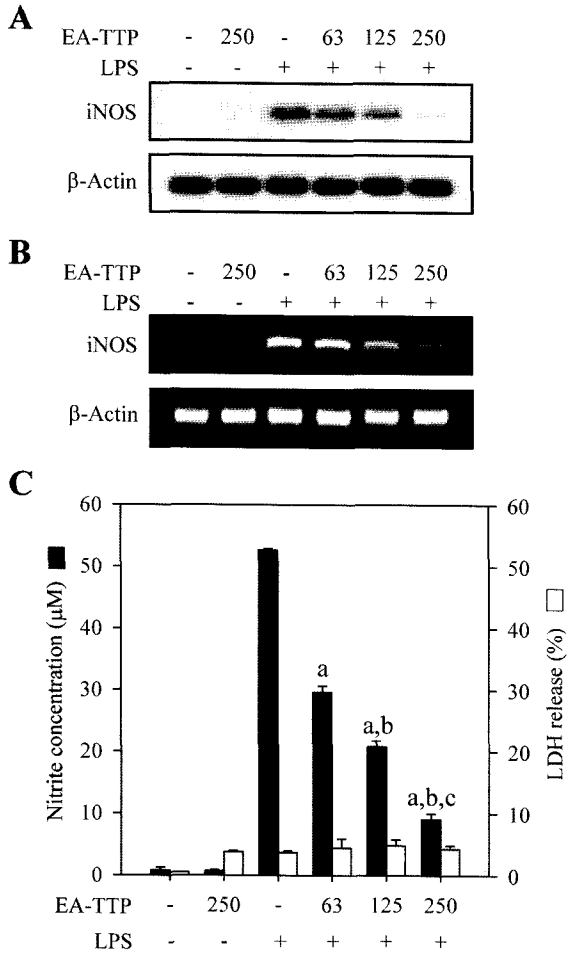

Fig. 2. Effects of the ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) on iNOS protein and mRNA expression and NO production in LPS-stimulated RAW 264.7 cells. (A) The protein levels (B) mRNA levels of iNOS and β-actin were determined by western and RT-PCR analyses, respectively. (C) The culture medium was harvested, and NO levels and LDH release were determined. The values are the means ± SD of triplicate experiments. a) $p< 0.05$ vs. the LPS alone-treated group, b) $p<0.05$ vs. the 63 μg/mL EA-TTP plus LPS-treated group, and c) $p<0.05$ vs. the 125 μg/mL EA-TTP plus LPS-treated group.

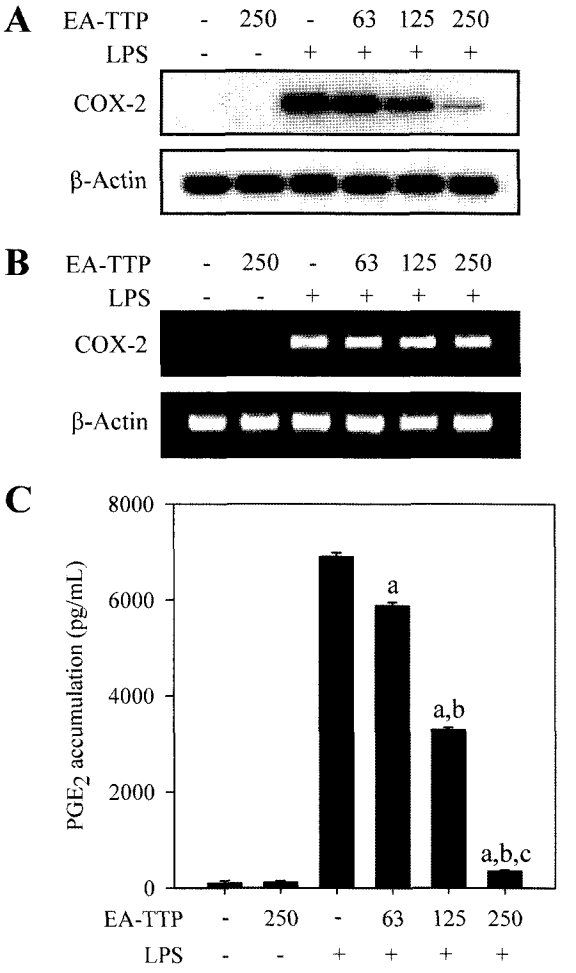

Fig. 3. Effects of the ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) on COX-2 protein and mRNA expression, and PGE_2 production in LPS-stimulated RAW 264.7 cells. (A) The protein levels and (B) mRNA levels of COX-2 and β-actin were determined by western and RT-PCR analyses, respectively. (C) The culture medium was harvested, and PGE_2 levels were determined. The values are the means ± SD of triplicate experiments. a) $p<0.05$ vs. the LPS alone-treated group, b) $p<0.05$ vs. the 63 μg/mL EA-TTP plus LPS-treated group, and c) $p<0.05$ vs. the 125 μg/mL EA-TTP plus LPS-treated group.

extract and its hexane and EtOAc fractions were 756.7, 77.8, and 84.3 μg/mL, respectively; the selectivity indices were > 1.3, 1.1, and 5.7, respectively (Table 1). The inhibitory effect of the hexane fraction was seemingly attributable to toxicity; therefore, we used the EtOAc fraction of the Tsunokaori tangor peel extract (EA-TTP), which displayed a comparable activity with much less toxicity, in the following experiments (Fig. 1).

Effects of EA-TTP on LPS-induced iNOS expression Western blot and RT-PCR analyses were performed to determine whether EA-TTP inhibited iNOS expression. In unstimulated RAW 264.7 cells, iNOS protein and mRNA were undetectable. However, the expression of iNOS protein was markedly augmented in response to LPS, and this augmentation was significantly inhibited by EA-TTP in a concentration-dependent manner (Fig. 2A). The RT-PCR analysis showed that the expression of iNOS mRNA correlated with its protein expression (Fig. 2B). The cytotoxicity of EA-TTP in the presence and absence of LPS was evaluated using the LDH assay. EA-TTP did not affect RAW 264.7 cell viability even at 250 μg/mL for 24 hr (Fig. 2C).

Effects of EA-TTP on LPS-induced PGE_2 synthesis and COX-2 expression The cells were treated with LPS for 24 hr in the absence or presence of EA-TTP to evaluate the effect of EA-TTP on LPS-induced PGE_2 production. As shown in Fig. 3C, the production of PGE_2 was significantly inhibited by EA-TTP in a concentration-dependent manner. The expression of COX-2 protein was inhibited in a similar manner (Fig. 3A). However, the level of COX-2 mRNA was not diminished by EA-TTP (Fig. 3B), suggesting that EA-TTP does not have an effect on the transcription and mRNA stability of the COX-2 gene. There is growing evidence that COX-2 inhibitors may be effective anti-inflammatory agents and useful in the prevention and treatment of colon

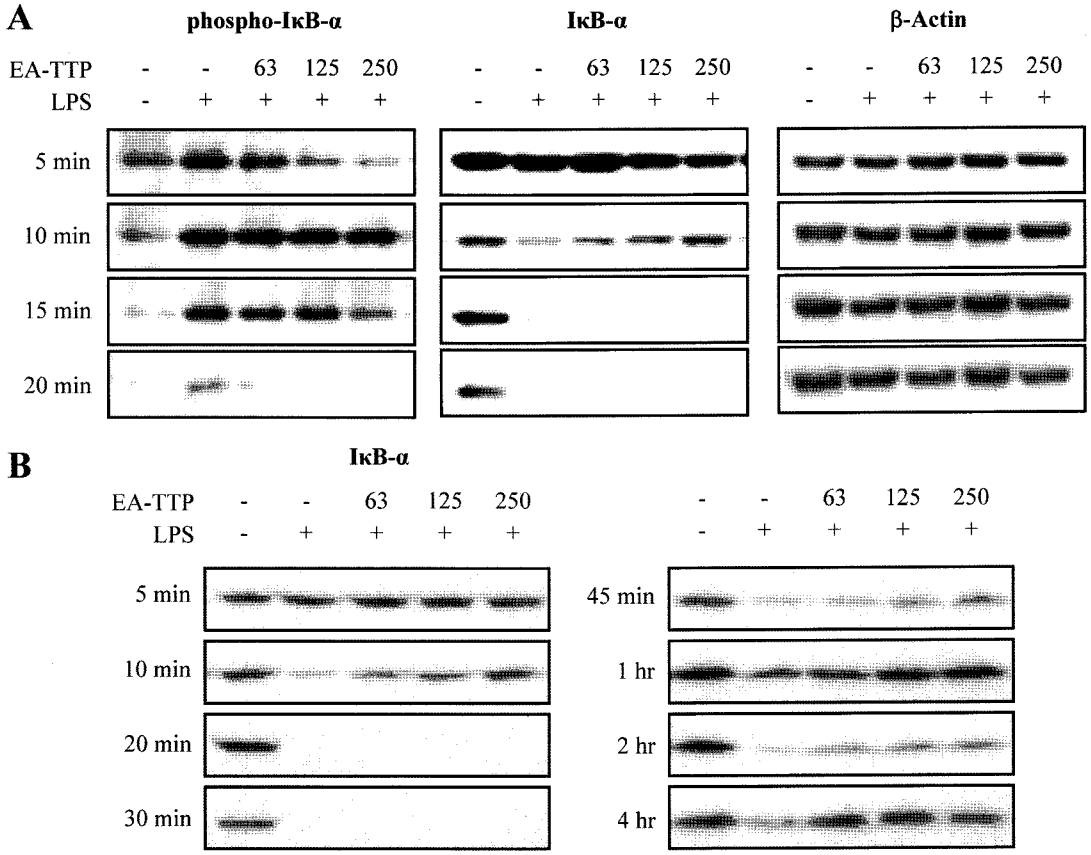

Fig. 4. Effects of the ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) on IκBα degradation and re-synthesis in LPS-stimulated RAW 264.7 cells. (A) Effects of EA-TTP on IκBα phosphorylation and degradation in LPS-stimulated RAW 264.7 cells. (B) Effects of EA-TTP on IκBα degradation and re-synthesis following degradation in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) alone or LPS plus the indicated concentrations (μg/mL) of EA-TTP for the indicated times. The protein levels of IκBα and phospho-IκBα were determined by Western analysis.

cancer (26). Furthermore, agents that interfere with the signaling mechanisms governing the expression of COX-2 may inhibit inflammation and tumorigenesis (27, 28).

Effects of EA-TTP on IκBα degradation and re-synthesis

We performed Western analyses to analyze the effects of EA-TTP on IκBα protein degradation and phosphorylation in LPS-stimulated RAW 264.7 cells. LPS induced the degradation of IκBα after 10 min, and EA-TTP treatment delayed the degradation (Fig. 4A). Western analysis of the LPS-induced phosphorylated form of IκBα revealed that LPS induced the phosphorylation of IκBα within 5 min and that EA-TTP dose-dependently suppressed IκBα phosphorylation (Fig. 4A). We next analyzed the effects of EA-TTP on the re-synthesis of IκBα following LPS-induced degradation. The IκBα protein level recovered to the basal level by 40-50 min after LPS-induced degradation, and EA-TTP enhanced the re-appearance of IκBα following its LPS-induced degradation (Fig. 4B) without producing a change in β-actin expression (data not shown). Aberrant NF-κB activity is a hallmark of chronic inflammatory diseases and it is known that activation of NF-κB in various cancers is a central anti-apoptotic event (29, 30). IκB phosphorylation alters NF-κB activation, thereby contributing to cancer and chronic inflammatory diseases. Constitutive IKK activity

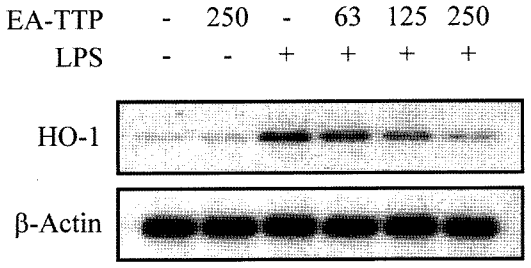

Fig. 5. Effects of the ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) on HO-1 protein expression in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) alone or LPS plus the indicated concentrations (μg/mL) of EA-TTP for 24 hr. The protein levels of HO-1 and β-actin were determined by western analysis.

has been described in inflammatory diseases (31) and in a variety of solid tumors (32). Therefore, the phosphorylation of IκB represents a promising target for the treatment of chronic inflammatory diseases and cancer. Selective IKK inhibitors, such as BMS-345541, have been shown to possess anti-inflammatory activity *in vivo*, making them potentially useful in the treatment of rheumatoid arthritis (33). Our data show that EA-TTP delays IκBα degradation,

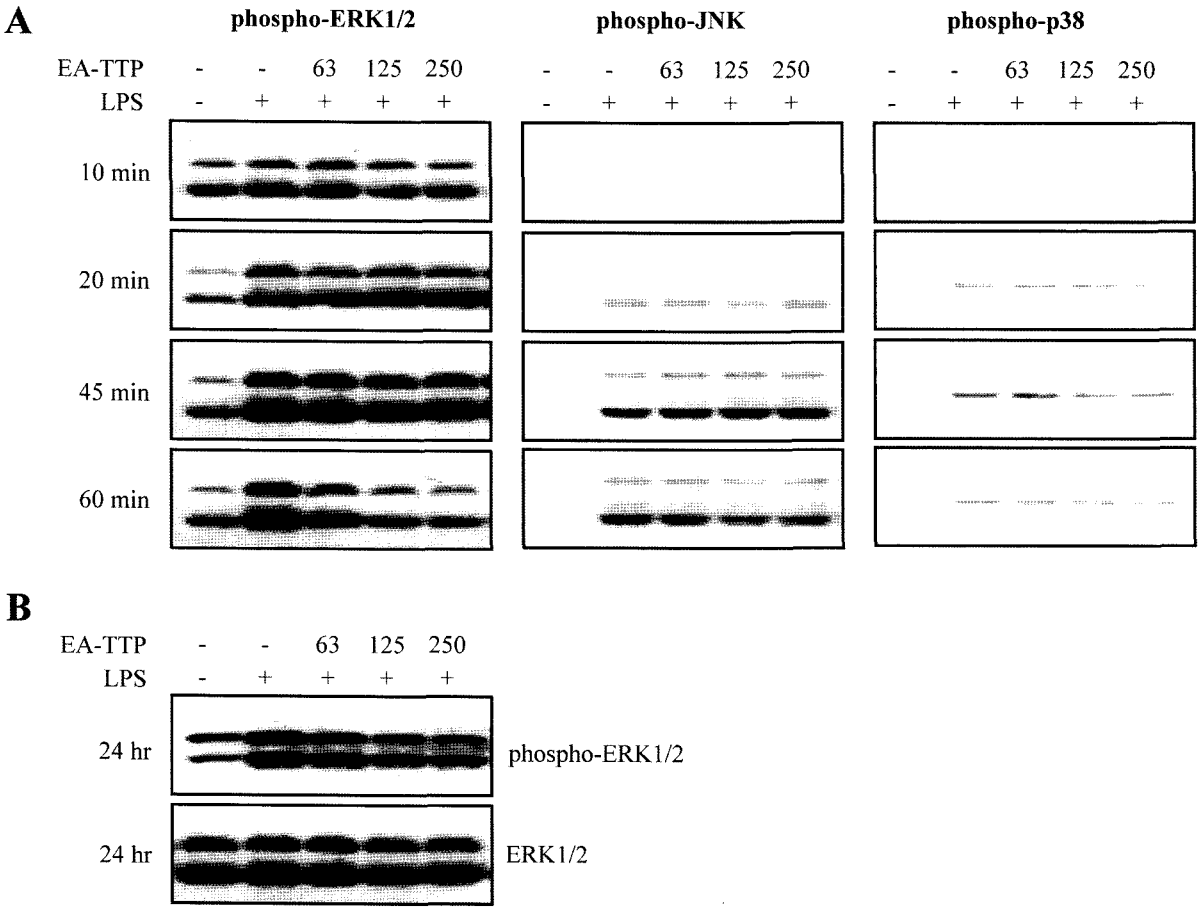

Fig. 6. Effects of the ethyl acetate fraction of Tsunokaori tangor peel (EA-TTP) on the phosphorylation of ERK, JNK, and p38 MAP kinases in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (100 ng/mL) alone or LPS plus the indicated concentrations (μg/mL) of EA-TTP for the indicated times. The protein levels of ERK, phospho-ERK, phospho-JNK, and phospho-p38 were determined by Western analysis.

probably through an inhibition of IKK activity. EA-TTP also facilitated the re-appearance of IκBα following LPS-induced degradation and prevented sequential rounds of IκBα degradation. This action may represent a crucial step in the anti-inflammatory effects of EA-TTP.

Effect of EA-TTP on LPS-induced HO-1 expression

Heme oxygenase-1 (HO-1) is an important cytoprotective enzyme and a widely accepted marker of oxidative stress. We used immunoblotting to determine whether EA-TTP decreases LPS-mediated HO-1 induction in RAW 264.7 cells. After treatment with 100 ng/mL LPS for 24 hr in RAW 264.7 cells, HO-1 protein expression increased remarkably versus the control. EA-TTP treatment dose-dependently decreased the LPS-induced HO-1 protein expression (Fig. 5). Cellular heme content is tightly regulated. In pathological conditions of increased hemoprotein turnover, it is important that low levels of free heme are maintained (34). As with the inducible proteins iNOS and COX-2, HO-1 protein expression was dose-dependently inhibited by EA-TTP in LPS-stimulated RAW 264.7 cells. This study is the first demonstration that citrus peel modulates LPS-induced HO-1 expression.

Effects of EA-TTP on LPS-induced MAPK activation

Given that EA-TTP significantly decreased iNOS, COX-2, and HO-1 protein expression in LPS-induced macrophages, we further investigated the signaling pathways affected by EA-TTP treatment. The activation of ERK, JNK, and p38 MAPK was measured as the relative amount of the phosphorylated proteins on western blots. As shown in Fig. 6A, LPS stimulation activated the ERK, JNK, and p38 MAPK signal pathways in RAW 264.7 cells. The activation became apparent at 20 min after LPS addition and was sustained for up to 1 hr. EA-TTP reduced ERK activation, but not JNK and p38 activation, at 1 hr following LPS stimulation (Fig. 6A). EA-TTP did not alter the total protein levels of ERK, JNK, and p38 (data not shown). Cells stimulated with 100 ng/mL LPS for 24 hr exhibited sustained activation of ERK for up to 24 hr. EA-TTP reduced this sustained ERK activation in a dose-dependent manner (Fig. 6B).

ERK plays a critical role in the regulation of cell growth and differentiation, and in the control of cellular responses to cytokines and stressors (35). EA-TTP dose-dependently reduced the LPS-induced sustained ERK activation in RAW 264.7 cells. Sustained ERK activation is required for the persistent activation of NF-κB by interleukin-1β (IL-1β) and subsequent iNOS expression in vascular smooth muscle cells. Two selective inhibitors of ERK activation,

PD98059 and U0126, did not influence the IL-1β-induced early activation of NF-κB, but did effectively reduce its prolonged activation and iNOS expression (36). These results suggest that EA-TTP may inhibit the inflammatory response through the inhibition of sustained ERK activation in LPS-stimulated RAW 264.7 cells. In conclusion, our study suggests that it may be possible to develop EA-TTP as a useful agent for the chemoprevention of cancer or inflammatory diseases. To develop chemopreventive agents based on EA-TTP, our ongoing study will focus on characterizing the downstream targets of IκBα and identifying the active ingredients in EA-TTP.

Acknowledgments

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