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Oolong Tea Theasinensins Attenuate Cyclooxygenase-2 Expression in Lipopolysaccharide (LPS)-Activated Mouse Macrophages: Structure−Activity Relationship and Molecular Mechanisms

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Oolong tea theasinensins are a group of tea polyphenols different from green tea catechins and black tea theaflavins. The present study reports the inhibitory effects of oolong tea theasinensins on the expression of cyclooxygenase-2 (COX-2) and underlying molecular mechanisms in lipopolysaccharide (LPS)-activated murine macrophage RAW264 cells. The structure−activity data revealed that the galloyl moiety of theasinensins played an important role in the inhibitory actions. Theasinensin A, a more potent inhibitor, caused a dose-dependent inhibition of mRNA, protein, and promoter activity of COX-2. An electrophoretic mobility shift assay (EMSA) revealed that theasinensin A reduced the complex of NF-κB− and AP-1−DNA in the promoter of COX-2. Signaling analysis demonstrated that theasinensin A attenuated IκB-α degradation, nuclear p65 accumulation, and c-Jun phosphorylation. Furthermore, theasinensin A suppressed the phosphorylation of MAPKs, IκB kinase α/β (IKKα/β), and TGF-β activated kinase (TAK1). These data demonstrated that the down-regulation of TAK1-mediated MAPKs and NF-κB signaling pathways might be involved in the inhibition of COX-2 expression by theasinensin A. These findings provide the first molecular basis for the anti-inflammatory properties of oolong tea theasinensins.

KEYWORDS: Oolong tea; theasinensins; cyclooxygenase-2; MAPK; NF-κB

INTRODUCTION

Cyclooxygenase (COX) is a rate-limiting enzyme that catalyzes the synthesis of prostaglandins from arachidonic acid. There are two isoforms of COX, designated COX-1 and COX-2, which are encoded by different genes (*1*, *2*). COX-1 is constitutively expressed in most tissues and believed to be responsible for normal physiological functions (*3*, *4*). In contrast, COX-2 is not detectable in most normal tissues or resting immune cells, but it is induced by bacterial lipopolysaccharide (LPS) (*2*), carcinogens (*3*), and inflammatory cytokines (*4*). Many types of cells associated with inflammation such as macrophages (*4*), endothelial cells, and fibroblasts, express COX-2 upon induction (*5*, *6*). It has been identified that AP-1 and NF-κB play a critical role in the regulation of COX-2 transcription (*5*, *7*). NF-κB is a transcription factor involved in many inflammatory responses including LPS- and cytokine-induced inflammation (*7*, *8*). AP-1 is essential for both basal and inducible COX-2 transcription (*9*, *10*).

LPS, a principal component of the outer membrane of Gram-negative bacteria, is a well-characterized inducer for COX-2 expression. Several lines of studies have indicated that LPS modulates toll-like receptor 4 (TLR4)-mediated signaling pathways by binding TLR4 in membrane (*11*, *12*). Although the molecular events are not fully understood in LPS-signaling, TGF-β activated kinase (TAK1) has been suggested to be an important factor for COX-2 expression (*13*). In unstimulated conditions, TAK1 binds TNF receptor-associated factor 6 (TRAF6) with TAK-binding protein (TAB) and forms a complex of TRAF6/TAK1/TAB1/TAB2 in membrane (*11*, *14*). Stimulation of cells with LPS results in the activation of TAK1, which subsequently activates the I-κ B kinase complex (IKK) and mitogen-activated protein (MAP) kinases, culminating in the activation of NF-κB and AP-1, respectively (*15*). NF-κB and MAPK further activate transcriptional factors of NF-κB and AP-1 to stimulate COX-2 expression (*2*, *9*).

Accumulated data indicate that COX-2 is involved in many inflammatory processes and is induced in various carcinomas, suggesting that COX-2 plays a key role in inflammation and tumorigenesis (*5*, *6*). Interestingly, some antioxidants with chemopreventive effects inhibit COX-2 expression by interfering with the signaling pathways that regulate COX-2 gene expression (*12*, *16*). Thus, the COX-2 gene has been used as a target to screen the cancer chemopreventive effects of phytochemicals. The identification of COX-2 inhibitor is considered to be a promising approach to protect against inflammation and tumorigenesis.

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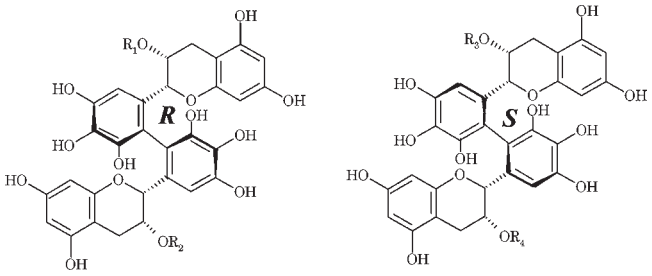

Theasinensin	R_1	R_2
A	G	G
B	G	H
C	H	H

Theasinensin	R_3	R_4
D	G	G
E	H	H

Figure 1. Chemical structures of theasinensins.

Through the fermentation during tea processing, tea polyphenols can be divided into two kinds (*17*, *18*). One is primary polyphenols present in green tea leaves without fermentation; the representative component is catechins such as (−)-epigallocatechin-gallate (EGCG). Another is secondary polyphenols, which are formed during the fermentation process. The secondary polyphenols are mainly present in black tea, and theaflavins are the representative components. Both catechins and theaflavins have been extensively studied on chemopreventive efficacy in multiple organs and have been considered to be potent compounds for chemoprevention (*19*, *20*). We have isolated a group of polyphenols, namely, theasinensins A−E, from oolong tea (*17*, *18*), which is consumed heavily in Asian and most Eastern countries. Oolong tea is a partially fermented tea between green tea and black tea according to the fermentation degree. Theasinensins are major secondary polyphenols formed during the partial fermentation processes. Some lines of studies have demonstrated that oolong tea theasinensin A showed antioxidative effects against lipid peroxidation (*21*), apoptosis induction in human U937 cells (*22*), and inhibitory effect on matrix metalloproteinases activities of human fibrosarcoma HT1080 cells (*23*). However, structure−activity relationships and the underlying mechanisms are not well understood. To clarify these points, five kinds of theasinesins were used to treat mouse macrophage RAW264 cells, which can be stimulated by LPS to mimic a state of inflammation. The inhibitory effects of COX-2 expression and underlying molecular mechanisms were then investigated in the present study. Structure−activity study indicated that theasinensins bearing galloyl moiety suppressed COX-2 expression. Molecular data further suggested that the down-regulation of TAK1-mediated MAPKs and NF-κB signaling pathways is involved in the inhibition of COX-2 expression by theasinensin A.

MATERIALS AND METHODS

Materials and Cell Culture. Theasinensins A−E (**Figure 1**) were isolated from oolong tea or synthesized as described in a former publication (*18*) and purified by HPLC with 99% purity. Their chemical structures were formerly identified according to their spectroscopic analyses (*18*). Theasinensins A−C have *R*-configuration, and theasinensins D and E have *S*-configuration in the biphenyl structure with different moieties at R_1, R_2, R_3, and R_4 (**Figure 1**). The final concentration of DMSO with theasinensin A was 0.2% in cell culture. LPS (*Escherichia coli* serotype 055: B5) was from Sigma (St. Louis, MO). Antibodies against phospho-ERK1/2, phospho-p38 kinase, phospho-c-Jun (Ser73), phospho-JNK, ERK1/2, p38 kinase, JNK, phospho-TAK1, phospho-IKKα/β, phospho-IκB-α, TAK1, and IκB-α were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against COX-2, COX-1, IKKα/β, α-tubulin, and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA).

Murine macrophage-like RAW264 cells were obtained from RIKEN BioResource Center Cell Bank (cell RCB0535), Tsukuba, Japan, and cultured at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

Measurement of Prostaglandin E_2 (PGE_2). PGE_2 in culture medium was measured with a PGE_2 enzyme immunoassay kit (Cayman Co., St. Louis, MO) according to manufacturer's manual (*24*). In brief, RAW264 cells (5×10^5 cells) were seeded into each well of 6-well plates. After incubation for 24 h, cells were starved by being cultured in serum-free medium for another 2.5 h to eliminate the influence of FBS. The cells were then treated with or without theasinensins for 30 min before exposure to 40 ng/mL LPS for 12 h. The level of PGE_2 released into culture medium was determined by measuring absorbance at 405 nm in a microplate reader.

Cell Survival Assay. A cell survival assay was performed as described previously (*24*). Briefly, RAW264 cells (2×10^4 cells/well) were plated into each well of 96-well microtiter plates. After incubation for 24 h, the cells were treated with 0−100 μM theasinensins for 16 h. MTT solution was then added to each well, and the cells were incubated for another 4 h. The resulting MTT−formazan product was dissolved by the addition of 100 μL of 0.04 N HCl−isopropanol. The amount of formazan was determined by measuring the absorbance at 595 nm in a microplate reader. The results were expressed as the optical density ratio of the treatment to control.

Plasmids and Transient Transfection Assay. The human COX-2 promoter-luciferase constructs (−327/+59) have been described previously (*9*, *25*). Transient transfection was performed according to the modified method as described previously (*24*). RAW264 cells (1×10^5) were plated into each well of 12-well plates and cultured for 24 h. The cells were then cotransfected with 0.5 μg of COX-2 promoter-luciferase plasmids and 0.12 μg of CMV-β-galactosidase plasmid, using Lipofect-AMINE2000 (Promega, Madison WI). After incubation for 5 h, the medium was changed with fresh medium and cultured for another 20.5 h. The cells were treated with or without theasinensin A (0−75 μM) for 30 min before exposure to 40 ng/mL LPS for 6 h. The activities of luciferase and β-galactosidase in cell lysate were measured in a luminometer (Berthold, Bad Wildbad, Gemany) according to the supplier's recommendations. Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and COX-2 transcription activity was expressed as fold induction relative to the control cells without LPS treatment.

RNA Extraction and RT-PCR. RNA extraction and RT-PCR were performed as described previously (*26*). Briefly, RAW264 cells (1×10^6) were precultured in a 6 cm dish for 24 h and then starved by being cultured in serum-free medium for another 2.5 h to eliminate the influence of FBS. The cells were treated with the concentration range of 0−75 μM theasinensin A for 30 min before exposure to 40 ng/mL LPS for 6 h. Cellular RNA was extracted with an ISOGEN RNA isolation kit (Nippon Gene, Tokyo, Japan) as described by the manufacturer. The oligonucleotide primers used to amplify mouse COX-2 were 5′-CAG CAA ATC CTT GCT GTT CC-3′ and 5′-TGG GCA AAG AAT GCA AAC ATC-3′ (*27*). The oligonucleotide primers used to amplify mouse COX-1 were 5′-ACT GGC TCT GGG AAT TTG TG-3′ and 5′-AGA GCC GCA GGT GAT ACT GT-3′ (*28*). The RT-PCR was done by one-step reaction with Read-to-Go RT-PCR beads (GE Healthcare, Buckinghamshire, U.K.) as described previously (*26*). Briefly, RNA (250 ng) was used for reverse transcription into cDNA at 42 °C for 30 min using oligo (dT) 12−18 primers. Amplifications were done at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s with a GenAmp PCR System 2400 machine (Perkin-Elmer, Boston, MA). Template and cycle dependences of the PCR products were confirmed, and the available cycle numbers of PCR for COX-2 and COX-1 were determined as 30 cycles, respectively. The PCR products were separated on 2% agarose gel, and digitally imaged after staining by ethidium bromide. The bands were quantified by Imager Gauge Software (Fuji Photo Film, Tokyo, Japan). The mRNA level in the control culture is arbitrarily set to 1.0, the basal level for subsequent mRNA comparisons.

Nuclear Protein Extraction. Nuclear extracts were prepared as described previously (*26*, *29*). Briefly, RAW264 cells (1×10^6) were cultured in a 6 cm dish for 24 h and then starved by being cultured in serum-free medium for another 2.5 h to eliminate the influence of FBS. The cells were treated with theasinensin A for 30 min before exposure to 40 ng/mL LPS for 4 h. Harvested cells were lysed by incubation in buffer A

(10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride) on ice for 15 min and then centrifuged at 13500*g* for 10 min at 4 °C. The nuclear pellets were resuspended in buffer B (20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM phenylmethanesulfonyl fluoride) for 15 min at 4 °C and then centrifuged at 13500*g* for 15 min at 4 °C. The supernatants containing nuclear extracts were stored at −80 °C until use.

Electrophoretic Mobility Shift Assay (EMSA). EMSA was performed as described previously (*30*). In brief, oligonucleotide probes were synthesized by Genenet Co., Ltd. (Fukuoka, Japan) and then annealed in TE buffer. Ten picomole probes were labeled with T4 polynucleotide kinase (TakaraBio Inc., Shiga, Japan) and (γ-^{32}P) ATP (5000 Ci/mmol; Amersham Biosciences, U.K.). The sequences of wild AP-1 probes were 5′-CGG AAA GAC AGA GTC ACC ACT AC-3′ and 5′-GTA GTG GTG ACT CTG TCT TTC CG-3′. The mutant AP-1 probes were 5′-CGG AAA GAC AGA GTT GCC ACT AC-3′ and 5′-GTA GTG GCA ACT CTG TCT TTC CG-3′. The sequences of wild NF-κB probes were 5′-GAG AGG TGA GGG GAT TCC CTT AGT TAG-3′ and 5′-CTA ACT AAG GGA ATC CCC TCA CCT CTC-3′. The mutant NF-κB probes were 5′-GAG AGG TGA GGG CCT TCC CTT AGT TAG-3′ and 5′-CTA ACT AAG GGA AGG CCC TCA CCT CTC-3′ (*31*). The labeled oligonucleotides were purified by Sephadex G-25 spin column (Amersham Biosciences, U.K.). Five micrograms of nuclear extract was incubated at 25 °C for 30 min with labeled or unlabeled competitor oligonucleotides in binding buffer (25 mM Tris-HCl, pH 7.5, 75 mM KCl, 0.3% Nonidet-40, 7.5% glycerol, 2.5 mM dithiothreitol, 1 mg/mL bovine serum albumin, and 1 μg of poly(dI)·poly(dC)). The products were separated at 4 °C on a 5% nondenaturing polyacrylamide gel in 0.5× Tris borate/EDTA buffer, and the gel was then dried on 3 MM chromatography paper. The paper was exposed to a radioactive imaging plate, and densitometry was then detected in an FLA-2000 machine (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Western Blotting. A Western blotting assay was performed as described previously (*26*). In brief, RAW264 cells (1×10^6) were cultured in a 6 cm dish for 24 h and then starved by being cultured in serum-free medium for another 2.5 h to eliminate the influence of FBS. The cells were treated with theasinensin A for 30 min before exposure to 40 ng or 1 μg/mL LPS for the different times. Cellular lysates were boiled for 5 min. Protein concentration was determined by dye-binding protein assay (Bio-Rad Hercules, Hercules, CA) according to the manufacturer's manual. Equal amounts of lysate protein (40 μg) were run on 10% SDS-PAGE and electrophoretically transferred to PVDF membrane (GE Healthcare, Buckinghamshire, U.K.). After blotting, the membrane was incubated with specific primary antibody overnight at 4 °C and further incubated for 1 h with HRP-conjugated secondary antibody. Bound antibodies were detected by ECL system with a Lumi Vision PRO machine (TAITEC Co., Saitama, Japan). The relative amount of proteins associated with specific antibody was quantified by Lumi Vision Imager software (TAITEC Co.).

Statistical Analyses. Differences among the treatments and the control were analyzed by ANOVA tests. A probability of $p < 0.05$ was considered to be statistically significant.

RESULTS

Theasinensins Suppress Production of COX-2 and PGE2 with a Structure−Activity Relationship. To investigate the effects of theasinensins on COX-2 and PGE_2 production, RAW264 cells were treated with 75 μM theasinensins A−E for 30 min, respectively, before exposure to 40 ng/mL LPS for 12 h. As shown in **Figure 2a**, LPS-induced COX-2 protein was significantly suppressed by theasinensins A, D, and B, but not by theasinensins E and C ($p < 0.05$) at 75 μM. The constitutive protein, COX-1, showed no change in such treatment. Moreover, theasinensins A, D, and B also suppressed LPS-induced PGE_2 production in the same fashion as COX-2 protein (**Figure 2b**). To determine and compare the potencies of theasinensins A, B, and D, we further examined their effects on COX-2 and PGE_2 at the lower concentration indicated. As shown in **Table 1**, theasinensins A and D suppressed significantly the production of both COX-2 and PGE_2 in the concentration range of 50−75 μM, whereas theasinensin B attenuated significantly the production of COX-2 and PGE_2 only at 75 μM. These results suggest the galloyl moiety at R_1, R_2, R_3, or R_4 played an important role in the inhibitory action. Theasinensins A and D, having two galloyl moieties, showed the strongest effect. Theasinensin B, having one galloyl moiety, showed an effect at higher dose, whereas theasinensins C and E, having no galloyl moiety, failed to show such an inhibitory effect in the same concentration range. To investigate whether the inhibition is due to the cytotoxicity of theasinensins, we investigated the viability of the cells treated by theasinensin A, which is the strongest inhibitor of these five compounds, by MTT assay. The results indicated that theasinensin A did not affect cell viability at the concentration range of 25−75 μM, whereas a 10.5% decrease of cell viability was observed in the cells treated by 100 μM (Supporting Information). Thus, the inhibitory effect of theasinensins on COX-2 and PGE_2 when used at < 75 μM was not caused by their cytotoxicity, suggesting that theasinensins A, D, and B might be potential inhibitors for COX-2 expression.

Theasinensin A Inhibits the Transcription Activity of COX-2 Gene. Because theasinensin A showed the strongest inhibition on COX-2 and PGE_2 production among five kinds of theasinensins, we used theasinensin A to investigate the molecular mechanism of inhibitory action in subsequent experiments. First, a dose-dependent inhibition of COX-2 protein was confirmed (**Figure 3a**). Then, we examined the levels of *COX-2* mRNA at the same concentration range by RT-PCR to determine whether the suppression of COX-2 protein by theasinensin A was due to transcriptional regulation. As shown in **Figure 3b**, theasinensin A suppressed *COX-2* mRNA expression in the same fashion, whereas *COX-1* showed no change in such treatment. We furthermore performed a promoter activity assay of *COX-2* gene with a core *COX-2* promoter-luciferase plasmid (−327/+59), which contains the *cis*-elements including NF-κB (−223/−214) and AP-1 (−67/−62) sites (*28*, *29*). As shown in **Figure 3c**, theasinensin A also suppressed *COX-2* promoter-driven luciferase activity in the same style as COX-2 mRNA and protein. Although CREB and C/EBP sites are also present in the promoter region of the *COX-2* gene, we observed that theasinensin A showed no inhibitory effect on the activation of both CREB and C/EBP in our preliminary works (Hou et al., unpublished data). These results suggest that theasinensin A might inhibit COX-2 expression by suppressing the activation of transcriptional factors including NF-κB and AP-1, which bind the *COX-2* promoter.

Theasinensin A Reduces the Bound Complex of AP-1− and NF-κB−DNA. Several lines of studies indicate that AP-1 and NF-κB are the principal transcriptional factors to regulate COX-2 expression stimulated by LPS or proinflammatory cytokines (*7*,*9*). To investigate whether theasinensin A suppressed the binding of AP-1 and NF-κB in their *cis*-elements of COX-2 promoter, EMSA assays were performed with the oligonucleotides of AP-1 and NF-κB binding sites. As indicated in **Figure 4**, LPS caused a notable increase in the complexes of AP-1−DNA (left) and NF-κB−DNA (right) (lane 2). Pretreatment with 50 μM theasinensin A reduced markedly the complexes of AP-1−DNA and NF-κB−DNA (lane 3) induced by LPS. Although theasinensin A alone decreased the endogenous complex of AP-1, but not NF-κB (lane 4), it was not due to the cytotoxicity because treatment with theasinensin A (50 μM, 16 h) did not affect cell viability as measured by MTT assay (Supporting Information). The specific interaction between DNA and AP-1 or NF-κB was demonstrated by competitive inhibition with excess unlabeled wild and mutant AP-1 or NF-κB oligonucleotides. Treatment with a 10-fold excess of unlabeled wild AP-1 or NF-κB oligonucleotides completely blocked the complex (**Figure 4**, lane C10) whereas treatment with

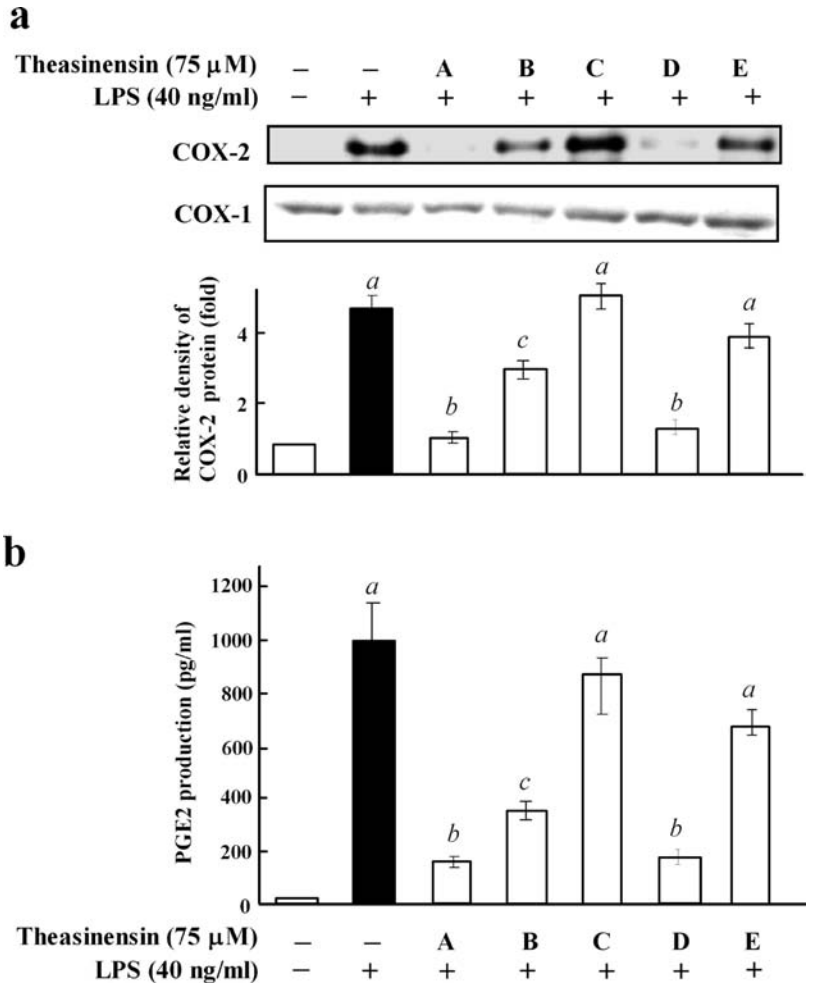

Figure 2. Effects of theasinensins on COX-2 (**a**) and PGE_2 production (**b**) in LPS-activated RAW264 cells. (**a**) COX-2 protein. RAW264 cells (1×10^6 cells) were pretreated with 75 μM concentrations of the indicated theasinensins for 30 min, respectively, and then exposed to 40 ng/mL LPS for 12 h. Western blotting was done as described under Materials and Methods. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1 protein. The data represent the mean ± SD of triplicate tests, and the picture is representative of those experiments, each with similar results. (**b**) PGE_2. Cell culture and treatment were done the same as for (**a**). The amount of PGE_2 in medium was measured as described under Materials and Methods. Values represent the mean ± SD of triplicate tests. Means with different letters differ at $p < 0.05$.

Table 1. Inhibition of Theasinesins on COX-2 and PGE_2 Production[a]

	0 μM	25 μM	50 μM	75 μM
	COX-2 (Fold Density)			
theasinensin A	4.3 ± 0.8 a	4.1 ± 0.7 a	2.2 ± 0.3 b	1.1 ± 0.1 c
theasinensin B	4.3 ± 0.7 a	4.2 ± 0.6 a	3.8 ± 0.4 a	2.1 ± 0.2 b
theasinensin D	4.3 ± 0.7 a	4.1 ± 0.5 a	2.3 ± 0.2 b	1.2 ± 0.2 c
	PGE_2 (pg/mL)			
theasinensin A	1010 ± 151 A	870 ± 70 A	450 ± 35 B	200 ± 18 C
theasinensin B	1010 ± 121 A	950 ± 78 A	860 ± 40 A	430 ± 20 B
theasinensin D	1010 ± 111 A	890 ± 80 A	460 ± 30 B	210 ± 16 C

[a] Means with different letters differ at $p < 0.05$.

a 10-fold excess of mutant AP-1 or NF-κB oligonucleotides did not block the complex (lane M10). These results indicated that theasinensin A might suppress expression of *COX-2* gene by blocking the complex of AP-1−DNA and NF-κB−DNA in COX-2 promoter.

Theasinensin A Suppresses c-Jun Phosphorylation, IκB Degradation, and Nuclear p65 Accumulation. To identify the effects of theasinensin A on these transcriptional factors, we pretreated RAW264 cells with 0−75 μM theasinensin A for 30 min before exposure to 40 ng/mL LPS and then detected these transcription factors using specific antibodies, respectively. As shown in **Figure 5a**, theasinensin A inhibited LPS-induced phosphorylation of c-Jun, which is a major component of AP-1 in c-Jun/c-Fos heterodimer form, in the concentration range of 50−75 μM.

NF-κB is inactivated in the cytosol associated with IκB and becomes active through translocation to the nucleus preceded by LPS-induced proteolytic degradation of IκB (*32*, *33*). Thus, we examined whether theasinensin A inhibits IκB-α degradation. In our previous study, we found that LPS caused IκB-α degradation at 15−45 min, which was then restored from 60 min (*26*). Thus, we pretreated RAW264 cells with 0−75 μM theasinensin A for 30 min and detected total IκB-α protein after exposure to 40 ng/mL LPS for another 30 min by Western blotting. As shown in **Figure 5b**, LPS significantly induced IκB-α degradation (lane 2). Pretreatment with 50 μM theasinensin A suppressed IκB-α degradation in a dose-dependent manner (lanes 3−5). These results demonstrated that theasinensin A might inhibit LPS-induced NF-κB activation by blocking IκB-α degradation. To confirm this, we further examined the nuclear p65, a part of the p65/p50 heterodimer, at the same time. In parallel with the result of IκB-α degradation, LPS markedly resulted in reduction of

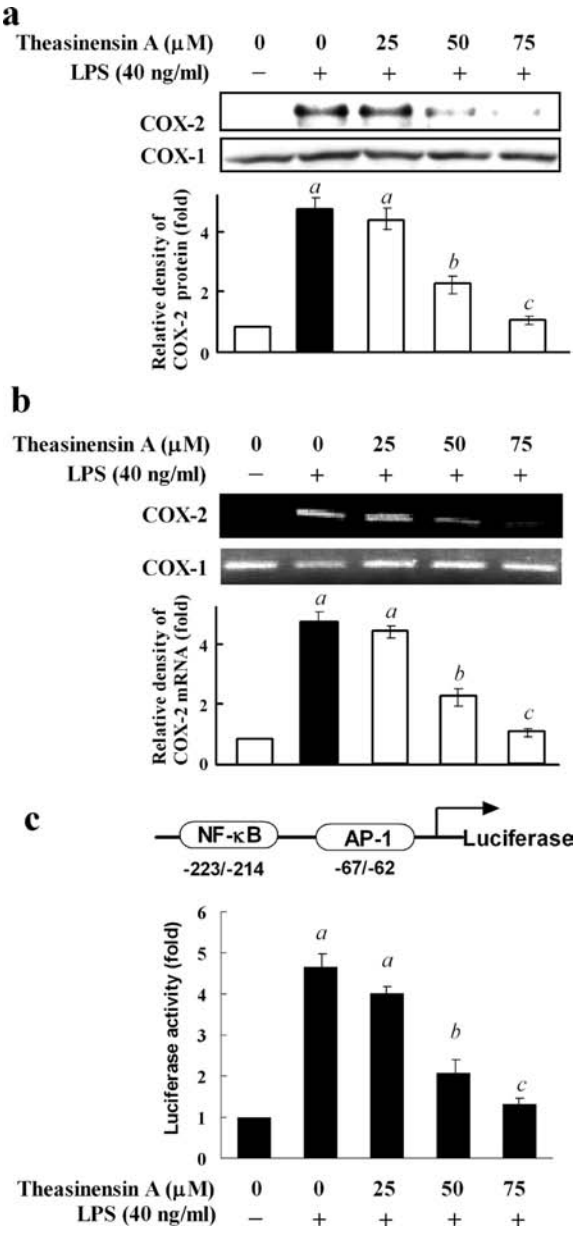

Figure 3. Theasinensin A causes a dose-dependent inhibition of COX-2 protein (**a**), mRNA (**b**), and promoter activity (**c**) in LPS-activated RAW264 cells. (**a**) COX-2 protein. RAW264 cells (1×10^6) were pretreated with 0−75 μM theasinensin A for 30 min and then exposed to 40 ng/mL LPS for 12 h. COX-2 and COX-1 were detected by Western blotting analysis with their antibodies, respectively. Histograms show the densitometric analysis of COX-2 protein normalized to COX-1. (**b**) COX-2 mRNA. RAW264 cells (1×10^6) were pretreated with 0−75 μM theasinensin A for 30 min and then exposed to 40 ng/mL LPS for 6 h. Cellular RNA extraction and RT-PCR were performed as described under Materials and Methods. Histograms show the densitometric analysis of COX-2 mRNA normalized to COX-1 mRNA. (**c**) COX-2 promoter activity. RAW264 cells (1×10^5) seeded on each well of a 12-well plate were transfected with 0.5 μg of COX-2 promoter (−327/+59)-luciferase reporter constructs and 0.12 μg of CMV-β-galactosidase plasmid. After 5 h of incubation, the medium was replaced with complete medium and cultured for another 20.5 h. The cells were then treated by 0−75 μM theasinensin A for 30 min before they were exposed to 40 ng/mL LPS for 6 h. The luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression, and COX-2 promoter activity is expressed as fold induction to the (−327/+59) construct without LPS treatment. The data represent mean ± SD of triplicate tests. Means with different letters differ at $p < 0.05$.

nuclear p65 after 30 min of treatment (**Figure 5c**, lane 2), and theasinensin A (50 μM) significantly reduced the nuclear level of p65 (**Figure 5c**, lanes 3−5). Lamin B was used as a control for nuclear protein.

Theasinensin A Inhibits MAPK Activation. Accumulated data have indicated that LPS causes the activation of MAPK including JNK, ERK1/2, and p38 kinase and subsequently activates the transcription factors with the attendant induction of COX-2 (*29*). Thus, we investigated the influence of theasinensin A on the activation of JNK, ERK1/2, and p38 kinase. As shown in **Figure 6**, theasinensin A caused a dose-dependent inhibition of LPS-induced phosphorylation of JNK, ERK1/2, and p38 kinase, suggesting that down-regulation of MAPK signaling pathways is involved in the inhibition of COX-2 expression by theasinensin A.

Theasinensin A Suppresses IKK and TAK1 Phosphorylation. Recent studies have shown that IκB phosphorylation is regulated by IκB kinases (IKKα and IKKβ) (*14*, *15*), and IKKα/β phosphorylation is further regulated by upstream TAK1 (*11*−*13*). Phosphorylated TAK1 can phosphorylate the IKK complex, which in turn stimulates IκB degradation. Thus, we investigated the effect of theasinensin A on IKKα/β phosphorylation and TAK1 activation. RAW264 cells were pretreated by 50 μM theasinensin A for 30 min and then treated with 40 ng/mL or 1 μg/mL LPS for 15 min. We found that treatment with 40 ng/mL LPS caused a low induction of IKKα/β and TAK1 (data not shown). To clarify the induction of IKKα/β and TAK1, we treated the cells with 1 μg/mL LPS as recommended previously (*31*). As shown in **Figure 7**, LPS induced remarkable phosphorylation of IKKα/β and TAK1 (lane 2). Treatment with theasinensin A significantly inhibited phosphorylation of IKKα/β and TAK1 without effect on total IKKα/β and TAK1 protein. The data indicated that the down-regulation of TAK1-mediated signaling pathways is involved in the inhibition of COX-2 expression by theasinensin A.

DISCUSSION

The chemopreventive effects of oolong tea theasinensins are poorly understood, although green tea and black tea polyphenols have been extensively studied with regard to their chemopreventive efficacy and molecular mechanisms (*19*, *20*). This study is the first report of the inhibitory effects of theasinensins on COX-2 and PGE_2 production and underlying molecular mechanisms.

As a result of structure−activity study, theasinensins A and D revealed strongest inhibition on both COX-2 expression and PGE_2 production among five kinds of theasinensins. Comparison of their structures suggests that the galloyl moiety may play an important role in the inhibitory action of theasinensins. Theasinensins A and D bearing two galloyl moieties showed strongest inhibitory effect, whereas theasinensins C and E bearing no galloyl moiety failed to show inhibitory effects under the same conditions. On the other hand, the configuration of theasinensins did not affect the inhibitory action because there was no difference in inhibitory effect between *S*- and *R*-forms bearing the same number of galloyl moieties. Several lines of studies have indicated that catechins bearing a galloyl moiety showed higher activity in many cases, such as growth inhibition of human lung cancer cell line (*34*), an inhibitory effect on the oxidative modification of low-density lipoprotein (*35*), antibacterial activity (*36*), and suppression of postprandial hypertriglyceridemia in rat (*37*). Recent studies have demonstrated that the presence of a galloyl moiety in catechins was responsible for their high affinity for lipid bilayers. Galloylated catechins revealed higher phospholipid/water partition coefficients when they were immersed in the phospholipid palisade intercalating within the hydrocarbon

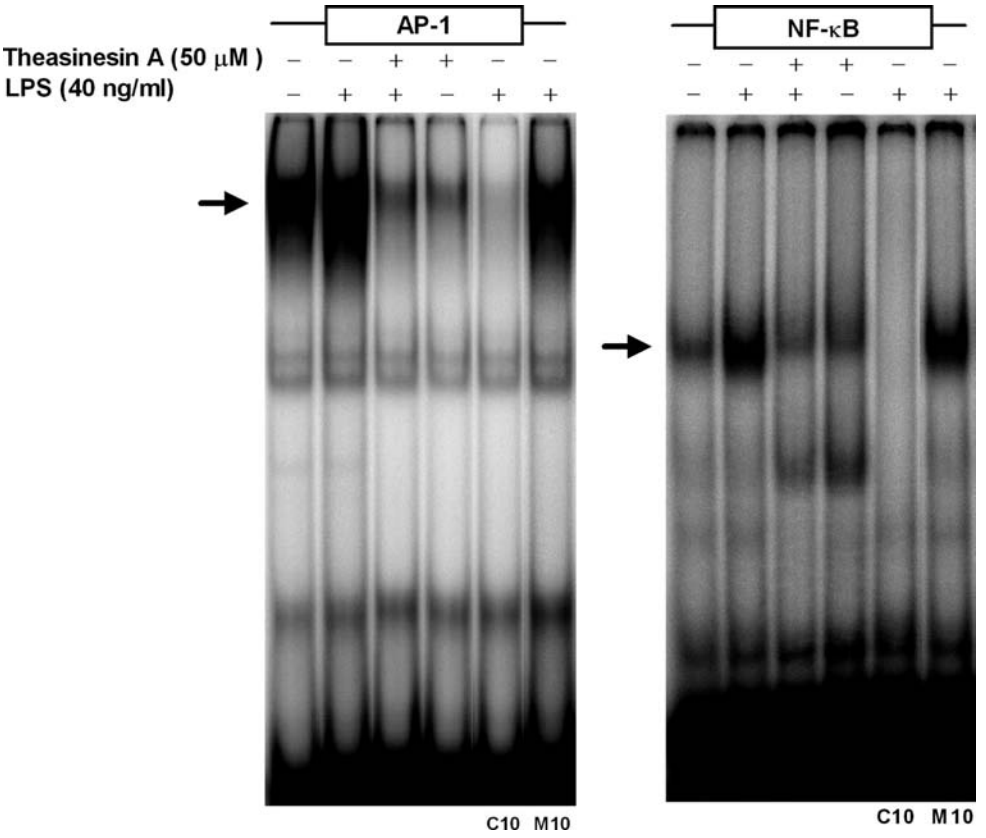

Figure 4. Theasinensin A reduces the complex of AP-1−DNA (left) and NF-κB−DNA (right). RAW264 cells was pretreated with or without 50 μM theasinensin A for 30 min and then exposed to 40 ng/mL LPS for another 30 min. Nuclear protein was extracted and then incubated with (γ-^{32}P)-labeled oligonucleotides of wild AP-1 and NF-κB (lanes 1−4) or with unlabeled oligonucleotides of 10-fold excess molar of wild AP-1 or NF-κB (lane C10) and mutant AP-1 or NF-κB (lane M10). After separation by 5% nondenaturing polyacrylamide gel, the gel was dried on 3 MM chromatography paper and exposed to a radioactive imaging plate. The signal was detected with an FLA-2000 machine. The shifted protein−DNA complexes are denoted with arrows.

chains. In contrast, nongalloylated catechins showed a shallow location close to the phospholipid/water interface (*38*). Moreover, the interaction of epigallocatechin gallate with lipid bilayers was directly demonstrated by solid-state nuclear magnetic resonance (*39*). Thus, tea polyphenols bearing a galloyl moiety can effectively affect cells based on the formation of membrane structures. According to these data, we presume that the higher inhibitory activity of theasinensins A and D on COX-2 expression may be associated with the higher affinity of its galloyl moiety to cellular membrane, although it is required to be proven in further works.

In our previous study, we also found that green tea proanthocyanidins could inhibit COX-2 expression in LPS-activated mouse macrophages at similar concentration ranges with theasinensin A. The results also revealed that the galloyl moiety of proanthocyanidins plays a critical role in their inhibitory action because prodelphinidin B_2 3,3′-di-*O*-gallate (PDGG), having two galloyl moieties, showed strongest effect, whereas prodelphinidin B_2 (PD), having no galloyl moiety, failed to show an inhibitory effect (*40*). However, EGCG, a major polyphenol of green tea, has been demonstrated to increase COX-2 expression in the same RAW264 macrophages (*41*, *42*). The reasons why tea EGCG has opposite effects from theasinesins and proanthocyanidins on COX-2 expression remain unclear. On the basis of its chemical structure, EGCG is a flavan-3-ol. Theasinensins and proanthocyanidins belong to condensed tannins; the building blocks of most theasinensins and proanthocyanidins are the flavan-3-ols (+)-catechin and (+)-epicatechin. It is worth identifying the structure−activity relationship in the inhibition of COX-2 expression between monomer and polymers of tea polyphenols in further study.

Although theasinensins D and E are isolated only from oolong tea, theasinensins A−C were also isolated from black tea (Hashimoto et al., unpublished data), and theasinensins A and B were also isolated in the fermented leaves of green tea (*17*). Moreover, theasinensins A−D, but not E, could be synthesized from EGCG and EGC by chemical reaction (*18*, *43*). Therefore, there is a wide potential resource for these functional theasinensins.

MAPKs including JNK, ERK1/2, and p38 have been reported to play a critical role in LPS-mediated COX-2 induction (*28*, *29*). JNK, ERK1/2, and p38 kinase can coregulate COX-2 transcription in LPS-treated macrophage/monocytic lineage (*24*, *26*). In the present study, oolong tea theasinensin A showed a dose-dependent inhibition on the activation of those three kinases (**Figure 6**) and AP-1 (**Figure 5a**), which is one of the transcriptional factors targeted by MAPK. EMSA data further revealed that theasinensin A inhibited the complex of AP-1−DNA present in the COX-2 promoter (**Figure 4**, left). These data suggest that the down-regulation of the MAPK signaling pathway was involved in the suppression of COX-2 by theasinensin A. Similar cases are also observed in other COX-2 inhibitors such as delphinidin (*26*), diarylheptanoid (*44*), and sesquiterpene lactones (*45*). They inhibit COX-2 expression by targeting MAPK signaling pathways.

NF-κB is another important player in the regulation of COX-2 expression induced by many cytokines and inflammatory products such as LPS (*5*, *7*, *31*). To clarify whether the NF-κB signaling pathway is involved in COX-2 expression inhibited by theasinensin A, we further investigated the effects of theasinensin A on NF-κB signaling. EMSA data revealed that theasinensin A

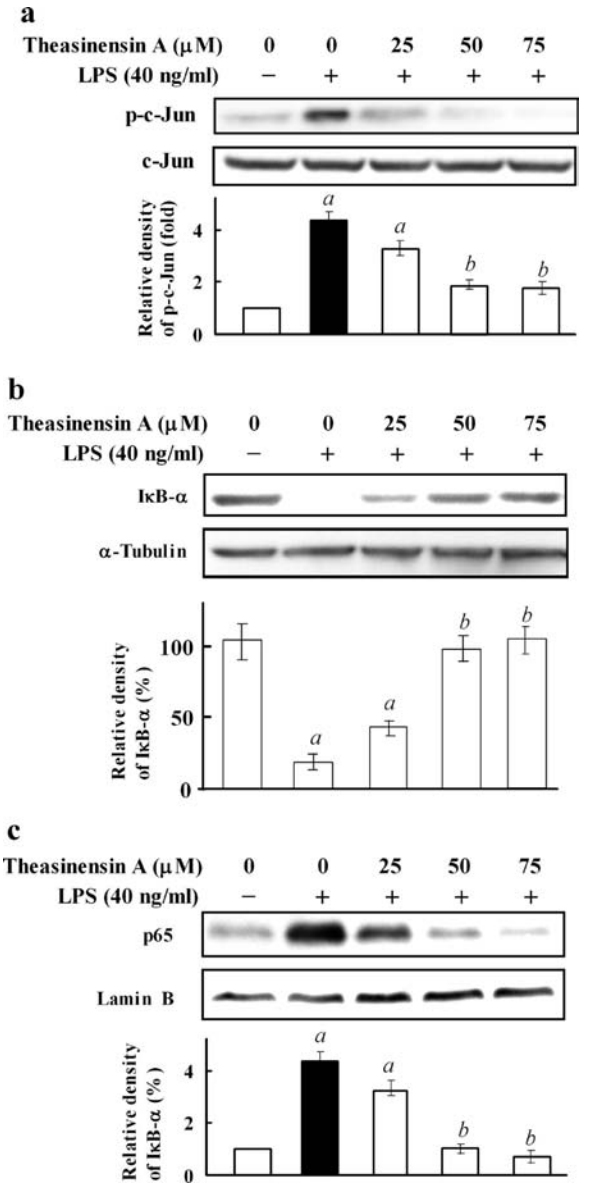

Figure 5. Effects of theasinensin A on the transcriptional factors binding to COX-2 promoter. (**a**) Phosphorylation of c-Jun. RAW264 cells were pretreated with 0−75 μM theasinensin A for 30 min and then exposed to 40 ng/mL LPS for 30 min. Phosphorylated c-Jun and total c-Jun were detected with their antibodies. Histograms show the densitometric analysis of phosphorylated c-Jun normalized to total c-Jun. (**b**) IκB-α degradation. RAW264 cells were treated with 0−75 μM theasinensin A for 30 min before exposure to 40 ng/mL LPS for 30 min. IκB-α protein were detected with its antibody. Histograms show the percentage of IκB-α protein to non-LPS treatment after normalization to α-tubulin. (**c**) Nuclear p65. Cell culture, treatment, and nuclear extraction were performed as for **Figure 4**. Nuclear p65 were detected with p65 antibody. Histograms show the densitometric analysis of p65 in nuclear lysates. Lamin B was used as a control of nuclear protein. The data represent the mean ± SD of triplicate tests, and the pictures are representative of those experiments, each with similar results. Means with different letters differ at $p < 0.05$.

reduced the complex of NF-κB−DNA in the COX-2 promoter (**Figure 4**, right). Western blotting results revealed that theasinensin A inhibited LPS-induced degradation of IκBα (**Figure 5b**) and subsequent reduction of nuclear p65 (**Figure 5c**). These data suggest that down-regulation of the NF-κB signaling pathway by theasinensin A might be due to the inhibition of IκBα degradation and reduction of nuclear p65.

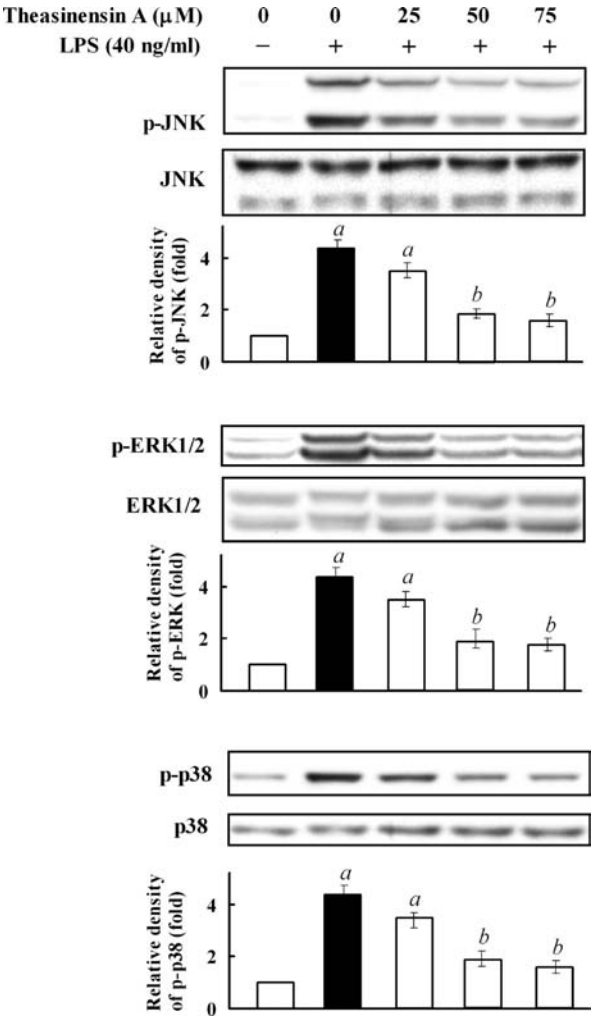

Figure 6. Theasinensin A suppresses the phosphorylation of JNK, ERK1/2, and p38 kinase. RAW264 cells were pretreated with 0−75 μM theasinensin A for 30 min and then exposed to 40 ng/mL LPS for 30 min. Total or phosphorylated JNK, ERK1/2, and p38 kinase were detected with their antibodies, respectively. Histograms show the densitometric analysis of phosphorylated kinase normalized to total kinase, respectively. The data represent the mean ± SD of triplicate tests, and the pictures are representative of those experiments, each with similar results. Means with different letters differ at $p < 0.05$.

Recent studies have suggested that IκB phosphorylation is regulated by both α and β isoforms of IKK (*14*, *15*), which are further regulated by upstream factors such as TAK1 (*13*). These kinases may represent novel sites for pharmacological intervention in a number of inflammatory conditions. Therefore, we examined the effects of theasinensin A on phosphorylation of IKK α/β and TAK1. In parallel with the inhibition of IκBα degradation, theasinensin A inhibited the phosphorylation of IKK α/β and TAK1 (**Figure 7**). Furthermore, TAK1 is also an upstream regulator of LPS-induced MAPK (*13*). Thus, theasinensin A might inhibit COX-2 expression through the suppression of TAK1-mediated NF-κB and MAPK pathways in LPS-activated RAW264 cells.

It is known that LPS modulates TLR4-mediated signaling pathways by binding TLR4 in membrane (*11*, *12*). Is it possible that theasinensin A binds the TLR4 competitively with LPS? For this, we treated RAW246 cells with FITC-conjugated LPS, cold LPS, and theasinensin A and then detected the binding by flow cytometric assay. Our results revealed that the addition of

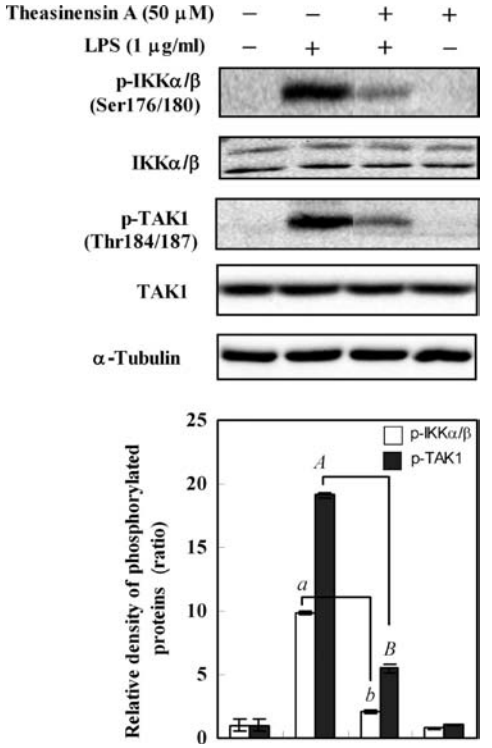

Figure 7. Theasinensin A suppresses the phosphorylation of IKKα/β and TAK1. RAW264 cells were pretreated with 50 μM theasinensin A for 30 min and then exposed to 1 μg/mL LPS for 10 min. p-IKKα/β, IKKα/β, p-TAK1, TAK1, and α-tubulin were detected with their antibodies, respectively. Histograms show the densitometric fold of phosphorylated IKKα/β or TAK1 to total IKKα/β or TAK1 normalized to α-tubulin, respectively. The data represent the mean ± SD of triplicate tests, and the pictures are representative of those experiments, each with similar results. Means with different letters differ at $p < 0.05$.

theasinensin A did not affect LPS−TLR4 binding. As a positive control, excess cold LPS reduced the signals of FITC-conjugated LPS binding to TLR4 (Hou et al., unpublished data). On the basis of our data and other information, theasinensin A did not bind TLR4 to suppressed COX-2 expression. The molecules between TLR4 and TAK1 signaling should be the targets by theasinensin A. TAK1 is, at least, one of them. Others are worthy of further investigation.

The effective concentrations of theasinensin A on the inhibition of COX-2 and PGE_2 used in this study were > 50 μM, which are similar to the effective concentrations of other polyphenols such as anthocyanidins (*26*) and tea proanthocyanidins (*31*) as reported previously. Accumulated data have shown that the effective concentrations of polyphenols in culture cells are, in general, quite higher than those measured in animal tissue or plasma. Although it is still hard to fill the gap between the two, some lines of studies have indicated that cells cultured under laboratory conditions of 95% air/5% CO_2 are in a state of hyperoxia, experiencing about 150 mmHg of O_2, whereas most cells in the human body are exposed to O_2 concentrations in the range of 1−10 mmHg (*46*). Polyphenols in higher O_2 concentration tend to be oxidized and broken down (*47*). This may partially explain why some polyphenols show cellular effects in culture cells with higher concentrations than in in vivo experiments. We will further clarify these facts in the case of theasinensins.

In summary, we showed data, for the first time, that oolong tea theasinensins bearing a galloyl moiety inhibited LPS-induced COX-2 expression in LPS-activated RAW264. The down-regulation of TAK1-mediated NF-κB and MAPK signaling pathways is demonstrated to be involved in the inhibition of COX-2 expression by theasinensins A. These data provide the primary molecular basis for the anti-inflammatory properties of oolong tea theasinensins.

ABBREVIATIONS USED

AP-1, activator protein-1; COX-2, cyclooxygenase-2; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; IKK, I-κB kinase; LPS, lipopolysaccharide; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PGE_2, prostaglandin E2; TAK1, TGF-β-activated kinase; TLR4, toll-like receptor.

Supporting Information Available: Effect of theasinensin A on cell survival. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Funk, C. D.; Funk, L. B.; Kennedy, M. E.; Pong, A. S.; Fitzgerald, G. A. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J.* **1991**, *5*, 2304−2312.

(2) Hempel, S. L.; Monick, M. M.; Hunninghake, G. W. Lipopolysaccharide induces prostaglandin H synthase-2 protein and mRNA in human alveolar macrophages and blood monocytes. *J. Clin. Invest.* **1994**, *93*, 391−396.

(3) Kelley, D. J.; Mestre, J. R.; Subbaramaiah, K.; Sacks, P. G.; Schantz, S. P.; Tanabe, T.; Inoue, H.; Ramonetti, J. T.; Dannenberg, A. J. Benzo(a)pyrene up-regulates cyclooxygenase-2 gene expression in oral epithelial cells. *Carcinogenesis* **1997**, *18*, 795−799.

(4) Mitchell, J. A.; Belvisi, M. G.; Akarasereenont, P.; Robbins, R. A.; Kwon, O. J.; Croxtall, J.; Barnes, P. J.; Vane, J. R. Induction of cyclooxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. *Br. J. Pharmacol.* **1994**, *113*, 1008−1014.

(5) Hla, T.; Ristimaki, A.; Appleby, S.; Barriocanal, J. G. Cyclooxygenase gene expression in inflammation and angiogenesis. *Ann. N.Y. Acad. Sci.* **1993**, *696*, 197−204.

(6) Mestre, J. R.; Chan, G.; Zhang, F.; Yang, E. K.; Sacks, P. G.; Boyle, J. O.; Shah, J. P.; Edelstein, D.; Subbaramaiah, K.; Dannenberg, A. J. Inhibition of cyclooxygenase-2 expression. An approach to preventing head and neck cancer. *Ann. N.Y. Acad. Sci.* **1999**, *889*, 62−71.

(7) D'Acquisto, F.; Iuvone, T.; Rombola, L.; Sautebin, L. D.; Rosa, M.; Carnuccio, R. Involvement of NF-κB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett.* **1997**, *418*, 175−178.

(8) Inoue, H.; Tanabe, T. Transcriptional role of the nuclear factor κB site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 143−148.

(9) Inoue, H.; Yokoyama, C.; Hara, S.; Tone, Y.; Tanabe, T. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. *J. Biol. Chem.* **1995**, *270*, 24965−24971.

(10) Subbaramaiah, K.; Chung, W. J.; Michaluart, P.; Telang, N.; Tanabe, T.; Inoue, H.; Jang, M.; Pezzuto, J. M.; Dannenberg, A. J. Resveratrol inhibits cyclooxygenase-2 transcription and activity in phorbol ester-treated human mammary epithelial cells. *J. Biol. Chem.* **1998**, *273*, 21875−21882.

(11) Doyle, S. L.; O'Neill, L. A. J. Toll-like receptors: from the discovery of NF-κB to new insights into transcriptional regulations in innate immunity. *Biochem. Pharmacol.* **2006**, *72*, 1102−1113.

(12) Gloire, G.; Legrand-Poels, S.; Piette, J. NF-κB activation by reactive oxygen species: fifteen years later. *Biochem. Pharmacol.* **2006**, *72*, 1493−1505.

(13) Pang, H. Y.; Liu, G.; Liu, G. T. Compound FLZ inhibits lipopolysaccharide-induced inflammatory effects via down-regulation of the TAK-IKK and TAK-JNK/p38 MAPK pathways in RAW264.7 macrophages. *Acta Pharmacol. Sin.* **2009**, *30*, 209−218.

(14) Wang, C.; Deng, L.; Hong, M.; Akkaraju, G. R.; Inoue, J.; Chen, Z. J. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* **2001**, *412*, 346−351.
(15) Adhikari, A.; Xu, M.; Chen, Z. J. Ubiquitin-mediated activation of TAK1 and IKK. *Oncogene* **2007**, *26*, 3214−3226.
(16) Chinery, R.; Beauchamp, R. D.; Shyr, Y.; Kirkland, S. C.; Coffey, R. J.; Morrow, J. D. Antioxidants reduce cyclooxygenase-2 expression, prostaglandin production, and proliferation in colorectal cancer cells. *Cancer Res*. **1998**, *58*, 2323−2327.
(17) Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. (1). *Chem. Pharm. Bull*. **1983**, *31*, 3906−3914.
(18) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. LXIX. Isolation and structure elucidation of B,B′-linked bisflavanoids, theasinensins D−G and oolongtheanin from oolong tea. (2). *Chem. Pharm. Bull*. **1988**, *36*, 1676−1684.
(19) Khan, N.; Afaq, F.; Saleem, M.; Ahmad, N.; Mukhtar, H. Targeting multiple signaling pathways by green tea polyphenol (−)-epigallocatechin-3- gallate. *Cancer Res*. **2006**, *66*, 2500−2505.
(20) Yang, C. S.; Lambert, J. D.; Hou, Z.; Ju, J.; Lu, G.; Hao, X. Molecular targets for the cancer preventive activity of tea polyphenols. *Mol. Carcinog*. **2006**, *45*, 431−435.
(21) Hashimoto, F.; Ono, M.; Masuoka, C.; Ito, Y.; Sakata, Y.; Shimizu, K.; Nonaka, G.; Nishioka, I.; Nohara, T. Evaluation of the antioxidative effect (*in vitro*) of tea polyphenols. *Biosci., Biotechnol., Biochem*. **2003**, *67*, 396−401.
(22) Pan, M. H.; Liang, Y. C.; Lin-Shiau, S. Y.; Zhu, N. Q.; Ho, C. T.; Lin, J. K. Induction of apoptosis by the oolong tea polyphenol theasinensin A through cytochrome *c* release and activation of caspase-9 and caspase-3 in human U937 cells. *J. Agric. Food Chem*. **2000**, *48*, 6337−6346.
(23) Maeda-Yamamoto, M.; Kawahara, H.; Tahara, N.; Tsuji, K.; Hara, Y.; Isemura, M. Effects of tea polyphenols on the invasion and matrix metalloproteinases activities of human fibrosarcoma HT1080 cells. *J. Agric. Food Chem*. **1999**, *47*, 2350−2354.
(24) Uto, T.; Hou, D. X.; Fujii, M. Inhibition of lipopolysaccharideinduced cyclooxygenase-2 transcription by 6-(methylsulfinyl) hexyl isothiocyanate, a chemopreventive compound from *Wasabia japonica* (Miq.) Matsumura, in mouse macrophages. *Biochem. Pharmacol*. **2005**, *70*, 1772−1784.
(25) Kang, Y. J.; Wingerd, B. A.; Arakawa, T.; Smith, W. L. Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. *J. Immunol*. **2006**, *177*, 8111−8122.
(26) Hou, D. X.; Yanagita, T.; Uto, T.; Masuzaki, S.; Fujii, M. Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: structure−activity relationship and molecular mechanisms involved. *Biochem. Pharmacol*. **2005**, *70*, 417−425.
(27) Sun, L. K.; Beck-Schimmer, B.; Oertli, B.; Wuthrich, R. P. Hyaluronaninduce cyclooxygenase-2 expression promotes thromboxane A2 production by renal cells. *Kidney Int*. **2001**, *59*, 190−196.
(28) Chen, J. C.; Huang, K. C.; Wingerd, B.; Wu, W. T.; Lin, W. W. HMG-CoA reductase inhibitors induce COX-2 gene expression in murine macrophages: role of MAPK cascades and promoter elements for CREB and C/EBPβ. *Exp. Cell Res*. **2004**, *301*, 305−319.
(29) Mestre, J. R.; Mackrell, P. J.; Rivadeneira, D. E.; Stapleton, P. P.; Tanabe, T.; Daly, J. M. Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophage/monocytic cells. *J. Biol. Chem*. **2001**, *276*, 3977−3982.
(30) Akimaru, H.; Hou, D. X.; Ishii, S. Drosophila CBP is required for dorsal-dependent twist gene expression. *Nat. Genet*. **1997**, *17*, 211−214.
(31) Hou, D. X.; Luo, D.; Tanigawa, S.; Hashimoto, F.; Uto, T.; Masuzaki, S.; Fujii, M.; Sakata, Y. Prodelphinidin B-4 3′-*O*-gallate, a tea polyphenol, is involved in the inhibition of COX-2 and iNOS via the downregulation of TAK1-NF-κB pathway. *Biochem. Pharmacol*. **2007**, *74*, 742−751.
(32) Baeuerle, P. A.; Baltimore, D. IκB: a specific inhibitor of the NFκB transcription factor. *Science* **1988**, *242*, 540−546.
(33) Zandi, E.; Rothwarf, D. M.; Delhase, M.; Hayakawa, M.; Karin, M. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NFκB activation. *Cell* **1997**, *91*, 243−252.
(34) Okabe, S.; Suganuma, M.; Hayashi, M.; Sueoka, E.; Komori, A.; Fujiki, H. Mechanisms of growth inhibition of human lung cancer cell line, PC-9, by tea polyphenols. *Jpn. J. Cancer Res*. **1997**, *88*, 639−643.
(35) Miura, S.; Watanabe, J.; Tomita, T.; Sano, M.; Tomita, I. The inhibitory effects of tea polyphenols (flavan-3-ol derivatives) on Cu^{2+} mediated oxidative modification of low density lipoprotein. *Biol. Pharm. Bull*. **1994**, *17*, 1567−1572.
(36) Kajiya, K.; Hojo, H.; Suzuki, M.; Nanjo, F.; Kumazawa, S.; Nakayama, T. Relationship between antibacterial activity of (+)-catechin derivatives and their interaction with a model membrane. *J. Agric. Food Chem*. **2004**, *52*, 1514−1519.
(37) Suzuki, Y.; Unno, T.; Kobayashi, M.; Nozawa, A.; Sagesaka, Y.; Kakuda, T. Dose-dependent suppression of tea catechins with a galloyl moiety on postprandial hypertriglyceridemia in rats. *Biosci., Biotechnol., Biochem*. **2005**, *69*, 1288−1291.
(38) Caturla, N.; Vera-Samper, E.; Villalain, J.; Mateo, C. R.; Micol, V. The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biol. Med*. **2003**, *34*, 648−662.
(39) Kumazawa, S.; Kajiya, K.; Naito, A.; Saito, H.; Tuzi, S.; Tanio, M.; Suzuki, M.; Nanjo, F.; Suzuki, E.; Nakayama, T. Direct evidence of interaction of a green tea polyphenol, epigallocatechin gallate, with lipid bilayers by solid-state nuclear magnetic resonance. *Biosci., Biotechnol., Biochem*. **2004**, *68*, 1743−1747.
(40) Hou, D. X.; Masuzaki, S.; Hashimoto, F.; Uto, T.; Tanigawa, S.; Fujii, M.; Sakata, Y. Green tea proanthocyanidins inhibit cyclooxygenase-2 expression in LPS-activated mouse macrophages: molecular mechanisms and structure−activity relationship. *Arch. Biochem. Biophys*. **2007**, *460*, 67−74.
(41) Park, J. W.; Choi, Y. J.; Suh, S. I.; Kwon, T. K. Involvement of ERK and protein tyrosine phosphatase signaling pathways in EGCG-induced cyclooxygenase-2 expression in RAW 264.7 cells. *Biochem. Biophy. Res. Commun*. **2001**, *286*, 721−725.
(42) Murakami, A.; Takahashi, D.; Hagihara, K.; Koshimizu, K.; Ohigashi, H. Combinatorial effects of nonsteroidal anti-inflammatory drugs and food constituents on production of prostaglandin E2 and tumor necrosis factor-α in RAW264.7 murine macrophages. *Biosci., Biotechnol., Biochem*. **2003**, *67*, 1056−1062.
(43) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. CXIV. Structures of novel fermentation products, theogallinin, theaflavonin and desgalloyl theaflavonin from black tea, and changes of tea leaf polyphenols during fermentation. *Chem. Pharm. Bull*. **1992**, *40*, 1383−1389.
(44) Yadav, P. N.; Liu, Z.; Rafi, M. M. A diarylheptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, p44/42, and transcription factor nuclear factor-κ B. *J. Pharmacol. Exp. Ther*. **2003**, *305*, 925−931.
(45) Hwang, D.; Fischer, N. H.; Jang, B. C.; Tak, H.; Kim, J. K.; Lee, W. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases. *Biochem. Biophys. Res. Commun*. **1996**, *226*, 810−818.
(46) Tang, S. Y.; Halliwell, B. Medicinal plants and antioxidants: what do we learn from cell culture and *Caenorhabditis elegans* studies? *Biochem. Biophys. Res. Commun*. **2010**, *394*, 1−5.
(47) Halliwell, B. Oxidative stress in cell culture: an under-appreciated problem? *FEBS Lett*. **2003**, *540*, 3−6.

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