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Modulation of arachidonic acid metabolism and nitric oxide synthesis by garcinol and its derivatives

Jungil Hong[1], Shengmin Sang[1], Hye-Jin Park[1], Seok Joo Kwon[1], Nanjoo Suh[1], Mou-Tuan Huang[1], Chi-Tang Ho[2] and Chung S.Yang[1,*]

[1]Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA and [2]Department of Food Science, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901, USA

*To whom correspondence should be addressed at: Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854-8020, USA. Tel: +1 732 445 5360; Fax: +1 732 445 0687; Email: csyang@rci.rutgers.edu

Garcinol, a polyisoprenylated benzophenone, from the fruit rind of *Garcinia* spp., has been shown to have anti-inflammatory and anticarcinogenic activities. To study its mechanism of action, we analyzed the effects of garcinol and its derivatives, including cambogin, garcim-1 and garcim-2, on arachidonic acid metabolism and nitric oxide (NO) synthesis in lipopolysaccharide (LPS)-stimulated RAW264.7 murine macrophages as well as in three intestinal cell lines. We also examined the effect of garcinol on cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), and related upstream signaling. At 1 μM, garcinol and its derivatives, added 1 h after LPS stimulation, significantly inhibited the release of arachidonic acid and its metabolites in macrophages; garcinol was the most effective, showing >50% inhibition. Similar inhibitory activity was also observed in intestinal cells, HT-29, HCT-116 and IEC-6 cells, showing 40–50% inhibition by 1 μM garcinol. In LPS-stimulated macrophages, garcinol inhibited the phosphorylation of $cPLA_2$ without altering its protein level, and the effect was related to the inhibition of ERK1/2 phosphorylation. Garcinol inhibited NFκB activation and COX-2 expression only when it was added to the cells before LPS stimulation. Garcinol (1 μM) also significantly decreased iNOS expression and NO release from LPS-stimulated macrophages; this is probably due to the inhibition of the signal transducer and activator of transcription-1 (STAT-1), an upstream event in the activation of iNOS synthesis. The results suggest that garcinol modulates arachidonic acid metabolism by blocking the phosphorylation of $cPLA_2$ and decreases iNOS protein level by inhibiting STAT-1 activation. These activities may contribute to the anti-inflammatory and anticarcinogenic actions of garcinol and its derivatives.

Abbreviations: COX, cyclooxygenase; $cPLA_2$, cytosolic PLA_2; ERK, extracellular signal related kinase; IκB, inhibitior of κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; LPS, lipopolysaccharides; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; PGE_2, prostaglandin E_2; NFκB, nuclear factor-κB; NO, nitric oxide; PLA_2, phospholipase A_2; STAT, signal transducer and activator of transcription.

Introduction

Garcinol, a polyisoprenylated benzophenone derivative, is found in Guttiferae plants (*Garcinia indica*, *Garcinia huillkensis* and *Garcinia cambogia*, etc.), which are shrubs native to India and South East Asia (1,2). Garcinol is one of the major constituents of an extract of rind of *G.indica*, known as Kokum. The extract has been used as a food ingredient, garnish and cosmetic constituent, as well as a traditional medicine for the treatment of inflammation and other disorders (1). Garcinol is structurally related to curcumin, a food coloring and flavoring agent, in that both contain phenolic hydroxyl groups and β-diketone moieties. Recent studies demonstrated the preventive effects of garcinol against azoxymethane-induced colon carcinogenesis and 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in models of rats (3,4). Garcinol also showed interesting biological activities in cell culture studies, including induction of apoptosis, suppression of COX-2 and inducible nitric oxide synthase (iNOS) expression, and inhibition of proteasome protease activity (5–7).

Aberrant arachidonic acid metabolism and nitric oxide (NO) synthesis are involved in inflammation and carcinogenesis. Arachidonic acid is released by phospholipase A_2 (PLA_2) from membrane phospholipids, and the released arachdionic acid is further metabolized by cyclooxygenases (COX), lipoxygenases (LOX) and cytochrome P450 in three different pathways. Modulation of arachidonic acid metabolism by inhibiting COX and LOX has been considered an effective approach for treating inflammation and for cancer chemoprevention (8–10). NO is involved in various physiological processes, including vasodilation, inhibition of platelet function, synaptic neurotransmission as well as host defense (11). The formation of NO from arginine is catalyzed by three different types of NO synthase (NOS): endothelial NOS, neuronal NOS and iNOS (12,13). iNOS is the enzyme stimulated by inflammatory cytokines for NO production in macrophages and many other cell types.

Previously, we studied the autoxidation mechanisms of garcinol, and three major oxidative products, cambogin, garcim-1 and garcim-2, were isolated (14,15). Their effects on iNOS and COX-2 expression in lipopolysaccharide (LPS)-stimulated macrophages were also evaluated (6). Although garcinol and its derivatives showed potent anti-inflammatory effects, the underlying mechanisms concerning arachidonic acid metabolism and iNOS expression are poorly understood. In the present study, the mechanisms by which physiologically relevant concentrations of garcinol modulate arachidonic acid metabolism and NO synthesis were investigated. Our results suggest that garcinol and its derivatives modulate arachidonic acid metabolism by retarding the phosphorylation of cytosolic PLA_2

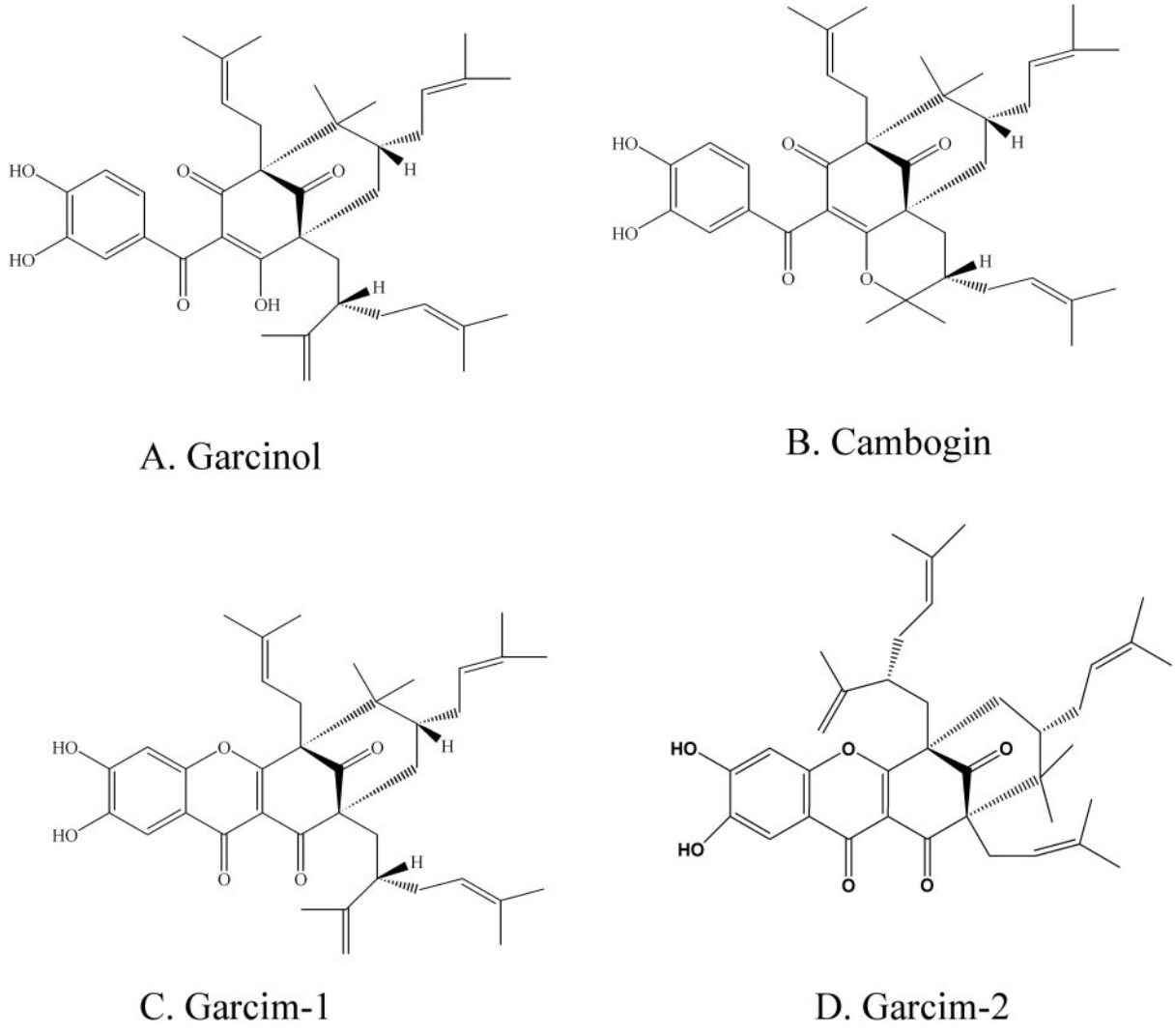

Fig. 1. (**A–D**) Structures of garcinol and its derivatives used in the present study. The structures of three garcinol derivatives were identified previously (14,15). The previously reported GDPPH-1 and GDPPH-2 are renamed as garcim-1 and garcim-2, respectively, in our laboratory.

($cPLA_2$) through the inhibition of extracellular signal related kinase ERK1/2 activation and suppressing iNOS expression through modulation of the Janus kinase (JAK)/STAT-1 signaling pathway.

Materials and methods

Chemicals and cell lines

[5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid was purchased from NEN Life Science (Boston, MA). $cPLA_2$, COX-2 and iNOS antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies of phospho-$cPLA_2$ (Ser^{505}), inhibitor of κB (IκB), ERK1/2, phospho-ERK1/2, signal transducer and activator of transcription-1 (STAT-1) and phospho-STAT-1 were from Cell Signaling Technology (Beverly, MA). U0126, PD98059 and Bay11-7082 were from Calbiochem (La Jolla, CA). Garcinol and its derivatives were prepared by a previous method (14,15). The purity of the compounds was determined to be >95% by using high performance liquid chromatography (HPLC). Structures of these compounds are shown in Figure 1. All the other chemicals were purchased from Sigma Chemical Co. (St Louis, MO). Murine macrophage RAW264.7 and other intestinal cell lines were obtained from American Type Culture Collection (Rockville, MD). RAW 264.7 macrophage cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and sodium bicarbonate supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37°C in 95% humidity and 5% CO_2. Other intestinal cells were maintained in recommended media by American Type Culture Collection.

Release of arachidonic acid and its metabolites and release of NO in intact cell system

The release of arachidonic acid and its metabolites from RAW264.7 cells was analyzed by a previously described method (16). For analyzing their release from intestinal cells, the cells were plated into a 24-well plate at $\sim 2.0 \times 10^5$ cells per well in the growth media. After 24 h, the media were removed and replaced with 1 ml of serum free media containing [5,6,8,9,11,12,14,15-^{3}H](N) arachidonic acid (0.1 μCi/ml) overnight. The cells were then washed twice with phosphate-buffered saline containing 0.1% BSA to remove unabsorbed arachidonic acid. Cells were then treated with fresh medium containing test compounds or vehicle [final concentration, 0.1% dimethyl sulfoxide (DMSO)]. After 24 h incubation, the culture medium was collected and centrifuged for 10 min at 10 000 *g*. Radioactivity of the cell culture medium was measured using a scintillation counter (Model LS3801, Beckman Coulter Inc., Fullerton, CA). For analyzing NO formation, RAW264.7 cells were treated as above without using radiolabeled arachidonic acid, and 50 or 100 μl of the culture medium was taken. The nitrite concentration was determined using Griess reagent [1% sulfanilamide in 5% H_3PO_4 and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride] by measuring the absorbance at 550 nm (17).

Effects on proteins as determined by western blotting

RAW 264.7 cells were plated into a 6-well plate at $\sim 2 \times 10^6$ cells per well. After 24 h, the media were replaced with serum free DMEM for 24 h, and the cells were stimulated with 2 μg/ml LPS (from *Escherichia coli*, serotype

055:b5) for 1 h, and then incubated with fresh medium containing test compounds or vehicle. The cells were washed with ice cold phosphate-buffered saline twice and lysed with cell lysis buffer (1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, in 20 mM Tris, pH 7.4). The cell lysate was sonicated and centrifuged at 10 000 *g* for 15 min at 4°C. The supernatant, containing 20–50 μg of the protein, was loaded onto 10% or 4–15% gradient SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred onto PVDF or nitrocellulose membrane and probed with the antibodies described above. Western blots were probed with secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes, Eugene, OR) or IRdye 800 (Rockland Immunochemicals, Gilbertsville, PA). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE). Protein concentration in the cell lysates was determined using the method of Bradford (Bio-rad, Hercules, CA).

Data analysis

Statistical significance was evaluated using the Student's *t*-test. One-way analysis of variance and the Tukey HSD test were used for comparing the effects of test compounds. Pearson's correlation coefficient with *P*-value was also determined to examine the association between concentration and efficacy.

Results

Effects of garcinol and its derivatives on the release of arachidonic acid metabolites

After stimulation of the cells with 2 μg/ml LPS for 1 h, the release of arachidonic acid and its metabolites from RAW264.7 macrophage cells to the culture media increased ~3-fold in an 18 h incubation. Based on HPLC profiles it was found that the radioactivity in the media released from the RAW264.7 cells as well as from the other intestinal cells was mainly due to arachidonic acid metabolites (>95%). Garcinol and its derivatives (1 μM) significantly decreased the release of arachidonic acid metabolites from RAW264.7 cells during the 18 h incubation (Figure 2A). Among the test compounds, garcinol apparently showed the most potent inhibitory effects (>50% inhibition). Cambogin, garcim-1 and garcim-2 were slightly less effective (30–45% inhibition). Inhibition of the release of arachidonic acid metabolites was observed even with 0.1 μM of garcinol (Figure 2B). At 1 μM, garcinol and

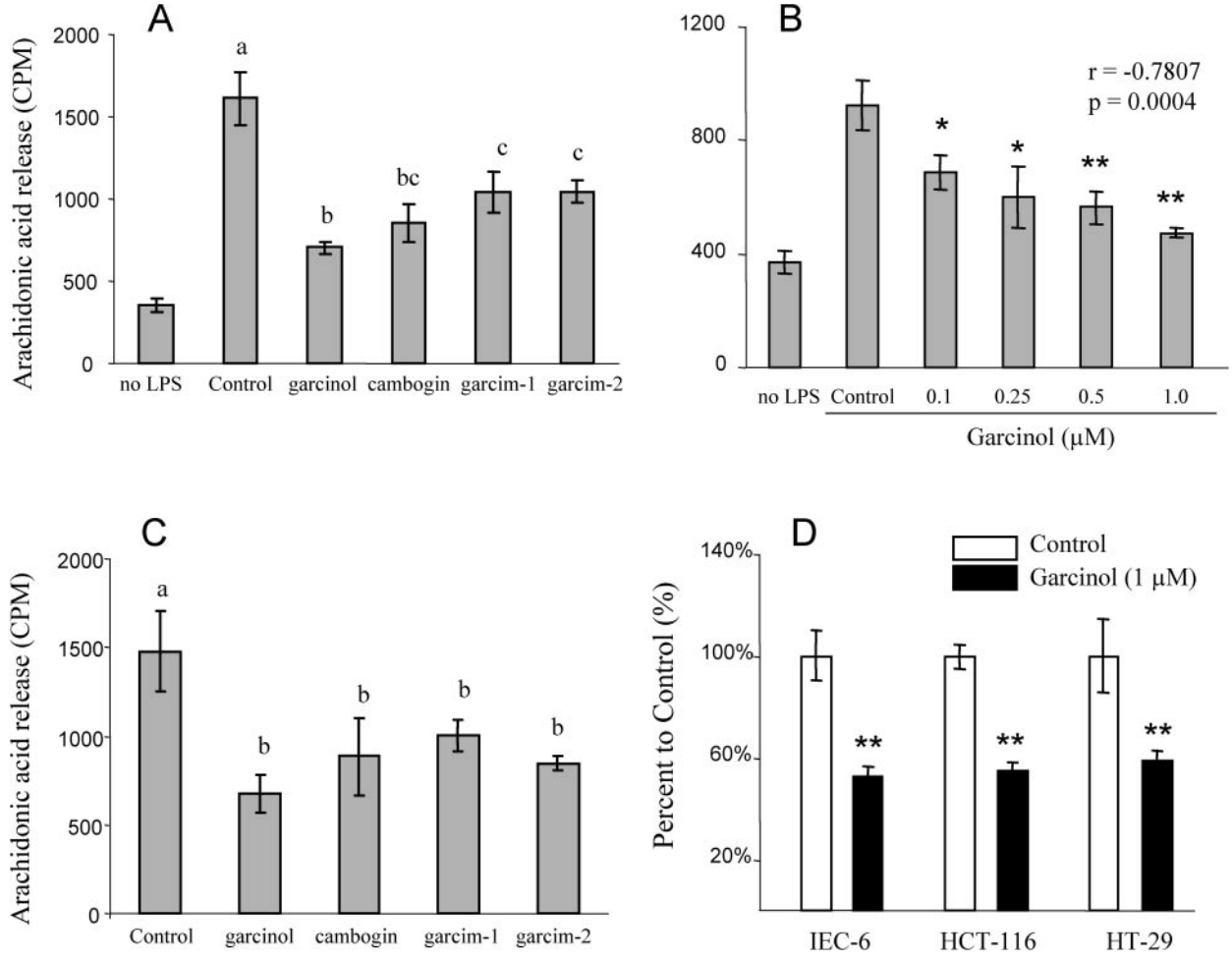

Fig. 2. Effects of garcinol and its derivatives on the release of arachidonic acid metabolites from LPS-stimulated RAW264.7 and intestinal cell lines. RAW254.7 cells labeled with [5,6,8,9,11,12,14, 15-^{3}H](N) arachidonic acid (0.1 μCi/ml) were stimulated with 2 μg/ml LPS for 1 h. Then the cells were given the fresh medium containing 1 μM of garcinol, other test compounds or the vehicle (final concentration, 0.1% DMSO), and incubated for 18 h. For the experiment with the intestinal cells, the cells labeled with arachidonic acid (0.1 μCi/ml) were treated with 1 μM of test compounds for 24 h. The release of arachidonic acid metabolites was determined by measuring the radioactivity in the culture medium. Inhibitory effects are shown on the release of arachidonic acid metabolites from RAW 264.7 cells by garcinol and its derivatives (**A**). Concentration-dependent effect of garcinol on the release of arachidonic acid metabolites. The Pearson's correlation coefficient (*r*) for concentration dependence and *P*-value are indicated (**B**). Effects of garcinol and its derivatives on the release of arachidonic acid metabolites from HCT-116 human colon cancer cells (**C**). Effect of garcinol (1 μM) on the release of arachidonic acid metabolites from different types of intestinal cells (**D**). Each bar represents the mean ± SD ($n = 4$–8). Different letters indicate a significant difference ($P < 0.05$) based on the one-way analysis of variance and the Tukey HSD test. *, ** Significantly different from control according to the Student's *t*-test ($^*P < 0.05$; $^{**}P < 0.01$, respectively).

its derivatives also markedly decreased the release of arachidonic acid metabolites in HCT-116 human colon adenocarcinoma cells (Figure 2C). The release of arachidonic acid metabolites from HT-29 and HCT-116 human colon adenocarcinoma cells and IEC-6 rat normal immortalized intestinal cells was inhibited by 1 μM garcinol by ~40–50% (Figure 2D).

Effects on cPLA$_2$ and ERK1/2

To elucidate the mechanisms for inhibition of the release of arachidonic acid and its metabolites from cells by garcinol, we investigated the effect of garcinol on the protein level and phosphorylation of cPLA$_2$. Treatment of RAW264.7 cells with LPS induced the phosphorylation of cPLA$_2$ at Ser^{505} without changing the protein level at an early time point of 1 h; garcinol inhibited cPLA$_2$ phosphorylation without influencing the cPLA$_2$ protein (Figure 3A). The effect of garcinol was concentration-dependent; significant inhibition was observed with 0.5 μM garcinol (Figure 3B). Incubation of garcinol for different periods of time with LPS-stimulated cells decreased the level of phospho-cPLA$_2$, and the inhibitory effect was prominent during a period of 2–22 h (Figure 3C). Phosphorylation of cPLA$_2$ can be catalyzed by several mitogen activated protein kinases (MAPKs), including ERK1/2, p38 and c-jun N-terminal kinase. Previously, we showed that the inhibition of ERK, rather than other MAPKs, decreased the release of arachidonic acid metabolites from LPS-stimulated RAW264.7 cells (16). Consistent to this report, U0126, a MAPK kinase (MEK) inhibitor, almost completely inhibited phosphorylation of ERK1/2 (data not shown) and inhibited the activation of cPLA$_2$ (Figure 3D). Both U0126 and PD98059 (another MEK inhibitor) decreased the release of arachidonic acid metabolites to the basal level in LPS-stimulated RAW264.7 cells (Figure 3E). In the present study, it was observed that after stimulation of RAW264.7 cells by LPS, ERK1/2 was readily activated and the activation was sustained for 24 h; garcinol decreased the phospho-ERK1/2 level concentration-dependently without affecting the protein level of ERK1/2 (Figure 4A). Incubation of garcinol (1 μM) with LPS-stimulated cells for different periods of time decreased the level of phospho-ERK1/2 (Figure 4B). The inhibitory effect of garcinol appeared to be more pronounced on the

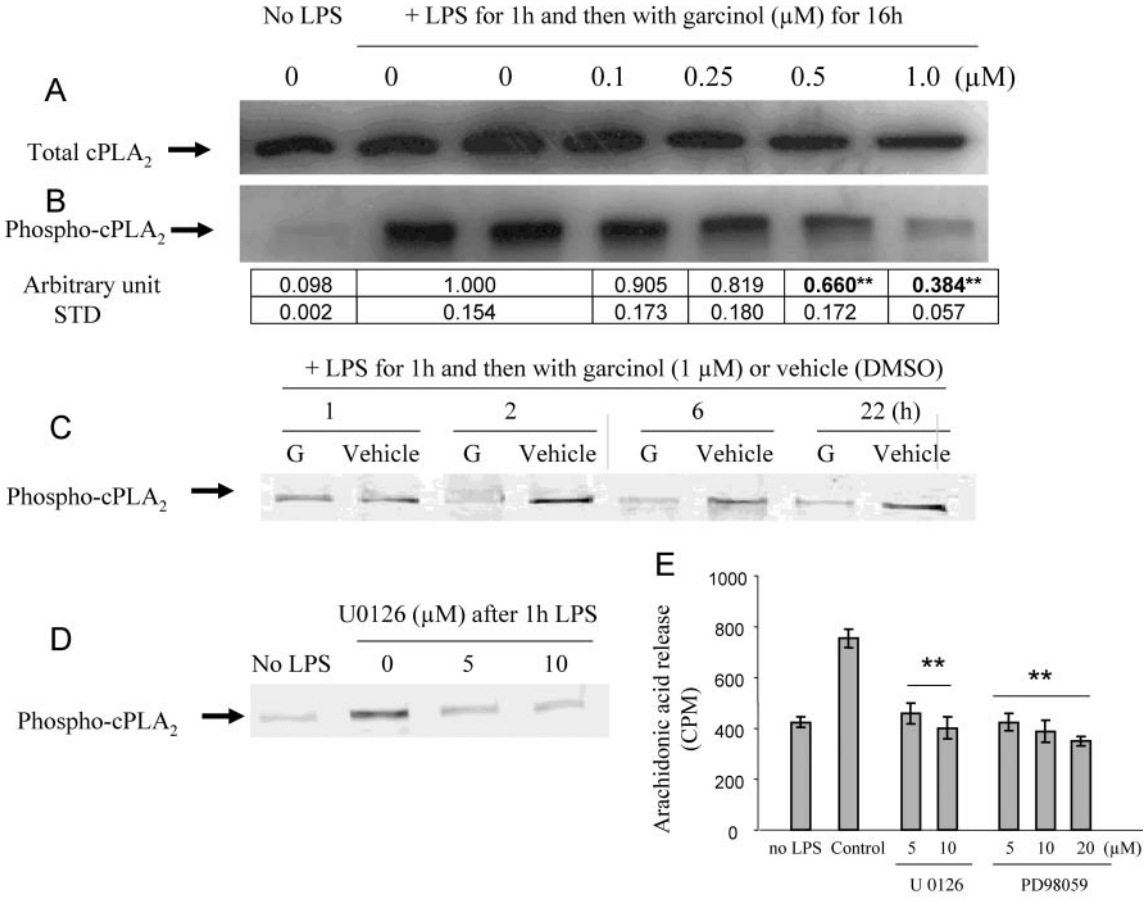

Fig. 3. Effects of garcinol and U0126 on the levels of cPLA$_2$ in RAW264.7 cells. After treatment with 2 μg/ml LPS for 1 h, RAW264.7 cells were incubated with different concentrations of garcinol or vehicle (0.1% DMSO) for 16 h. Western blot analysis was performed on cell lysates with antibodies against cPLA$_2$ (**A**) or phospho-cPLA$_2$ (Ser^{505}) (**B**). After stimulating with LPS for 1 h, the effects of garcinol (1 μM) on the level of phospho-cPLA$_2$ at different time points were analyzed (**C**). The effects of U0126 (a MEK inhibitor, 5 and 10 μM) on the level of phospho-cPLA$_2$ are shown (**D**). The effects of MEK inhibitors (U0126 and PD98059) on the release of arachidonic acid metabolites from LPS-stimulated RAW264.7 cells analyzed at 16 h after the addition of the inhibitors (**E**). The results are representative of two (A, C and D) or three (B) independent experiments. Lower panel in (B) shows the densitometry quantification of phospho-cPLA$_2$ level normalized to each control (mean ± SD $n = 3$). Each bar (E) represents the mean ± SD ($n = 4$). ** Significantly different from the control according to the Student's *t*-test ($^{**}P < 0.01$).

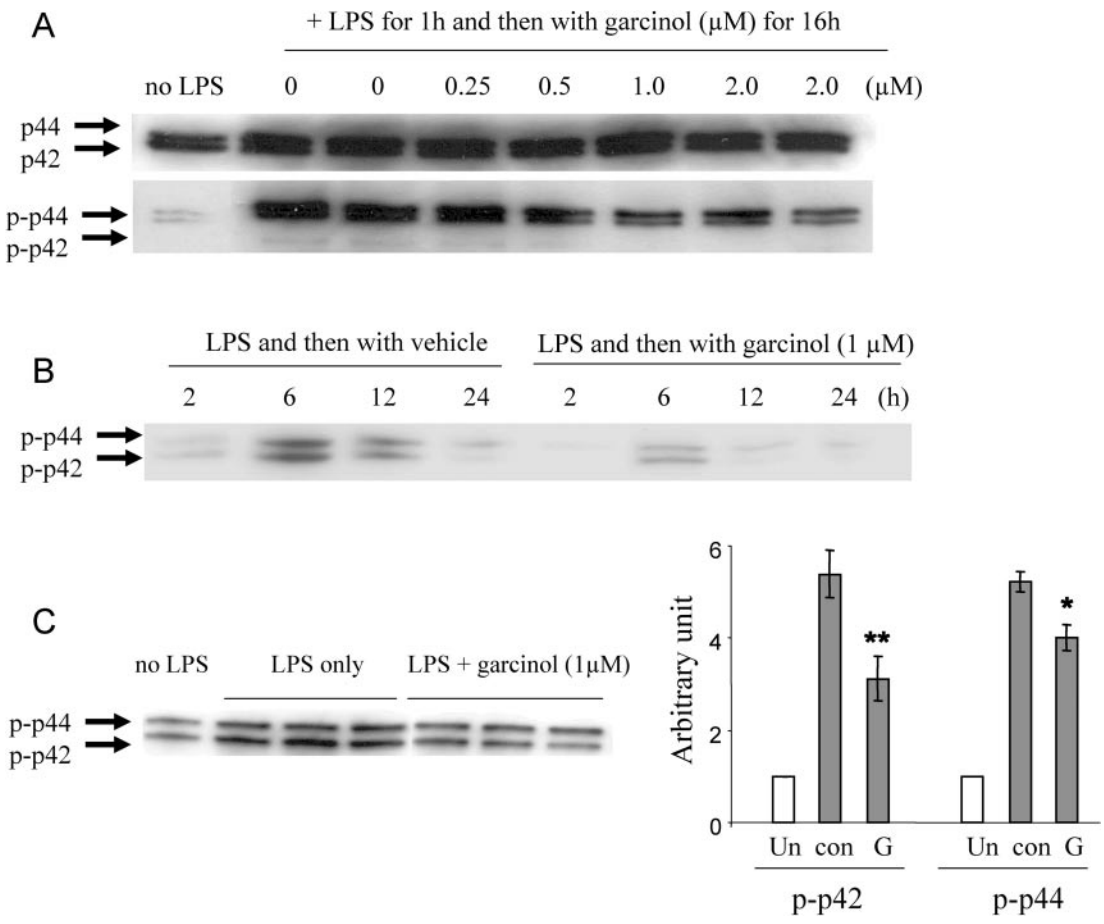

Fig. 4. Effects of garcinol on the levels of ERK1/2 and phospho-ERK1/2 in LPS-stimulated RAW264.7 cells. After stimulating with 2 μg/ml LPS for 1 h, cells were incubated with different concentrations of garcinol or vehicle (0.1% DMSO) for 16 h (**A**) or with 1 μM garcinol for different time periods (**B**). Western blot analysis was performed on cell lysates with antibodies against ERK1/2 (upper panel in A) or phospho-ERK1/2 (low panel in A, and B). The effect of garcinol (1 μM) on phospho-ERK1/2 at 6 h was analyzed in triplicated assays (**C**); the right panel shows the densitometry quantification of phospho-ERK1/2 level normalized to each unstimulated control. The results are representative of two (A and B) independent experiments and triplicate experiments for (C) (mean ± SD, $n = 3$). *, ** Significantly different from control ($^{*}P < 0.05$; $^{**}P < 0.01$).

phosphorylation of ERK2 (p42) than ERK1 (p44) at 6 h incubation (Figure 4C).

Effects on COX-2 and NFκB pathway

Stimulation of RAW264.7 cells with LPS increased the COX-2 protein level, which was detectable at 2 h, more obvious at 6 h and more pronounced at 22 h. The COX-2 expression was not affected by 1 μM garcinol (Figure 5A). A second experiment, using a range of 0.25–2.0 μM of garcinol and an incubation period of 18 h, also showed that there was no significant inhibition of COX-2 expression (Figure 5B). With garcinol at concentrations >2 μM and with 18 h incubation, decreased COX-2 expression was observed, but the changes in cell morphology suggest that it is a consequence of cytotoxicity (data not shown). On the other hand the addition of garcinol to RAW264.7 cells after LPS stimulation did not affect the levels of IκB and COX-2, addition of garcinol before LPS stimulation resulted in a higher IκB level and lower COX-2 level in the macrophages (Figure 5C and D). Bay 11-7082, an IκB kinase (IKK) inhibitor, is known to prevent IκB degradation. When added to the RAW264.7 cells before or after activation by LPS, Bay 11-7082 increased the cellular level of IκB, and almost completely abolished the COX-2 protein levels. Curcumin, another known IKK inhibitor, also markedly decreased COX-2 level when added 1 h after LPS treatment. Using LPS-stimulated RAW264.7 cell lysates as the enzyme source, the effects of garcinol and its derivatives on COX-2 activity were analyzed. These compounds, up to 10 μM, did not inhibit COX-2 activity significantly (data not shown).

Effects on iNOS and JAK/STAT-1 pathway

After stimulation of RAW264.7 cells with LPS, significant NO accumulation in culture medium was observed at 18 h, and much higher accumulation of NO was observed at 24 and 36 h. The presence of garcinol (0.5 and 1 μM) significantly decreased NO accumulation (Figure 6A). The inhibition was concentration-dependent, with significant inhibition observed at a concentration as low as 0.25 μM (Figure 6B). Other garcinol derivatives (1 μM) also significantly inhibited NO formation in LPS-stimulated RAW264.7 cells, showing 20–30% inhibition (Figure 6C). After stimulation with LPS, the induction of iNOS was a slower event as compared with the induction of COX-2; the expression of iNOS was observed at 22 h but not at 6 h or earlier (Figure 7A). At 22 h, the expression was inhibited by garcinol when added 1 h after the addition of LPS. The inhibitory effect on iNOS expression by garcinol was also concentration-dependent, and the effect

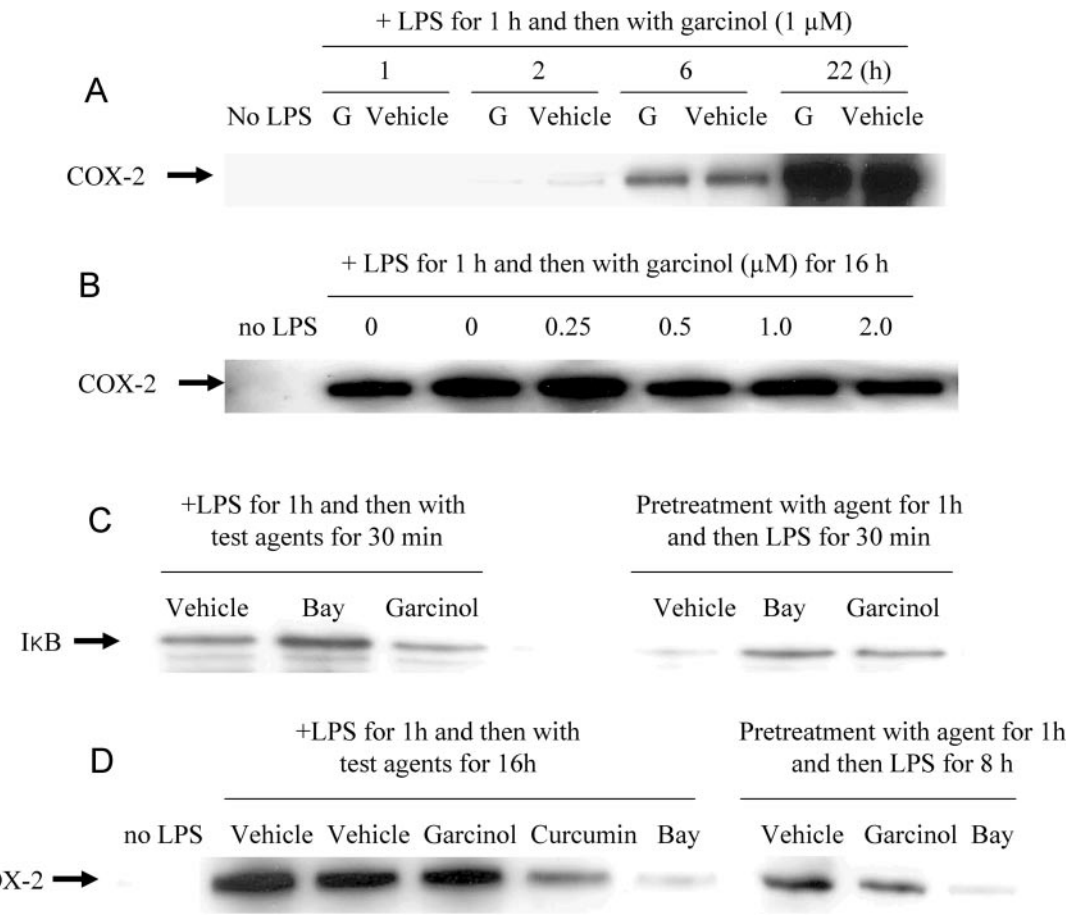

Fig. 5. Effects of garcinol on COX-2 expression and IκB degradation in RAW264.7 cells. After treatment with 2 μg/ml LPS for 1 h, RAW264.7 cells were incubated with 1 μM of garcinol or vehicle (0.1% DMSO) for different time periods (**A**), or different concentrations of garcinol for 16 h (**B**). The effect of garcinol (1 μM) or Bay11-7082 (2 μM) on IκB degradation when added before or after LPS stimulation was analyzed (**C**). After treatment of 2 μg/ml LPS for 1 h, RAW264.7 cells were treated with fresh medium containing test compounds for 30 min (left panel). After treatment with test compounds for 1 h, the RAW cells were stimulated with LPS for 1 h (right panel). The effect of garcinol (1 μM), Bay11-7082 (2 μM) or curcumin (20 μM) on COX-2 expression in different sequences of LPS stimulation is shown (**D**). The results are representative of two (C and D) and four (A and B) independent experiments, which showed a similar pattern.

was oberved at a concentration of 0.5 μM of garcinol (Figure 7B). In order to investigate the mechanisms of action involved in the inhibition of iNOS expression, the effect of garcinol on JAK/STAT-1 pathway was investigated. Phosphorylation of STAT-1 was observed at 3 h after the addition of LPS, and garcinol (1 μM) inhibited the activation of STAT-1 (Figure 7C). Garcinol, however, did not affect total STAT-1 protein. The results indicate that garcinol may inhibit iNOS expression by affecting JAK/STAT-1 signaling.

Discussion

Due to its anti-inflammatory and anticarcinogenic effects garcinol has received much attention recently (3,4,6). In the present study, we investigated possible mechanisms of action by garcinol and its derivatives on arachidonic acid metabolism and NO synthesis at concentrations (>1 μM) that may be achievable *in vivo*. Our preliminary result indicates that peak plasma and urine levels of garcinol in CD-1 female mice were 12 and 2.7 μM, respectively, after oral gavage of garcinol (10 mg dose per mouse) (S.Sang, J.Hong, M.J.Lee, M.T.Huang, C.T.Ho and C.S.Yang, unpublished data). Our study indicates that garcinol and its metabolites effectively decrease the release of arachidonic acid and its metabolites from murine macrophage RAW264.7 cells and several intestinal cells. Since $cPLA_2$ plays a major role in catalyzing the release of arachidonic acid from membrane phospholipids in most tissues (18), we investigated the effect of garcinol on this enzyme and the upstream events that activate this enzyme (19–22). Garcinol significantly inhibited LPS-stimulated phosphorylation of ERK1/2 and $cPLA_2$, without affecting the protein levels of these two enzymes. Herein, we report a mechanistic relationship between ERK1/2 and $cPLA_2$, showing that blocking of ERK activation inhibited phosphorylation of $cPLA_2$, and consequently decreased the release of arachidonic acid metabolites in LPS-stimulated RAW264.7 cells. The results agree with our previous report that inhibition of ERK, rather than p38 and c-jun N-terminal kinase, is important for inhibiting arachidonic acid release in this cell (16). Accordingly, we suggest that the inhibitory effects of garcinol on the released arachidonic acid metabolites are mainly due to the inhibition of $cPLA_2$ phosphorylation through modulating ERK1/2 activation. Since cambogin, garcim-1 and garcim-2 also showed similar effects for inhibiting the release of arachidonic acid metabolites, these compounds are also expected to have a similar mechanism of action as garcinol. In related studies, it was shown that garcinol (0.5 μmol/ear) inhibited phorbol ester-stimulated

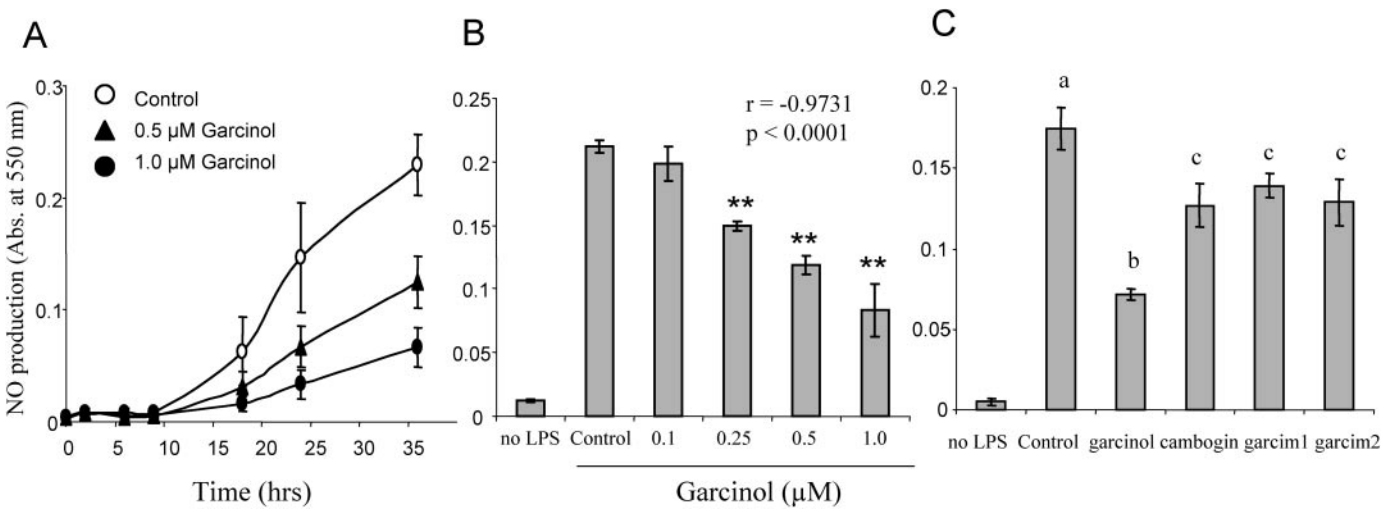

Fig. 6. Effects of garcinol and its derivatives on NO production. The level of NO in the culture medium of LPS-stimulated RAW264.7 cell analyzed at different time points and effects of 0.5 and 1 μM of garcinol (**A**). Concentration-dependent effects of garcinol on NO release after 24 h of incubation. The Pearson's correlation coefficient (r) for concentration dependence and P-value are indicated (**B**). Effect of garcinol or its derivatives (1 μM) on NO release from RAW264.7 cells after 24 h of incubation (**C**). RAW264.7 cells were stimulated with 2 μg/ml LPS for 1 h, and fresh medium containing garcinol or its derivatives was then added. NO level in the medium was determined using the Griess reagent, measuring the absorbance at 550 nm. Each value represents the mean ± SD ($n = 4$–8). **, Significantly different from control according to the Student's t-test ($P < 0.01$). Different letters indicate a significant difference ($P < 0.05$) based on one-way analysis of variance and the Tukey HSD test.

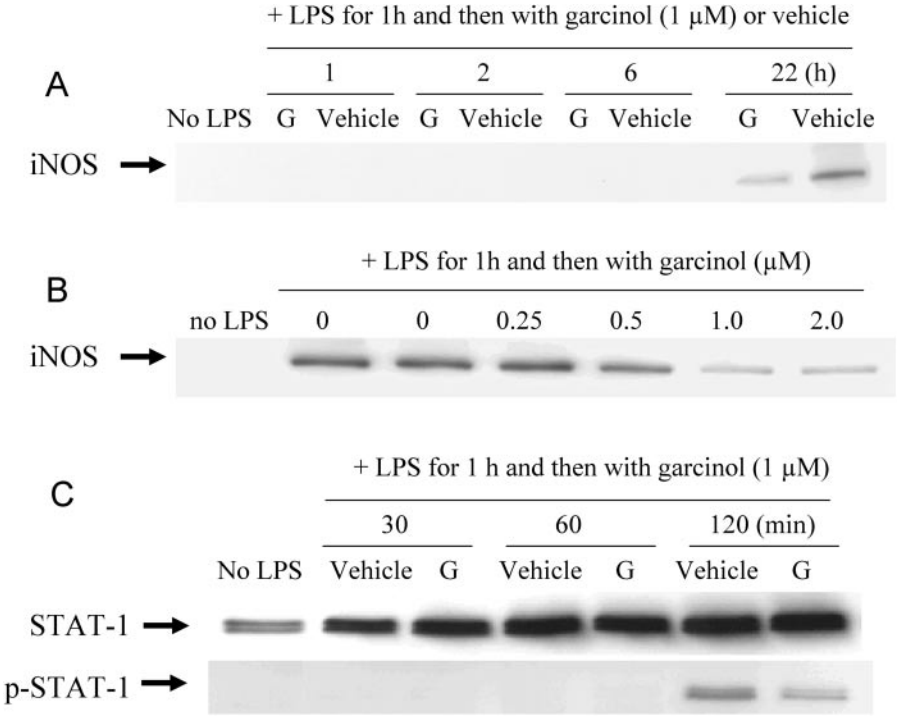

Fig. 7. Effects of garcinol on iNOS expression and STAT-1 activation. iNOS level at different time points in LPS-stimulated RAW264.7 cells and effects by garcinol (**A**). Concentration-dependent effects of garcinol on levels of iNOS in RAW264.7 cells after 16 h incubation (**B**). After stimulating with 2 μg/ml LPS for 1 h the cells were incubated with the fresh medium containing 1 μM garcinol for different time periods or with different concentrations of garcinol. Effects of garcinol (1 μM) on STAT-1 and phospho-STAT-1 level after stimulation with 2 μg/ml LPS for 1 h (**C**). The results are representative of two independent experiments, which showed a similar pattern.

prostaglandin E_2 and leukotriene B_4 formation as well as ear inflammation in mice by >50% (M.T.Huang, Y.Liu, S.Sang, C.T.Ho and C.S.Yang, unpublished data). Inhibition of $cPLA_2$ phosphorylation appears to be a key mechanism for decreasing arachidonic acid metabolites by garcinol.

In the activation of nuclear factor-κB (NFκB) pathway in macrophages, LPS activates IKK through binding to toll-like receptors (e.g. toll-like receptor 4) (23). The activated IKK then phosphorylates IκB, which is then released from the inactive NFκB complex and subjected to proteasomal degradation (24). The activated NFκB (p65 and p50) then translocates to the nucleus and functions as a transcription factor to induce many inflammation-related gene products, including COX-2 (25). Our results indicated that garcinol added to activated macrophages did not affect IκB degradation or COX-2 expression. Garcinol, however, inhibited IκB degradation

and COX-2 expression when it was added to the macrophage cells before stimulation by LPS. It is likely that garcinol interrupts LPS binding to its receptor (e.g. toll-like receptors) in the macrophage cells, rather than directly inhibiting IKK. Known IKK inhibitors, such as Bay11-7082 and curcumin, inhibited IκB degradation and COX-2 expression regardless of whether they were added before or after the LPS treatment. It was previously reported that pretreatment with garcinol before LPS stimulation inhibited the phosphorylation of IκB and COX-2 expression in RAW264.7 cells (6). The effect might be due to the interruption of LPS binding to its receptors by garcinol. The LPS-stimulated macrophage system has been commonly used for testing anti-inflammatory effects of various agents. Many experiments in previous reports used simultaneous treatment of LPS with test compounds or pretreatment of test compounds before LPS stimulation (26–28). Agents that interrupt LPS binding to its receptor would affect the entire series of downstream events. The downstream events, therefore, cannot be interpreted as direct targets for the test compounds. By comparing the results of the test agents when they are added before and after stimulation by LPS, we were able to obtain more mechanistic information. The precise mechanism regarding how garcinol interrupts the binding of LPS to its receptors (e.g. either garcinol directly binds to its receptors or indirectly interrupts LPS binding) needs to be investigated further.

Garcinol strongly inhibited iNOS expression and NO formation in LPS-stimulated RAW264.7 cells. The induction of COX-2 and iNOS by LPS is known to involve toll-like receptors and is mediated via NFκB activation (23,29,30). On the other hand the activation of NFκB is required and is sufficient for LPS-induced COX-2 expression, the induction of iNOS requires stimulation by secondary cytokines produced after stimulation of RAW264.7 cells by LPS. These secondary cytokines include tumor necrosis factor-α, interleukin-1β, interferon-α/β and interferon-γ (23,31–33). Consistent with this concept, the stimulation of iNOS expression and NO accumulation occur later than the induction of COX-2 (Figures 6 and 7). The signal responsible for iNOS induction by interferons is known to be through the JAK/STAT pathway (33), and there is no evidence that LPS directly activates JAK/STAT-1. Since the NFκB pathway was not affected by garcinol when added after LPS stimulation, we suspected that the effect of garcinol on iNOS is due to the inhibition of JAK/STAT-1 signaling. Activation of STAT-1 was observed after stimulation with LPS for 3 h, possibly mediated by secondary cytokines produced after LPS stimulation. Garcinol decreased STAT-1 phosphorylation without affecting the total STAT-1 level. Therefore, inhibition of STAT-1 phosphorylation may be a key mechanism for the suppression of iNOS expression and NO formation by garcinol.

Involvement of COX-2 and iNOS in the carcinogenic process has been extensively studied. A previous report indicated that combinatory inhibition of COX-2 and iNOS effectively modulated the carcinogenic process of azoxymethane-induced animal models (34). The possible connection between NO and COX-2 through β-catenin and polyoma enhancer activator 3 and their oncogenic potential were also reported (35). Garcinol showed inhibitory effects on iNOS and COX-2 (both directly and through inhibiting $cPLA_2$), and these effects may contribute to its anticarcinogenic action. The roles of iNOS and NO in intestinal tumorigenesis, however, are not clear (36), and further studies are required in this respect.

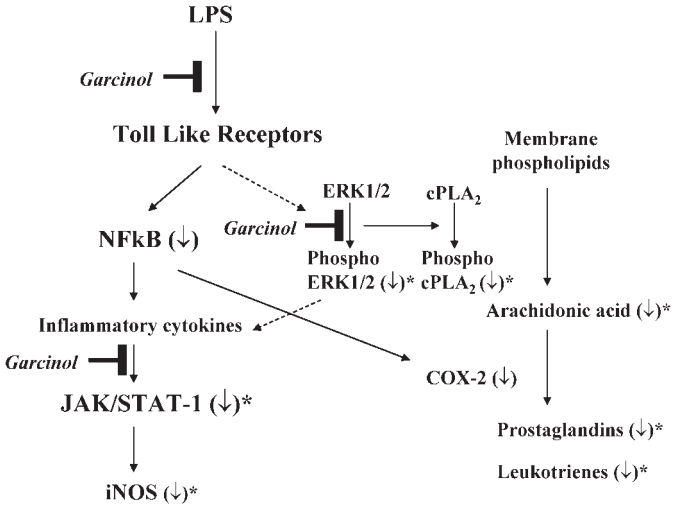

Fig. 8. Proposed mechanisms for the action of garcinol in LPS-stimulated macrophages. Garcinol interrupts LPS binding to its toll-like receptors when added before LPS; in such case, garcinol would affect the entire series of downstream events, including inhibition of NFκB activation and COX-2 expression. Garcinol added after LPS stimulation modulates arachidonic acid metabolism by inhibiting phosphorylation of $cPLA_2$ (the activation process of the enzyme) by inhibiting the activation of ERK1/2. Garcinol also inhibits NO synthesis by suppressing iNOS expression through the interruption of the JAK/STAT-1 pathway. **T**, Possible inhibitory targets of garcinol; *, changes observed with garcinol added to the macrophages after LPS stimulation.

Our hypothesis on the mechanism of action of garcinol on arachidonic acid metabolism and NO synthesis is summarized in Figure 8. Garcinol modulates arachidonic acid metabolism by inhibiting the activation of $cPLA_2$ through the inhibition of ERK1/2 phosphorylation, and suppresses iNOS expression (and NO formation) by inhibiting JAK/STAT-1 activation. When added before LPS, garcinol suppressed NFκB activation and COX-2 expression through the interruption of LPS binding to toll-like receptors. These inhibitory effects may be the key mechanisms for the anti-inflammatory and anticarcinogenic actions of garcinol.

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References

1. Krishnamurthy,N., Lewis,Y.S. and Ravindranath,B. (1981) On the structures of garcinol, isogarcinol and camboginol. *Tetrahedron Lett.*, **22**, 793–796.
2. Bakana,P., Claeys,M., Totte,J., Pieters,L.A., Van Hoof,L., Tamba,V., Van den Berghe,D.A. and Vlietinck,A.J. (1987) Structure and chemotherapeutical activity of a polyisoprenylated benzophenone from the stem bark of *Garcinia huillensis*. *J. Ethnopharmacol.*, **21**, 75–84.
3. Tanaka,T., Kohno,H., Shimada,R., Kagami,S., Yamaguchi,F., Kataoka,S., Ariga,T., Murakami,A., Koshimizu,K. and Ohigashi,H. (2000) Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. *Carcinogenesis*, **21**, 1183–1189.
4. Yoshida,K., Tanaka,T., Hirose,Y., Yamaguchi,F., Kohno,H., Toida,M., Hara,A., Sugie,S., Shibata,T. and Mori,H. (2005) Dietary garcinol inhibits 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats. *Cancer Lett.*, **221**, 29–39.
5. Pan,M.H., Chang,W.L., Lin-Shiau,S.Y., Ho,C.T. and Lin,J.K. (2001) Induction of apoptosis by garcinol and curcumin through cytochrome *c*

release and activation of caspases in human leukemia HL-60 cells. *J. Agric. Food Chem.*, **49**, 1464–1474.
6. Liao,C.H., Sang,S., Liang,Y.C., Ho,C.T. and Lin,J.K. (2004) Suppression of inducible nitric oxide synthase and cyclooxygenase-2 in downregulating nuclear factor-kappa B pathway by Garcinol. *Mol. Carcinog.*, **41**, 140–149.
7. Balasubramanyam,K., Altaf,M., Varier,R.A., Swaminathan,V., Ravindran,A., Sadhale,P.P. and Kundu,T.K. (2004) Polyisoprenylated benzophenone, garcinol, a natural histone acetyltransferase inhibitor, represses chromatin transcription and alters global gene expression. *J. Biol. Chem.*, **279**, 33716–33726.
8. Kuehl,F.A.Jr and Egan,R.W. (1980) Prostaglandins, arachidonic acid, and inflammation. *Science*, **210**, 978–984.
9. Subbaramaiah,K., Zakim,D., Weksler,B.B. and Dannenberg,A.J. (1997) Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc. Soc. Exp. Biol. Med.*, **216**, 201–210.
10. Steele,V.E., Holmes,C.A., Hawk,E.T., Kopelovich,L., Lubet,R.A., Crowell,J.A., Sigman,C.C. and Kelloff,G.J. (1999) Lipoxygenase inhibitors as potential cancer chemopreventives. *Cancer Epidemiol. Biomarkers Prev.*, **8**, 467–483.
11. Nussler,A.K. and Billiar,T.R. (1993) Inflammation, immunoregulation, and inducible nitric oxide synthase. *J. Leukoc. Biol.*, **54**, 171–178.
12. Nathan,C. and Xie,Q.W. (1994) Nitric oxide synthases: roles, tolls, and controls. *Cell*, **78**, 915–918.
13. Xie,Q. and Nathan,C. (1994) The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.*, **56**, 576–582.
14. Sang,S., Pan,M.-H., Cheng,X., Bai,N., Stark,R.E., Rosen,R.T., Lin-Shiau,S.-Y., Lin,J.-K. and Ho,C.T. (2001) Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol and their antitumor activities. *Tetrahedron*, **57**, 9931–9938.
15. Sang,S., Liao,C.-H., Pan,M.-H., Rosen,R.T., Lin-Shiau,S.-Y., Lin,J.-K. and Ho,C.-T. (2002) Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities. *Tetrahedron*, **58**, 10095–10102.
16. Hong,J., Bose,M., Ju,J., Ryu,J.H., Chen,X., Sang,S., Lee,M.J. and Yang,C.S. (2004) Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase. *Carcinogenesis*, **25**, 1671–1679.
17. Green,L.C., Wagner,D.A., Glogowski,J., Skipper,P.L., Wishnok,J.S. and Tannenbaum,S.R. (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.*, **126**, 131–138.
18. Leslie,C.C. (1997) Properties and regulation of cytosolic phospholipase A2. *J. Biol. Chem.*, **272**, 16709–16712.
19. Bouvier,G., Hergenhahn,M., Polack,A., Bornkamm,G.W. and Bartsch,H. (1993) Validation of two test systems for detecting tumor promoters and EBV inducers: comparative responses of several agents in DR-CAT Raji cells and in human granulocytes. *Carcinogenesis*, **14**, 1573–1578.
20. Kramer,R.M., Roberts,E.F., Um,S.L., Borsch-Haubold,A.G., Watson,S.P., Fisher,M.J. and Jakubowski,J.A. (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J. Biol. Chem.*, **271**, 27723–27729.
21. Fouda,S.I., Molski,T.F., Ashour,M.S. and Sha'afi,R.I. (1995) Effect of lipopolysaccharide on mitogen-activated protein kinases and cytosolic phospholipase A2. *Biochem. J.*, **308**, 815–822.
22. Gijon,M.A. and Leslie,C.C. (1999) Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J. Leukoc. Biol.*, **65**, 330–336.
23. Rhee,S.H. and Hwang,D. (2000) Murine TOLL-like receptor 4 confers lipopolysaccharide responsiveness as determined by activation of NF kappa B and expression of the inducible cyclooxygenase. *J. Biol. Chem.*, **275**, 34035–34040.
24. Tebo,J.M., Chaoqun,W., Ohmori,Y. and Hamilton,T.A. (1994) Murine inhibitory protein-kappa B alpha negatively regulates kappa B-dependent transcription in lipopolysaccharide-stimulated RAW 264.7 macrophages. *J. Immunol.*, **153**, 4713–4720.
25. Mercurio,F. and Manning,A.M. (1999) Multiple signals converging on NF-kappaB. *Curr. Opin. Cell Biol.*, **11**, 226–232.
26. Tsao,L.T., Lin,C.N. and Wang,J.P. (2004) Justicidin A inhibits the transport of tumor necrosis factor-alpha to cell surface in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Mol. Pharmacol.*, **65**, 1063–1069.
27. Kang,J.S., Yoon,Y.D., Cho,I.J., Han,M.H., Lee,C.W., Park,S.K. and Kim,H.M. (2005) Glabridin, an isoflavan from licorice root, inhibits inducible nitric-oxide synthase expression and improves survival of mice in experimental model of septic shock. *J. Pharmacol. Exp. Ther.*, **312**, 1187–1194.
28. Pan,M.H., Lin-Shiau,S.Y., Ho,C.T., Lin,J.H. and Lin,J.K. (2000) Suppression of lipopolysaccharide-induced nuclear factor-kappaB activity by theaflavin-3,3′-digallate from black tea and other polyphenols through down-regulation of IkappaB kinase activity in macrophages. *Biochem. Pharmacol.*, **59**, 357–367.
29. Jones,B.W., Means,T.K., Heldwein,K.A., Keen,M.A., Hill,P.J., Belisle,J.T. and Fenton,M.J. (2001) Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.*, **69**, 1036–1044.
30. Jones,B.W., Heldwein,K.A., Means,T.K., Saukkonen,J.J. and Fenton,M.J. (2001) Differential roles of Toll-like receptors in the elicitation of proinflammatory responses by macrophages. *Ann. Rheum. Dis.*, **60** (Suppl. 3), iii6–12.
31. Pfeilschifter,J., Rob,P., Mulsch,A., Fandrey,J., Vosbeck,K. and Busse,R. (1992) Interleukin 1 beta and tumour necrosis factor alpha induce a macrophage-type of nitric oxide synthase in rat renal mesangial cells. *Eur. J. Biochem.*, **203**, 251–255.
32. Lorsbach,R.B., Murphy,W.J., Lowenstein,C.J., Snyder,S.H. and Russell,S.W. (1993) Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J. Biol. Chem.*, **268**, 1908–1913.
33. Jacobs,A.T. and Ignarro,L.J. (2001) Lipopolysaccharide-induced expression of interferon-beta mediates the timing of inducible nitric-oxide synthase induction in RAW 264.7 macrophages. *J. Biol. Chem.*, **276**, 47950–47957.
34. Rao,C.V., Indranie,C., Simi,B., Manning,P.T., Connor,J.R. and Reddy,B.S. (2002) Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. *Cancer Res.*, **62**, 165–170.
35. Liu,Y., Borchert,G.L. and Phang,J.M. (2004) Polyoma enhancer activator 3, an ets transcription factor, mediates the induction of cyclooxygenase-2 by nitric oxide in colorectal cancer cells. *J. Biol. Chem.*, **279**, 18694–18700.
36. Scott,D.J., Hull,M.A., Cartwright,E.J., Lam,W.K., Tisbury,A., Poulsom,R., Markham,A.F., Bonifer,C. and Coletta,P.L. (2001) Lack of inducible nitric oxide synthase promotes intestinal tumorigenesis in the Apc(Min/+) mouse. *Gastroenterology*, **121**, 889–899.

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