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

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Abbreviations: COX, cyclooxygenase; cPLA2, cytosolic PLA2; ERK, extracellular signal regulated kinase; IκB, inhibitor of κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JAK, Janus kinase; LPS, lipopolysaccharides; MAPK, mitogen activated protein kinase; MEK, MAPK kinase; PGE2, prostaglandin E2; NFκB, nuclear factor-κB; NO, nitric oxide; PLA2, phospholipase A2; STAT, signal transducer and activator of transcription.

Introduction

Garcinol, a polyisoprenylated benzophenone derivative, is found in Guttiferae plants (Garcinia indica, Garcinia huintakensis 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 α,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 from membrane phospholipids, and the released arachidonic 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 PLA2.
(cPLA2) 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**

[5,6,8,9,11,12,14,15-3H](N) arachidonic acid was purchased from NEN Life Science (Boston, MA). cPLA2, COX-2 and iNOS antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies of phospho-cPLA2 (Ser505), 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 1-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% CO2. 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 ~2.0 x 10⁵ cells per well in the growth media. After 24 h, the media were removed and replaced with 1 ml of serum free media containing [5,6,8,9,11,12,14,15-3H](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% H3PO4 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 ~2 x 10⁶ 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...
(55:5) 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 Na3VO4, 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, 15-3H](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 (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 cPLA2 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 cPLA2. Treatment of RAW264.7 cells with LPS induced the phosphorylation of cPLA2 at Ser505 without changing the protein level at an early time point of 1 h; garcinol inhibited cPLA2 phosphorylation without influencing the cPLA2 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-cPLA2, and the inhibitory effect was prominent during a period of 2–22 h (Figure 3C). Phosphorylation of cPLA2 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 cPLA2 (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

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**Fig. 3. Effects of garcinol and U0126 on the levels of cPLA2 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 cPLA2 (A) or phospho-cPLA2 (Ser505) (B). After stimulating with LPS for 1 h, the effects of garcinol (1 μM) on the level of phospho-cPLA2 at different time points were analyzed (C). The effects of U0126 (a MEK inhibitor, 5 and 10 μM) on the level of phospho-cPLA2 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-cPLA2 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).
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
was observed 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 the 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.I.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 cPLA2 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 cPLA2, without affecting the protein levels of these two enzymes. Herein, we report a mechanistic relationship between ERK1/2 and cPLA2, showing that blocking of ERK activation inhibited phosphorylation of cPLA2, 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 cPLA2 phosphorylation through modulating ERK1/2 activation. Since cambogin, garcin-1 and garcin-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. 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.

Anti-inflammatory mechanism of garcinol

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prostaglandin E_{2} and leukotriene B_{4} formation as well as ear inflammation in mice by 450% (M.T.Huang, Y.Liu, S.Sang, C.T.Ho and C.S.Yang, unpublished data). Inhibition of cPLA2 phosphorylation appears to be a key mechanism for decreasing arachidonic acid metabolites by garcinol.

In the activation of nuclear factor-\kappaB (NF\kappaB) pathway in macrophages, LPS activates IKK through binding to toll-like receptors (e.g. toll-like receptor 4) (23). The activated IKK then phosphorylates I\kappaB, which is then released from the inactive NF\kappaB complex and subjected to proteasomal degradation (24). The activated NF\kappaB (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 I\kappaB degradation or COX-2 expression. Garcinol, however, inhibited I\kappaB 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 IkB 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 IkB 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 and COX-2 expression by LPS. It was previously reported that pretreatment with garcinol before LPS stimulation inhibited the phosphorylation of IkB 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.

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Involvement of COX-2 and iNOS in the carcinogenic process has been extensively studied. A previous report indicated that combinatorial 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 cPLA2), 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.

**References**


