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γ-Mangostin Inhibits Inhibitor-κB Kinase Activity and Decreases Lipopolysaccharide-Induced Cyclooxygenase-2 Gene Expression in C6 Rat Glioma Cells

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ABSTRACT

We investigated the effect of γ-mangostin purified from the fruit hull of the medicinal plant *Garcinia mangostana* on spontaneous prostaglandin E_2 (PGE_2) release and inducible cyclooxygenase 2 (COX-2) gene expression in C6 rat glioma cells. An 18-h treatment with γ-mangostin potently inhibited spontaneous PGE_2 release in a concentration-dependent manner with the IC_{50} value of approximately 2 μM, without affecting the cell viability even at 30 μM. By immunoblotting and reverse-transcription polymerase chain reaction, we showed that γ-mangostin concentration-dependently inhibited lipopolysaccharide (LPS)-induced expression of COX-2 protein and its mRNA, but not those of constitutive COX-1 cyclooxygenase. Because LPS is known to stimulate inhibitor κB (IκB) kinase (IKK)-mediated phosphorylation of IκB followed by its degradation, which in turn induces nuclear factor (NF)-κB nuclear translocation leading to transcriptional activation of COX-2 gene, the effect of γ-mangostin on the IKK/IκB cascade controlling the NF-κB activation was examined. An in vitro IKK assay using IKK protein immunoprecipitated from C6 cell extract showed that this compound inhibited IKK activity in a concentration-dependent manner, with the IC_{50} value of approximately 10 μM. Consistently γ-mangostin was also observed to decrease the LPS-induced IκB degradation and phosphorylation in a concentration-dependent manner, as assayed by immunoblotting. Furthermore, luciferase reporter assays showed that γ-mangostin reduced the LPS-inducible activation of NF-κB–and human COX-2 gene promoter region-dependent transcription. γ-Mangostin also inhibited rat carrageenan-induced paw edema. These results suggest that γ-mangostin directly inhibits IKK activity and thereby prevents COX-2 gene transcription, an NF-κB target gene, probably to decrease the inflammatory agent-stimulated PGE_2 production in vivo, and is a new useful lead compound for anti-inflammatory drug development.

Prostaglandins (PGs), arachidonic acid (AA) metabolites of the cyclooxygenase (COX) pathway, are major mediators in the regulation of inflammation and immune function (Smith et al., 2000). In the brain, the prostaglandin E_2 (PGE_2) is the most abundant PG. PGE_2 levels are very low or undetectable in normal conditions but can rise in response to inflammatory processes, multiple sclerosis, and AIDS-associated de-

mentia (Fretland, 1992; Griffin et al., 1994). High levels of PGE_2 can modulate the activities of multiple cell types, including neurons, glial, and endothelial cells, as well as microglia/macrophage and lymphocyte functions during inflammatory and immune processes (Weissmann, 1993). Astrocytes are a known important source of PGE_2 in the CNS (Katsuura et al., 1989). Their ability to produce PGE_2 upon stimulation with interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), or bacterial wall protein lipopolysaccharide (LPS) has been extensively documented (Fontana et al., 1982; Mollace et al., 1998; Molina-Holgado et al., 2000).

Cyclooxygenase (COX) is well known to be responsible for PG production and the rate-limiting enzymes. This enzyme

ABBREVIATIONS: PG, prostaglandin; AA, arachidonic acid; COX, cyclooxygenase; COX-1, constitutive cyclooxygenase; COX-2, inducible cyclooxygenase; PGE_2, prostaglandin E_2; LPS, lipopolysaccharide; IκB, inhibitor κB; IKK, inhibitor κB kinase; CNS, central nervous system; IL, interleukin; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; RT, reverse transcription; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; ODS, octadecylsilyl; HPLC, high-performance liquid chromatography; DMSO, dimethyl sulfoxide; NS398, *N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methane sulfonamide.

exists as two isoforms, namely constitutive COX (COX-1) and inducible COX (COX-2). COX-2 gene is an early-inducible gene in response to many inflammatory cytokines, including IL-1, TNF-α, and LPS. COX-2 gene expression is controlled at the transcriptional and/or the post-transcriptional levels (Dixon et al., 2000). The promoter regions of COX-2 gene of rat, human, and mouse have been extensively analyzed (Sirois et al., 1993; Appleby et al., 1994; Kosaka et al., 1994; Inoue et al., 1995) and have been shown to contain potential binding sites for various transcriptional factors, some of which have been demonstrated to be indeed functional (Goppelt-Struebe, 1995). For example, activation of nuclear factor-κB (NF-κB) has been reported recently to actually participate in the transcriptional activation of COX-2 gene induced by IL-1 (Newton et al., 1997), TNF-α (Yamamoto et al., 1995), and LPS (Inoue and Tanabe, 1998). Furthermore, the LPS-induced activation of COX-2 gene evidently has been shown to be mediated by inhibitor κB (IκB) kinase (IKK), which specifically catalyzes IκB phosphorylation followed by its degradation and the subsequent NF-κB nuclear translocation, leading to a stimulation of the *cis*-acting κB element-mediated transcription (Griseavage et al., 1996).

Because the fruit hull of mangosteen, *Garcinia mangostana*, has been used for many years as traditional medicine for the treatment of skin infection, wounds, and diarrhea in Southeast Asia—that is, the fruit hull exhibits an anti-inflammatory action—we pharmacologically studied the anti-inflammatory components of the fruit hull of mangosteen. In an earlier study, we examined the effect of γ-mangostin, a tetraoxygenated diprenylated xanthone from the fruit hull of this plant (Fig. 1A), on AA cascade in C6 rat glioma cells, which is well known to be a useful model system for the study of PG production in the astrocytes, and demonstrated that this natural product reduces PGE$_2$ release from C6 glioma cells with an IC$_{50}$ of approximately 5 μM and potently inhibits the activities of both COX-1 and COX-2 enzymes with the IC$_{50}$ values of approximately 0.8 and 2 μM, respectively, in vitro (Nakatani et al., 2002).

Herein, we describe the first evidence that γ-mangostin directly inhibits IKK activity, which specifically phosphorylates IκB, and thereby prevents its degradation and, as a result, induces a decrease in the expression of COX-2 protein and its mRNA by a suppression of NF-κB–dependent transcription. This study also demonstrated that γ-mangostin inhibited rat carrageenan-induced hind paw edema, an in vivo acute model of inflammation.

Materials and Methods

Materials. PGE$_2$ was a generous gift from Ono Pharmaceuticals (Osaka, Japan). Fetal bovine serum, horse serum, Ham's F-10 medium, Eagle's minimum essential medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and NS398, a selective inhibitor of COX-2, were purchased from Cell Culture Laboratory (Cleveland, OH), Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan), Invitrogen (Carlsbad, CA), Nissui (Tokyo, Japan), Dojindo (Kumamoto, Japan), and Calbiochem (San Diego, CA), respectively. Anti-COX-1, anti-COX-2, anti-IκB, anti-phospho-IκB, anti-IKKα/β, and anti-actin antibodies and recombinant IκBα fusion protein (1–317), anti-PGE$_2$ antibody, and protein A Sepharose 4B were obtained from Santa Cruz Biochemicals (Santa Cruz, CA), Chemicon International (Temecula, CA), and Zymed Laboratories (South San Francisco, CA), respectively. Alkaline phosphatase-conjugated affinity-purified anti-

goat IgG and horseradish peroxidase-conjugated affinity-purified anti-rabbit IgG were from Rockland (Gilbertsville, PA) and Cell Signaling Technology Inc. (Beverly, MA), respectively. Total RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) kits, EndoFree Plasmid Maxi Kit, and pRG-TK vector and Dual-Luciferase Reporter Assay System were purchased from Toyobo Engineering (Osaka, Japan), QIAGEN K.K. (Tokyo, Japan), and Promega (Madison, WI), respectively. [^{3}H]PGE$_2$ (200 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA) and Amersham Biosciences Inc. (Piscataway, NJ), respectively. Other chemicals and drugs were of reagent grade or of the highest quality.

Extraction and Isolation of γ-Mangostin. γ-Mangostin (Fig. 1A) was obtained from the fruit hull of *G. mangostana* L. as reported previously (Jefferson et al., 1970). In brief, the dried fruit hull (1 kg) was ground and then extracted with ethanol (10 liters) for 1 h. The ethanol extract was partitioned between ethyl acetate and water to afford an ethyl acetate fraction (87.4 g). The ethyl acetate soluble fraction was dissolved in hexane/ethyl acetate (4:1) and subjected on a silica gel column chromatography (Wakogel C-200, 800 g, a diameter of 11 cm; Wako Pure Chemicals, Tokyo, Japan), and the sample was eluted with a step-wise gradient of hexane/ethyl acetate [3:1 (2 liters), 3:2 (1 liter), 1:1 (1 liter), 2:3 (1 liter), and 3:7 (2 liters)]. After collecting 1400 ml of the first eluent, the subsequent eluent was
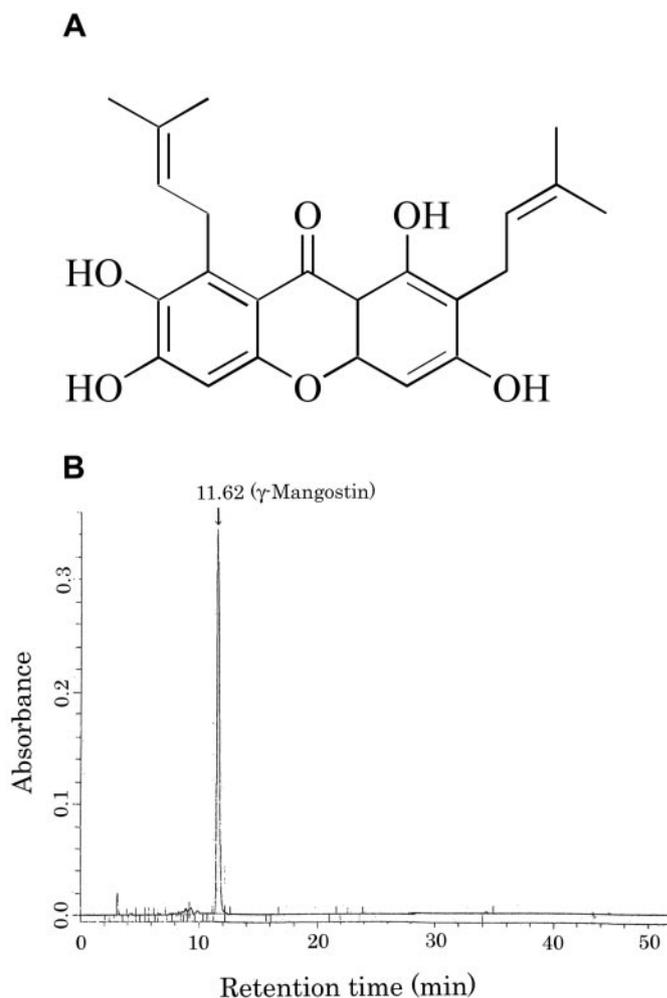
Fig. 1. Chemical structure of γ-mangostin (A) and analysis of purified γ-mangostin by HPLC (B). The elution profile was obtained by monitoring at UV absorbance of 280 nm using an HPLC column (SenshuPak ODS-1251-SS, 4.5 $\times$ 250 mm; elution: a linear gradient of 65 to 100% CH$_3$CN in H$_2$O at 1 ml/min before which the column was washed with 65% CH$_3$CN in H$_2$O for 60 min).

fractionated (200 ml each). Each fraction was monitored by thin layer chromatography [ODS silica gel; acetonitrile/water (8:2) as the developing solvent], and thereby the γ-mangostin-rich fractions, which contained γ-mangostin (R_f = 0.49) but not α-mangostin (R_f = 0.38), were combined. This silica gel column chromatography was performed two times additionally to further obtain the γ-mangostin fraction. The combined γ-mangostin fractions (17.8 g) underwent chromatography on a Senshupak PEGSIL ODS column (20 × 250 mm; Senshu Scientific Co., Tokyo, Japan) and were eluted with methanol/water (4:1 to 1:0) to yield partially purified γ-mangostin (9.0 g). The partially purified γ-mangostin fraction was subjected to an Ultra Pack Silica gel column chromatography (50 mm x 300 mm) (Yamazen, Schaumburg, IL) and eluted with hexane/ethyl acetate (1:1) followed by recrystalization in hexane to finally give purified γ-mangostin (6.0 g). Its purity was more than 90%, as determined by HPLC (SenshuPak ODS-1251-SS, 4.5 × 250 mm; Senshu Scientific) (Fig. 1B). As shown in Fig. 1B, each of the other constituents was less than 3%. Purified γ-mangostin was dissolved in dimethyl sulfoxide (DMSO) to make a concentration of 20 mM as a stock solution and diluted to appropriate concentrations before use.

Cell Culture. C6 rat glioma cells were grown in Ham's F-10 medium containing 15% horse serum and 2.5% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO_2 in air (Nakatani et al., 2002).

PGE_2 Assay. For PGE_2 assay, C6 cells were seeded onto 12-well plates at a cell density of $1.0 × 10^5$ cells per well. Two days after cell seeding, cells were subjected to the experiment. Cells were cultured for 18 h in culture medium containing vehicle (0.1% DMSO), γ-mangostin, or NS398, a selective inhibitor of COX-2. The conditioned medium was acidified to pH 4.0 by the addition of 1 N HCl, and PGE_2 was extracted twice with ethyl acetate. After ethyl acetate was evaporated under a stream of N_2 gas, the sample was dissolved in 10 mM Tris-HCl, pH 7.6. PGE_2 was determined by radioimmunoassay, as described previously (Nakatani et al., 2002).

Cell Viability Assay. Cell viability for C6 cells was measured using the MTT method as reported previously (Taglialatela et al., 1997). Cells were seeded onto 96-well plates at a cell density of $1.0 × 10^4$ cells per well and cultured with vehicle or γ-mangostin. Twenty-four hours later, cells were subjected to MTT assay. Product formation was monitored by reading the absorbance at 595 nm using a microplate reader (model 450; Bio-Rad, Hercules, CA).

Semi Quantitative RT-PCR. For RT-PCR, C6 cells were seeded onto six-well plates at a cell density of $2.0 × 10^5$ cells per well. Two days later, cells were pretreated with vehicle or γ-mangostin for 1 h and were incubated subsequently with or without 10 μg/ml LPS for 1 h. The total RNA from cells was prepared by using a total RNA extraction kit. Both the COX-1 and COX-2 mRNA levels were semi-quantitatively assayed using an RT-PCR kit as reported previously (Yang et al., 1998) as follows: The sense primers (5′-CTG CTG AGA AGG GAG TTC AT-3′, 602–621 of rat COX-1 cDNA; and 5′-ACA CTC TAT CAC TGG CAT CC-3′, 1229–1248 of rat COX-2 cDNA) and the antisense primers (5′-GTC ACA CAC ACG GTT ATG CT-3′, 981-1000 of rat COX-1 cDNA; and 5′-GAA GGG ACA CCC TTT CAC AT-3′, 11794-1813 of rat COX-2 cDNA) were complementary to the conserved regions of the cDNAs (Yang et al., 1998). The cDNA fragments were amplified 32 cycles (94°C for 60 s, 62°C for 60 s, and 72°C for 60 s). It was demonstrated that this condition for RT-PCR quantitatively yielded PCR product by our preliminary experiments (data not shown). Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal control for the present semiquantitative RT-PCR. PCR products for COX-1 and COX-2 mRNAs were separated by 2% agarose gel electrophoresis, detected by ethidium bromide staining, and subjected to quantitative analysis using an image scanner (Foto/Eclipse; Fotodyne, Hartland, WI). Furthermore, after purification of the PCR products by electrophoresis and filtration, the nucleotide sequences were determined by the dideoxy nucleotide chain termination method to verify that these PCR products are derived from COX-1 and COX-2 mRNAs.

Immunoblotting. For immunoblotting, C6 cells were seeded onto six-well plates at a cell density of $2.0 × 10^5$ cells per well. Two days after seeding, the cells were incubated with vehicle or γ-mangostin for 1 h at 37°C. After cells were incubated with or without 10 μg/ml LPS for an additional 1 h, the medium was aspirated. The cells were lysed by addition of SDS-PAGE sample buffer (187.5 mM Tris-HCl, 6% SDS, 30% glycerol, and 15% 2-mercaptoethanol, pH 6.8). These protein samples were boiled for 5 min, subjected to SDS-PAGE (8–11% gel), and then transferred electrically onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Bedford, MA) by the semidry blotting method. The blots were incubated with 2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 at 25°C for 2 h and incubated with goat anti-COX-1 antibody (0.1 μg/ml), goat anti-COX-2 (0.1 μg/ml), rabbit anti-IκB (0.2 μg/ml), rabbit anti-phospho-IκB (0.2 μg/ml), or rabbit anti-actin (0.2 μg/ml) antibody at 25°C for 2 h. The blots were washed several times and incubated with a 1:1000 to 2000 dilution of alkaline phosphatase-conjugated affinity-purified anti-goat IgG or horseradish peroxidase-conjugated affinity-purified anti-rabbit IgG in Tris-buffered saline/Tween 20 containing 2% bovine serum albumin at 4°C overnight. Immunoreactive signals were visualized by incubation of the blots with chemiluminescence assay reagents followed by exposing them to Hyperfilm ECL (Amersham Biosciences).

In Vitro IKK Assay. After 10 min of treatment with 10 μg/ml LPS, C6 cells were washed with phosphate-buffered saline, lysed with ice-cold lysis buffer (2 mM EGTA, 150 mM NaCl, 2 mM dithiothreitol, 1 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na_3VO_4, and 10 mM Tris-HCl, pH 7.5), and sonicated to prepare cell extract. IKK proteins were prepared by immunoprecipitation as follows: the cell extract was incubated with 6 μg of anti-IKKα/β antibody at 4°C for 4 h, and the immunocomplex was recovered using protein A Sepharose 4B beads. The IKK protein-bound beads were then washed three times with lysis buffer and were aliquoted to five reaction tubes, including 25 μl of the following kinase reaction mixture: 10 mM $MgCl_2 · 6H_2O$, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 5 mM β-glycerolphosphate, [γ-^{32}P]ATP, and 25 mM Tris-HCl, pH 7.5. After a 10-min incubation with or without tested concentrations of γ-mangostin at 30°C, 1 μg of IκBα was added as a substrate to each reaction tube, and the reaction mixtures were further incubated for 30 min. The reaction was terminated by the addition of SDS-PAGE sample buffer and fractionated by SDS-PAGE. Phosphorylated IκBα (1–317) was visualized as radioluminogram and quantitatively analyzed with the use of Molecular Imager (GS363; Bio-Rad).

Transient Transfection and Dual Luciferase Assay. C6 cells were plated at a cell density of $3.0 × 10^4$ cells per well on a 24-well plate or $1.2 × 10^4$ cells per well on a 48-well plate. Two days later, the cells were transfected using LipofectAMINE 2000 in serum-free medium according to the manufacturer's recommended method. Cells plated onto 24-well plates were subjected to transfection with 0.475 μg/well pNF-κB-Luc, a firefly luciferase reporter construct containing five repeated NF-κB–responsive elements, or dN-Luc, a reporter plasmid that is deficient in the repeated NF-κB–responsive elements in the pNF-κB-Luc (Hirai et al., 1994). Cells plated onto 48-well plates were also subjected to transfection with 0.4 μg/well of phPES2(−327/+59)-Luc, a firefly luciferase reporter construct containing the human COX-2 gene promoter fragment including NF-κB–responsive element (−223/−214) (Inoue et al., 1995). In addition, to normalize transfection efficiency, C6 cells were cotransfected with 0.025 μg of a *Renilla reniformis* luciferase control vector (phRG-TK). After transfection, cells were cultured in the culture medium for 13 or 20 h and then preincubated with vehicle or γ-mangostin for 3 h. Cells were incubated with or without 1 μg/ml LPS for an additional 13 or 18 h, and then the cells were harvested. Determination of both the firefly and *R. reniformis* luciferase activities was performed using a MiniLumat LB 9506 (Berthold Technologies, Bad Wildbad, Germany) with Dual-Luciferase Reporter Assay System (Promega).

Relative luciferase activity represents the ratio of the activity of firefly luciferase to that of *R. reniformis* luciferase.

Animals. 7-week-old male Wistar rats (weighing 150–170 g) were obtained from Charles River Japan (Yokohama, Japan). All experimental procedures were approved by the Laboratory Animal Care and Use Local Committee of Graduate School of Pharmaceutical Sciences, Tohoku University, and were in accordance with the principles and guidelines on animal care of Tohoku University.

Paw Edema. Paw edema was induced on the left hind paw in each rat by a subplantar injection of 75 μl of sterile saline (0.9% NaCl) containing 1% carrageenan. γ-Mangostin at doses ranging from 1 to 30 mg/kg or the vehicle control (DMSO) was given i.p. 30 min before the carrageenan injection. According to the procedure described previously (Planas et al., 1995), the presence of edema was assessed by measuring the volume of the left hind paw before (V_0) and 5 h after carrageenan injection (V_5). The increase in volume in the inflamed paw was obtained by subtracting the volume measured before the carrageenan injection from the observed value at 5 h and expressed as a percentage: % edema = $[(V_5 - V_0)/V_0] \times 100$.

Data Analysis. IC_{50} values were calculated from nonlinear regression analysis of the data. The data are expressed as the means $\pm$ S.E.M., and a significant difference ($P < 0.05$) was analyzed by analysis of variance.

Results

Effect of γ-Mangostin on Spontaneous PGE$_2$ Release from C6 Cells. Glial cells are an important source of PGs in the CNS (Katsuura et al., 1989). In our earlier report, a short-term (10-min) treatment of C6 cells with γ-mangostin has been demonstrated to reduce A23187 (10 μM)-induced PGE$_2$ release from the cells (Nakatani et al., 2002). From this finding, we hypothesized that if long-term treatment with γ-mangostin might effectively reduce spontaneous PGE$_2$ release and/or its production, this compound could be used as a potent anti-inflammatory agent for clinical control of PGE level. Therefore, to first test the possibility, C6 cells were treated with γ-mangostin for 18 h to examine the effect of this compound on the spontaneous release of endogenous PGE$_2$ from C6 cells. It was revealed that γ-mangostin potently reduced the PGE$_2$ release in a concentration-dependent manner (Fig. 2A) and that NS398, a selective COX-2 inhibitor, markedly reduced the PGE$_2$ release at 30 μM (Fig. 2B). γ-Mangostin also had no effect on the cell viability in the

concentrations ranging from 0.1 to 30 μM, but at 100 μM, it caused an 80% decrease in the cell viability (data not shown), indicating that this appreciable inhibition of the COX-2–dependent spontaneous PGE$_2$ release from the cells does not result from a reduction in the cell viability caused by γ-mangostin.

Inhibitory Effect of γ-Mangostin on the Expression of Protein and mRNA for COX-2 but Not COX-1 in C6 Cells. COX is a key enzyme for prostaglandin production, because this enzyme is involved in the rate-limiting step in the conversion of AA to prostaglandins (Rosen et al., 1989). Because expression of COX-2 protein and its mRNA are enhanced in response to inflammatory stimuli lasting for several hours (Yamamoto et al., 1995; Inoue and Tanabe, 1998), we next examined whether γ-mangostin affects the expression of COX-1 and COX-2 proteins in C6 cells treated with LPS by immunoblotting. In untreated cells, expressions of COX-1 and COX-2 proteins were detected (Fig. 3, A and B). When treated with LPS, C6 cells showed an increase in protein expression of COX-2 but not COX-1 (Fig. 3, A and B). γ-Mangostin was observed to inhibit this LPS-induced increase in the expression of COX-2 protein in a concentration-dependent manner (Fig. 3B). At 10 μM, this compound also showed a 70% inhibition of the LPS-induced increase in expression of COX-2 protein. In contrast, this compound did not affect the expression of COX-1 protein (Fig. 3A). Furthermore, to examine whether this inhibition of LPS-induced COX-2 protein expression by γ-mangostin results from a reduction in the mRNA level, we analyzed mRNA levels for COX-1 and COX-2 by using RT-PCR. For this assay of mRNA expression of COX-1 and COX-2, an established semiquantitative RT-PCR technique that has been developed recently for determining the regional and temporal profiles of COX-1 and COX-2 mRNA distributions (Yang et al., 1998) was used. Consistent with the results of Western blot analysis described above, γ-mangostin prevented the LPS-induced stimulation of COX-2 mRNA expression in a concentration-dependent manner, with an IC_{50} value of approximately 10 μM in C6 cells, whereas this compound exerted no effect on COX-1 mRNA expression level despite the absence or presence of LPS (Fig. 4, A and B). These results indicate that γ-mangostin may inhibit the expression of COX-2 gene at the transcription level.

Inhibition of NF-κB–Dependent Transcriptional Activation via IKK by γ-Mangostin in C6 Cells. Activation of NF-κB has recently been demonstrated to participate in the induction of expression of COX-2 mRNA by LPS (D'Acquisto et al., 1997) or inflammatory cytokines, including TNF-α (Yamamoto et al., 1995). Therefore, the finding that γ-mangostin prevents the LPS-induced augmentation of expression of COX-2 mRNA in C6 cells raises the possibility that this reduction in expression of COX-2 mRNA by γ-mangostin is the consequence of an inhibition of LPS-induced activation of COX-2 gene transcription through the NF-κB/IκB system. It was thus examined whether γ-mangostin directly influences IKK activity, which phosphorylates IκB protein, using an in vitro IKK assay system. As shown in Fig. 5, γ-mangostin exhibited a concentration-dependent inhibition of the IKK activity with an IC_{50} of approximately 10 μM in vitro, demonstrating that this compound directly inhibits the IKK activity. Next, the phosphorylation and accumulation of IκBα in C6 cells treated with LPS in the absence or presence
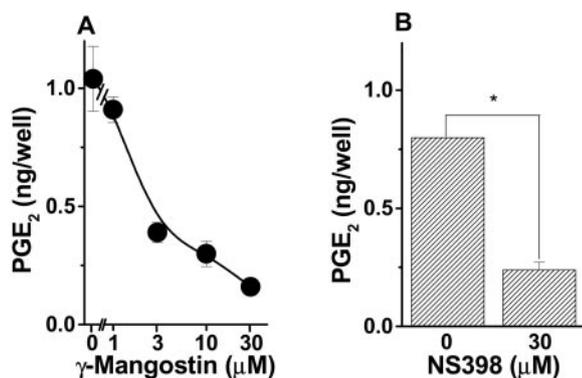

Fig. 2. Concentration-dependent inhibition of endogenous PGE$_2$ release from C6 cells by γ-mangostin (A) and the effect of NS398 on endogenous PGE$_2$ release from C6 cells (B). A and B, C6 cells were incubated with the indicated concentrations of γ-mangostin or 30 μM NS398 for 18 h. The released PGE$_2$ into the culture medium was determined by radioimmunoassay. Each point represents the mean $\pm$ S.E.M. ($n = 3$). *, $P < 0.05$ compared with the value for cells treated with 0.1% DMSO (as vehicle control).

of γ-mangostin was analyzed by Western blotting. γ-Mangostin was shown to inhibit the LPS-induced IκB phosphorylation in a concentration-dependent manner and cause an 80% inhibition of the LPS-induced IκB phosphorylation at 10 μM (Fig. 6A). When assayed by Western blotting, it was also shown that γ-mangostin inhibited LPS-induced IκB degradation in a concentration-dependent manner, with an IC_{50} of 10 μM (Fig. 6B). It has been well-documented that proteolytic degradation of IκB is involved in an activation of NF-κB. Thus, to further determine whether treatment with γ-mangostin actually inhibits NF-κB–dependent transcription activity in LPS-induced C6 cells, we conducted transient transfection of C6 cells with pNFκB-Luc, an NF-κB–dependent luciferase reporter plasmid, or dN-Luc, the NF-κB–responsive element-deficient pNFκB-Luc, and assayed the luciferase activity in C6 cells treated with vehicle or γ-mangostin in the absence or presence of LPS. As shown in Fig. 7A, LPS increased the luciferase activity in C6 cells transfected with pNFκB-Luc, whereas LPS-treated cells did not show such an increase in the luciferase activity when transfected with dN-Luc, demonstrating that the LPS-induced stimulation of luciferase activity is NF-κB–dependent. Furthermore, it was shown that at 10 μM, γ-mangostin inhibited the NF-κB–dependent transcriptional activation induced by LPS (Fig. 7A). γ-Mangostin also reduced LPS-induced augmentation of the luciferase activity in C6 cells transfected with phPES2 (−327/+59)-Luc, a human COX-2 reporter gene containing an NF-κB–responsive element, at 30 μM (Fig. 7B).

Inhibition of Rat Carrageenan-Induced Paw Edema by γ-Mangostin. To evaluate the anti-inflammatory activity of γ-mangostin in vivo, rat carrageenan-induced hind paw edema was used as an acute model of inflammation. γ-Mangostin exhibited a concentration-dependent inhibition of the edema and produced remissions of the inflammatory reaction

at the doses of 10 and 30 mg/kg, demonstrating that this compound has an anti-inflammatory activity in vivo (Fig. 8).

Discussion

The fruit hull of mangosteen, *G. mangostana*, has been widely used as an anti-inflammatory medicine in Southeast Asia for many years (Mahabusarakam et al., 1987). γ-Mangostin is one of the main constituents contained in the fruit hull of mangosteen. In the present study, we revealed that long-term treatment with γ-mangostin effectively reduced spontaneous PGE_2 release from C6 rat glioma cells, which was shown to be appreciably inhibited by NS398, a selective COX-2 inhibitor, without affecting the cell viability, and that this natural product, like aspirin (Yin et al., 1999), inhibited IKK activity in vitro and consistently reduced LPS-induced NF-κB–dependent transcriptional activation responsible for COX-2 gene transcription, which resulted in the decrease in LPS-induced COX-2 gene expression in C6 cells. Moreover, as expected, γ-mangostin was shown to prevent rat carrageenan-induced hind paw edema used as an acute model of inflammation. We thus suggest that γ-mangostin serves as not only a potent inhibitor of the release of an inflammatory chemical mediator, PGE_2, but also a new inhibitor of COX-2 gene activation and, as a result, acts as an anti-inflammatory agent in vivo.

In this study, we observed that both COX-1 and COX-2 proteins and their mRNA were expressed in the untreated cells. More than 60% of spontaneous PGE_2 release from C6 glioma cells was inhibited by an 18-h treatment with 30 μM NS398, demonstrating that COX-2 mainly contributes to this spontaneous PGE_2 release. In our earlier study, it was demonstrated that suppression of A23187-induced PGE_2 release by γ-mangostin results from its direct inhibition of the COX-1
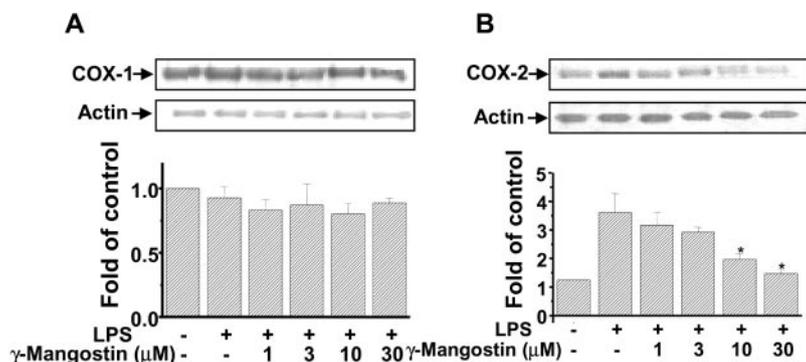

Fig. 3. Decreasing effect of γ-mangostin on the expression of COX-1 (A) and COX -2 (B) proteins as assayed by immunoblotting. After preincubation with the indicated concentrations of γ-mangostin or without this compound for 1 h, cells were incubated in the absence or presence of 10 μg/ml of LPS for 1 h. Shown are representative expression patterns from three independent experiments (top). The densitometric data on the expression of COX-1 and COX -2 proteins (bottom) were calculated as the fold increase of the value for untreated cells. Each column represents the mean ± S.E.M. ($n = 3$). *, $P < 0.05$ compared with the value for cells treated with 0.1% DMSO (as vehicle control). Actin protein was used as the loading control.
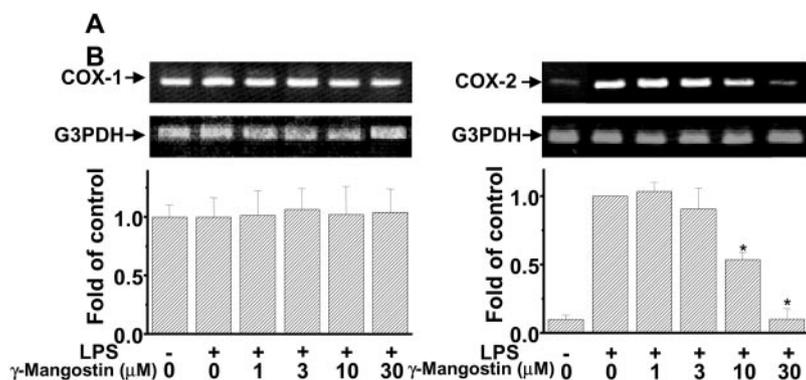

Fig. 4. Decreasing effects of γ-mangostin on COX-1 (A) and COX-2 (B) mRNA expression in C6 cells as measured by RT-PCR. Cells were preincubated with the indicated concentrations of γ-mangostin or without this compound for 1 h and thereafter were incubated in the absence or presence of 10 μg/ml LPS for 1 h. Total RNA from C6 cells was used as a template for cDNA synthesis and then subjected to PCR as described under *Materials and Methods*. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as the loading control. Shown are representative expression patterns from three independent experiments (top). The densitometric data on COX-1 and COX-2 mRNA expression (bottom) are calculated as the fold increase of the value for cells not treated with γ-mangostin. Each point represents the mean ± S.E.M. ($n = 3$). *, $P < 0.05$ compared with the value for cells treated with 0.1% DMSO (as vehicle control).

and COX-2 activities in C6 cells, like aspirin and sodium salicylate, nonsteroidal anti-inflammatory drugs (NSAIDs) (Nakatani et al., 2002). COX is the rate-limiting enzyme in the conversion of AA to prostanoids (Rosen et al., 1989). Therefore, we suggest that a marked reduction in the spontaneous PGE_2 release from C6 glioma cells by long-term treatment with γ-mangostin mainly results from a direct inhibition of the COX-2 activity by this natural compound.

Activation of COX-2 gene transcription is mediated by several *cis*-acting promoter elements that respond to multiple intracellular signaling pathways (Mestre et al., 2001); that is, the specific factors involved in the activation of COX-2 gene transcription depend on the cell types and the stimuli. In C6 cells, LPS was observed to stimulate NF-κB– but not activator protein-1–, cAMP-responsive element-, and glucocorticoid response element-dependent transcription (K. Aoki, T. Yamakuni, K. Nakatani, N. Kondo, H. Oku, K. Ishiguro, and Y. Ohizumi, unpublished data). Furthermore, as described above, it was found that LPS induced an increase in the expression of protein and mRNA for COX-2 but not COX-1 in C6 Cells, and that γ-mangostin inhibited the LPS-induced increase in the expression of protein and mRNA for COX-2 in a concentration-dependent manner. The most important finding of the present study is that γ-mangostin has a pharmacological activity of preventing stimulation of NF-κB, a central mediator of inflammation by LPS, by directly inhibiting IKK activity, which resulted in a decrease in

Fig. 5. Concentration-dependent inhibitory effect of γ-mangostin on IKK activity. C6 cells were harvested, lysed, and immunoprecipitated with anti-IKKα/β antibody. The kinase assay was carried out in 25 μl of kinase buffer containing 5 μM ATP, 10 μCi [γ-^{32}P]ATP (5000 Ci/mmol), and 1 μg IκBα (1–317) as a substrate and incubated with the indicated concentrations of γ-mangostin at 25°C for 30 min. The samples were subjected to SDS-PAGE (11% gel). At the top is a representative radioluminogram detected by using a molecular imager (GS363; Bio-Rad). At the bottom, data obtained by densitometry are shown. The results are shown as the fold increase from the value for a sample excluding the substrate and γ-mangostin (0.1% DMSO alone). Each column represents the mean $\pm$ S.E.M. (n = 3). *, P < 0.05 compared with the value for the sample containing the substrate but not γ-mangostin (as vehicle control).

LPS-induced phosphorylation and degradation of IκB. This finding raises the possibility that γ-mangostin inhibits IKK activity in vivo to decrease the LPS-induced augmentation of COX-2 gene expression. It was indeed observed that γ-mangostin produced remission of the inflammatory reaction in a concentration-dependent manner, when assayed in an in vivo model of inflammation. It has been shown thus far that aspirin and sodium salicylate inhibit NF-κB activation and IKKβ kinase activity with IC_{50} values of 30 to 50 μM in vitro (Yin et al., 1999) and that these agents suppress LPS-inducible COX-2 gene transcription (Xu et al., 1999). As evaluated using an in vitro assay system, the inhibition of IKK kinase activity by γ-mangostin showed an IC_{50} of approximately 10 μM, although γ-mangostin and these NSAIDs exhibited no chemical structure similarities. On the other hand, antioxidants such as pyrrolidinedithiocarbamate and N-acetylcysteine have been reported to inhibit IKK activation in endothelial cells (Spiecker et al., 1998). Taken together, these pharmacological characteristics of this natural product thus provide a plausible explanation for the anti-inflammatory action of the fruit hull of mangosteen, although the precise dual mechanisms of inhibitory actions of γ-mangostin remain to be elucidated.

Astrocytes are a known important, although not the only, source of PGE_2 in the CNS (Katsuura et al., 1989). Their ability to produce PGE_2 upon stimulation with IL-1β, TNF-α, or LPS has been extensively documented (Fontana et al., 1982; Mollace et al., 1998; Molina-Holgado et al., 2000). The increase in PGE_2 levels has been observed in some diseases, including AIDS-associated dementia (Minghetti et al., 1998). Expression of COX-2 in astrocytes has been demonstrated in vitro as well as in vivo. COX-2 expression has been detected in astrocytes of patients suffering from amyotrophic lateral sclerosis (Almer et al., 2001) as well as in astrocytes surrounding the plaques in a mouse model of AD (Matsuoka et al., 2001). Furthermore, the level of astrocytic COX-2 expression in brain tumors was shown to be among the best indicator of tumor progression and severity (Shono et al., 2001). In addition, increased COX-2 expression by tissue macrophages is responsible for the accumulation of large amounts of PGE_2 in local tissues (Smith et al., 2000). Secreted PGE_2 promotes inflammation by increasing vascular permeability and vasodilation and by directing cell migration into the site of inflammation through the induction of inflammatory cytokines (Muraoka et al., 1999). Therefore, the control of PGE_2 production is a critical step in clinically regulating inflammatory reactions during bacterial infection and tissue injury. From the fact that γ-mangostin inhibits stimulation of
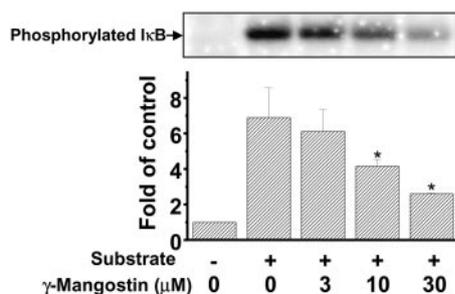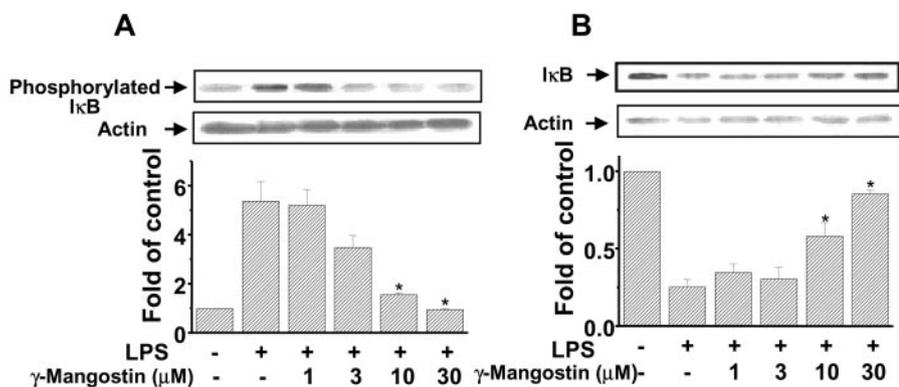

Fig. 6. Concentration-dependent inhibition of LPS-induced phosphorylation (A) and degradation (B) of IκB protein by γ-mangostin in C6 cells. Cells were preincubated with the indicated concentrations of γ-mangostin or without this compound for 1 h and thereafter were incubated in the absence or presence of 10 μg/ml LPS for 0.5 h (A) and 1 h (B). Shown are representative phosphorylation and degradation patterns from three independent experiments (top). The densitometric data on the phosphorylation and degradation of IκB protein (bottom) were calculated as the fold increase of the value for untreated cells. Each column represents the mean $\pm$ S.E.M. (n = 3). *, P < 0.05 compared with the value for cells treated with 0.1% DMSO (as vehicle). Actin protein was used as the loading control.

COX-2 gene expression as well as the enzyme activity in C6 cells, this natural product is suggested to be valuable for drug development for the treatment of these brain diseases and inflammations.

In conclusion, we for the first time demonstrated that γ-mangostin, a tetraoxygenated diprenylated xanthone isolated from mangosteen, suppresses IKK activity to inhibit LPS-induced NF-κB activation without affecting cell viability in C6 glioma cells and thereby decreases COX-2 induction.
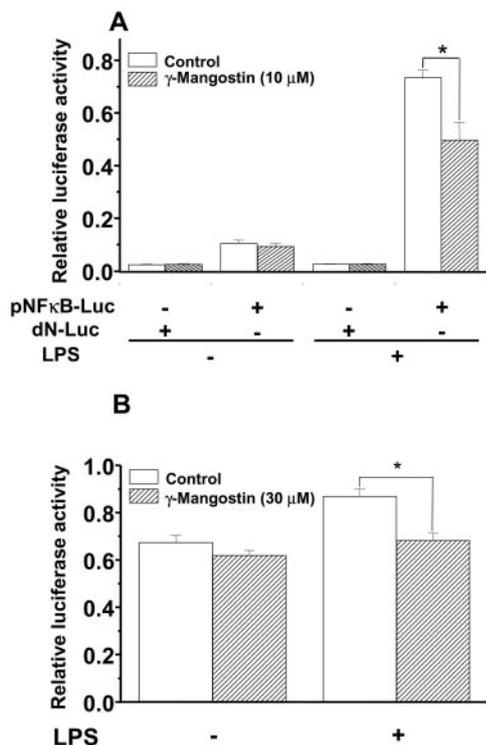

Fig. 7. Inhibitory effect of γ-mangostin on LPS-induced enhancement of luciferase activity of pNFκB-Luc (A) or phPES2(−327/+59)-Luc (B) in C6 cells. Cells were transfected with 0.475 μg/well pNFκB-Luc or dN-Luc (the NF-κB–responsive element-deficient pNFκB-Luc), or 0.4 μg/well phPES2(−327/+59)-Luc, and 0.025 μg/well of pRG-TK plasmid. After transfection, cells were preincubated with the indicated concentration of γ-mangostin or without this compound for 3 h and then incubated in the absence or presence of 1 μg/ml LPS for 8 h. Cells were harvested, and thereafter the luciferase activity was determined as described under *Materials and Methods*. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *R. reniformis* luciferase activity. Each column represents the mean ± S.E.M. ($n = 3$). ★, $P < 0.05$ compared with the value for cotransfected cells with pNFκB-Luc (A), or phPES2(−327/+59)-Luc (B) and pRG-TK plasmid, which were treated with LPS alone (0.1% DMSO).
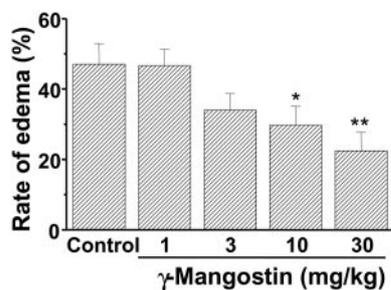

Fig. 8. Concentration-dependent inhibition of rat carrageenan-induced paw edema by γ-mangostin. Rats were injected i.p. with the indicated doses of γ-mangostin 30 min before carrageenan injection. Each column represents the mean ± S.E.M. ($n = 6$). *, $P < 0.05$ and **, $P < 0.01$ compared with the value for the vehicle (DMSO).

This study also demonstrated that γ-mangostin had an anti-inflammatory activity in vivo. These anti-inflammatory properties of this natural compound revealed by our present study using the combined methods of biochemistry and molecular biology sufficiently account for the anti-inflammatory action of the fruit hull of mangosteen. The chemical structure of this natural product is totally different from those of the NSAIDs reported so far, including aspirin and sodium salicylate. These findings thus suggest that γ-mangostin serves not only as a new attractive pharmacological tool for studying the molecular mechanism underlying inflammation but also as a new lead compound for drug development for the prevention and/or treatment of inflammation and brain diseases, including amyotrophic lateral sclerosis and brain tumors.

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