INTRODUCTION

GA (gambogic acid) is a polyphenylated xanthone isolated from the resin of *Garcinia morella* and *Garcinia hanburyi* with a long history of use as a complementary and alternative medicine. The antitumour activity of GA has been well demonstrated and is thought to arise partly from the associated anti-inflammatory activity. Recent studies have indicated that the antitumour activity of GA is mediated by its ligation of TfR1 (transferrin receptor-1). Since the cellular expression of TfR1 is down-regulated by LPS (lipopolysaccharide), we hypothesized that an alternative pathway exists in immune cells, such as macrophages, where GA could mitigate the expression of pro-inflammatory genes. Here we demonstrate that GA inhibits the LPS-dependent expression of NF-κB (nuclear factor κB) target pro-inflammatory genes in macrophages. Western immunoblot, NF-κB-luciferase reporter and gel-shift analyses revealed that GA strongly blocked the activation of NF-κB induced by LPS, whereas 9,10-dihydro-GA, which lacks the reactive α,β-unsaturated carbonyl group, was ineffective. Moreover, GA was able to decrease nuclear p55 levels in RAW264.7 macrophages, where the expression of TIR1 was down-regulated by RNA interference. In vitro kinase assays coupled with interaction studies using biotinylated GA as well as proteomic analysis demonstrated that IKKβ [IkB (inhibitory κB) kinase-β], a key kinase of the NF-κB signalling axis, was covalently modified by GA at Cys-179, causing significant inhibition of its kinase activity. Taken together, these results demonstrate the potent anti-inflammatory activity of GA.

Key words: anti-inflammatory activity, gambogic acid, inhibitory κB (IkB) kinase-β (IKK β), plant-derived bioactive, polyphenylated xanthone, cyclo-oxygenase-2 (COX-2).

GA (gambogic acid) is a polyphenylated xanthone abundant in the resin of *Garcinia morella* and *Garcinia hanburyi* with a long history of use as a complementary and alternative medicine. The antitumour activity of GA has been well demonstrated and is thought to arise partly from the associated anti-inflammatory activity. Recent studies have indicated that the antitumour activity of GA is mediated by its ligation of TIR1 (transferrin receptor-1). Since the cellular expression of TIR1 is down-regulated by LPS (lipopolysaccharide), we hypothesized that an alternative pathway exists in immune cells, such as macrophages, where GA could mitigate the expression of pro-inflammatory genes. Here we demonstrate that GA inhibits the LPS-dependent expression of NF-κB (nuclear factor κB) target pro-inflammatory genes in macrophages. Western immunoblot, NF-κB-luciferase reporter and gel-shift analyses revealed that GA strongly blocked the activation of NF-κB induced by LPS, whereas 9,10-dihydro-GA, which lacks the reactive α,β-unsaturated carbonyl group, was ineffective. Moreover, GA was able to decrease nuclear p55 levels in RAW264.7 macrophages, where the expression of TIR1 was down-regulated by RNA interference. In vitro kinase assays coupled with interaction studies using biotinylated GA as well as proteomic analysis demonstrated that IKKβ [IkB (inhibitory κB) kinase-β], a key kinase of the NF-κB signalling axis, was covalently modified by GA at Cys-179, causing significant inhibition of its kinase activity. Taken together, these results demonstrate the potent anti-inflammatory activity of GA.

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Figure 1 Structure of GA
The putative site of addition to cysteine thiol is shown. Reduction of the unsaturation (C9–C10) converts GA into DGA.

(Curcuma longa) [20], caffeic acid phenethyl ether from the propolis (resinous mixture that honey-bees collect from botanical sources) of honeybee (Apis mellifera) [21], zerumbone from ginger (Zingiber officinale) [22] and many others cause transcriptional down-regulation of pro-inflammatory genes by inhibiting the pathway of NF-κB activation [23]. In addition, endogenous Michael acceptors with α,β-unsaturated carbonyl moiety such as 15-deoxy-Δ12,14-prostaglandin J2 and 4-HNE [10] inhibit pro-inflammatory gene expression by targeting the NF-κB-dependent transcription via covalent interaction with the IKKβ subunit [24], Cys-62 in p50 [25], Cys-38 in p65 [26], in addition to its interaction with cysteine in c-Jun to promote homodimerization [27]. We hypothesized that GA could inhibit the NF-κB pathway through the α,β-unsaturated carbonyl group to impart potent anti-inflammatory activity. Here we demonstrate that the anti-inflammatory effect of GA occurs via the inhibition of IKKβ activity by covalent modification, leading to the consequent inhibition of NF-κB-dependent transcription of pro-inflammatory genes.

EXPERIMENTAL
Cell culture
The mouse RAW264.7 macrophage cell line, HEK-293 cells (human embryonic kidney cells) and human monocytic U937 cells, obtained from A.T.C.C. (Manassas, VA, U.S.A.), were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 5% (v/v) FBS (fetal bovine serum; HyClone), 2 mM L-glutamine (Invitrogen) and 10 μg/ml ciprofloxacin (Sigma) at 37°C in a humidified atmosphere of 5% CO₂. The cells were subcultured in either 6-well or 12-well tissue culture plates.

Flow cytometric analysis of TIR1 expression
The expression of TIR1 (CD71) in RAW264.7 macrophages before and after LPS treatment (1 μg/ml; 12 h) was quantified by flow cytometry. The cells were incubated with blocking antibody (CD16/CD32; BD Biosciences) for 2 h, followed by incubation with anti–CD71–FITC antibody (BD Biosciences). Both anti-CD16/CD32 and anti–CD71–FITC were kindly provided by Dr Robert Paulson (Penn State University). Results were analysed using FlowJo® software program (Tree Star).

Preparation of total cell lysates and nuclear extracts
GA and its inactive dihydro-derivative, DGA (9,10-dihydro-GA), were purchased from Gaia Chemical (Gaylordsville, CT, U.S.A.). To study the effect of GA on the expression of pro-inflammatory genes, RAW264.7 and U937 cells were grown to 80% confluence and treated with various concentrations of GA for 30 min. After pretreatment with GA, cells were stimulated with LPS (1 μg/ml) for 2 h. Cells were washed with PBS and lysed in M-PER (mammalian protein extraction reagent; Pierce) at 4°C for 20 min. Lysates were centrifuged at 16,000 g for 10 min and the resulting supernatants were frozen until further analyses. Protein concentrations in the supernatants were determined by using a BCA (bicinchoninic acid) protein assay kit (Pierce). Nuclear extracts were isolated using an NE-PER kit according to the manufacturer’s instructions (Pierce).

Western-blot analysis
Nuclear and cytosolic proteins (∼10 μg) were resolved by SDS/PAGE (10% polyacrylamide) and subjected to Western immunoblotting as we have described previously in [9]. Primary antibodies for COX-2 (cyclo-oxygenase-2; Cayman Chemicals), iNOS (inducible nitric oxide synthase; Cayman Chemicals), p65 (Santa Cruz Biotechnology), pIkBa (phosphorylated IkB α-subunits; Cell Signaling Technology), pTIR1 (Cell Signaling Technology) and pIKKβ (Igenex) were used. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and RNA polymerase II (Santa Cruz Biotechnology) were used as loading controls. In pull-down experiments, neutravidin–agarose (Pierce) or anti-α-HA (haemagglutinin)–agarose beads (Santa Cruz Biotechnology) were employed. The bands were visualized by using an ECL (enhanced chemiluminescence) assay kit (Pierce). The membranes were also stained with Ponceau Red to verify equal loading and uniform transfer of proteins.

Quantification of nitrite production
Nitric oxide production in culture media supernatant was assayed by measuring the stable degradation product of nitric oxide, nitrite, using the Griess reagent (Sigma). RAW264.7 cells were grown in 12-well plates and incubated with different concentrations of GA (0.1, 0.5, 1.0 and 1.5 μM) for 30 min. After 12 h of LPS stimulation, the culture media supernatants were isolated and the accumulation of TNF-α secretion was determined by using a commercially available TNF-α ELISA kit (Diaclone Research).

Immunoprecipitation of p65
The nuclear translocation of p65 was examined by immunoprecipitation. Nuclear proteins (50 μg) were immunoprecipitated with 0.5 μg of anti-p65 polyclonal agarose conjugate (Santa Cruz Biotechnology) overnight at 4°C. The agarose beads were extensively washed with PBS (4 × 400 μl) and subjected to SDS/PAGE (10% polyacrylamide) followed by Western-blot analysis as described above.
Transient transfection assays

The effect of GA on NF-κB-dependent reporter gene transcription induced with LPS was analysed by the luciferase assay. Briefly, RAW264.7 cells were seeded at a concentration of 2 × 10^4 cells per well in 6-well plates. After overnight culture, the cells in each well were transfected with 1 μg of DNA [0.75 μg of COX-2, COX-2 double mutant or 5 × multimerized NF-κB-luciferase reporter plasmid (Stratagene) and 0.25 μg of pRLTK, Renilla luciferase control plasmid (Promega)] along with 6 μl of Lipofectamine™ 2000 (Invitrogen Life Technologies) in serum-free DMEM. After a 6 h exposure to the transfection mixture, complete medium with 5% FBS was added to the cells and incubated at 37°C for an additional 16 h. The transfected cells were treated with various concentrations of GA (0.1–1.5 μM) for 30 min and the cells were stimulated with LPS (1 μg/ml) for 6 h. The cells were harvested and luciferase activity was measured by the dual luciferase assay (Promega). Renilla luciferase activity was used to normalize transfection efficiency.

RNA interference

RAW264.7 cells were plated on to 6-well plates and allowed to adhere overnight. These cells were transfected with TIR1 siRNA (small interfering RNA; 1 μg/well) or si-control (Dharmacon) using the Mirus SiQuest TransIT™ reagent (Mirus Bio). After 48 h, cells were treated with 1 μM GA for 30 min at 37°C followed by stimulation with 1 μg/ml LPS for 2 h. Cytoplasmic and nuclear fractions were separated from cells using the protocol described above. The cytoplasmic and nuclear fractions were used in Western-blot analyses using specific antibodies. GAPDH and tubulin were used as controls for cytoplasmic and nuclear fractions respectively.

EMSAs (electrophoretic mobility-shift assays)

NF-κB activation was analysed by EMSA as described previously [9]. Briefly, nuclear extracts (10 μg) were incubated with 40 000 c.p.m. of [32P]-labelled NF-κB double-stranded oligonucleotide (Promega) and 0.1 μg of poly(dI-dC)-(dI-dC) for 30 min and subjected to 5% PAGE under non-denaturing conditions in Tris/borate buffer (Bio-Rad). To confirm specificity of NF-κB binding, unlabelled oligonucleotide (3.5 pmol) was used as a specific competitor. For supershift experiments, the nuclear extract from LPS-treated cells was incubated with 2 μg of anti-p50 (Santa Cruz Biotechnology) followed by [32P]-labelled oligonucleotides as described above.

Preparation of biotinylated GA

The carboxy group of GA was modified by amidation with EZ-link (5-biotinamido)pentylamine (Pierce). Briefly, GA (∼2 mg) and EZ-link (5-biotinamido)pentylamine (5 mg) were dissolved in DMSO (300 μl) and were allowed to react with EDAC [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide] in 100 mM Mes (pH 5.5) at 37°C for 3 h. The reaction was stopped by extracting the product with ethyl acetate and Mes buffer (1:1, v/v). The extracted biotinylated GA was purified using reverse-phase HPLC with a linear gradient of acetonitrile (10–100%). The biotinylated GA was dried under nitrogen and dissolved in acetonitrile for further use.

Interaction of biotinylated GA with IKKβ in HEK-293 cells

To determine the interaction of biotinylated GA with IKKβ, HEK-293 cells were transfected with 5 μg of HA-tagged IKKβ WT (wild-type) mammalian expression construct along with 20 μl of Lipofectamine™ for 16 h. After transfection, the cells were washed with PBS, harvested and lysed in M-PER. Cell lysates containing 50 μg of protein were treated with 1 mM DTT (dithiothreitol) and/or 1.5 μM biotinylated GA for 2 min at 37°C and immunoprecipitated with anti-HA–agarose beads (Santa Cruz Biotechnology) overnight at 4°C with constant shaking. The beads were washed three times with TBST [Tris-buffered saline (0.1 M Tris/NaCl, pH 7.5, containing 0.2% Tween 20] buffer by centrifugation at 16 000 × g for 10 min. The proteins were eluted by boiling the beads in SDS sample buffer for 5 min and analysed by SDS/PAGE. The biotinylated proteins were electrotransferred and immunodetected using the chemiluminescent detection kit (Pierce). The cell lysates, where IKKβ–HA was overexpressed, were incubated with biotinylated GA mixed with increasing amounts of GSH (0.1–3 mM) to test the effect of GSH on the interaction with IKKβ. To test whether Cys-179 was being modified by GA, we used the HA-tagged human IKKβ WT and IKKβ C179A (Cys179→Ala) mutant to compare the reactivity of GA–bion. The expression constructs were kindly provided by Dr Michael Karin at University of California (San Diego, CA, U.S.A.). The human HA-tagged IKKβ WT or C179A mutant expression constructs were transfected into RAW264.7 macrophages and cell lysates were treated with biotinylated GA as described above.

MS analysis of post-translational modification of IKKβ

To further confirm the modification of IKKβ by GA, murine IKKβ peptide-(173–186), LDQGSLUCUTFVGTL (synthesized at the Peptide Synthesis Facility, Penn State College of Medicine, Hershey, PA, U.S.A.) was incubated with DMSO and equimolar purified GA for 30 min at 37°C in PBS. The samples were analysed by MALDI–TOF-MS (matrix-assisted laser desorption ionization–time-of-flight MS) for modification at the Mass Spectrometry Facility, Penn State University (University Park, PA, U.S.A.).

Inhibition of IKKβ activity by GA in vitro

To determine whether GA inhibits IKK activation, RAW264.7 cells were transfected with HA-tagged IKKβ WT expression vector for 24 h as described above. The cells were lysed and the clarified lysates were then incubated with anti-HA prebound agarose at 4°C overnight. The beads were washed with TBST containing phosphatase inhibitors and treated with GA (1.0 μM) for 2 h at 37°C. The kinase reaction was performed on the immunoprecipitates in kinase buffer with GST (glutathione transferase)–IκBz as the substrate for 2 h at 30°C. The reaction was stopped by adding 10 μl of 1 × SDS sample buffer and the reaction mixtures were subjected to SDS/PAGE followed by Western-blot analysis. The membrane was probed with monoclonal anti-p-IκBz antibodies and reprobed with anti-IKKβ after treating the membrane with the stripping reagent (Pierce).

Cell viability assay

Cell viability was determined using cell counting Kit-8 to count the number of living cells (Dojindo Molecular Technologies, Gaithersburg, MD, U.S.A.). CCK-8 utilizes WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt], which produces a water-soluble formazan dye on bioreduction by cellular dehydrogenases to an orange formazan product that is soluble in the tissue culture medium. The amount of formazan produced is directly
proportional to the number of living cells. RAW264.7 cells were plated at a concentration of 5000 cells in 100 μl of complete medium per well in 96-well plates for 24 h before treatment. The cells were treated with the indicated concentrations of GA for different time points. Thereafter, 10 μl of CCK-8 reagent was added to each well and incubated for an additional 3 h. The attenuation was read in a Packard microplate reader at 450 nm and the cell viability was calculated. In addition, RAW264.7 cells were treated with 4 log orders of GA (0.01–10 μM) for 24 h and the cell viability was assessed using the Trypan Blue exclusion method. The ratio of live to total cells was calculated using a haemocytometer.

Statistical analysis

When necessary, results are expressed as means ± S.D., and the Student’s t test was used in statistical analysis for comparison. P < 0.05 was used as the criterion for statistical significance.

RESULTS

Effect of GA on the LPS-induced expression of COX-2, TNF-α and iNOS

To investigate the anti-inflammatory effects of GA, the expressions of three prototypical inflammatory markers, COX-2, TNF-α and iNOS, were analysed in RAW macrophages. As shown in Figure 2(A), GA pretreatment markedly reduced the expression of COX-2 in LPS-treated macrophages in a concentration-dependent manner. The EC50 was found to be ∼0.5 μM. At 1 μM, there was complete inhibition of COX-2 expression. Along the same lines, the production of TNF-α in the extracellular media was analysed in RAW264.7 macrophages. As shown in Figure 2(B), the production of TNF-α was inhibited in a time-dependent manner in GA-pretreated RAW macrophages. The inhibitory effect was apparent as early as 30 min post LPS stimulation, whereas at later time points (2–4 h), the inhibition of TNFα was even more conspicuous. We also analysed the expression of iNOS in RAW264.7 macrophages before and after treatment with GA. As shown in Figure 3(A), pretreatment of RAW264.7 cells with increasing concentrations of GA promoted a dose-dependent inhibition of the amount of nitrite produced by these cells in response to LPS. The EC50 was calculated to be ∼0.5 μM GA. Western-blot analysis of the corresponding cell extracts showed that GA pretreatment inhibited the LPS-induced stimulation of iNOS expression. The EC50 in this case was calculated to be ∼0.5 μM of GA, which corroborates well with those obtained with activity. The fact that the expression of all three pro-inflammatory genes was inhibited by GA suggested that the effect of GA was likely mediated at the level of transcription.

Kasibhatla et al. [6] and Pandey et al. [7] have demonstrated that GA binds to TIR1 to negatively affect transcription of pro-inflammatory genes in cancer cells. However, flow-cytometric studies indicated a > 85 % down-regulation of TIR1 (also called CD71) in LPS-treated RAW264.7 cells (Figure 4A). To address the fact that GA targeted the NF-κB pathway independently of TIR1, we examined the LPS-dependent nuclear translocation of p65 in TIR1-knockdown cells. Results shown in Figure 4(B) clearly indicate that even in the absence of TIR1, GA caused a significant decrease in the nuclear levels of p65 in cells treated with LPS. Thus, given the possibility of the existence of alternative cellular targets other than TIR1, we examined the effect of GA on the activation of the NF-κB pathway.
Anti-inflammatory function of gambogic acid

GA blocks LPS-induced nuclear translocation of p65 subunit of NF-κB

We determined the effect of GA on the LPS-induced nuclear translocation of the p65 subunit of NF-κB by immunoprecipitation of nuclear protein extracts. LPS treatment elicited a rapid nuclear accumulation of p65 in control and DMSO-treated cells. Pre-incubation with GA abolished the LPS-stimulated p65 translocation to the nucleus in RAW264.7 macrophages (Figure 5). RAW264.7 cells showed enhanced sensitivity towards GA in that the inhibition of p65 translocation was robust at concentrations as low as 0.5 μM, whereas at higher concentrations (1.0 and 1.5 μM), there was complete inhibition of nuclear translocation of p65. Taken together, these results indicate that GA blocks the nuclear translocation of the p65 subunit of NF-κB.

Effect of GA on NF-κB DNA binding

To determine whether GA inhibited LPS-induced NF-κB transcriptional activity, transient transfections with a multimerized NF-κB-driven luciferase vector, murine COX-2 WT and COX-2ΔκB promoter luciferase vectors were carried out (Figures 6A and 6B). We tested the modulation of murine COX-2 promoter reporter construct by GA, which has two NF-κB sites that are pivotal to LPS-induced transcriptional activation [28,29]. Pre-incubation of such COX-2 WT transfected cells with various concentrations of GA clearly indicated that the LPS-induced NF-κB activation was significantly decreased at ≥0.5 μM GA, and at concentrations ≥1 μM, there was hardly any activity observed (Figure 6A). The NF-κB double mutant COX-2 reporter was inactive and exhibited no effect on LPS and/or GA treatment (Figure 6A). Furthermore, we studied the effect of GA on the LPS-dependent activation of the multimerized NF-κB-luciferase reporter, (κB)5-Luc (Figure 6B). The results of the inhibition of LPS-dependent luciferase activity by GA corroborate well with those obtained earlier with the COX-2 reporter assays, with the EC50 in the range of 0.3–0.5 μM (Figure 6B). Interestingly, DGA was unable to repress the NF-κB activity at 1 μM.

In support of the reporter studies, we analysed nuclear extracts from LPS-stimulated RAW264.7 macrophages (Figure 6C) and human U937 monocytic cells (Figure 6D) before or after GA and DGA treatment for binding to NF-κB oligonucleotide in gel-shift assays. LPS at a concentration of 1 μg/ml strongly activated NF-κB activity in both the macrophage cell types. Pretreatment

Figure 4 Tfr1-independent modulation of p65 in GA-pretreated macrophages

(A) Flow cytometry histograms of RAW264.7 cells treated with or without LPS (1 μg/ml) for 12 h. Cells were blocked with FcR (Fc receptor) blocking antibody (CD16/CD32), stained for CD71 (Tfr1)–FITC and analysed by flow cytometry. The histograms represent unstained (grey shaded), treated with LPS (dotted line) and untreated with LPS (solid line) respectively. Cell numbers described above are averages of triplicate experiments ± S.D. (B) RAW264.7 cells were plated on to 6-well plates and were transfected with Tfr1 siRNA or si-control at 1 μg/well. After 48 h, cells were treated with 1 μM GA or vehicle (DMSO) for 30 min at 37 °C followed by treatment with 1 μg/ml LPS for 2 h. Cytoplasmic and nuclear extracts were prepared from these cells. Results shown are representative of n = 3 experiments. IB, immunoblot.

Figure 5 GA inhibits LPS-induced nuclear translocation of p65

RAW264.7 cells were pretreated with 0.1–1.5 μM GA for 30 min and then stimulated with LPS (1 μg/ml) for 2 h. Nuclear proteins were analysed for the presence of p65 by immunoprecipitation followed by Western-blot analysis. IgG heavy chain was used to confirm near equal loading of the immunoprecipitate. Cell lysates (pre-immunoprecipitation) were analysed for Western blotting for GAPDH and RNA polymerase II as markers of cytosolic and nuclear fractions respectively. Results shown are representative of n = 3 experiments. IP, immunoprecipitate; IB, immunoblot.
with GA markedly inhibited LPS-dependent NF-κB activation in both cell types. As seen earlier, pretreatment with DGA was ineffective in inhibiting the activation of NF-κB. Taken together, these studies unequivocally indicate that GA inhibits the activation of NF-κB and that the α,β-unsaturated moiety is essential for the inhibitory activity.

**In vitro kinase assays**

Based on our previous experiments with 15-Δ^12,14^-PGJ_2 (15-Δ^12,14^-prostaglandin J_2) [9], bioactive compounds with an α,β-unsaturated moiety have the potential to interact covalently with a key thiol in IKKβ. Therefore we first examined whether IKK activity was affected by GA. To determine the effect of GA on LPS-induced IKK activation, we analysed the cellular levels of pIκBα before and after LPS treatment (Figure 7). GA pretreatment significantly decreased pIκBα in a dose-dependent manner in RAW264.7 cells (Figure 7A) at ≤ 0.5 μM GA. To further investigate whether GA targeted IKKβ, IKKβ-HA was overexpressed in RAW264.7 cells and the immunoprecipitates were examined for the effect of GA (1.0 μM) on kinase activity. As shown in Figure 7(B), GA significantly inhibited the IKK activity, suggesting the possibility of covalent interaction with IKKβ to inhibit the enzymatic activity.

**Covalent binding of GA with IKKβ**

To determine whether GA covalently reacted with IKKβ, a biotinylated derivative of GA was added to the lysates from HEK-293 cells that were transfected with a pCMV-IKKβ-HA expression construct. The GA–IKKβ adduct was pulled down with anti-HA–agarose beads and the biotinylation of IKKβ was examined. It appeared that GA reacted with IKKβ and treatment with 1 mM DTT disrupted the binding of GA with IKKβ (Figure 8A, upper panel). Alternatively, the biotinylated proteins from the cell lysates were pulled-down with neutravidin–agarose beads. The affinity pull-downs indicated the presence of
Anti-inflammatory function of gambogic acid

Figure 7 Effect of GA on LPS-induced IKK activity

(A) RAW264.7 cell lysates from RAW264.7 cells treated with DMSO or GA (0.1–1.5 μM) followed by stimulation with LPS for 2 h. (B) IKKβ–HA-tagged protein was expressed in RAW264.7 cells and the immunoprecipitate (IKKβ–anti-HA complex) was incubated with DMSO or GA (1.0 μM) for 30 min followed by an in vitro kinase activity assay (KA) using the GST–IκBα-(1–55) protein and ATP. In all experiments, the blots were reprobed for GAPDH or IKKβ to normalize for equal protein loading. Results shown are representative of n = 3 experiments.

IKKβ as one of the proteins that interacted with GA (Figure 8A, lower panel). In addition, we examined whether the interaction between IKKβ and GA was modulated by physiological cellular thiols such as GSH. Although increasing GSH concentration from 0.1 mM to 1.0 and 3 mM decreased the ability of IKKβ to bind GA, we were still able to detect interaction even at 3 mM (Figure 8B). Both these experiments clearly indicated that GA covalently interacted with IKKβ and that the interaction withstood reducing and denaturing conditions during electrophoresis except when DTT or GSH was included during reaction. To further confirm the reaction, GA was incubated with the IKKβ-(173–186) peptide and subjected to qualitative MALDI–TOF-MS analysis. The results clearly demonstrated an increase in the mass of the peptide from 1440.7 to 2066.2, a difference of 625.5 m/z units, indicating that the peptide was covalently modified by GA (Figure 8C). To address the importance of Cys179 in the interaction with GA, an IKKβ(Cys/Ala) mutant expression construct was transfected into RAW264.7 cells. The cell lysates were incubated with GA as described above. Immunoprecipitation with anti-HA followed by Western immunoblotting with streptavidin–HRP (horseradish peroxidase) clearly showed lack of binding of GA to IKKβ(Cys/Ala) mutant compared with the WT IKKβ (Figure 8D). Taken together, these studies indicate that GA interacts with the Cys179 residue of IKKβ.

Cytotoxicity of GA

To further test whether GA exhibited any effect on the viability of RAW264.7 cells used in the present study, cytotoxic assays were performed. Results shown in Figure 9 indicate that in RAW264.7 cells, no cytotoxic effect was observed even at 24 h of incubation.
is an intracellular target. The interaction of GA with IKKβ was inhibited to a significant extent by increasing levels of GSH, a cellular thiol antioxidant. It could be predicted that imbalances in the [GSH]/[GSSG] ratio towards the oxidizing state, as in many inflammatory disease pathologies that activate NF-κB [31], may in fact allow GA to impart its anti-inflammatory activity. Thus glutathionylation represents a key cellular metabolic event in the regulation of the anti-inflammatory activity of GA.

It is known that the electrophilic α,β-unsaturated carbonyl group in bioactive compounds selectively reacts with protein thiols. Electrophiles such as 15-deoxy-Δ12,14-PGJ2 [9,24] and 4-HNE [10] interact with IKKβ leading to complete abrogation of its kinase activity. To test whether the interaction of GA and 15-deoxy-Δ12,14-PGJ2 with cellular proteins overlapped, we analysed the ability of GA to modify thioredoxin and p50, which are already established as key protein targets of 15-deoxy-Δ12,14-PGJ2 [25,32]. Studies showed that GA interacted with both these proteins (results not shown). Molecular modelling studies have provided some insight into the interaction of 15-deoxy-Δ12,14-PGJ2 with cysteine thiols in the above proteins being controlled by molecular recognition by fit of shape and complementarity in addition to the accessibility and environment of the cysteine thiol [33]. Thus it is likely that a subset of cellular proteins may meet these requirements to interact with hydrophobic electrophiles such as 15-deoxy-Δ12,14-PGJ2 and GA. Needless to say, the specificity of covalent interaction of GA with cysteine thiols in target proteins will need to be further characterized using proteomics, which might provide clues regarding the environment of the cysteine thiol and the structural features that are necessary for interaction. Based on these studies, we believe that the spectrum of activities exhibited by GA could be partly attributed to interaction with proteins covalently, as shown here, in addition to the ability to interact non-covalently with TIR1 to effect downstream gene expression.

In summary, our results indicate the existence of an alternative pathway of inhibition of NF-κB activation by GA via specifically modifying Cys179 of IKKβ, leading to the down-regulation of expression of COX-2, iNOS and TNFα, which are implicated in inflammation.

ACKNOWLEDGEMENTS

We are grateful to the Macromolecular Core Facility, Penn State College of Medicine (Hershey, PA, U.S.A.), for the synthesis of the IKKβ peptide and to the Penn State Mass Spectrometry Facility and the Center for Quantitative Cell Analysis for the mass spectral and flow cytometric analyses respectively. We thank Dr Michael Karin (UCSD School of Medicine, University of California San Diego, La Jolla, CA, U.S.A.) for the IKK plasmid constructs.

FUNDING

This work was supported by the National Institutes of Health [grant number AT 004350 (a Public Health Service grant to K.S.P.)]

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