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A mechanism of benefit of soy genistein in asthma: inhibition of eosinophil p38-dependent leukotriene synthesis

R. Kalhan, L. J. Smith, M. C. Nlend, A. Nair, J. L. Hixon, and P. H. S. Sporn

Division of Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Summary

Background—Dietary intake of the soy isoflavone genistein is associated with reduced severity of asthma, but the mechanisms responsible for this effect are unknown.

Objective—To determine whether genistein blocks eosinophil leukotriene C₄ (LTC₄) synthesis and to evaluate the mechanism of this effect, and to assess the impact of a 4-week period of soy isoflavone dietary supplementation on indices of eosinophilic inflammation in asthma patients.

Methods—Human peripheral blood eosinophils were stimulated in the absence and presence of genistein, and LTC₄ synthesis was measured. 5-lipoxygenase (5-LO) nuclear membrane translocation was assessed by confocal immunofluorescence microscopy. Mitogen-activated protein (MAP) kinase activation was determined by immunoblot. Human subjects with mild-to-moderate persistent asthma and minimal or no soy intake were given a soy isoflavone supplement (100 mg/day) for 4 weeks. The fraction of exhaled nitric oxide (FE_{NO}) and *ex vivo* eosinophil LTC₄ production were assessed before and after the soy isoflavone treatment period.

Results—Genistein inhibited eosinophil LTC₄ synthesis (IC₅₀ 80 nM), blocked phosphorylation of p38 MAP kinase and its downstream target MAPKAP-2, and reduced translocation of 5-LO to the nuclear membrane. In patients with asthma, following 4 weeks of dietary soy isoflavone supplementation, *ex vivo* eosinophil LTC₄ synthesis decreased by 33% (*N* = 11, *P* = 0.02) and FE_{NO} decreased by 18% (*N* = 13, *P* = 0.03).

Conclusion—At physiologically relevant concentrations, genistein inhibits eosinophil LTC₄ synthesis *in vitro*, probably by blocking p38- and MAPKAP-2-dependent activation of 5-LO. In asthma patients, dietary soy isoflavone supplementation reduces eosinophil LTC₄ synthesis and eosinophilic airway inflammation. These results support a potential role for soy isoflavones in the treatment of asthma.

Keywords

asthma; eosinophils; genistein; 5-lipoxygenase; soy isoflavones

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Correspondence: Ravi Kalhan, Division of Pulmonary and Critical Care Medicine, Northwestern University Feinberg School of Medicine, 676 N St Clair Street, #1400, Chicago, IL 60611, USA. r-kalhan@northwestern.edu.

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Introduction

Asthma is a complex disease whose prevalence and severity are determined by multiple genetic and environmental factors. The prevalence of asthma has increased during the past few decades [1-5]. Although causal relationships have not been proven, changing dietary practices have paralleled the increase in asthma prevalence. Several surveys have found an association between reduced lung function and decreased consumption of antioxidants, flavonoids, and essential vitamins in a variety of respiratory conditions, including asthma [6-10]. These findings suggest that diet may be a factor that impacts asthma and its clinical manifestations [11, 12].

One dietary component of interest is soy, which contains the biologically active isoflavone genistein, a potent tyrosine kinase inhibitor. Soy and soy isoflavones have been studied in cardiovascular disease, osteoporosis, prostate cancer, and other diseases [13-17], but only recently have they received attention in asthma and allergy.

Epidemiologic studies have associated higher dietary consumption of soy isoflavones with decreased self-report of cough [10, 18] and other allergic respiratory symptoms [6]. We have shown that asthma patients in the United States with high dietary genistein consumption have less severe airflow obstruction than those who consume little or no genistein [19]. The molecular mechanisms by which consumption of genistein might attenuate asthma and allergy have not been elucidated.

In animal models, genistein blocks allergen-induced airway inflammation. Intratracheal instillation of genistein reduces lung levels of cysteinyl-leukotrienes and histamine in *ex vivo* allergen-challenged guinea-pig bronchi and chopped lung specimens [20]. Genistein also reduces bronchoconstriction, airway hyper-reactivity, bronchoalveolar lavage eosinophil peroxidase levels, and airway inflammation in an *in vivo* guinea-pig model of allergic asthma [21]. When provided in the diet, soy isoflavones reduce allergen-induced lung tissue eosinophilia in guinea-pigs [22].

Because cysteinyl-leukotrienes are key inflammatory mediators produced by eosinophils in asthma and other allergic diseases [23], we investigated the ability of genistein to block the synthesis of leukotriene C₄ (LTC₄) by freshly isolated human blood eosinophils. While treatment with genistein has previously been shown to reduce eosinophil protein tyrosine kinase phosphorylation and leukotriene production [24], the specific intracellular mechanisms of this inhibition have not been investigated. We sought, therefore, to determine whether genistein blocks calcium-ionophore A23187-stimulated synthesis of LTC₄ in freshly isolated human peripheral blood eosinophils, whether it inhibits 5-lipoxygenase (5-LO) activation, and the biochemical mechanism responsible for its effect on the 5-LO pathway of arachidonic acid metabolism. To validate the effects that we observed *in vitro*, we administered a dietary soy isoflavone supplement to patients with asthma for 4 weeks and measured *ex vivo* A23187-stimulated eosinophil LTC₄ production and the fraction of exhaled nitric oxide (FE_{NO}) before and at the end of the period of supplementation.

Materials and methods

Human subjects

For the *in vitro* eosinophil experiments, blood was obtained from volunteers with a clinical history of atopic disease (in most cases allergic rhinitis) and a peripheral eosinophilia of 3%. The subjects were all non-smokers and were not taking oral or inhaled steroids, non-steroidal anti-inflammatory drugs, leukotriene synthesis inhibitors, or leukotriene receptor antagonists.

In the soy isoflavone dietary supplementation study, subjects aged 18–65 years old had asthma as defined by an forced expiratory volume in 1 s (FEV₁) between 60% and 90% of predicted, and at least a 12% increase in FEV₁ 15 min after inhaling two puffs of albuterol via a metered dose inhaler. Subjects were excluded if they were treated with oral corticosteroids during the antecedent 3 months, if they reported a history of cigarette smoking within the past 6 months or reported a >10 pack-year history of tobacco use, if they were regular consumers of soy products, or if they did not use a daily inhaled corticosteroid. All study protocols were approved by the Institutional Review Board at Northwestern University, and the clinical pilot study of dietary supplementation with soy isoflavones in asthma patients was registered with <http://www.clinicaltrials.gov>

Eosinophil isolation

Eosinophils were isolated by a modification of the method of Hansel et al. [25] in a manner previously described by our group [26]. Briefly, heparinized venous blood was centrifuged through Percoll (Pharmacia & Upjohn, Kalamazoo, MI, USA) (density 1.084 g/mL), and erythrocytes were removed by hypotonic lysis. The remaining granulocytic fraction was washed, counted, and incubated with MACS CD16 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) on ice for 30 min. The granulocyte suspension was then applied to type CS magnetic columns (Miltenyi Biotec) in a 0.6 T magnetic field, and the eluate containing eosinophils was collected. Eluates were washed and resuspended in RPMI 1640 medium containing 10% foetal calf serum (FCS). The resulting cell suspensions derived by this method have resulted in 97% pure eosinophils and >98% viability [26].

Eosinophil culture and stimulation

Individual wells in tissue culture plates and glass cover slips were coated with human plasma fibronectin (5 µg/mL) (Collaborative Biomedical Products, Bedford, MA, USA) by incubation overnight at 4 °C. Freshly purified eosinophils were allowed to adhere to either tissue culture plates or glass cover slips for 1 h at 37 °C in 5% CO₂/95% air in RPMI 1640 containing 10% FCS. Genistein (derived from soybean and 98% pure by HPLC; Sigma, St Louis, MO, USA), the p38 inhibitor SB203580 (10 µM) (EMD Biosciences, San Diego, CA, USA), or the ERK1/2 inhibitor U0126 (20 µM) (EMD Biosciences) was added, and cells were incubated for an additional hour. Each inhibitor was dissolved in 0.1% DMSO, and control conditions were treated with this concentration of vehicle. Eosinophils were then stimulated by the addition of 2 µM calcium ionophore A23187 (Sigma) for 15 min or 1 µM formyl-methionylleucylphenylalanine (fMLP; Sigma) for 30 min.

Measurement of leukotriene C₄ production by eosinophils

LTC₄ was quantified in cell-free supernatants by an enzyme-linked immunoassay (Cayman Chemical, Ann Arbor, MI, USA). Every condition in which LTC₄ was measured was performed in triplicate, and the results from replicate samples from individual donors were averaged and counted as a single *N*.

Determination of eosinophil viability

Eosinophils that adhered to fibronectin-coated wells were cultured in the absence or in the presence of 10 µM genistein and/or recombinant human IL-5 (R&D Systems, Minneapolis, MN, USA) for 24 h. Adherent cells were then lifted from culture wells with ice-cold phosphate-buffered saline, and cell suspensions were incubated in 1 µg/mL propidium iodide (PI) solution on ice for 15 min. PI-positive and -negative cells were then immediately quantified by flow cytometry using a Beckman Coulter (Fullerton, CA, USA) Epics XL-MCL flow cytometer. For each sample, the percent viability was calculated as the ratio of PI-negative cells to total cells multiplied by 100.

Immunoblotting

Eosinophils were lysed in ice-cold RIPA buffer with protease and phosphatase inhibitors (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and sonicated. Samples of whole-cell lysate containing equal quantities of protein were subjected to SDS-PAGE in 10% polyacrylamide gels (Biorad Laboratories, Hercules, CA, USA) and transferred onto nitrocellulose membranes using a semi-dry transfer system (Biorad Laboratories). Membranes were incubated with antibodies specific for phosphorylated and total ERK1/2, p38, and MAPKAP-2 (all from Cell Signaling Technology, Danvers, MA, USA) and developed using the SuperSignal West Dura ECL detection kit (Pierce Biotechnology, Rockford, IL, USA). Densitometry was performed using NIH Image J software, and the ratio of phosphorylated to total ERK1/2, p38, and MAPKAP-2 was determined for each sample.

Evaluation of 5-lipoxygenase nuclear membrane translocation via immunofluorescence

Eosinophils adhered to fibronectin-coated glass cover slips were fixed in 10% buffered formalin for 7 min, and then permeabilized with 0.1% Triton-X 100 for 20 min at 37 °C. Cells were subsequently incubated with anti-human 5-LO antiserum (Cayman Chemical) at a dilution of 1 : 100 for 30 min at 37 °C. After washing, the cover slips were incubated with an AlexaFluor 555 goat anti-rabbit antibody (Molecular Probes, Carlsbad, CA, USA) at a dilution of 1 : 200 for 30 min at 37 °C. The cover slips were mounted on glass slides using Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA, USA) as a marker for cell nuclei. Immunostained cells were imaged using a Zeiss (Thornwood, NY, USA) LSM510 confocal laser scanning microscope to evaluate sub-cellular localization of 5-LO.

Effects of dietary supplementation with soy isoflavones on eosinophilic inflammation in patients with asthma

Subjects consumed 100 mg of soy isoflavones (two 50 mg tablets once daily) (NOVASOY, ArcherDanielsMidland, Decatur, IL, USA), containing genistein (29 mg), daidzein (28 mg), and glycitein (8 mg) as aglycone for 4 weeks. Immediately before and at the end of the 4-week period of dietary supplementation, peripheral blood eosinophils were isolated, plated in triplicate (2.5×10^4 eosinophils/well), and stimulated with A23187 as described above, after which LTC₄ was measured in cell-free supernatants. FE_{NO} was measured before and after the 4-week supplementation period using a NIOX analyser (Aerocrine, Solna, Sweden) according to American Thoracic Society guidelines [27]. Assuming a mean baseline value of 50 p.p.b. and a standard deviation of the response of 13 p.p.b., in order to have 80% power to detect a 20% reduction in FE_{NO} using a paired *t*-test, we planned to enroll 15 patients with asthma.

Statistical analysis

For experiments in which LTC₄ was measured, data were normalized to the value obtained from cells stimulated with A23187 or fMLP in the absence of inhibitors. For experiments in which activation of ERK1/2, p38, and MAPKAP-2 was assessed, densitometric ratios were normalized to the value obtained from the unstimulated cells in the absence of inhibitors. In all cases, *N* refers to the number of independent experiments using eosinophils from different donors. Data are presented as means $\pm$ SEM. Differences between groups were assessed by one-way ANOVA or, in the case of the fMLP stimulation experiments, an unpaired *t*-test. *Post hoc* testing for multiple comparisons was performed via the Student's–Newman–Keuhls method (for ERK1/2, p38, and MAPKAP-2 immunoblots) or Dunnett's test (for LTC₄) for multiple comparisons against a single control. Data from the human study were assessed via paired *t*-tests comparing results for individual subjects at the

beginning and end of the period of soy supplementation. Statistical analysis was performed using GraphPad Prism for Windows 4.00 (GraphPad Software, San Diego, CA, USA) and Intercooled Stata 9.2 (StataCorp, College Station, TX, USA).

Results

Effect of genistein on eosinophil leukotriene C₄ synthesis *in vitro*

To investigate the effect of genistein on LTC₄ production, eosinophils that adhered to fibronectin were incubated for 1 h in the absence or in the presence of genistein at concentrations from 1 nM to 10 μM, and then stimulated for 15 min with calcium ionophore A23187. In the absence of genistein, eosinophils (100 000 cells per condition) stimulated with A23187 synthesized and released 17.59 ± 2.92 ng/mL of LTC₄ ($N = 4$). Eosinophils that were not stimulated with A23187 produced no detectable LTC₄ (data not shown). As shown in Fig. 1, genistein inhibited A23187-induced eosinophil LTC₄ synthesis in a concentration-dependent manner, with significant inhibition at concentrations of 100 nM and higher. The estimated concentration to achieve 50% inhibition (IC₅₀) of LTC₄ synthesis was 80 nM.

To assess the effect of genistein on eosinophil LTC₄ synthesis in response to a receptor-mediated stimulus, we stimulated eosinophils with fMLP. As with A23187 stimulation, genistein (10 μM) inhibited LTC₄ synthesis by eosinophils stimulated with fMLP (31.0 ± 8.6 pg/mL/250 000 eosinophils without genistein vs. 8.219 ± 0.4 pg/mL/250 000 eosinophils after treatment with genistein; $69 \pm 40\%$ inhibition, $P < 0.01$; $N = 3$).

To evaluate whether inhibition of LTC₄ synthesis resulted from an effect of genistein on cell viability, we exposed eosinophils to genistein, as above, and then stained them with PI, and quantified viable cells using flow cytometry. The mean percentage of viable cells after 24 h in the untreated condition was 58.9% ($N = 4$). Exposure to genistein at 10 μM (the highest concentration used in our studies) for 24 h did not reduce eosinophil viability either in the absence or in the presence of IL-5 (Table 1).

Determination of mitogen-activated protein kinase phosphorylation after treatment with genistein

Because the MAP kinases ERK1/2 and p38 play a role in leukotriene synthesis [28-30], and genistein inhibits both ERK1/2 and p38 in other cell types [31-34], we investigated whether genistein's effect on LTC₄ synthesis in eosinophils might be due to inhibition of these kinases. First, we assessed whether blocking ERK1/2 with U0126 (20 μM) or blocking p38 with SB203580 (10 μM) would inhibit LTC₄ synthesis to a degree similar to that seen with genistein (10 μM). As shown in Fig. 2, all three compounds similarly reduced A23187-stimulated LTC₄ synthesis by more than 80%.

We next determined whether genistein blocks A23187-induced phosphorylation of ERK1/2 in eosinophils. Immunoblots of both phosphorylated and total ERK1/2 were performed after treatment with genistein. In contrast to the MEK inhibitor U0126 (10 μM), which effectively blocked A23187-induced activation of ERK1/2, treatment with genistein (10 μM) did not have any effect (Fig. 3a).

Immunoblots of phosphorylated and total p38 were also performed after treatment with genistein. In contrast to its lack of effect on ERK1/2, genistein (10 μM) decreased p38 phosphorylation (Fig. 3b), similar to the effect of SB203580 (20 μM). Because MAPKAP-2, a downstream target of p38, has been implicated as the kinase directly responsible for phosphorylation and activation of 5-LO [28, 29], we examined whether genistein also affected A23187-induced phosphorylation of MAPKAP-2. As shown in Fig. 3c, genistein

blocked A23187-induced MAPKAP-2 phosphorylation, again to the same degree as the p38 inhibitor SB203580.

Effect of genistein on activation of 5-lipoxygenase

Leukotriene synthesis in eosinophils requires the catalytic activity of three enzymes acting in sequence: cytosolic phospholipase A₂ (cPLA₂), 5-LO, and LTC₄ synthase. To evaluate whether inhibition of LTC₄ production by eosinophils occurred at the level of cPLA₂, we stimulated eosinophils with A23187 in the absence and presence of exogenous arachidonic acid, which provides a substrate for 5-LO without the need for cPLA₂-mediated phospholipid hydrolysis. As shown in Fig. 4, exogenous arachidonic acid (10 µg/mL) lessened, but did not eliminate, the inhibitory effect of genistein, indicating that a major portion of genistein's inhibitory effect occurs downstream of cPLA₂.

In eosinophils, translocation of 5-LO to the nuclear membrane [26] and lipid bodies [35, 36] is essential for its ability to catalyse the synthesis of leukotrienes. We therefore assessed the effect of genistein on the intracellular distribution of 5-LO in A23187-stimulated eosinophils. Cells stimulated with A23187 in the absence and presence of genistein were fixed and immunostained for 5-LO, and viewed with a confocal laser microscope. Unstimulated cells, in the absence or in the presence of genistein (10 µM), demonstrated diffuse, mainly cytoplasmic 5-LO staining, and did not have evidence of nuclear membrane or lipid body localization (Figs 5a and b). Cells stimulated with A23187 in the absence of genistein showed prominent outlining of the nuclear envelope and a punctate staining pattern in the cytoplasm consistent with 5-LO localization at the nuclear membrane and in lipid bodies (Fig. 5c). Treatment with genistein (10 µM) before A23187 stimulation attenuated localization of immunoreactive 5-LO to the nuclear membrane and lipid body-like cytoplasmic structures (Fig. 5d).

Effects of dietary supplementation with soy isoflavones on measures of eosinophilic inflammation in patients with asthma

In order to establish the relevance to asthma of the *in vitro* effects of genistein, we assessed A23187-stimulated eosinophil LTC₄ synthesis *ex vivo* and FE_{NO}, a marker of eosinophilic airway inflammation [27, 37, 38], in asthma patients before and at the end of a 4-week period of treatment with a soy isoflavone dietary supplement. Thirteen subjects completed the study. Two additional subjects were enrolled, but did not return for the post-supplement study visit and were lost to follow-up. The clinical characteristics of the participants in this pilot study are described in Table 2. Of the 13 subjects who completed the study, eosinophil isolation was successful at both the pre- and post-supplementation visits in 11. Following 4 weeks of soy isoflavone supplementation, *ex vivo* A23187-stimulated LTC₄ synthesis decreased by 33%, from 3.11 ± 1.63 to 2.07 ± 1.17 ng/mL ($P = 0.02$) (Fig. 6a). Likewise, FE_{NO} was decreased by 17%, from 67.0 ± 54.6 to 54.8 ± 49.2 p.p.b. ($P = 0.03$) (Fig. 6b). There was no significant change in pre-bronchodilator FEV₁ (2.59 ± 0.77 – 2.56 ± 0.92 L, $P = 0.88$). The Juniper Asthma Control Score was 16% lower after soy isoflavone supplementation (1.60 ± 0.75 – 1.34 ± 0.74 , $P = 0.12$), although the difference did not reach statistical significance.

Discussion

In this study, we have identified a plausible cellular mechanism, whereby genistein, a biologically active isoflavone found predominantly in soy, inhibits human blood eosinophil LTC₄ synthesis. We have shown that genistein decreases the activation of p38 MAP kinase and its downstream target MAPKAP-2, and that this is associated with decreased activation of 5-LO. Inhibition of eosinophil LTC₄ synthesis by genistein was concentration dependent,

with an IC₅₀ in the range that can be attained in circulating blood by dietary consumption [17]. We extended these *in vitro* findings by documenting that a 4-week period of soy isoflavone supplementation in patients with asthma results in decreased *ex vivo* eosinophil LTC₄ synthesis and reduced FE_{NO}, illustrating the biologic activity of soy isoflavones *in vivo*. Given that epidemiologic and animal data have identified this soy isoflavone as a potential modifier of asthma and allergic disease, our findings provide both a mechanistic and a clinical rationale for pursuing additional studies of its therapeutic potential.

Our findings are consistent with other reports of the effects of tyrosine kinase inhibitors on the 5-LO pathway of arachidonic acid metabolism. Dent et al. [24] reported that genistein and other tyrosine kinase inhibitors block platelet-activating factor (PAF)-induced eosinophil LTC₄ synthesis. This study, however, did not examine the steps downstream from PAF receptor activation where genistein might be exerting its effects. In contrast, using the non-receptor-mediated stimulus A23187 to activate eosinophils, we have shown that a major locus for the inhibitory action of genistein is 5-LO, the first committed step in the conversion of arachidonic acid to leukotrienes. A previous report also documented that genistein, as well as other tyrosine kinase inhibitors, blocked activation of 5-LO and its localization to a generic membrane fraction in differentiated HL-60 cells and human blood neutrophils [39]. We have extended this finding by demonstrating that genistein blocks 5-LO translocation specifically to the nuclear membrane in eosinophils, a key effector cell in asthma and other allergic diseases.

We further investigated the mechanism whereby genistein inhibits eosinophil LTC₄ synthesis by focusing on the MAP kinases p38 and ERK1/2. ERK1/2 and p38 activities are necessary for 5-LO activation in human blood neutrophils [28-30], and ERK1/2 inhibition blocks IL-5 priming of LTC₄ synthesis in eosinophils [40]. In the current study, we have demonstrated that genistein inhibits A23187-induced LTC₄ synthesis in eosinophils to the same degree as pharmacologic inhibitors of p38 and ERK1/2. While genistein had no inhibitory effect on activation of ERK1/2, we found that it blocked A23187-stimulated activation of p38 and phosphorylation of its downstream target MAPKAP-2, which activates 5-LO in neutrophils [28]. Our finding that genistein inhibits p38-mediated intracellular signalling in eosinophils is new, and is consistent with results in other cell types. For example, in a cell line derived from prostate cancer, a condition in which genistein has been investigated as a chemoprevention agent, genistein blocked TGF-β₁-induced p38 activation, which is required for matrix metalloproteinase-2 release and cell invasion [32]. Our data do not clarify whether genistein blocks activation of p38 directly or acts upstream in the p38 activation cascade. Activation of p38 requires phosphorylation of both a tyrosine (Tyr182) and a threonine (Thr180) residue, which is catalysed by the dual-specificity MAP kinase kinases, MKK3 and MKK6 [41]. Genistein may exert its inhibitory effect by blocking MKK3- or MKK6-mediated p38 phosphorylation on Tyr182. Alternatively, genistein may block upstream kinases required for activation of MKK3 and MKK6 [41]. In an ischaemic heart model, genistein blocked MAPKAP-2 and p38 phosphorylation by inhibiting MKK3 and MKK6 [42], suggesting the possibility of a similar effect in eosinophils.

While we have shown a close association between inhibition by genistein of LTC₄ synthesis, 5-LO activation, and phosphorylation of p38 and MAPKAP-2, our data do not prove definitively that inhibition of p38 and MAPKAP-2 is the mechanism by which genistein blocks activation of 5-LO and formation of LTC₄. In principle, more conclusive evidence of this mechanism might be demonstrated by transfection experiments using constitutively active and/or dominant-negative kinase constructs. These genetic approaches, however, are not feasible in human eosinophils, which are highly resistant to transfection. We have therefore relied on widely used pharmacologic inhibitors of p38 and ERK1/2, which implicate the former and exclude the latter kinase as a locus of genistein's effect. While

SB203580 and U0126 are not entirely specific, at the concentrations used they are highly selective for their intended MAP kinase targets [43, 44].

The use of calcium ionophore A23187 as a stimulus of 5-LO activation may be viewed as a weakness of our study because it is a non-physiologic trigger of LTC₄ synthesis. However, we do not consider this to be the case. By directly and rapidly increasing intracellular calcium, stimulation with A23187 provides a means to study the effects of genistein on the LTC₄ biosynthetic pathway and its immediate upstream regulators, independent of receptor activation. This approach allowed us to uncover genistein's inhibitory effects on p38, MAPKAP-2, and 5-LO itself. The ability to inhibit LTC₄ synthesis by blocking activation of 5-LO indicates that genistein should reduce LTC₄ synthesis by eosinophils regardless of the specific stimulus. We confirmed this by showing that genistein strongly inhibited LTC₄ synthesis stimulated by the chemotactic peptide fMLP, which triggers eosinophils through a G-protein-coupled receptor. These findings suggest that genistein would inhibit leukotriene synthesis *in vivo* in asthma, where cytokines, chemokines, and other stimuli may activate eosinophils through multiple mechanisms.

While we have focused the present study on the mechanism by which genistein inhibits 5-LO, our data suggest that genistein may also inhibit cPLA₂. This is supported by the observation that exogenous arachidonic acid, which bypasses the need for PLA₂-mediated phospholipid hydrolysis, overcame genistein inhibition of LTC₄ synthesis by approximately 50% (Fig. 4). An effect on cPLA₂ might also be a consequence of p38 inhibition, as p38 has been shown to activate this enzyme in several cell types [45, 46], including eosinophils [47]. cPLA₂, therefore, may be another locus of genistein's effects on LTC₄ synthesis in eosinophils. The specific effects of genistein on cPLA₂ activation represent an area that warrants further investigation.

In addition, we did not evaluate the effects of genistein on LTC₄ synthase in eosinophils. Existing data, however, point to protein kinase C (PKC) as the major kinase regulating LTC₄ synthase, and show that active PKC inhibits this enzyme, whereas tyrosine kinase inhibitors have no effect [48, 49]. Given this information, we think it is unlikely that genistein modulates the activation of LTC₄ synthase.

The half-maximal inhibitory concentration (IC₅₀) of genistein (80 nM) that we have demonstrated for eosinophil LTC₄ synthesis is in the range of levels attainable in the circulation through dietary supplementation with soy isoflavones [17]. In fact, for our study of the effects of soy isoflavone supplementation on indices of eosinophilic inflammation in patients with asthma, we chose a dose that we estimated would achieve blood levels close to this IC₅₀ [17]. Our results document the biological activity of dietary soy isoflavones in patients with asthma by showing that 4 weeks of isoflavone treatment reduced both *ex vivo* eosinophil LTC₄ production and FE_{NO}, which reflects eosinophilic airway inflammation [27, 38, 50, 51] (Fig. 6). The reduction in FE_{NO} that we report in this study is similar to that seen with the use of moderate-dose inhaled corticosteroids, in which an approximately 20% reduction in FE_{NO} was accompanied by a nearly 60% reduction in sputum eosinophilia [52]. We therefore think that the 17% reduction in FE_{NO} seen in the current study represents a meaningful biological response. In fact, given that all the subjects in the present study were using inhaled corticosteroids, an additional 17% reduction in FE_{NO} seems quite meaningful. While we did not document an improvement in FEV₁ and only showed a trend towards improvement in asthma symptom scores, the actual clinical impact of soy isoflavone consumption on asthma cannot be assessed from this small pilot study, which was designed to identify the specific biological effects on eosinophil function rather than the clinical parameters of the disease.

In summary, we have shown that genistein inhibits human blood eosinophil LTC₄ synthesis in a concentration-dependent manner, and that this inhibition results from blockade of 5-LO activation, in association with inhibition of p38 and MAPKAP-2. We conclude that genistein's effect on leukotriene synthesis is a consequence of impaired activation of p38 MAP kinase, which may be due to direct inhibition of p38 phosphorylation on Tyr182 or to blockade of the upstream activators of p38, MKK3, and MKK6. Diminished p38 activity results in decreased phosphorylation/activation of MAPKAP-2, and decreased nuclear translocation and activation of 5-LO. We have tested the potential clinical relevance of these *in vitro* findings by documenting that dietary supplementation with soy isoflavones in patients with asthma has biological effects on eosinophil effector functions. These studies therefore elucidate a biochemical and molecular mechanism that provides an insight into how dietary intake of soy isoflavones may influence allergy and asthma, and further establish that dietary supplementation with these compounds may influence airway inflammation in asthma. These findings form the rationale for future clinical investigation of dietary supplementation with soy isoflavones, or novel inhibitors of the pathway we have identified, as a strategy to ameliorate asthma and other eosinophil- or leukotriene-mediated diseases.

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Fig. 1.

Genistein reduces stimulated eosinophil leukotriene C₄ (LTC₄) synthesis in a concentration-dependent fashion. Freshly isolated human blood eosinophils were adhered to fibronectin-coated wells for 1 h, treated with medium alone or genistein for an additional hour, and stimulated with 2 μ M A23187 for 15 min in the absence or presence of genistein at the indicated concentrations. LTC₄ released into culture medium was quantified by enzyme-immunoassay, and data were normalized to the amount of LTC₄ detected in the absence of genistein. The concentration of genistein that resulted in 50% inhibition of LTC₄ synthesis is 80 nM. * $P < 0.05$ compared with the condition without genistein ($N = 4$).

Fig. 2.

Pharmacologic inhibitors of the mitogen-activated protein kinases ERK 1/2 and p38 block eosinophil leukotriene C₄ (LTC₄) synthesis to the same degree as genistein. Eosinophils adhered to fibronectin-coated wells for 2 h were stimulated with 2 μ M A23187 for 15 min in the absence (vehicle alone) and presence of 10 μ M genistein, the p38 inhibitor SB203580 (10 μ M), or the ERK1/2 inhibitor U0126 (20 μ M). LTC₄ release was normalized to the amount detected in the absence of inhibitors. * P < 0.05 compared with the condition without inhibitors (N = 3).

Fig. 3.

Genistein blocks A23187-induced phosphorylation of p38 and MAPKAP-2, but not ERK 1/2, in eosinophils. Unstimulated eosinophils or eosinophils stimulated with $2 \mu\text{M}$ A23187 for 15 min in the absence (vehicle alone) or presence of genistein ($10 \mu\text{M}$), U0126 ($20 \mu\text{M}$), or SB203580 ($10 \mu\text{M}$) were lysed and subjected to immunoblot analysis using antibodies against phosphorylated and total ERK1/2, p38, and MAPKAP-2 (MK2). Protein content in bands was quantified by densitometry, and in each case the phosphorylated-to-total kinase ratio was normalized to the unstimulated control. (a) Representative immunoblot and histogram showing ratios for phospho-ERK1/2/total ERK1/2. * $P < 0.05$ compared with A23187 alone ($N = 3$). (b) Representative immunoblot and histogram showing ratios for phospho-p38/total p38. * $P < 0.05$ compared with A23187 alone ($N = 4$). (c) Representative immunoblot and histogram showing ratios for phospho-MAPKAP-2/total MAPKAP-2. * $P < 0.05$ compared with A23187 alone ($N = 3$).

Fig. 4.

The addition of exogenous arachidonic acid does not prevent inhibition of leukotriene C₄ (LTC₄) synthesis caused by genistein. Eosinophils adhered to fibronectin coated wells were incubated with or without 10 μ g/mL arachidonic acid for 20 min, then stimulated with 2 μ M A23187 in the absence and presence of 10 μ M genistein (Gen). LTC₄ synthesis was normalized to amount detected without genistein or exogenous arachidonic acid. * $P < 0.05$ compared with the corresponding condition without genistein ($N = 3$).

Fig. 5.

Genistein blocks eosinophil 5-lipoxygenase (5-LO) nuclear membrane and lipid body localization. Eosinophils were adhered to fibronectin-coated cover slips for 1 h, incubated in the absence or presence of 10 μM genistein for an additional hour, and stimulated with 2 μM A23187 or exposed to vehicle alone (no stim) for 15 min. Cells were then fixed, permeabilized, and immunostained for 5-LO, counterstained with DAPI, and viewed with a confocal laser microscope. (a and b) Vehicle (no stim) and genistein-treated cells not stimulated with A23187 show diffuse 5-LO staining throughout the cytoplasm and to a lesser extent within the nucleus. (c) Eosinophils stimulated with A23187 in the absence of genistein show prominent redistribution of 5-LO to the nuclear membrane (arrowheads) as well as a punctate cytoplasmic staining pattern (arrows) consistent with localization to lipid bodies. (d) Treatment with genistein largely abrogated redistribution of immunoreactive 5-LO to the nuclear membrane and lipid bodies in A23187-stimulated eosinophils. Representative immunostained eosinophils from one of four independent experiments, all with similar results, are shown.

Fig. 6.

A 4-week period of dietary supplementation with soy isoflavones in asthma patients results in reduced *ex vivo* synthesis of leukotriene C₄ (LTC₄) by eosinophils and reduced fraction of exhaled nitric oxide. Patients with asthma consumed a 100 mg soy isoflavone supplement containing 35 mg of genistein daily for 4 weeks. (a) Before and at the end of the 4-week supplementation period, eosinophils were isolated, plated on fibronectin-coated culture wells (2.5×10^4 cells/well), and stimulated with $2 \mu\text{M}$ A23187 for 15 min, after which LTC₄ in culture supernatants was measured. Each line represents an individual subject. At the end of the soy isoflavone treatment, *ex vivo* eosinophil LTC₄ synthesis decreased by 33% ($N = 11$, $P = 0.02$). (b) Fraction of expired nitric oxide (FE_{NO}) was determined before and at the end of the 4-week supplementation period using a NIOX analyser. Each line represents an individual subject. At the end of the soy isoflavone treatment, FE_{NO} decreased by 17% ($N = 13$, $P = 0.03$).

Table 1

Eosinophil viability at 24 hours is not affected by treatment with genistein

Condition	Fold change in viability (relative to untreated condition) Mean $\pm$ SEM, $N = 4$
Untreated	1.00
Genistein (10 μ m)	1.20 $\pm$ 0.106
IL-5 (2 ng/mL)	1.74 $\pm$ 0.05*
IL-5+Genistein	1.77 $\pm$ 0.08*
25% EtOH	0.11 $\pm$ 0.02*

* $P < 0.05$ compared with untreated (control) condition.

Table 2Baseline characteristics of subjects in the clinical pilot study (mean $\pm$ SD, unless otherwise indicated)

Age (range)	37.7 $\pm$ 12.1 (20–60)
Gender	
Male (N, %)	6, 46%
Female (N, %)	7, 54%
Pre-bronchodilator FEV ₁ (L)	2.59 $\pm$ 0.77
Pre-bronchodilator FEV ₁ % predicted	74.4 $\pm$ 12.1
Pre-bronchodilator FEV ₁ /FVC	0.67 $\pm$ 0.06
Post-bronchodilator FEV ₁ (L)	2.95 $\pm$ 0.81
Post-bronchodilator FEV ₁ % predicted	85.1 $\pm$ 10.6
Post-bronchodilator FEV ₁ /FVC	0.71 $\pm$ 0.07
Juniper Asthma Control Score	1.6 $\pm$ 0.75

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.