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Summative Interaction Between Astaxanthin, *Ginkgo biloba* Extract (EGb761) and Vitamin C in Suppression of Respiratory Inflammation: A Comparison with Ibuprofen

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Short Title: Phytochemicals cooperatively suppress inflammation.

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ABSTRACT

Here, combinations of *Ginkgo biloba* leaf extract (EGb761) plus the carotenoid antioxidant astaxanthin (ASX) and vitamin C are evaluated for summative dose effect in inhibition of asthma-associated inflammation in asthmatic Guinea Pigs. Ovalbumin-sensitized Hartley Guinea pigs challenged with ovalbumin aerosol to induce asthma, were administered EGb761, ASX, vitamin C, or ibuprofen. Following sacrifice, Bronchoalveolar lavage (BAL) fluid was evaluated for inflammatory cell infiltrates and lung tissue cyclic nucleotide content. Each parameter measured was significantly altered to a greater degree by drug combinations, than by each component acting independently. An optimal combination was identified that included astaxanthin (10 mg/kg), vitamin C (200 mg/kg), and EGb761 (10 mg/kg), resulting in counts of eosinophils and neutrophils each 1.6-fold lower; macrophages 1.8-fold lower, cAMP 1.4-fold higher; and cGMP 2.04-fold higher than levels in untreated, asthmatic animals ($p<0.05$). In conclusion, EGb761, ASX and vitamin C are shown here to interact summatively to suppress inflammation with efficacy equal to or better than ibuprofen, a widely used non-steroidal anti-inflammatory drug (NSAID). Such combinations of non-toxic phytochemicals constitute powerful tools for prevention of onset of acute and chronic inflammatory disease if consumed regularly by healthy individuals; and may also augment effectiveness of therapy for those with established illness.

Key words: Inflammation, Astaxanthin, Ginkgolide, ibuprofen, cAMP, cGMP.

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INTRODUCTION

This report describes progress toward creation of dietary phytochemical formulations ("functional foods") containing bioactive components that interact summatively to strengthen host immunoregulatory mechanisms which prevent inflammation from becoming pathological; and augment drug therapies for age-related illnesses such as arthritis, cardiovascular syndromes, dementia, autoimmunity and many other severe conditions with underlying inflammatory pathogenesis. The products are designed to mediate therapeutic and preventive clinical outcomes equivalently or superior to commonly-used non-steroidal anti-inflammatory drugs (NSAIDs), immunosuppressants, corticosteroids and other pharmaceuticals, but with negligible adverse effects and at low cost to patients. Beyond their medical use it is anticipated that such products may be consumed for general health as food supplements and additionally provide protection against debilitating effects of a diverse range of illness. Here the anti-inflammatory capacity of the NSAID ibuprofen is compared with formulations incorporating three naturally-occurring products: the dietary carotenoid astaxanthin (ASX), *Ginkgo biloba* whole leaf extract (EGb761) and vitamin C, for the ability to independently or interactively affect major indicators of inflammatory disease severity in ovalbumin-sensitized asthmatic guinea pigs.

Asthma was used as a disease model in this study, although this disorder was not our focus. Asthma pathogenesis is a cascade of progressively severe inflammatory processes initiated by an immune imbalance (Umetsu et al. 2002) and provides a very good general paradigm for severe chronic inflammation. Moreover, the commonality of

signaling pathways leading to tissue damage in asthma and most other inflammatory syndromes means that treatment strategies affecting major indicators of asthma-associated inflammation are likely to be relevant for many conditions in which inflammation plays an underlying role.

The emphasis here on general inflammatory, rather than asthma-specific processes, led to the choice of ibuprofen for comparison with the other agents in the context of anti-inflammatory potency. Ibuprofen is a widely-used NSAID that acts by strong inhibition of cyclooxygenases (COX), enzymes which metabolize membrane phospholipids into mediators called prostanoids, small molecules regulating a diverse range of homeostatic functions including inflammation (Soberman and Christmas 2003). The drug acts on both major isoforms of the enzyme: COX-1 which is constitutively expressed in most cells and acts to protect tissues such as lining of small airways and the gastrointestinal tract (Chandrasekharan et al. 2002; Soberman and Christmas 2003; Warner and Mitchell 2002); and an inducible variant, COX-2, responsible for production of inflammatory mediators in macrophages, neutrophils and other cells participating in inflammation (Grosser 2009).

Ibuprofen has clinical utility in many diseases for which inflammation is a major component (Hawkey 2001; Wallace 1999) and therefore, serves as a valuable benchmark against which the therapeutic potential of other NSAIDs may be compared. However, unlike the phytochemical products used in these experiments, ibuprofen cannot be administered long-term as prophylaxis against inflammatory disease owing to its toxicity (Bjarnason 2007; Moore 2007). For this reason, formulations with anti-inflammatory potency in the same range as ibuprofen, but without adverse side effects,

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constitute extremely valuable prophylaxes and therapy for persons at-risk of, or diagnosed with illness such as asthma, allergies, arthritis and other chronic inflammatory conditions.

Activation of many cell types during inflammation typically results in a cascade of processes in which autocrine and paracrine expression of platelet activating factor (PAF) is a major event. This molecule interacts with PAF receptors (PAFR) to cause release of cytosolic calcium from cellular stores, constituting a positive feedback process that amplifies immune cell activation and the severity of associated inflammatory pathologies (Asako et al. 1992; Sasakawa et al. 2000; Wenzel-Seifert and Seifert 1993). Compounds in Ginkgo extract (ginkgolides) that block PAFR thereby inhibiting increases of intracellular calcium, interrupt this process (Brochet et al. 1999), thereby allowing lower doses of drug to suppress calcium-dependent activation events resulting in enhanced therapeutic outcomes (Bagnis et al. 1996; Brochet et al. 1999; Haines et al. 2000).

The present investigation builds on previous work by the authors demonstrating synergism between EGb761 and the immunosuppressant FK506 in an *ex vivo* rat heart model (Haines et al. 2000); and between ASX and Ginkgolide B (GB) a purified, PAFR-inhibitory ginkgolide, in a cultured human peripheral blood mononuclear cell (PBMC) model (Mahmoud et al. 2002; Mahmoud et al. 2004). Neither ASX nor EGb761 are independently potent enough to be used as stand-alone treatment of serious inflammatory disease. However, it is here hypothesized that both compounds summate when co-administered, to mediate therapeutic effects in the same range as currently available single-molecule drugs, with ibuprofen used in these experiments as a comparative model.

The therapeutic effects of ginkgolides are due substantially to their ability to block PAF-PAFR-dependent calcium signaling, leading to prevention of inflammatory tissue damage (Bagnis et al. 1996; Brochet et al. 1999). ASX-mediated cytoprotection occurs through a different mechanism, involving highly efficient scavenging of toxic reactive oxygen compounds by ASX, with the additional capacity to induce host antioxidant defenses (Kang, Kim, and Kim 2001; Wang, Willen, and Wadstrom 2000). The biological action of both ginkgolides and ASX result in several common anti-inflammatory effects, including down-regulation of potentially pathological T cell activity (Mahmoud et al. 2002; Mahmoud et al. 2004). Thus, ginkgolides and ASX potentially exhibit additive and possibly synergistic interaction, an outcome seen in many drug combinations that act via different physiological pathways resulting in therapeutic benefits greater than that of each drug acting independently (Evans 2003; Haines et al. 2000; Mahmoud et al. 2002; Mahmoud et al. 2004).

MATERIALS AND METHODS

Animals (ethical background and institutional policy):

Male Hartley guinea pigs weighing between 250-350 grams and kept under pathogen-free conditions were used for all experiments in this study. Animals were maintained at the Animal Care facility at the School of Pharmacy, University of Debrecen, in Debrecen, Hungary. All activities in this study involving animal transport, care, experimentation and sacrifice have been reviewed and confirmed by University of Debrecen's committee for animal experiments. Standards of animal use demanded by this committee include an experimental design conducted on a valid scientific and ethical

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basis, with particular attention given to animal welfare. Animal use protocols at this institution emphasize respect and humane treatment of experimental animals, to include three main principles: (i) Minimization of stress and discomfort (ii) Conduct non-animal experiments where possible; and (iii) Minimization of the number of animals used to statistically valid sample sizes.

Drug administration and treatment groups:

Pharmacological agents used for this investigation include *Ginkgo biloba* whole leaf extract (EGb761), astaxanthin (ASX), vitamin C (Natural Alternatives International Inc., San Marcos California, USA) and ibuprofen (Abbott Laboratories, Abbott Park Illinois, USA) – hereafter for the purpose of brevity referred to as “drugs”. Drugs were given orally with feed to groups of 6 animals, segregated on the basis of dosage and type of drug. Five studies were conducted, each defined by administration of a particular drug. The treatment groups included animals fed: (1) astaxanthin; (2) ginkgo extract/EGb761; (3) vitamin C; (4) combinations of the previous 3 agents; and (5) ibuprofen. Controls included one drug-free, non-asthmatic control group; and one drug-free asthmatic cohort. Animals in both control groups were OA-sensitized and given the same feed vehicle as treatment groups, but non-drug supplemented. One of these was subsequently challenged with inhaled OA to induce an asthmatic response. The second received no OA challenge and remained asthma free.

In initial studies of the effect of each agent on asthma-associated disease indicators, independent of the action of the other drugs, guinea pigs were administered a daily regimen of 5-200 mg astaxanthin per kg body weight; 5-100 mg/kg of ginkgo

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extract; 50-400 mg/kg vitamin C; and various combined dosages of astaxanthin, ginkgo and vitamin C. Each drug was dispersed into a suspension of 1% methylcellulose in physiological saline, which was then added to standard rodent chow. Drug treatments for each study covered a time period of 26 days and ran concurrently with allergen sensitization (see asthmatic response induction protocol below). Animals were monitored throughout the 26 day preconditioning period for any signs of adverse reaction to treatments with special attention being given to unexplained death, tremors, agitation, breathing difficulties, lethargy random body movements or spasms, and precipitous gain or loss of weight.

Induction of Asthmatic response:

Asthmatic responses were induced in animals by sensitization to ovalbumin (OA) and subsequent aerosol challenge with this antigen by the method of Underwood *et al*, and Santing *et al*, (Santing et al. 1992; Underwood et al. 1993). Briefly, each animal was sensitized by intramuscular injections of 0.35 ml of a 5% (W/V) ovalbumin (OA)/saline solution into each thigh on days 1 and 4 of each study. On day 25, the animals were challenged with ovalbumin aerosol in a specially designed animal cage, in which the guinea pigs may move freely. Habituation of each animal to the cage environment was initiated 2 days before exposure to OA. Challenge with the allergen was performed by inhalation of increasing aerosol concentrations containing 1.0, 3.0, 5.0 and 7.0 mg/ml ovalbumin in saline for 3 min, separated by 7-min intervals. Only those animals displaying prominent asthmatic symptoms were selected for further evaluation. These included: i. obvious wheezing, ii. copious nasal discharge of clear, viscous fluid. Average

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magnitude of disease indicators in animals in groups receiving ASX, EGb761, vitamin C and combinations thereof, then was measured as described below compared with treatment groups receiving 10-1,000 mg/kg ibuprofen per day.

Evaluation of lung inflammatory cells:

Animal sacrifice and collection of biological samples was conducted according to the methods described in Underwood *et al* (Underwood et al. 1993). Briefly, twenty-four hours after aerosol OA challenge (day 26 of each study), guinea pigs were sacrificed by decapitation conducted rapidly to minimize pain and discomfort (a departure from the method used by Underwood *et al*, who used cervical dislocation (Underwood et al. 1993). Within a timeframe of no more than 8 seconds so as to prevent contamination of the lungs with peripheral blood, the chest was opened, trachea severed directly above the bronchi and lungs were removed and lavaged with 50 ml of DulBecco's phosphate-buffered saline (aliquots of 10 ml), which were aspirated after gentle massage of the organ. Bronchoalveolar lavage (BAL) fluid was collected and centrifuged at 2200 rpm (1100 x g) for 10 min, supernatant was aspirated, and pellets were resuspended in 5 ml 0.25% NaCl to lyse residual erythrocytes. This dispersion was centrifuged at 2200 rpm (1100 x g) for 10 min, supernatant was aspirated, and pellets were resuspended 5 ml 0.9% NaCl. Total cell counts were done by hemocytometry using trypan blue stain. Slides were prepared on a Shandon Cytospin 2 (Pittsburgh, PA) at 300 rpm for 5 min, fixed and stained. Differential cell counts were performed using standard morphologic criteria to classify cells as eosinophils, neutrophils, or macrophages, and the results were expressed in cell numbers.

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Measurement of cAMP and cGMP:

Biopsies from lung tissues were harvested for measurement of cyclic nucleotide content. cAMP and cGMP were measured using commercially available radioimmunoassay kits (Amersham). Immediately after sampling, lung biopsies were frozen by means of a Wollenberger clamp pre-chilled in liquid nitrogen. Samples were powdered with a pestle and mortar in liquid nitrogen and trichloroacetic acid (TCA) was added to the powdered frozen samples (10 ml to every mg of tissue). Samples were further homogenized in frozen TCA using a drying mortar and then centrifuged at 14,000 x g for 10 min at 4°C. The supernatants were extracted 6 times in water-saturated diethyl ether, evaporated and assayed for cAMP and cGMP by radioimmunoassay using liquid scintillation counter (Packard, Tri-Carb 2100TR).

Dosage selection for drug combination studies:

After evaluating the changes mediated by each drug independently on BAL inflammatory cells and lung tissue cAMP and cGMP content, the effect of drug co-administration on these parameters was measured. As shown in Table 1 and Figures 1-5, three different combinations of astaxanthin, ginkgo extract and vitamin C were used to estimate the minimum dosage of each agent necessary to maximally suppress each disease-associated parameter. The dose ranges used in these studies (shown in Table 1 and Figures 1-5) was in excess of what would reasonably be expected to correspond to veterinary clinical use; and would correspond to unrealistically high dosages if scaled linearly to the average weight of a human (Feldman and McMahon 1983). However,

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small animals with high metabolic rates typically metabolize a drug much more rapidly than larger animals; hence the effects seen at the dosages used in this study are expected to be reasonable predictors of the responses in humans (Feldman and McMahon 1983). In planning these experiments it was also anticipated that response trends of disease indicators that may be difficult to detect at lower dosage (but are nonetheless of potential therapeutic significance), are better-defined at the dosages used here (Feldman and McMahon 1983).

Statistics:

The data were expressed as the mean ± SEM. One-way analysis of variance test was first carried out to test for any differences between the mean values of all groups. If differences were established, the values of the treated groups were compared with those of the drug-free control group by multiple t-test followed by Bonferroni correction. A change of $p<0.05$ between the drug-free control and treated groups was considered to be significant.

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RESULTS

No unexpected deaths or other adverse effects occurred during the preconditioning period. This was somewhat surprising in the case of animals administered ibuprofen since there is toxicity associated with its chronic use (Bjarnason 2007; Moore 2007) and doses administered were high. It is likely that the animals administered this drug suffered some degree of gastrointestinal damage, however an assessment for this was not made and none of the ibuprofen-treated animals exhibited obvious adverse effects.

Following preconditioning, the 5 inflammation-associated indicators of asthma severity described above in Methods were evaluated in the treated animals and compared with outcomes in two control groups: non asthmatic guinea pigs; and a group of OA-challenged asthmatic animals that had received no drug pre-treatment. As shown in Table 1 and Figures 1-5, significant inhibition of disease indicators was observed in animals administered each product independently and in combination. Comparison of the average magnitude of indicators in treated animal groups with the untreated asthmatic group demonstrated significantly favorable effects ($p<0.05$) of all products and their combinations in the upper dosage range tested. The indicators: levels of BAL inflammatory cell infiltrate and lung tissue cyclic nucleotide content exhibited significantly favorable dose-responsive correlation to pre-treatment of animals with ASX (Table 1A); *Ginkgo biloba* leaf extract (Table 1B); vitamin C (Table 1C); combined administration of ASX, Ginkgo and vitamin C (Table 1D); and ibuprofen (Table 1E). As shown in Figures 1-5, when compared with drug-free asthmatic controls, animals pretreated with each agent or their combinations showed significant suppression of the

disease severity indicators: BAL fluid inflammatory cell content (Figures 1-3); and lung tissue cyclic nucleotides (Figures 4 and 5) ($p<0.05$). Treatment of animals with ASX, Ginkgo and vitamin C or their combinations resulted in suppression of each disease indicator to the same range as ibuprofen (Table 1, Figures 1-5), demonstrating that under the conditions of the present study, the three natural products (ASX, Ginkgo and vitamin C) mediated an anti-inflammatory effect equal or superior to that of ibuprofen.

Summative enhancement of drug effects between ASX, EGb761 and vitamin C was observed (Table 1) but was not an easily identified feature of the experimental outcomes. Figures 1-5 show average magnitudes of disease indicators in treatment groups administered the lowest dose of each agent or combination of agents significantly different ($p<0.05$) from the untreated asthmatic control group. At the dosages used, average disease indicator levels in animals receiving combined treatments do not appear obviously improved versus groups treated with single agents (Figures 1-5). Indeed, qualitative comparison of inflammatory cell infiltrate numbers between treatment groups shown in Figures 1-3 suggest that some of the single-agent treated groups experienced better improvement than those treated with 10 mg/kg each of and EGb761 plus 200 mg/kg of vitamin C. Statistical analysis did however reveal significant additive interaction between these three agents suggesting benefits to co-administration in prevention or treatment of asthma. Animals receiving 10 mg/kg ASX (Table 1A), or 10 mg/kg EGb761 (Table 1B), or 200 mg/kg vitamin C (Table 1C), or less independently of treatment with other agents did not exhibit BAL inflammatory cell numbers or lung tissue cyclic nucleotide levels significantly different from asthmatic controls. However,

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combined administration of these agents at the dosages stated, correlated with significant improvement in all disease indicators ($p<0.05$) (Table 1D).

DISCUSSION

The present study demonstrates that in comparison with untreated controls, animals fed astaxanthin, *Ginkgo biloba* extract and vitamin C alone or in combination exhibited significantly lower BAL fluid inflammatory cell numbers and enhancement of lung tissue content of cAMP and cGMP (Figures 1-5). The magnitude of these anti-inflammatory effects mediated by each phytonutrient product independently was observed to be in the same range as outcomes in ibuprofen-treated animal groups (Figures 1-5).

The data suggest that the anti-inflammatory effects of ASX, EGb761 and vitamin C acting in concert counteracts pathological inflammation to a more significant degree than any component acting independently (Table 1). Although summative enhancement of drug effects was not a striking characteristic of the experimental outcomes, the significantly reduced dosage of each agent needed to achieve therapeutic effects when co-administered is clinically meaningful since products for human use in place of, or augmenting ibuprofen may be configured to contain lower dosage of each agent, thereby reducing risk of adverse reaction to persons who may be sensitive to any of the three agents.

The independent effect of ASX, EGb761, vitamin C and ibuprofen on selected disease indicators was expected based on the known mechanisms of inflammation underlying the pathogenesis of asthma and the pharmacology of each of the products. The

highly potent free radical scavenging properties of ASX is expected to systemically suppress the intensity of oxidative processes in ASX-treated animals. Such a shift, particularly when it occurs in the lungs is observed to activate antioxidant/reactive oxygen signaling processes that favorably alter major indicators of asthma and related respiratory syndromes (Fan et al. 1998; Kirkham and Rahman 2006; Kurashige et al. 1990).

The experimental endpoints chosen for this study were selected on the basis of their relevance to asthma-associated inflammation, which is a complex process involving activation of cytokine networks tissue damage and many other pathological features. Nevertheless BAL content of inflammatory cells and lung tissue cyclic nucleotide levels are established indicators of the severity of inflammatory lung disease (Busse 1998; Hai 2007; Hamad et al. 2003; Underwood et al. 1993; Wenzel 1996) and changes in the magnitude of these indicators provide a simple and direct picture of the effectiveness of a particular therapy.

The ability of EGb761 to mediate the same favorable changes in major disease indicators as ASX is most likely due primarily to components of the mixture that block the PAF receptor, principally the terpene trilactones such as ginkgolide B (GB) (Bagnis et al. 1996; Brochet et al. 1999). Systemic inhibition of PAF-dependent processes is expected to interrupt the pathogenesis of asthma at several checkpoints. Of particular importance is the probable effect on pathogenic T lymphocytes that act as triggers for the disease. These cells express high levels of IL-5 which act in an autocrine and paracrine manner to induce expression of PAF and its cognate receptor on T cells, inflammatory cells and several other categories of tissue involved in asthmatic inflammation

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(Calabresse et al. 1992). PAF thus acts as an amplifier of a wide range of inflammation-associated processes (Calabresse et al. 1992), hence blockade of its receptor is expected to significantly attenuate the two major indicators of disease studied here, and a host of others. Other components of EGb761 undoubtedly also contributed to the observed effects. *Ginkgo biloba* leaf extract, contains bioflavones which inhibit COX-2 and cAMP-phosphodiesterase (Saponara and Bosisio 1998), the latter property possibly acting as a contributor to EGb761's ability to enhance cyclic nucleotide levels in lung tissue. Here cAMP and cGMP content of lung tissue were evaluated as asthma-associated biomarkers, reflecting efficacy of drug treatments. This is a consequence of the role of both cAMP and cGMP as second messengers in airway smooth muscle cells (SMC) that act to decrease contractility thus relaxing the airways and contributing to lessening of disease severity (Hai 2007; Hamad et al. 2003; Underwood et al. 1993). It is therefore likely that phosphodiesterase inhibitors present in EGb761 contributed to anti-inflammatory effects by preservation or enhancement of SMC cyclic nucleotide levels.

Vitamin C was included in our formulations as a water-soluble dietary antioxidant, expected to act as a physiologic relay, allowing scavenging and urinary elimination of reactive oxygen metabolites. It has been shown to augment the activity of astaxanthin in suppression of oxidative damage secondary to *helicobacter pylori* infection (Wang, Willen, and Wadstrom 2000), a result which suggested it would interact well with ginkgolides and astaxanthin in suppression of asthma-associated inflammation.

The decision to use ibuprofen as a reference drug against which to compare the anti-inflammatory capacities of the other agents was made with some reservation. Its therapeutic properties are due substantially to its ability to strongly inhibit COX-2.

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However, the compound also inhibits COX-1, a feature which contributes to disregulated platelet aggregation and gastric damage (Kakuta et al. 2008; Rao and Knaus 2008). COX-1 inhibition additionally promotes bronchoconstriction in asthmatics (Harrington et al. 2008), thereby contributing to lung inflammation and potentially adding a built-in confounder to the study given the use of an asthma model. Rofecoxib (brand name Vioxx) was also considered for use as a reference drug, since as a strongly anti-inflammatory, specific inhibitor of COX-2, with no associated effect on COX-1 (Kose et al. 2009) it was expected to favorably alter asthma inflammation-associated indicators without counteracting these outcomes through exacerbation of the disease process. Two other COX-2 selective NSAIDs, celecoxib (brand name Celebrex) and valdecoxib (brand name Bextra) were also considered for use in the study.

Although Vioxx, Celebrex, Bextra or other COX-2-selective NSAIDs would have been more appropriate to our model, using any of these as a reference drug would not have been consistent with the major aim of this research, which is to compare the anti-inflammatory capacity of phytochemical formulations that may be consumed regularly for general health, with a widely used NSAID that can be taken only for brief periods as a therapeutic measure. The three aforementioned drugs are associated with occurrence of serious, potentially lethal side effects (Chan et al. 2009; Dajani and Islam 2008; El et al. 2009; Ray 2009) and only Celebrex remains in clinical use, making them irrelevant to the aims of this study due to their very limited use in healthcare. Anti-asthmatic drugs were likewise not considered for the present investigation since the objective was not to develop an asthma-specific therapy, but establish groundwork for a phytochemical-based NSAID with clinical application in multiple disorders.

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Ultimately it was decided that the advantages of ibuprofen outweighed its drawbacks. The drug is available without prescription, is one of the most widely-used anti-inflammatory compounds ever manufactured and in 2005 was designated by the World Health Organization (WHO) as an essential public health resource for all nations (*WHO Model List of Essential Medicines. World Health Organization.* 2005). Moreover, despite its potential to exacerbate bronchoconstriction, it has known clinical utility in preventing asthma-associated morbidity in children (Mazur 2002), raising the possibility that use of ibuprofen as a reference for these studies might yield new insight into the ability of the drug to complement existing strategies for management of asthma.

CONCLUSIONS:

Results of the present study suggest that a product based on the combined activity of ASX, EGb761 and vitamin C would be of substantial benefit for a large percentage of the World's population at risk of or suffering from chronic inflammatory disease. The product would be as potent as ibuprofen, but could be consumed prophylactically for general health to decrease the overall risk of chronic inflammatory disease; and would also be valuable for augmenting the effects of other therapies for persons who become ill.

ASX and ginkgo extracts are minimally toxic (Bagnis et al. 1996; Haines et al. 2000; Mahmoud et al. 2002; Mahmoud et al. 2004; Wang, Willen, and Wadstrom 2000) and are marketed in the U.S. without prescription as components of food or food supplements. The present study demonstrates that oral administration of these phytonutrients to asthmatic animals along with vitamin C, significantly improved major indicators of asthma severity. Specifically, the therapeutic agents reduced lung infiltration

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by polymorphonuclear leukocytes; and increased levels of cyclic nucleotides in lung tissue. Both effects correlate strongly with reduced inflammation and improved prognosis in asthma (Busse 1998; Hai 2007; Hamad et al. 2003; Wenzel 1996). These observations suggest that the phytochemical formulations evaluated here, have potential to decrease risk of, and improve therapy for a diverse spectrum of inflammatory pathologies and could greatly improve quality-of-life for millions of people Worldwide.

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Figure 1. Magnitude of inflammation-associated responses in animals treated with phytonutrients or ibuprofen: Bronchoalveolar lavage (BAL) fluid content of eosinophils.
OA-sensitized Guinea pig groups (6 animals in each group) treated as described previously with 0-200 mg/kg astaxanthin (ASX), 0-100 mg/kg *Ginkgo biloba* leaf extract (EGb761), 0-400 mg/kg vitamin C (VitC), combinations of ASX, Ginkgo and vitamin C and 0-1,000 mg/kg ibuprofen (IBP). Data shown are restricted to outcomes from treatment groups receiving the lowest dose of each product resulting in values of each inflammation indicator significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

* Inflammation indicator magnitudes significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

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Figure 2. Magnitude of inflammation-associated responses in animals treated with phytonutrients or ibuprofen: Bronchoalveolar lavage (BAL) fluid content of neutrophils.
OA-sensitized Guinea pig groups (6 animals in each group) treated as described previously with 0-200 mg/kg astaxanthin (ASX), 0-100 mg/kg *Ginkgo biloba* leaf extract (EGb761), 0-400 mg/kg vitamin C (VitC), combinations of ASX, Ginkgo and vitamin C and 0-1,000 mg/kg ibuprofen (IBP). Data shown is restricted to outcomes from treatment groups receiving the lowest dose of each product resulting in values of each inflammation indicator significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

* Inflammation indicator magnitudes significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

Figure 3. Magnitude of inflammation-associated responses in animals treated with phytonutrients or ibuprofen: Bronchoalveolar lavage (BAL) fluid content of macrophages.
OA-sensitized Guinea pig groups (6 animals in each group) treated as described previously with 0-200 mg/kg astaxanthin (ASX), 0-100 mg/kg *Ginkgo biloba* leaf extract (EGb761), 0-400 mg/kg vitamin C (VitC), combinations of ASX, Ginkgo and vitamin C and 0-1,000 mg/kg ibuprofen (IBP). Data shown is restricted to outcomes from treatment groups receiving the lowest dose of each product resulting in values of each inflammation indicator significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

* Inflammation indicator magnitudes significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

Figure 4. Magnitude of inflammation-associated responses in animals treated with phytonutrients or ibuprofen: enhanced lung tissue content of cAMP.
OA-sensitized Guinea pig groups (6 animals in each group) treated as described previously with 0-200 mg/kg astaxanthin (ASX), 0-100 mg/kg *Ginkgo biloba* leaf extract (EGb761), 0-400 mg/kg vitamin C (VitC), combinations of ASX, Ginkgo and vitamin C

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and 0-1,000 mg/kg ibuprofen (IBP). Data shown is restricted to outcomes from treatment groups receiving the lowest dose of each product resulting in values of each inflammation indicator significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

* Inflammation indicator magnitudes significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

Figure 5. Magnitude of inflammation-associated responses in animals treated with phytonutrients or ibuprofen: enhanced lung tissue content of cGMP.
OA-sensitized Guinea pig groups (6 animals in each group) treated as described previously with 0-200 mg/kg astaxanthin (ASX), 0-100 mg/kg *Ginkgo biloba* leaf extract (EGb761), 0-400 mg/kg vitamin C (VitC), combinations of ASX, Ginkgo and vitamin C and 0-1,000 mg/kg ibuprofen (IBP). Data shown is restricted to outcomes from treatment groups receiving the lowest dose of each product resulting in values of each inflammation indicator significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

* Inflammation indicator magnitudes significantly different ($p<0.05$) than results from asthmatic, drug-free control animals.

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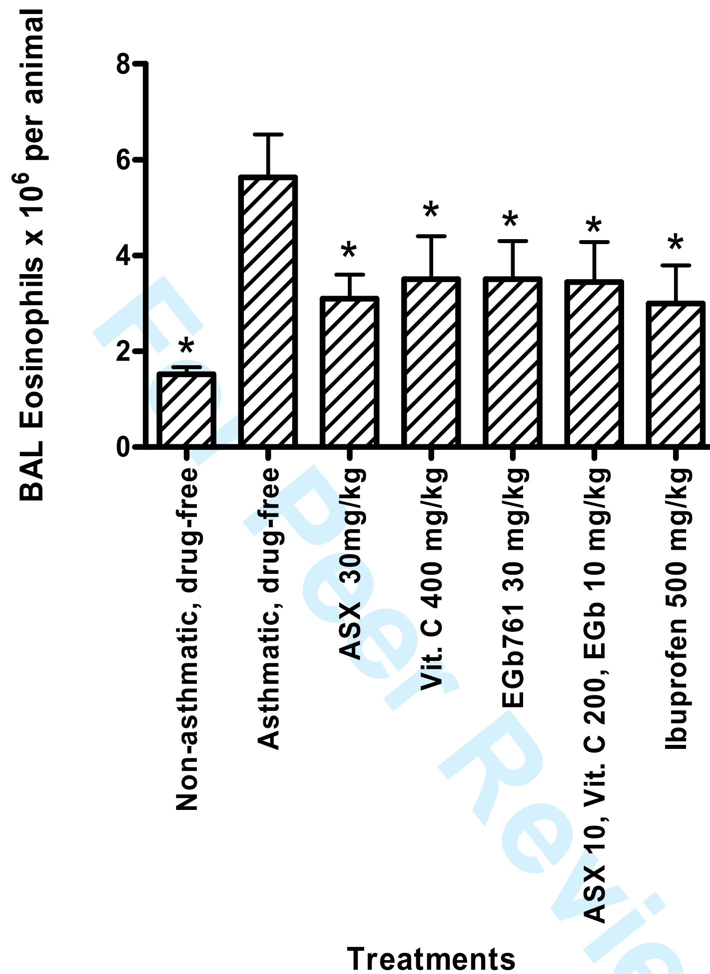
Figure 1.
Bronchoalveolar lavage (BAL) eosinophil content



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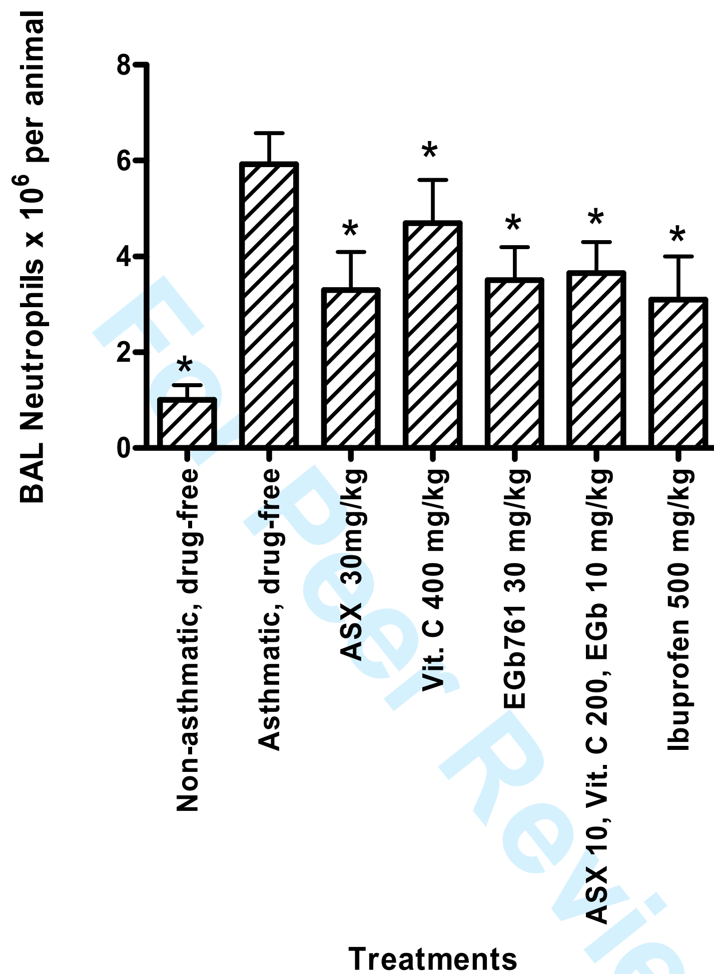

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Figure 3.
Bronchoalveolar lavage (BAL) macrophage content

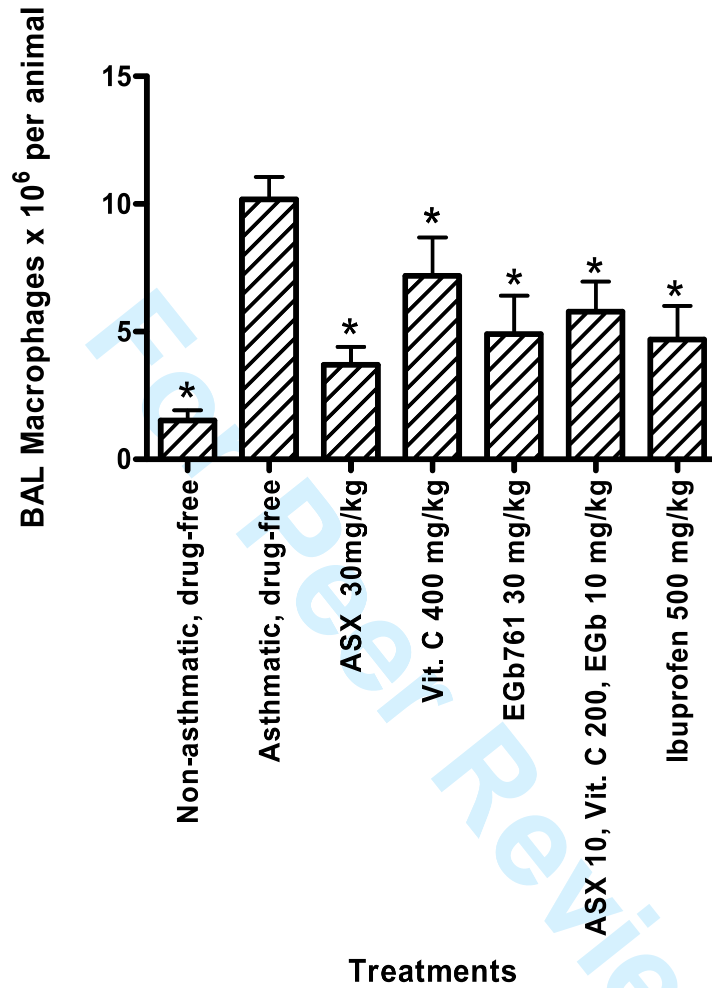

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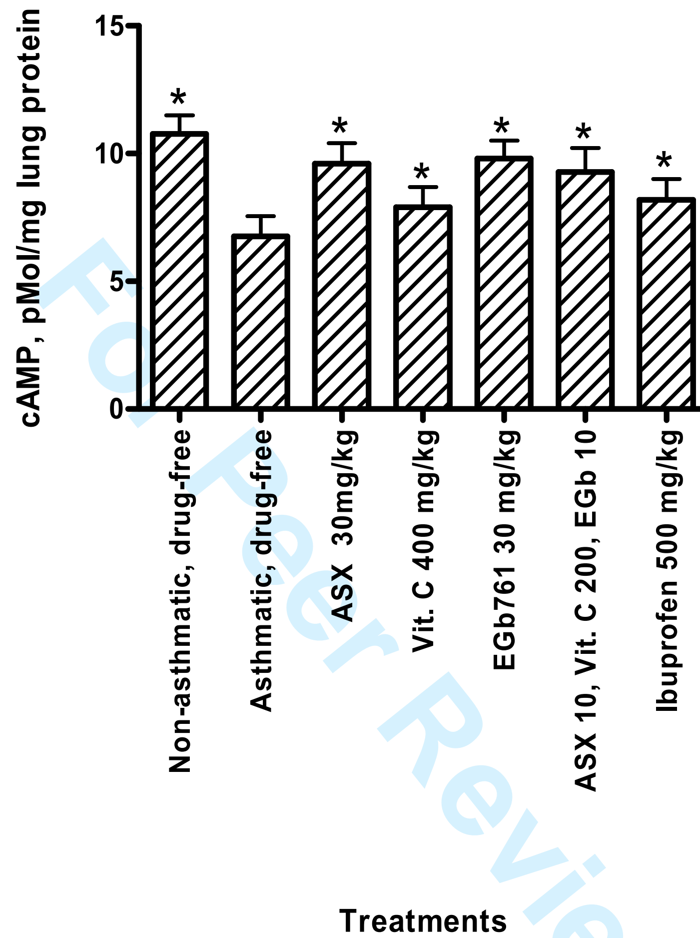
Figure 4.
Lung Tissue cAMP content



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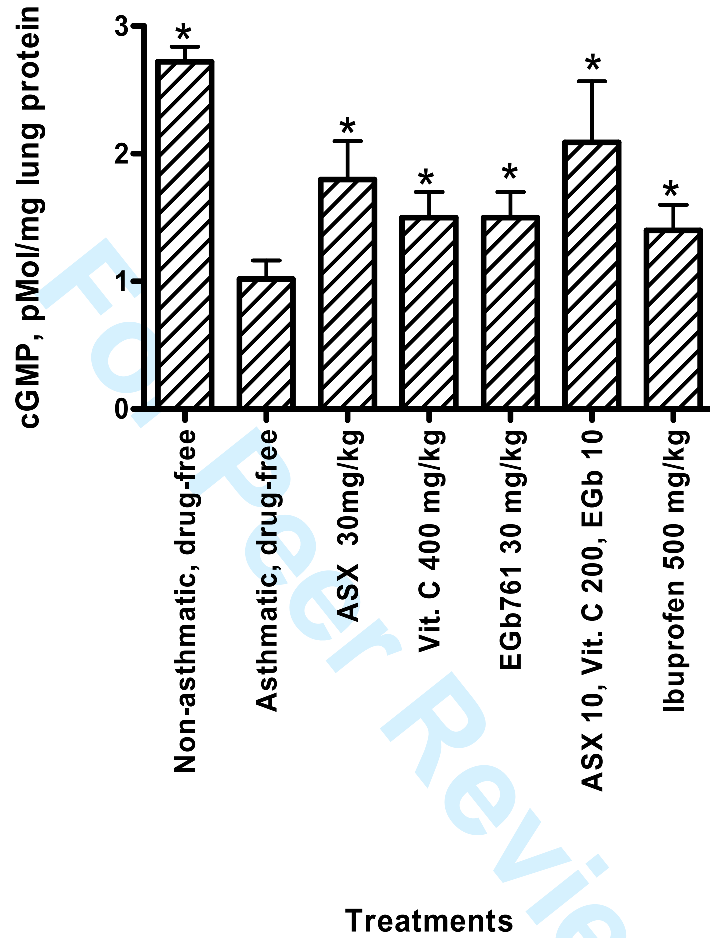

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Table 1. Lung inflammation-associated parameter values in treatment groups (basic data). Numbers of inflammatory cells in bronchoalveolar lavage (BAL) fluid and levels of the cyclic nucleotides cAMP and cGMP in lung tissue were measured in ovalbumin (OA)-sensitized guinea pigs, segregated into dosage groups of 6 animals each and administered feed containing the following supplements (referred to here as "drugs): astaxanthin (1A), *Ginkgo biloba* leaf extract (EGb761) (1B), vitamin C (1C), selected combinations of each (1D), or ibuprofen (1E), daily for 26 days. Two groups, designated as drug-free controls were given feed without phytochemicals or ibuprofen. One of these two control groups was exposed to vehicle aerosol (nebulized saline) only, without OA challenge and is designated below: "OA-neg, non-asthma, no drugs". The other control group which received an OA challenge is designated below: "OA+ asthma+, no drugs". Twentyfour hours following the 26-day drug treatment period, asthma was induced by OA inhalation, animals were sacrificed and BAL inflammatory cell levels and lung tissue cyclic nucleotide content was measured in each animal. Data is reported as mean ± standard error of means (SEM) of measurements taken in the 6 animals constituting each treatment group.

1A. Astaxanthin (ASX)-treated animals.

Cohort size: n = 6	**OA-neg non-asthma no drugs**	**OA+ asthma+ no drugs**	**OA+ 5mg/kg ASX**	**OA+ 10mg/kg ASX**	**OA+ 30mg/kg ASX**	**OA+ 100mg/kg ASX**	**OA+ 200mg/kg ASX**
Eosinophils (cells x 10^6/animal).	1.4±0.2	5.4±0.8	5.0±0.7	4.6±0.6	3.1±0.5*	2.4±0.6*	2.3±0.5*
Neutrophils (cells x 10^5/animal)	1.1±0.3	5.8±0.7	5.4±0.8	5.1±0.6	3.3±0.8*	2.4±0.5*	2.0±0.4*
Macrophages (cells x 10^6/animal)	1.7±0.5	9.9±1.1	9.6±1.0	8.0±0.8	3.7 ±0.7*	2.70±0.7*	2.6±0.5*
cAMP pMol/mg lung protein	10.9±0.98	6.7±0.58	6.9±0.5	8.1±0.8	9.6±0.8*	12.6±0.8*	12.1±0.5*
cGMP pMol/mg lung protein	2.6±0.26	1.1±0.17	1.2±0.1	1.4±0.1	1.8±0.3*	2.8±0.3*	2.7±0.2*

1B. *Ginkgo biloba* (EGb761)-treated animals.

Cohort size: n = 6	**OA-neg non-asthma no drugs**	**OA+ asthma+ no drugs**	**OA+ 5mg/kg EGb761**	**OA+ 10mg/kg EGb761**	**OA+ 30mg/kg EGb761**	**OA+ 100mg/kg EGb761**
Eosinophils (cells x 10^6/animal).	1.4±0.2	5.4±0.8	5.1±0.9	5.0 ±0.8	3.5±0.8*	3.1±0.5*
Neutrophils (cells x 10^5/animal)	1.1±0.3	5.8±0.7	5.6±0.6	5.4±0.6	3.5±0,7*	3.3±0.6*
Macrophages (cells x 10^6/animal)	1.7±0.5	9.9±1.1	9.8±1.1	9.4±1.1	4.9 ±1.5*	4.2±0.6*
cAMP pMol/mg lung protein	10.9±0.98	6.7±0.58	6.9±0.6	8.0±0.9	9.8±0.7*	12.0±1.3*
cGMP pMol/mg lung protein	2.6±0.26	1.1±0.17	1.1±0.2	1.1±0.2	1.5±0.2*	1.8±0.3*

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1C. Vitamin C-treated animals.

Cohort size: n = 6	OA-neg non-asthma no drugs	OA+ asthma+ no drug	OA+ 50mg/kg Vit. C	OA+ 100mg/kg Vit. C	OA+ 200mg/kg Vit. C	OA+ 400mg/kg Vit. C
Eosinophils (cells x 10^6/animal).	1.4±0.2	5.4±0.8	5.5±0.5	5.5±0.8	4.8±1.1	3.5±0.9*
Neutrophils (cells x 10^5/animal)	1.1±0.3	5.8±0.7	5.4±0.6	5.6±0.8	5.0±0.8	4.7±0.9
Macrophages (cells x 10^6/animal)	1.7±0.5	9.9±1.1	9.5±0.9	9.8±0.9	9.2 ±0.8	7.2±1.5*
cAMP pMol/mg lung protein	10.9±0.98	6.7±0.58	6.8±0.5	7.1±0.8	7.9±0.9	7.9±0.8
cGMP pMol/mg lung protein	2.6±0.26	1.1±0.17	1.1±0.2	1.0±0.2	1.2±0.2	1.5±0.2*

1D. Combination treatments: Animals simulataneously administered astaxanthin, *Gingko biloba* (EGb761) and vitamin C

Cohort size: n = 6	OA-neg non-asthma no drugs	OA+ asthma+ no drug	OA+ 5 mg/kg ASX + 5 mg/kg EGb761 + 50 mg/kg Vit C	OA+ 10 mg/kg ASX + 10 mg/kg EGb761 + 200 mg/kg Vit C	OA+ 30 mg/kg ASX + 30 mg/kg EGb761 + 400 mg/kg Vit C
Eosinophils (cells x 10^6/animal).	1.4±0.2	5.4±0.8	5.07±0.63	3.44±0.84*	2.95±0.62*
Neutrophils (cells x 10^5/animal)	1.1±0.3	5.8±0.7	5.27±0.74	3.65±0.65*	2.60±0.69*
Macrophages (cells x 10^6/animal)	1.7±0.5	9.9±1.1	9.40±0.96	5.78±1.18*	2.98±0.71*
cAMP pMol/mg lung protein	10.9±0.98	6.7±0.58	7.17± 0.61	9.28±0.94*	13.00±0.76*
cGMP pMol/mg lung protein	2.6±0.26	1.1±0.17	1.17± 0.19	2.09±0.48*	2.84±0.28*

1E. Ibuprofen-treated animals.

Cohort size: n = 6	Asthma-free control (OA neg)	OA+ asthma, no drug	OA+ IB 10mg/kg	OA+ IB 100mg/kg	OA+ IB 500mg/kg	OA+ IB 1000mg/kg
Eosinophils (cells x 10^6/animal).	1.4±0.2	5.4±0.8	5.4±0.8	4.7±0.6	3.0±0.8*	2.9±0.5*
Neutrophils (cells x 10^5/animal)	1.1±0.3	5.8±0.7	5.7±0.7	5.0±0.7	3.1±0.9*	3.1±0.5*
Macrophages (cells x 10^6/animal)	1.7±0.5	9.9±1.1	9.5±0.8	9.0±1.3	4.7±1.3*	4.3±0.9*
cAMP pMol/mg lung protein	10.9±0.98	6.7±0.58	7.1±0.6	7.3±0.9	8.2±0.8*	8.1±0.9*
cGMP pMol/mg lung protein	2.6±0.26	1.1±0.17	1.1±0.2	1.1±0.2	1.4±0.2	1.6±0.2*

*$p<0.05$ compared to corresponding values of OA antigen-challenged, asthmatic drug-free control group.

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