

MAG-EPA resolves lung inflammation in an allergic model of asthma

C. Morin^{1,2}, S. Fortin¹, A. M. Cantin³ and É. Rousseau²

¹SCF Pharma, Ste-Luce, QC, Canada, ²Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Sherbrooke, QC, Canada and

³Department of Medicine, Respiratory Division, Université de Sherbrooke, Sherbrooke, QC, Canada

Clinical & Experimental Allergy

Summary

Background Asthma is a chronic disease characterized by airways hyperresponsiveness, inflammation and airways remodelling involving reversible bronchial obstruction.

Omega-3 fatty acids and their derivatives are known to reduce inflammation in several tissues including lung.

Objectives The effects of eicosapentaenoic acid monoacylglyceride (MAG-EPA), a newly synthesized EPA derivative, were determined on the resolution of lung inflammation and airway hyperresponsiveness in an *in vivo* model of allergic asthma.

Methods Ovalbumin (OVA)-sensitized guinea-pigs were treated or not with MAG-EPA administered *per os*. Isometric tension measurements, histological analyses, homogenate preparation for Western blot experiments or total RNA extraction for RT-PCR were performed to assess the effect of MAG-EPA treatments.

Results Mechanical tension measurements revealed that oral MAG-EPA treatments reduced methacholine (MCh)-induced bronchial hyperresponsiveness in OVA-sensitized guinea-pigs. Moreover, MAG-EPA treatments also decreased Ca²⁺ hypersensitivity of bronchial smooth muscle. Histological analyses and leucocyte counts in bronchoalveolar lavages revealed that oral MAG-EPA treatments led to less inflammatory cell recruitment in the lung of OVA-sensitized guinea-pigs when compared with lungs from control animals. Results also revealed a reduction in mucin production and MUC5AC expression level in OVA-sensitized animals treated with MAG-EPA. Following MAG-EPA treatments, the transcript levels of pro-inflammatory markers such as IL-5, eotaxin, IL-13 and IL-4 were markedly reduced. Moreover, *per os* MAG-EPA administrations reduced COX2 over-expression in OVA-sensitized animals.

Conclusion and Clinical Relevance We demonstrate that MAG-EPA reduces airway hyperresponsiveness and lung inflammation in OVA-sensitized animals, a finding consistent with a decrease in IL-4, IL-5, IL-13, COX-2 and MUC5AC expression levels in the lung. The present data suggest that MAG-EPA represents a new potential therapeutic strategy for resolving inflammation in allergic asthma.

Keywords asthma, COX-2, EPA, MUC5AC, pro-inflammatory cytokines

Submitted 22 January 2013; revised 12 June 2013; accepted 14 June 2013

Correspondence:

Dr Éric Rousseau, Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, Sherbrooke, QC, Canada J1H 5N4.

E-mail: eric.rousseau@usherbrooke.ca

Cite this as: C. Morin, S. Fortin, A. M. Cantin and É. Rousseau, *Clinical & Experimental Allergy*, 2013 (43) 1071–1082.

Introduction

Afflicting more than 60 million North Americans and costing over 18 billion dollars annually [1], allergic asthma is a primary public health burden. Furthermore, the incidence of asthma has been steadily rising in North America over the past two decades [1] with a number of potential reasons accounting for this increase. Allergic asthma is the result of an excessive adaptive immune response to an inhaled antigen and is typically characterized by airway inflammation with

eosinophil and lymphocyte infiltration, increased levels of Th2 cytokines, circulating IgE, airway goblet (mucus-producing) cell metaplasia and airway hyperresponsiveness (bronchoconstriction in response to inhalation of a specific (allergen) or non-specific (methacholine, cold air) agonist [2, 3]. Despite the availability of several classes of asthma medication such as beta-agonists, leukotriene antagonists and corticosteroids, nearly 50% of asthmatics fail to benefit from one or more of these drugs [4]. Alternative treatment aimed at reducing the dose requirements of pharmacological interventions

would henceforth be beneficial, in addition to potentially reducing the public health and socio-economic burden of this disease [1, 4].

In asthma, there is increased expression of multiple inflammatory proteins in the respiratory tract, including cytokines, adhesion molecules, inflammatory enzymes, mediators and receptors. The majority of these inflammatory proteins are regulated at the level of gene transcription [5]. NF κ B is the main transcription factor involved in up-regulation of inflammatory cytokines, COX2 genes and adhesion molecules [6]. As airway macrophages and bronchial epithelial cells from asthmatics exhibit increased NF κ B activity compared with cells from healthy individuals [6], it has been suggested that NF κ B plays a pivotal role in the pathogenesis of asthma [6, 7]. Several studies demonstrated that omega-3 fatty acids such as EPA and DHA decrease NF κ B activation and related pro-inflammatory gene expression [see review 8]. Moreover, in previous studies, we have demonstrated that MAG-DHA mediates its anti-inflammatory effects through the activation of the nuclear receptor PPAR γ resulting in a decrease in NF κ B activation and in related pro-inflammatory gene expression such as cytokines and COX2 in an *in vitro* model of TNF α -stimulated human bronchi and [9].

Asthma is directly associated with increased levels of omega-6 (n-6) fatty acid levels, such as arachidonic acid, in blood circulation and tissues [10, 11]. Other studies have reported that the physiological levels of omega-3 fatty acid derivatives are relatively low in blood circulation, suggesting the importance of omega-3 supplementation [8, 11–14]. The protective effects of diets rich in very long chain ω -3 polyunsaturated fatty acid (PUFA) [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)] against asthma or wheezing illnesses in childhood have been shown in epidemiologic studies [11, 15]. EPA and DHA compete with arachidonic acid (AA) as substrates for the formation of leukotrienes (LTs) and prostaglandins (PGs), although EPA-derived metabolites have a much weaker pro-inflammatory activity compared with the 4-series cysteinyl LTEs and 2-series prostanoid products of AA metabolism [15]. Moreover, ω -3 PUFA-derived mediators, including E-series resolvins such as resolvin E1 from EPA, D-series resolvins and protectin D1 from DHA, appear to exert potent anti-inflammatory and pro-resolving actions both *in vitro* and *in vivo* [9, 16–20]. In light of the above, a new EPA sn1-monoacylglyceride, MAG-EPA, was synthesized to evaluate its anti-inflammatory properties in an allergic model of asthma. This compound is well absorbed by the GI tract, is non-toxic and its metabolites are found in blood circulation and lung tissues [9, 21–23]. The aim of this study was to determine the ability of MAG-EPA to resolve lung inflammation and reduce airway hyperresponsiveness

using an *in vivo* guinea-pig model of allergic asthma. Herein, we report the first evidence that MAG-EPA displays pro-resolving properties and reduces lung inflammation in an allergic model of asthma.

Materials and methods

Synthesis of omega-3 monoglyceride

MAG-EPA was synthesized as previously described using ethyl eicosapentaenoate as starting material (Fig. 1) [21, 22].

Sensitization and challenge protocols

Male or female albino guinea-pigs, (Hartley 200–250 g) were obtained from Charles River Laboratories (Montreal, QC, Canada). All procedures involving animal tissues were performed according to current Canadian Council for Animal Care (CCAC) guidelines. Guinea-pigs were sensitized with i.p. injections of 10 μ g ovalbumins (OVA) (Grade V, Sigma Chemical, St Louis, MO, USA) plus 1 mg of Al(OH) $_3$ as an adjuvant in 0.4 mL PBS, on days 0 and 7. On days 14–21, the OVA-sensitized guinea-pigs received an aerosol challenge containing 1.5% OVA for 10 min. Control animals were challenged with aerosolized PBS. MAG-EPA was given orally directly to the mouth with a pipette tip at 231 mg/kg, on days 19–21, 30 min after the aerosol challenge. The oral dose of 231 mg/kg was chosen according to Health Canada Draft Guidelines [9, 24] to have a human equivalent dose of 3.0 g/day (maximum allowed by Health Canada). The aerosols were generated with a small particle aerosol generator model SPAG-2 (ICN, Montreal, QC, Canada). On day 22, 24 h after the last aerosol challenge, the OVA-sensitized guinea-pigs, either untreated or treated with MAG-EPA, were killed, the external bronchi removed and used immediately for isometric tension measurements, while lung tissues were harvested for histological analysis, homogenate preparation for Western blot experiments or total RNA extraction for RT-PCR. As previously described [9], no behavioural or physical signs of toxicity with MAG-EPA treatments were observed. The time line illustrated in Fig. 2 summarizes the sensitization protocol as a function of time.

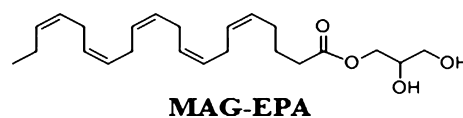


Fig 1. Chemical structure of eicosapentaenoic acid monoacylglyceride (MAG-EPA). MAG-EPA was synthesized from highly purified EPA attached to a monoglycerol in sn-1 position (17).

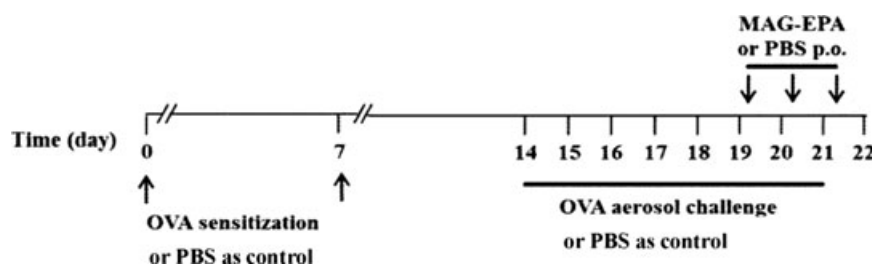


Fig 2. Sensitization protocol. Time line illustration summarizes the ovalbumin sensitization protocol for guinea-pigs as a function of time.

Isometric tension measurements

The mechanical effects induced by MCh were measured as previously described [9, 23, 25]. Briefly, bronchial rings were mounted in isolated organ baths (Radnoti Glass Tech., Monrovia, CA, USA), containing 6 ml of Krebs solution at 37°C, pH 7.4 bubbled continuously with a 95% O₂/5% CO₂ mixture and to which an initial load of 0.8 g was applied. Tissues were allowed to equilibrate for 1 h in Krebs solution and washed out every 15 min. Passive and active tensions were assessed using transducer systems coupled to Polyview software (Grass-Astro-Med Inc., West Warwick, RI, USA) for facilitating data acquisition and analysis. The measurement of resulting induced myofilament Ca²⁺ sensitivity was performed as previously reported by using a β -escin permeabilization protocol on guinea-pig bronchi [9, 25].

Fatty acid composition of plasma and lung tissues using high-performance liquid chromatography coupled to mass spectrometry

Plasma and lung tissue fatty acid compositions were measured using a high-performance liquid chromatography–mass spectrometry (HPLC/MS) method [26] modified to obtain complete fatty acid composition. Briefly, 5 μ L of KOH solution (9N) was added to a 25 μ L aliquot of plasma or homogenized lung tissue sample and heated at 65°C for 45 min. After cooling the samples to room temperature, 975 μ L of extraction solution [150 mM ammonium formate buffer pH = 3.0/acetonitrile (25 : 75 v/v)] was added and the samples were vortexed 15 s and centrifuged (12 000 g) for 5 min. Supernatant was transferred to a HPLC vial and 5 μ L was injected onto an HPLC column. Analyses were performed using an Agilent 1200 series HPLC system at room temperature with a reversed-phase column (Zorbax, C8, 50 $\times$ 2.1 mm, 3.5 μ m, Agilent Technologies Canada, Mississauga, Canada). The binary gradient mobile phase [mobile phase A (5 mM ammonium acetate) and mobile phase B (acetonitrile with 0.01% formic acid)] was pumped at a flow rate of 1.0 ml/min using the following gradient: 0 min (65% B), 1.5 min

(65% B), 1.7 min (99% B), 2.9 min (99% B), 2.95 min (65% B) and 3.5 min (65% B). Run time was 3.5 min with a post run time of 0.5 min. The mass spectrometry detector was operated in ESI negative mode, the capillary voltage was set at 3 kV, the fragmentor at 140 V, the gas temperature at 350°C, the gas flow at 13 L/min and the nebulizer pressure at 60 psi. Data were acquired in SIM mode corresponding to the following fatty acids: C14:0, C14:1, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3, C18:4, C20:0, C20:1, C20:2, C20:3, C20:4 (AA), C20:5 (EPA), C22:0, C22:1, C22:2, C22:3, C22:4, C22:5 (DPA), C22:6 (DHA), C24:0, C24:1, C24:2, C24:3, C24:4, C24:5, C24:6. The linear calibration curves were obtained in the concentration range from 2 to 500 μ g/mL. The chemstation LC/MSD software Rev.B.02.01 (Agilent) was used for instrument control and data analysis.

Allergen-initiated lung inflammation

Guinea-pig lungs were fixed in 10% buffered formalin and paraffin embedded, after which thin sections (3 μ m) were stained with haematoxylin–eosin and Alcian blue according to standard protocols [9]. Images were acquired with a Hamamatsu ORCA-ER digital camera attached to a Nikon Eclipse TE-2000 inverted microscope (Nikon-Canada, Mississauga, ON, Canada). Image analyses were performed for immuno-positive pixel staining of Alcian Blue in lung sections using MetaMorph 7.6 software (Molecular Devices, Silicon Valley, CA, USA). The pixel size was unrestricted and the automatic find function was set to search for positive pixels using smooth edges. Images were obtained (objective $\times$ 20), from lung sections derived from control, OVA and OVA+MAG-EPA-treated guinea-pigs. Bilateral bronchoalveolar lavages (BAL) were performed in control animals as well as in OVA-sensitized guinea-pigs untreated or treated with MAG-EPA (two aliquots of 2 mL of PBS plus 0.6 mM Ethylenediaminetetraacetic acid). Cells in BAL fluids were suspended in PBS, enumerated by haemocytometer counting and concentrated onto microscope slides by cytocentrifugation at 265 g. Cells were stained with a Wright-Giemsa stain (Sigma-Aldrich, Oakville, ON, Canada) to determine leucocyte differentials. Measurement of inflammatory mediator

was determined in lungs by real-time RT-PCR. Total RNA from the lung was extracted. mRNA encoding the indicated genes IL-4, IL-5, IL-13, eotaxin and MUC5AC were examined using real-time RT-PCR analysis. The expression levels of each gene were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels and reported as fold-change relative to normal control animals.

Western blot analysis

Lung homogenates were prepared from control as well as OVA-sensitized animals untreated or treated with MAG-EPA. Lung tissues were removed, weighed and promptly transferred in a buffer containing 0.3 M sucrose, 20 mM K-PIPES, 4 mM EGTA and a cocktail of protease and phosphatase inhibitors (Protease-inhibitor pellets from Roche Diagnostics, Indianapolis, IN, USA, +10 μ M Na_2VO_3). Tissues were then homogenized on ice, frozen in liquid nitrogen and stored at -80°C . Samples containing equal amounts of protein were separated on SDS-PAGE, and the proteins subsequently transferred by electroblotting onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada). Western blots were performed using specific antibodies against COX-2, ChemR23 and β -actin proteins [9]. Membranes were incubated for 1 h at 23°C with peroxidase-conjugated donkey anti-rabbit IgG1 antiserum (Amersham, QC, Canada) and revealed by Enhanced Chemiluminescence (Roche, QC, Canada). Blot immuno-stainings were digitized and analysed with Lab-Image software 2.7-2.

Data analysis and statistics

Results are expressed as means $\pm$ SEM with n indicating the number of experiments. Statistical analyses were performed using a one-way analysis of variance (ANOVA) and Holm-Sidak as *post hoc* test. Differences were considered statistically significant when $P < 0.05$. Data curve fittings were performed using Sigma Plot 11 (SPSS-Science, Chicago, IL, USA) to determine EC_{50} values [25].

Results

Effect of MAG-EPA on airway hyperresponsiveness in an in vivo model of allergic asthma

The effect of MAG-EPA treatment on the resolution of inflammation and airway hyperresponsiveness was assessed in OVA-sensitized guinea-pigs. MAG-EPA was administered *per os* at pharmacological doses (231 mg/mL). Experiments were designed to assess the mechanical properties of bronchial smooth muscles to cumulative concentrations of methacholine (MCh).

Figure 3a illustrates the cumulative concentration response curve (CCRC) to MCh in bronchial preparations derived from control, OVA-sensitized and OVA-sensitized + MAG-EPA treated guinea-pigs. Data revealed hyper-reactivity of the bronchi from OVA-sensitized guinea-pigs to MCh with an apparent EC_{50} value of 0.13 ± 0.03 μM , whereas oral MAG-EPA treatment reduced the reactivity of airway smooth muscle to MCh, with an EC_{50} value of 0.77 ± 0.03 μM . Increased Ca^{2+} sensitivity of bronchial smooth muscle contributes to maintaining airway hyperresponsiveness in asthma

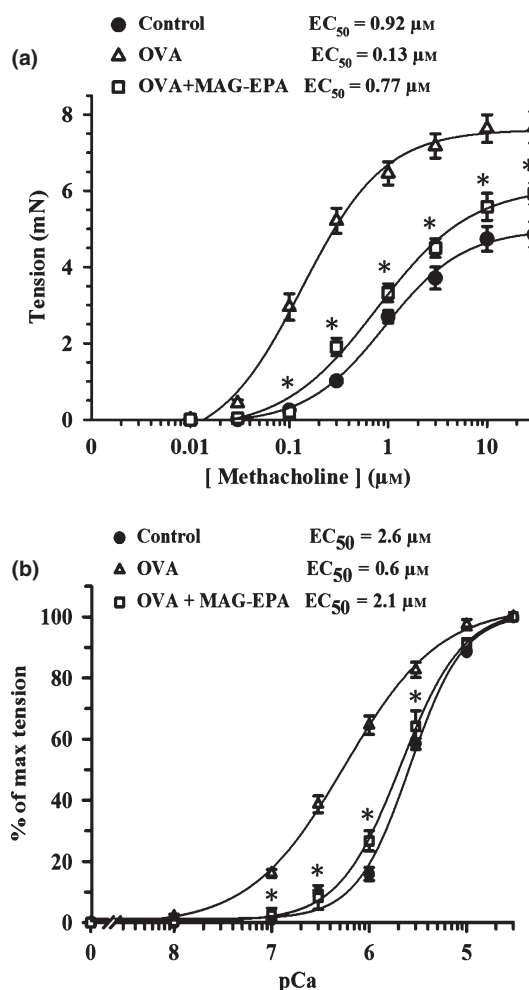


Fig 3. Effect of eicosapentaenoic acid monoacylglyceride (MAG-EPA) treatments on airway hyperresponsiveness. (a) Cumulative concentration response curves (CCRC) to Methacholine generated in Control (closed circles), OVA-sensitized (open triangles) and OVA-sensitized + MAG-EPA-treated guinea-pigs (open squares). Each point represents the mean $\pm$ SEM, with $n = 8$ for each experimental condition (* $P < 0.05$). (b) Normalized cumulative concentration response curves (CCRC) to free Ca^{2+} generated in Control (closed circles), OVA-sensitized (open triangles) and OVA-sensitized + MAG-EPA-treated guinea-pigs (open squares). Each point represents the mean $\pm$ SEM, with $n = 8$ for each experimental condition (* $P < 0.05$).

[27]. Comparative analyses were therefore performed on β -escin-permeabilized guinea-pigs bronchial rings to assess the effect of MAG-EPA administration on Ca^{2+} sensitivity of myofilaments. Figure 3b depicts CCRC to free Ca^{2+} concentrations on permeabilized bronchi obtained from control, OVA-sensitized and OVA-sensitized + MAG-EPA-treated animals. When compared with control condition (closed circles), an enhanced Ca^{2+} sensitivity to pre-calibrated Ca^{2+} steps was recorded in OVA-sensitized tissues (open triangles). However, MAG-EPA treatments (open squares) significantly reduced the Ca^{2+} sensitivity developed by OVA-sensitized bronchi. Data analyses demonstrated that MAG-EPA treatments induced a shift in EC_{50} value ($2.1 \pm 0.02 \mu\text{M}$) towards higher Ca^{2+} concentrations when compared with OVA-sensitized untreated tissues ($0.6 \pm 0.03 \mu\text{M}$). Difference was considered significant with a P value of 0.033 for OVA+MAG-EPA-treated and OVA-sensitized animals.

Effect of MAG-EPA treatment on lung and plasma fatty acid content in an allergic model of asthma

Asthma is directly associated with an increase in omega-6 (ω -6) fatty acid levels, such as arachidonic acid, in blood circulation and tissues [10]. Fatty acid content was thus determined by HPLC/MS on lung samples derived from control, OVA-sensitized as well as OVA-sensitized + MAG-EPA-treated animals. Lipid analyses revealed a decrease in AA and linoleic acid (LA) ω 6 and an increased level of EPA in lung sections derived from MAG-EPA-treated guinea-pigs when compared with lungs from control OVA-sensitized animals (Fig. 4a). Consequently, the ω 6/ ω 3 ratio was significantly decreased by twofold in lungs derived from OVA-sensitized + MAG-EPA-treated animals when compared with the ratio calculated in untreated OVA-sensitized guinea-pigs (Fig. 4a, inset). Moreover, plasma lipid analyses also demonstrated a decrease in

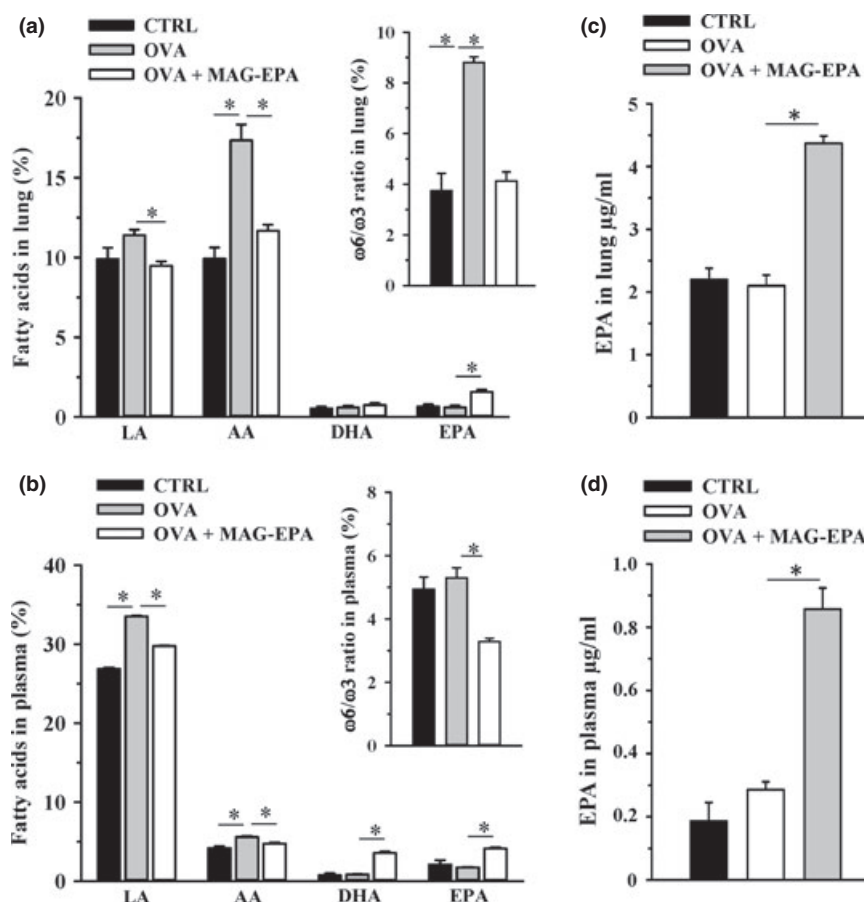


Fig 4. Effect of eicosapentaenoic acid monoacylglyceride (MAG-EPA) treatments on lung fatty acid content in an allergic model of asthma. Quantitative analysis of relative fatty acid content (%) and ω 6/ ω 3 ratio (insets) in lung (a) and in plasma (b) derived from control, OVA-sensitized and OVA-sensitized + MAG-EPA treated guinea-pigs ($n = 6$ per group, $*P < 0.05$). LA, linolenic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid. Quantitative analysis of the endogenous EPA levels ($\mu\text{g}/\text{mL}$) in lung tissue (c) and plasma (d) derived from control, OVA-sensitized and OVA-sensitized + MAG-EPA treated animals ($n = 6$ per group, $*P < 0.05$).

LA and AA as well as an increased level of $\omega 3$ fatty acids such as DHA and EPA fatty acids in plasma derived from MAG-EPA-treated guinea-pigs when compared with lungs from OVA-sensitized animals (Fig. 4b). Figures 4c and d show the endogenous levels of EPA in lung and plasma derived from control, OVA-sensitized and OVA-sensitized + MAG-EPA-treated animals. Results demonstrated that the dose of MAG-EPA used was able to increase four times the level of EPA in plasma and more than double the endogenous level of the lung of OVA-sensitized guinea-pigs. Together, these results demonstrate that following absorption of MAG-EPA, consistent increases in the levels of EPA and other long chain PUFA as well as decreased levels of AA and LA were consistently measured in lung tissues and plasma. This increased EPA level may contribute to reducing lung inflammation induced by OVA in this allergic model of asthma.

Effect of MAG-EPA treatments on lung inflammation in OVA-sensitized guinea-pig

The extent of inflammation in lung tissues was determined in control and OVA-sensitized guinea-pigs treated or not with oral doses of MAG-EPA. Histological analyses performed on lung sections stained with haematoxylin-eosin revealed that animals receiving MAG-EPA had substantially less eosinophils and lymphocytes in the peribronchial regions and airspaces compared with OVA-sensitized animals (Figs 5a–c). In BAL fluids, MAG-EPA treatments decreased total leucocytes, namely eosinophils and lymphocytes, when compared with the number of eosinophils and lymphocytes found in BAL fluids of untreated OVA-sensitized guinea-pigs (Fig. 5d). To assess the expression level of pro-inflammatory mediator transcripts, real-time polymerase chain reaction analyses were performed from messenger RNA (mRNA) isolated from lung samples of control and both OVA-treated groups. As illustrated in Fig. 5e, mRNA expression of IL-5, eotaxin, IL-13 and IL-4 cytokines in lung was strongly induced by OVA-sensitization compared with the mRNA expression levels of cytokines detected in control guinea-pigs. However, following MAG-EPA treatment, data analyses revealed a significant decrease in IL-5, eotaxin, IL-13 and IL-4 transcript levels relative to the expression in OVA-sensitized guinea-pigs.

Effect of oral MAG-EPA administration on COX-2 and CMKLR1 expression levels

To determine whether MAG-EPA reduces allergen-induced airway inflammation, the activation of nuclear factor kappa B (NF κ B) was investigated [7]. Western blot analysis revealed that MAG-EPA treatments

decrease phosphorylation of p65 NF κ B subunit induced by the allergen when compared with OVA-sensitized guinea-pigs (Figs 6a and b). COX-2 has been shown to be up-regulated under pro-inflammatory conditions and to contribute to increasing production of several PGs involved in the pathogenesis of allergic asthma [7]. We therefore evaluated the expression of COX-2 in lung homogenates derived from control, OVA-sensitized untreated as well as in OVA-sensitized MAG-EPA-treated guinea-pigs. Western blot analysis revealed a significant increase in COX-2 protein expression in OVA-sensitized animals when compared with the expression levels in control animals (Fig. 6a). However, analysis demonstrated that MAG-EPA treatment reduced the COX-2 expression level comparatively to expression levels in OVA-sensitized animals (Fig. 6a). Quantitative analysis of identical immunoblot membrane areas was normalized as a function of total β -actin staining in the corresponding fractions (Fig. 6b). As shown in Fig. 6b, MAG-EPA treatment significantly reduced the COX-2/ β -actin staining density ratio when compared with untreated OVA-sensitized guinea-pigs. Chemokine receptor-like 1 (CMKLR1), also known as ChemR23, has previously been identified as a receptor for RvE1 [28, 29] and shown to regulate the ability of RvE1 to inhibit TNF-induced NF- κ B activation in cells [14]. We thus assessed the expression level of CMKLR1 by Western blot in lung derived from control, OVA-sensitized untreated and OVA-sensitized MAG-EPA-treated animals (Fig. 6c). Data analyses revealed that MAG-EPA treatments significantly increased CMKLR1 expression level in lung tissue compared with the expression level detected in untreated OVA-sensitized guinea-pig lung (Figs 6c and d).

Effect of oral MAG-EPA treatment on mucin production in OVA-sensitized guinea-pigs

Histological analysis performed on lung sections derived from control, OVA-sensitized as well as in OVA-sensitized MAG-EPA-treated guinea-pigs were stained with Alcian blue to visualize mucin production. Histological and semi-quantitative analyses of stained lung sections revealed an increased mucus production in OVA-sensitized animals when compared with the production level in control animals (Figs 7a and b). However, MAG-EPA treatment markedly reduced goblet cell hyperplasia and airway mucus production as determined by Alcian blue staining (Figs 7c and d). To assess the expression level of MUC5AC transcripts, real-time PCR analyses were performed from mRNA isolated from lung samples of control and both OVA-treated groups. As illustrated in Fig. 7e, mRNA expression of MUC5AC in lung was strongly induced by OVA-sensitization compared with mRNA expression levels in control

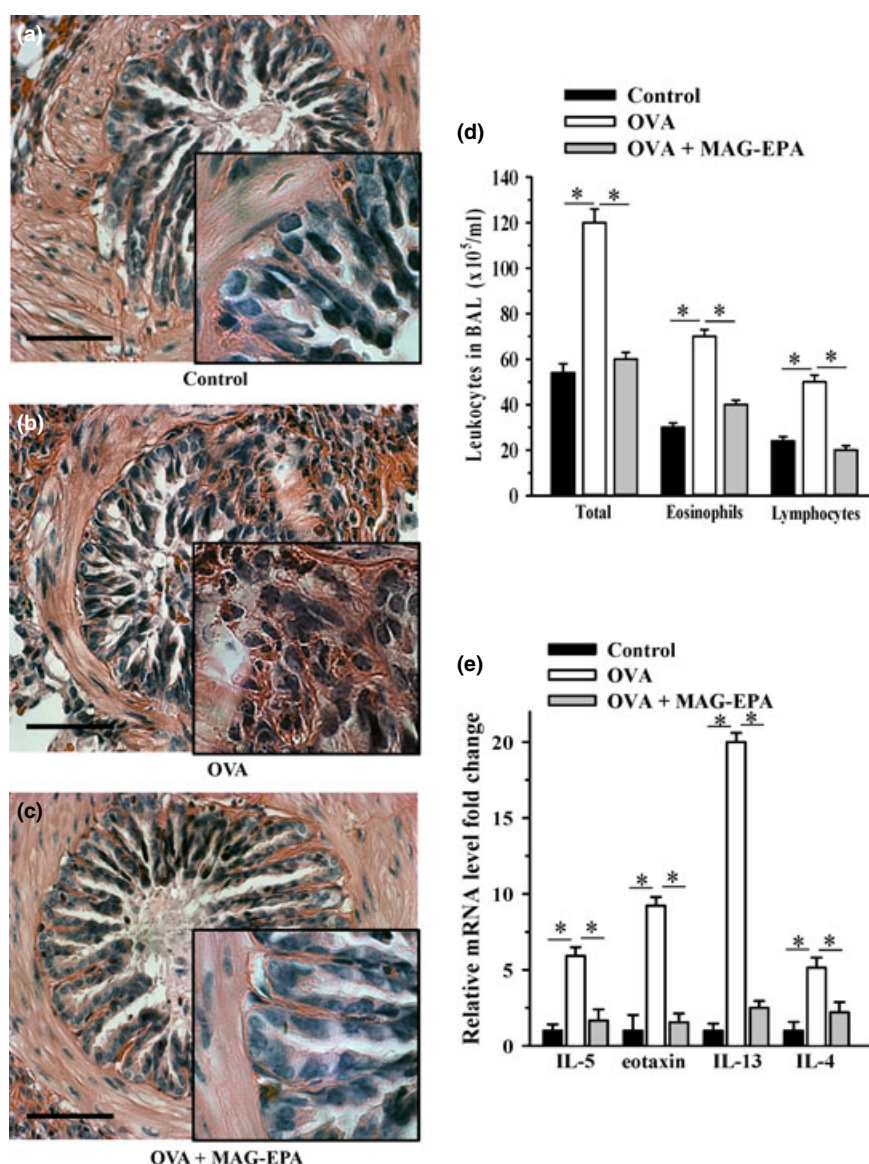


Fig 5. Eicosapentaenoic acid monoacylglyceride (MAG-EPA) reduces airway inflammation in ovalbumin-sensitized lung tissue. (a–d) Representative lung tissue sections obtained from a non-sensitized guinea-pig (control) (a) as well as from OVA-sensitized (b) and OVA-sensitized + MAG-EPA-treated animals (c). The degree of inflammation was assessed by determining the extent of inflammatory infiltrates present in lung sections stained with haematoxylin-eosin. Scale bar, 50 μ m. Images are representative of $n = 6$ per group. (d) Total leucocytes, eosinophils and lymphocytes in BAL fluid were enumerated and identified after Wright-Giemsa staining. Results are expressed as mean $\pm$ SEM, ($n = 6$), $*P < 0.05$. (e) mRNAs encoding four identified genes were examined using real-time RT-PCR analysis. Total RNA was extracted from the guinea-pig lung. The expression levels of each gene were normalized to glyceraldehyde-3-phosphate dehydrogenase expression levels and reported as fold-change relative to normal control animals. Results represent the mean $\pm$ SEM of six animals per group. [$*P < 0.05$ significantly different from the values for normal (control) and OVA-treated animals].

guinea-pigs. However, following MAG-EPA treatments, data analyses revealed a significant decrease in MUC5AC transcript levels comparatively to expression in OVA-sensitized guinea-pigs (Fig. 7e).

Discussion

In this study, we investigated the ability of *per os* administered MAG-EPA to resolve airway inflammation

in an allergic model of asthma. MAG-EPA was found to reduce airway hyperresponsiveness to MCh and Ca²⁺ hypersensitivity of smooth muscle cells. The anti-inflammatory effects of MAG-EPA were correlated with a decrease in expression levels of pro-inflammatory mediators such as IL-4, IL-5, eotaxin, IL-13, MUC5AC and COX-2. Thus, we propose that MAG-EPA is able to fine tune the transcription processes of regulatory proteins involved in the modulation of airway inflammation.

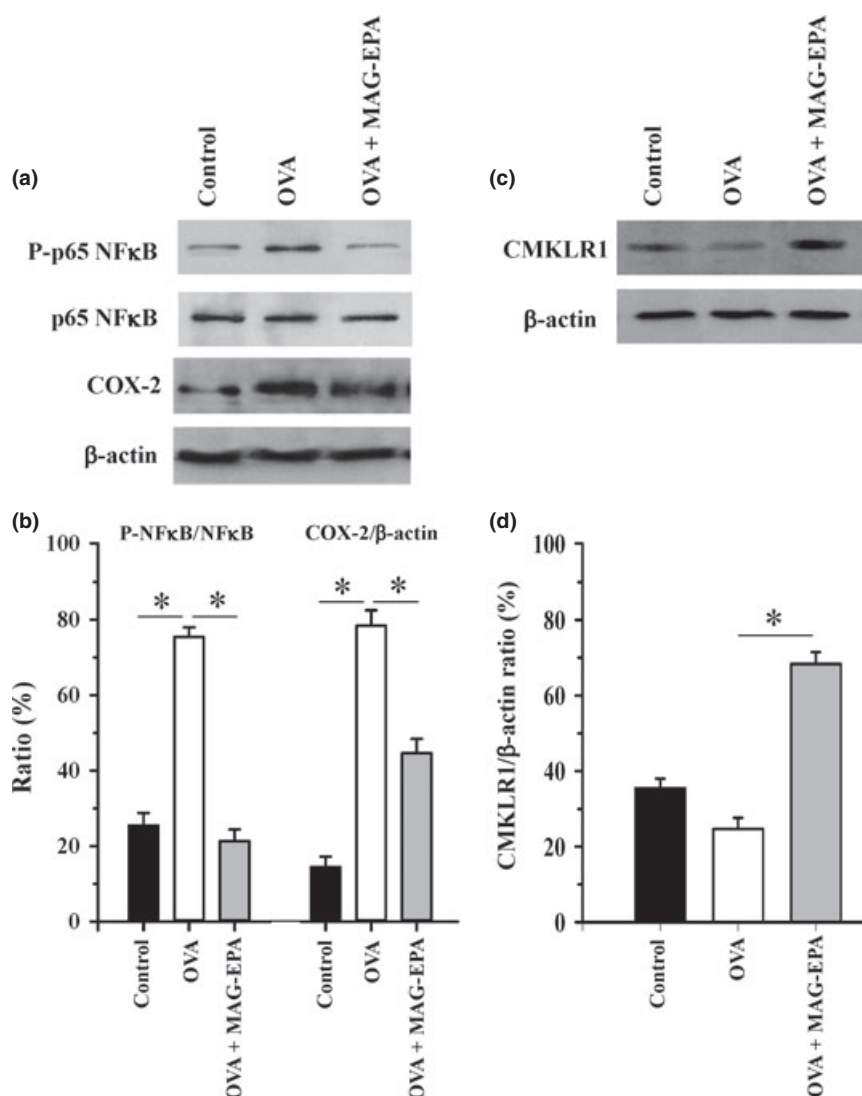


Fig 6. Effect of eicosapentaenoic acid monoacylglyceride (MAG-EPA) on COX-2 and CMKLR1 expressions in lung tissue. (a) Typical Western blot and (b) subsequent quantitative analysis of lung homogenate fractions derived from Control, OVA-sensitized and OVA-sensitized + MAG-EPA guinea-pigs, using specific antibodies against phosphorylated form of p65 NFκB (P-p65 NFκB) and total NFκB as well COX-2 and β-actin. Staining densities in the homogenates were expressed as a function of NFκB signals for P-p65 NFκB and β-actin for COX-2 ($n = 6$ for each experimental condition; $*P < 0.05$). (c) Typical Western blot and (d) subsequent quantitative analysis of lung homogenate fractions derived from Control, OVA-sensitized and OVA-sensitized + MAG-EPA guinea-pigs, using specific antibodies against CMKLR1 and β-actin. Staining densities in the homogenates were expressed as a function of β-actin signals ($n = 7$, $*P < 0.05$).

Anti-inflammatory effects of MAG-EPA in an allergic model of asthma

Over the past three decades, there has been considerable interest in the therapeutic potential of fish oils rich in omega-3 such as DHA and EPA for various inflammatory conditions. Epidemiologic evidence suggests that diets rich in omega-3 fatty acids can protect against asthma, cystic fibrosis, heart disease and cancer [8, 11, 12, 30]. Moreover, in fat-1 mouse allergic model of asthma, the changes in lung tissue levels of omega-3 fatty acids reduced the airway inflammation and hyperresponsiveness [31]. Indeed, airway epithelial cells

in cystic fibrosis and asthma display abnormalities in fatty acid metabolism, with low levels of omega-3 fatty acids [10]. At present, the exact mechanisms underlying the anti-inflammatory effects of DHA and EPA remain unclear, but may be related to altered gene transcription and translation via direct or indirect action on intracellular signalling pathways [12, 30]. In addition, PUFA-derived mediators such as lipoxinA4, resolvins and protectins may also have potent anti-inflammatory and pro-resolving properties [9, 16, 17, 19, 32, 33]. Our current strategy was to synthesize an EPA monoacylglyceride and to assess its effect in the resolution of inflammation and airway hyperresponsiveness in an

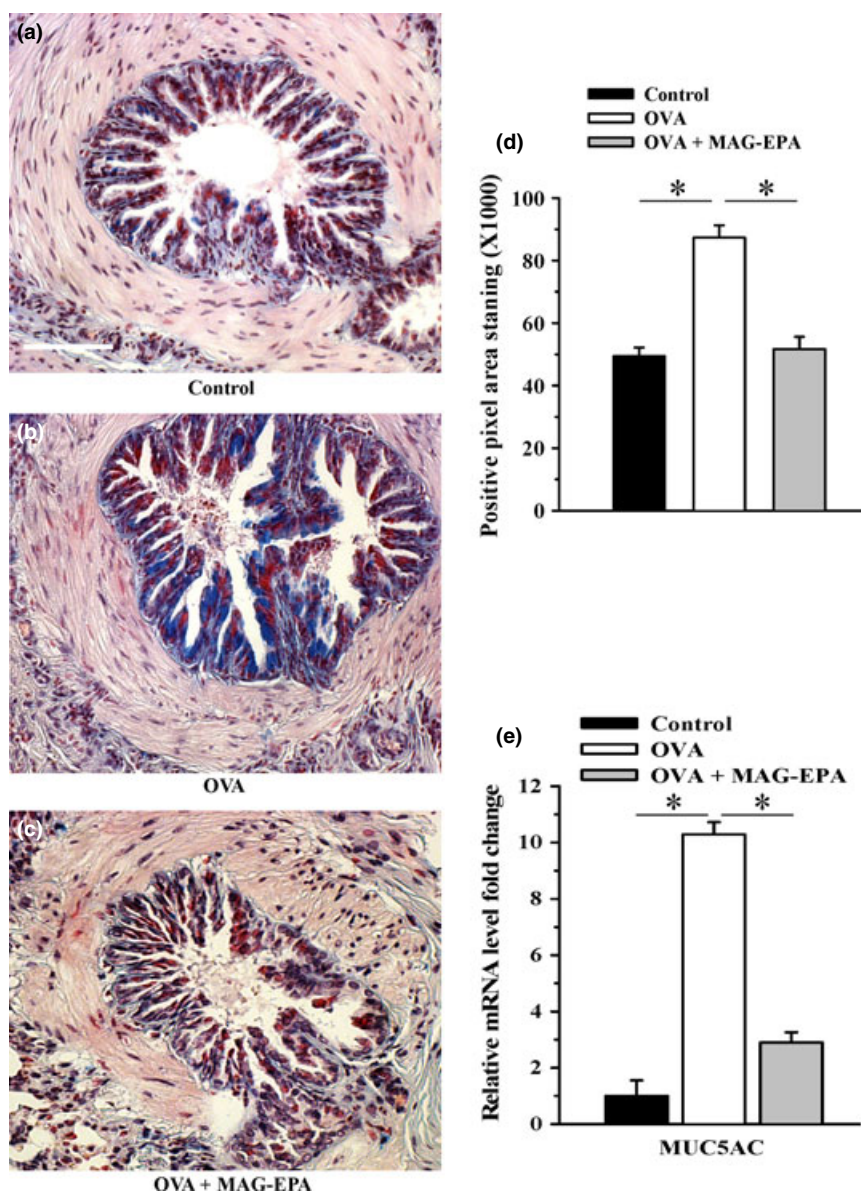


Fig 7. Eicosapentaenoic acid monoacylglyceride (MAG-EPA) treatments reduce mucin production in OVA-sensitized lung tissue. Lung thin sections obtained from non-sensitized Control (a), OVA-sensitized (b) and OVA-sensitized + MAG-EPA-treated guinea-pigs (c) were stained with Alcian blue. Mucus secretion and goblet cells containing mucus are stained in blue. a, b and c: Scale bar, 100 μm. Images are representative of $n = 6$ per group. (d) Quantitative analysis displaying the mean immunopositive pixel area staining of Alcian blue from lung tissue sections ($n = 6$ per group, $*P < 0.05$). (e) mRNA encoding the MUC5AC gene was examined using real-time RT-PCR analysis. The expression levels of MUC5AC were normalized to glyceraldehyde-3-phosphate dehydrogenase expression levels and reported as fold-change relative to normal control animals ($n = 6$ animals per group, $*P < 0.05$).

allergic model of asthma. Fatty acids in monoacylglyceride form are generally recognized as safe and are widely used as emulsifying agents in the food industry. Moreover, studies have shown that EPA in the form of monoacylglyceride increases the oral bioavailability of EPA compared with fish oil [34, 35]. Furthermore, comparatively to fish oil which contains 18% EPA and 12% DHA, MAG-EPA is enriched in EPA (95%), thus the use of our formulation represents an improved way to increase daily intake of EPA with a reasonable amount of oil.

Our previous studies demonstrated that omega-3 fatty acids in monoglyceride form are known to display an increased systemic bioavailability of omega-3 fatty acids *in vivo* in an allergic model of asthma and in a xenograft mice model of lung cancer [9, 21, 22]. In a previous study, we had shown that oral administration of either MAG-DHA or MAG-EPA prevented allergen-induced pulmonary inflammation and airway hyperresponsiveness in a guinea-pig model of allergic asthma [9]. Herein, we show that MAG-EPA treatment,

introduced in the last 3 days of a series of 8-day OVA challenges, was able to reduce MCh-induced airway hyperresponsiveness and Ca^{2+} sensitivity of airway smooth muscle cells. Airway infiltration of eosinophils, T-cells and mast cells with excess mucus secretion are common features of allergic airway inflammation and have been correlated clinically with airway hyperresponsiveness [36]. In this study, our data demonstrate a reduced number of eosinophils and lymphocytes in BAL fluid recovered from OVA-sensitized guinea-pigs receiving MAG-EPA. Moreover, airway mucus production and MUC5AC transcript levels were lowered in MAG-EPA-treated animals. An increased expression of multiple inflammatory proteins and lipid mediators has been reported in the respiratory tract of asthmatic patients, including cytokines, adhesion molecules, inflammatory enzymes and receptors [7, 36]. The majority of these inflammatory proteins are regulated at the transcription level [7, 12, 30], with NF κ B being the main transcription factor involved in up-regulation of inflammatory cytokines, COX2 genes and adhesion molecules [3, 7]. In this study, MAG-EPA treatments were observed to reduce the expression levels of COX-2, eotaxin, IL-4, IL-5 and IL-13, thus demonstrating the ability of this omega-3 monoglyceride to curtail the up-regulation of these inflammatory mediators.

Enhanced role of ω -3 PUFA in pulmonary pathophysiology

In this study, lipid analyses revealed an increased level of EPA in lung tissues derived from MAG-EPA-treated animals when compared with mean levels in lung from control and OVA-sensitized guinea-pigs. The resulting ω 6/ ω 3 ratio was decreased by twofold in lung derived from OVA-sensitized + MAG-EPA-treated guinea-pigs thereby reaching the baseline ratio of the control group. Our measurements also demonstrated that following absorption of MAG-EPA, increased levels of long chain ω -3 fatty acids such as DHA, EPA and DPA and a concomitant decrease in AA and LA level in blood likely contributed to reducing airway inflammation. Our data suggest that decreased AA and increased EPA concentrations might both contribute to decrease airway inflammation and hyperresponsiveness in OVA-sensitized animals. These observations could be of clinical relevance as it has been shown that the ratio of ω -6/ ω -3 PUFA may be the most important factor in determining lung inflammation in asthma and cystic fibrosis. Furthermore, several studies have demonstrated that the intake of fish oil or cod liver oil containing DHA and EPA over several days decreases AA levels in plasma and tissues [for review, see 37]. EPA and DHA can also suppress eicosanoid synthesis by competitive binding on cyclooxygenase enzymes and thus exclude

AA from the active site [37]. Recently, new findings on the initiation and resolution of inflammation (i.e. inflammasomes and resolvins) have been reported [14, 38]. Recent evidence suggests that resolution of inflammation is actively mediated by new resolving lipid classes such as resolvins, protectins, lipoxins and maresin [14]. Therefore, the failure of resolution is considered as one of the causes in chronic inflammatory diseases such as age-related asthma, macular degeneration, atherosclerosis, chronic pulmonary disease, inflammatory bowel disease, multiple sclerosis, rheumatic arthritis and cancer [39]. Resolvins and protectins are synthesized from omega-3 fatty acids such as DHA and EPA. Enzymes involved in the biosynthesis of these mediators include 5-lipoxygenase (5-LO), 12-LO and 15-LO [14]. We propose that following MAG-EPA treatment, the increased EPA bioavailability may lead to a production of tri hydroxyl derivative metabolites such as Resolvin E, thus putatively contributing to reducing lung inflammation in our *in vivo* allergic model of asthma. Indeed, several pharmacological activities of these lipids have been demonstrated to be effective in animal models of inflammation and cultured blood cells. For example, in a murine allergic inflammation model, Resolvin E1 (RvE1), in nanogram quantities, was found to promote resolution of inflammatory airway responses in part by directly suppressing IL-23 and IL-6 production in the lung and increasing the concentrations of interferon- γ in the lungs of RvE1-treated animals [40]. Moreover, RvE1 displayed anti-inflammatory effects when administered in a murine model of allergic asthma by decreasing leucocyte recruitment, specific Th2 cytokine, IL-13, ovalbumin-specific IgE and airway hyperresponsiveness to inhaled MCh [19, 20].

Membrane receptors and hydroxyl-PUFA

CMKLR1 is a signalling receptor activated by two ligands, chemerin and resolvin E1, and appears to play an anti-inflammatory role. The reported effects of CMKLR1/RvE1 interaction include the reduction in cytokine and chemokine production and phagocytosis [41]. At nanomolar concentrations, RvE1 has been shown to reduce leucocyte infiltration in a TNF α -induced air pouch or zymosan-induced mouse peritonitis model by 50–70% [42]. In a model of acute lung inflammation induced by LPS, intra-tracheal co-administration of recombinant chemerin, a known CMKLR1 ligand, with LPS reduced infiltrating PMN counts by 70% 12 h after challenge, an effect which persisted at 24 and 72 h within the resolution phase in WT mice. This reduced PMN infiltration was associated with reduced concentrations of KC/CXCL1, IL-6, TNF and IL-1 β in bronchoalveolar lavage fluids (BALFs) [43]. In CMKLR1-deficient mice, LPS was observed to induce a

greater PMN infiltration and chemokine and cytokine release in BAL fluids as compared with WT mice; in addition, in this model, chemerin treatment had no effect on the amount of chemokines and cytokines released into BAL fluids following LPS challenge [43]. To our knowledge, results of this study are the first to demonstrate a decreased CMKLR1 expression level in an allergic model of asthma whereas, in the presence of omega-3 derivative treatments, an increased CMKLR1 expression level was observed. These findings thus suggest a role for CMKLR1 in the control of lung inflammation in allergic asthma.

Lastly, as attested previously, MAG-EPA is well absorbed and metabolized in glycerol and EPA; which in turn could be transformed into active lipid mediators, including RvE1. One noteworthy characteristic of resolvins, however, is that they contain several double bonds and three hydroxyl groups such that this unstable structure is vulnerable to metabolic inactivation [16]. It is therefore difficult to produce resolvins in large amounts from synthetic pathways [16]. Novel approaches are hence required to overcome such metabolic and structural features of resolvins, thus further justifying the use of biochemical precursors or stable analogues such as MAG-EPA. Further investigation using high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) will help to determine the production of specific metabolites, including resolvins of the E-series. These measurements should putatively explain the beneficial effects of MAG-EPA in inflammatory tissues.

In conclusion, we propose that EPA monoacylglyceride derivatives, possessing anti-inflammatory properties, may help to resolve inflammation and provide an interesting new approach in the treatment of asthma and other inflammatory conditions. Furthermore, when administered *per os*, MAG-EPA represents a stable compound that could serve as a precursor to generate a variety of PUFA-derived mediators, such as resolvins and protectins known to mediate anti-inflammatory and pro-resolving effects. In this respect, it is proposed that MAG-EPA could lead to the production of bioactive metabolites, which represent new, prospective pharmacological compounds of low toxicity and high medicinal interest in modulating inflammation and bronchoconstriction in asthma.

Acknowledgements

This work was supported in by a grant from the Canadian Institutes of Health Research CIHR: MOP 111-112. The authors thank Mr. Pierre Pothier for critical review of the manuscript.

Conflict of interest

Samuel Fortin is the owner of SCF Pharma, which has an exclusive world-wide license of the patented uses of MAG-EPA.

References

- 1 Bahadori K, Doyle-Waters MM, Marra C *et al.* Economic burden of asthma: a systematic review. *BMC Pulm Med* 2009; **9**:24.
- 2 Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med* 2000; **161**:1720-45.
- 3 Mitzner W, Brown RH. Potential mechanism of hyperresponsive airways. *Am J Respir Crit Care Med* 2000; **161**:1619-23.
- 4 Holgate ST, Polosa R. Treatment strategies for allergy and asthma. *Nat Rev Immunol* 2008; **8**:218-30.
- 5 Murdoch JR, Lloyd CM. Chronic inflammation and asthma. *Mutat Res* 2010; **690**:24-39.
- 6 Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am J Respir Crit Care Med* 1998; **158**:1585-92.
- 7 Gagliardo R, Chanez P, Mathieu M *et al.* Persistent activation of nuclear factor-kappaB signaling pathway in severe uncontrolled asthma. *Am J Respir Crit Care Med* 2003; **168**:1190-8.
- 8 Connor WE. Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr* 2000; **71**(1 Suppl):171S-5S.
- 9 Morin C, Fortin S, Cantin AM, Rousseau E. Docosahexaenoic acid derivative prevents inflammation and hyperactivity in lung: implication of PKC-Potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kD in asthma. *Am J Respir Cell Mol Biol* 2011; **45**:366-75.
- 10 Freedman SD, Blanco PG, Zaman MM *et al.* Association of cystic fibrosis with abnormalities in fatty acid metabolism. *N Engl J Med* 2004; **350**:560-9.
- 11 Peat JK, Miharshahi S, Kemp AS *et al.* Three-year outcomes of dietary fatty acid modification and house dust mite reduction in the Childhood Asthma Prevention Study. *J Allergy Clin Immunol* 2004; **114**:807-13.
- 12 Jump DB. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol* 2002; **13**:155-64.
- 13 Serhan CN, Hong S, Gronert K *et al.* Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. *J Exp Med* 2002; **196**:1025-37.
- 14 Serhan CN, Petasis NA. Resolvins and protectins in inflammation resolution. *Chem Rev* 2011; **111**:5922-43.
- 15 Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. *Proc Nutr Soc* 2007; **66**:237-59.

- 16 Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosa-hexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. *J Biol Chem* 2003; **278**:14677–87.
- 17 Levy BD, Kohli P, Gotlinger K et al. Protectin D1 is generated in asthma and dampens airway inflammation and hyperresponsiveness. *J Immunol* 2007; **178**:496–502.
- 18 Serhan CN, Gotlinger K, Hong S et al. Anti-inflammatory actions of neuro-protectin D1/protectin D1 and its natural stereoisomers: assignments of dihydroxy-containing docosatrienes. *J Immunol* 2006; **176**:1848–59.
- 19 Aoki H, Hisada T, Ishizuka T et al. Resolvin E1 dampens airway inflammation and hyperresponsiveness in a murine model of asthma. *Biochem Biophys Res Commun* 2008; **367**:509–15.
- 20 Arita M, Bianchini F, Aliberti J et al. Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1. *J Exp Med* 2005; **201**:713–22.
- 21 Fortin S. Compositions comprising polyunsaturated fatty acid monoglycerides or derivatives thereof and uses thereof. US819690 (2012) & US8222295 (2012).
- 22 Fortin S. Polyunsaturated fatty acid monoglycerides, derivatives, and uses thereof. CA2677670 (2010) & US8119690 (2011).
- 23 Morin C, Fortin S, Cantin AM et al. Anti-Cancer Effects of a New Docosa-hexaenoic Acid Monoacylglyceride in Lung Adenocarcinoma. *Recent Pat Anticancer Drug Discov* 2013; **8**:319–34.
- 24 Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 2008; **22**:659–61.
- 25 Morin C, Sirois M, Echave V, Albadine R, Rousseau E. 17,18-epoxyeicosatetraenoic acid targets PPARgamma and p38 mitogen-activated protein kinase to mediate its anti-inflammatory effects in the lung: role of soluble epoxide hydrolase. *Am J Respir Cell Mol Biol* 2010; **43**:564–75.
- 26 Gagne S, Crane S, Huang Z, Li CS, Bateman KP, Levesque JF. Rapid measurement of deuterium-labeled long-chain fatty acids in plasma by HPLC-ESI-MS. *J Lipid Res* 2007; **48**:252–9.
- 27 King GG, Paré PD, Seow CY. The mechanics of exaggerated airway narrowing in asthma: the role of smooth muscle. *Respir Physiol* 1999; **118**:1–13.
- 28 Arita M, Ohira T, Sun YP, Elangovan S, Chiang N, Serhan CN. Resolvin E1 selectively interacts with leukotriene B4 receptor BLT1 and ChemR23 to regulate inflammation. *J Immunol* 2007; **178**:3912–7.
- 29 Ohira T, Arita M, Omori K, Recchiuti A, Van Dyke TE, Serhan CN. Resolvin E1 receptor activation signals phosphorylation and phagocytosis. *J Biol Chem* 2010; **285**:3451–61.
- 30 Calder PC. Fatty acids and gene expression related to inflammation. *Nestle Nutr Workshop Ser Clin Perform Programme* 2002; **7**:19–36.
- 31 Bilal S, Haworth O, Wu L, Weylandt KH, Levy BD, Kang JX. Fat-1 transgenic mice with elevated omega-3 fatty acids are protected from allergic airway responses. *Biochim Biophys Acta* 2011; **1812**:1164–9.
- 32 Marcheselli VL, Hong S, Lukiw WJ et al. Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. *J Biol Chem* 2003; **278**:43807–17.
- 33 Kohli P, Levy BD. Resolvins and protectins: mediating solutions to inflammation. *Br J Pharmacol* 2009; **158**:960–71.
- 34 Dyerberg J, Madsen P, Møller JM, Aardestrup I, Schmidt EB. Bioavailability of marine n-3 fatty acid formulations. *Prostaglandins Leukot Essent Fatty Acids* 2010; **83**:137–41.
- 35 Cruz-Hernandez C, Thakkar SK, Moulin J et al. Benefits of structured and free monoacylglycerols to deliver eicosapentaenoic (EPA) in a model of lipid malabsorption. *Nutrients*. 2012; **4**:1781–93.
- 36 Siddiqui S, Hollins F, Saha S, Brightling CE. Inflammatory cell microlocalisation and airway dysfunction: cause and effect? *Eur Respir J* 2007; **30**:1043–56.
- 37 Kinsella JE, Lokesh B, Stone RA. Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *Am J Clin Nutr* 1990; **52**:1–28.
- 38 Kinsella JE, Dixit VM. Modulation of inflammasome pathways by bacterial and viral pathogens. *J Immunol* 2011; **187**:597–602.
- 39 Nathan C, Ding A. Nonresolving inflammation. *Cell* 2010; **140**:871–82.
- 40 Haworth O, Cernadas M, Yang R, Serhan CN, Levy BD. Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat Immunol* 2008; **9**:873–9.
- 41 El Kebir D, Gjorstrup P, Filep JG. Resolvin E1 promotes phagocytosis-induced neutrophil apoptosis and accelerates resolution of pulmonary inflammation. *Proc Natl Acad Sci U S A* 2012; **109**:14983–8.
- 42 Haworth O, Cernadas M, Levy BD. NK cells are effectors for resolvin E1 in the timely resolution of allergic airway inflammation. *J Immunol* 2011; **186**:6129–35.
- 43 Luangsang S, Wittamer V, Bondue B et al. Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. *J Immunol* 2009; **183**:6489–99.