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New Omega-3 Derivatives Reduce Airway Inflammation and Prevent Rho-Kinase Activation in an Allergic Model of Asthma

Caroline Morin[1,2], Samuel Fortin[1] and Éric Rousseau[2]*

[1]*SCF Pharma, Ste-Luce, Quebec, Canada*
[2]*Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada*

Abstract

Asthma is a chronic inflammatory airways disease characterized by early and late asthmatic reactions that are associated with infiltration and activation of inflammatory cells in the airways. Bronchial hyperresponsiveness to a variety of stimuli is part of the symptomology which include neurotransmitters and inflammatory mediators. Omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are known to reduce inflammation in the lung. In the present study, we synthesized new omega-3 polyunsaturated fatty acid (n3-PUFA) derivatives, namely DHA monoglyceride (MAG-DHA) and EPA monoglyceride (MAG-EPA), and investigated their effects on lung inflammation and RhoA activation in an *in vivo* guinea pig model of allergic asthma. Histological analyses and leukocyte count in bronchoalveolar lavages revealed that oral MAG-DHA and MAG-EPA treatments led to less inflammatory cell recruitment in the lung of ovalbumine (OVA)-sensitized guinea pigs when compared to lung from control animals. Results also revealed a reduction in mucus production in OVA-sensitized animals treated with either MAG-DHA or MAG-EPA. Moreover, *per os* n-3 PUFA administrations prevented NFκB activation and COX2 over-expression in OVA-sensitized animals. Mechanical tension measurements revealed that oral MAG-DHA and MAG-EPA treatments prevented U-46619-induced bronchial hyperresponsiveness in OVA-sensitized guinea pigs. MAG-DHA and MAG-EPA also prevented U-46619-induced Ca^{2+} hypersensitivity of bronchial smooth muscle through a decrease in RhoA activation. Together, these findings provide key evidences regarding the mode of action of MAG-DHA and MAG-EPA in the lung and point to new potential therapeutic strategies for modulating inflammation and airway hyperresponsiveness in asthma.

Keywords: Airway hyperresponsiveness; Asthma; DHA; EPA; Inflammation; RhoA

Introduction

Asthma is a chronic disease characterized by airways hyperresponsiveness, inflammation, remodelling of the media and reversible bronchial obstruction. Airway structural cells, recruited inflammatory cells as well as numerous mediators such as cytokines, chemokines and adhesion molecules are involved in the pathogenesis of asthma [1,2]. Despite the availability of several classes of asthma medications such as beta-agonists, leukotriene antagonists and corticosteroids, nearly 50% of asthmatics fail to benefit from one or more of these drugs. Moreover, none of the treatments are preventive or curative such that the disease has reached epidemic proportions worldwide and its incidence continuously increasing, especially in developing countries [3].

Airway hyperresponsiveness (AHR) considered as the hallmark of the asthma phenotype, is defined as the ease with which the airways narrow in response to a bronchoconstrictive challenge and manifests as a combination of increased sensitivity and reactivity for a given stimulus [4]. Several mechanisms have been suggested to explain AHR, such as alterations in neural control of airway smooth muscles (ASM) [4], inflammatory process resulting in the release of cytokines and lipid mediators [3] and abnormal calcium handling by ASM cells [5,6]. The contraction of smooth muscles including airway smooth muscles is mediated by both Ca^{2+}-dependent and Ca^{2+}-independent pathways. The latter independent pathway, termed Ca^{2+} sensitization, is mainly regulated by a small monomeric GTP-binding protein, RhoA, and its downstream target Rho-kinase. Inactive RhoA exists in the cytosol with its partner molecule, GDP dissociation inhibitor (RhoGDI). G protein-coupled receptors, upon binding their respective ligands, activate the heterotrimeric $G_{12/13}$ protein [7], which in turn triggers one or more tyrosine kinases and other signaling molecules, culminating in the activation of a Rho-specific guanine nucleotide exchange factor (RhoGEF). These signaling molecules displace RhoGDI and stimulate the exchange of GDP for GTP. Once activated, RhoA translocates to the membrane and activates Rho associated kinase (ROCK), which in turn targets myosin light chain phosphatase (MLCP); phosphorylation of the constitutively active MLCP at its myosin binding region leads to its inhibition, favoring a larger quantity of phosphorylated myosin at lower levels of $[Ca^{2+}]i$, a phenomenon termed Ca^{2+} sensitization. Following deactivation of the receptor, RhoA inactivates by hydrolyzing GTP and reassociates with RhoGDI [7]. In animal models of allergic bronchial asthma, an augmented agonist-induced and RhoA-mediated contraction of bronchial smooth muscle has been demonstrated [8]. Moreover, the specific Rho-kinase inhibitor (Y-27632) has been shown to suppress AHR in mice repeatedly challenged with ovalbumin sensitization [9]. Hence, changes in contraction mediated by the Rho/Rho-kinase pathway upon allergen sensitization could contribute to the development of AHR under these conditions.

Clinical assessment of dietary supplementation of omega-3 (n-3) polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid

***Corresponding author:** Éric Rousseau, PhD, Department of Physiology and Biophysics, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12e Avenue Nord, Sherbrooke, J1H 5N4, QC, Canada, Tel: (819) 564-5306; Fax: (819) 564-5399; E-mail: Eric.Rousseau@USherbrooke.ca

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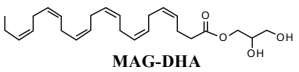
(EPA) and docosahexaenoic acid (DHA), indicates their beneficial impact in a wide range of human diseases in which unresolved inflammation is suspected to be a key component in disease pathogenesis [10,11]. Several putative mechanisms have been proposed to explain the benefits of n-3 PUFAs: (i) via substrate competition, preventing conversion of arachidonic acid into pro-inflammatory eicosanoids such as prostaglandins (PGs) and leukotrienes (LTs); (ii) serving as an alternative substrate to produce less potent 5-series LTs and 3-series PGs and thromboxanes [12-14]. Systematic metabolomic studies of lipid mediators in the course of acute inflammatory responses have revealed that n-3 PUFA-derived mediators are produced within inflammatory exudates, including E-series resolvins such as resolvin E1 from EPA and D-series resolvins and protectin D1 from DHA [15-17]. These newly identified chemical mediators appear to exert potent anti-inflammatory and pro-resolving actions both *in vitro* and *in vivo* [18].

Asthma is directly associated with an increase levels in omega-6 (n-6) fatty acid levels, such as arachidonic acid, in blood circulation and tissues [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 [10,16,19-21]. Thus, new EPA and DHA sn1-monoacylglycerides were synthesized. These compounds are non-toxic and their metabolites are found in blood circulation and lung tissues [22-24]. The aim of the present study was to determine the ability of MAG-DHA and MAG-EPA to reduce inflammation and AHR using an *in vivo* model of allergic asthma. Herein we report the first evidence that MAG-DHA and MAG-EPA display anti-inflammatory properties and prevent RhoA activation leading to AHR in lung tissues.

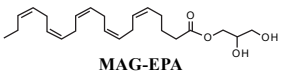
Materials and Methods

Synthesis of omega-3 monoglyceride

MAG-EPA and MAG-DHA were synthesized as previously described using either ethyl docosahexaenoate or ethyl eicosapentaenoate as starting material [22].



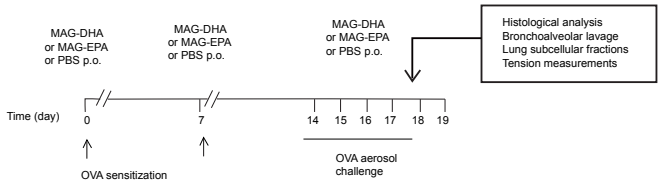
MAG-DHA



MAG-EPA

Sensitization and challenge protocols

Male or female albino guinea pigs, (Hartley 200-250 g) were obtained from Charles Rivers 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 µg ovalbumin (OVA) (Grade V, Sigma Chemical, St Louis, MO) plus 1 mg of $Al(OH)_3$ as an adjuvant in 0.4 ml PBS, on day 0 and 7. Controls animals received PBS by i.p. injection. MAG-DHA or MAG-EPA were administrated *per os* (231 mg/kg, according to Health Canada Draft Guidelines) [25] on day 0 and 7, 30 minutes before OVA sensitization. On days 14 to 18, the OVA-sensitized guinea pigs received MAG-DHA or MAG-EPA 30 minutes before an aerosol challenge containing 1.5% OVA for 10 min or until suffocation. Control animals were challenged with aerosolized PBS. The aerosols were generated with a small particle aerosol generator model SPAG-2 (ICN, Montreal, QC, Canada). On day 19, 24 hours after the last aerosol challenge, the OVA-sensitized guinea pigs, either untreated or treated with either MAG-DHA or MAG-EPA, were anesthetized, the bronchi removed and used immediately for isometric tension measurements while lung tissues were harvested for histological analysis or homogenate preparation for Western Blot experiments. Bilateral bronchoalveolar lavages (BAL) (2 aliquots of 2 ml of PBS plus 0.6 mM EDTA) were performed on control animals and on OVA-sensitized guinea pigs untreated or treated with either MAG-DHA or MAG-EPA (231 mg/kg). No BAL or histological analysis was performed on animals undergoing tension measurement or lung homogenate preparations. Moreover, no behavioral or physical signs of toxicity with MAG-DHA and MAG-EPA treatments were observed. The illustration below summarizes the sensitization protocol as a function of time:



Allergen-initiated lung inflammation

Guinea pig lungs were fixed in 10 % buffered formalin and paraffin embedded after which thin sections (3µm) were stained with hematoxylin-eosin and Alcian blue according to standard protocols. Measurement of inflammatory mediator was determined in cell-free BAL fluid (2000g, 10 min) by sensitive and specific ELISA, for TNFα (Cusabio Biotech, Newark, DE). Cells in BAL fluids were resuspended in PBS, enumerated by hemocytometer, and concentrated onto microscope slides by cytocentrifugation at 265 *g*. Cells were stained with a Wright-Giemsa stain (Sigma-Aldrich) to determine leukocyte differential counts [23].

Western blot analysis

Lung homogenates were prepared from control as well as OVA-sensitized animals untreated or treated with either MAG-DHA or 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 µM 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-polyacrylamide gels, and the proteins subsequently transferred by electroblotting onto nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada). Western blots were performed using specific antibodies against COX2, IκBα, p65-NFκB, its phosphorylated form (Anti P-NFκB) and β-actin proteins [23]. Membranes were incubated for 1h at 23°C with peroxidase-conjugated donkey anti-rabbit IgG1 antiserum (Amersham, QC, Can) and revealed by Enhanced Chemiluminescence (Roche, QC, Can). Blot immuno-stainings were digitized and analyzed with Lab-Image software 2.7-2.

Isometric tension measurements

The mechanical effects induced by U46619 (a thromboxane A2 analog) were measured as previously described [23,26]. Briefly, bronchial rings were mounted in isolated organ baths (Radnoti Glass Tech., Monrovia, CA), containing 6 ml of Krebs solution at 37°C, bubbled continuously with a 95% O_2/5% CO_2 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) for facilitating data acquisition and analysis. The measurement of resulting induced myofilament Ca^{2+} sensitivity was performed as previously reported

by using a β-escin permeabilization protocol on guinea pig bronchi [23,26].

Measurement of RhoA activity

RhoA activity was assessed in homogenized lung tissue samples by pulldown assays using the Rho-binding domain of the Rho effector: Rhotekin, according to the manufacturer's instructions (RhoA activation assay kit, Cell Biolabs, Cedarlane laboratories, Burlington, ON, Canada) [27].

Data analysis and statistics

Results are expressed as means ± S.E.M. with n indicating the number of experiments. Statistical analyses were performed using a Student t test or a one-way analysis of variance (ANOVA). Differences were considered statistically significant when $p<0.05$. Data curve fittings were performed using Sigma Plot 11 (SPSS-Science, Chicago, IL) to determine EC_{50} values [23].

Results

Effect of oral MAG-DHA and MAG-EPA administration on allergen-induced lung inflammation

An *in vivo* model of allergic asthma was used to determine the impact of MAG-DHA and MAG-EPA on lung inflammation and AHR in OVA-sensitized guinea pigs. The n-3 PUFA monoacylglycerides

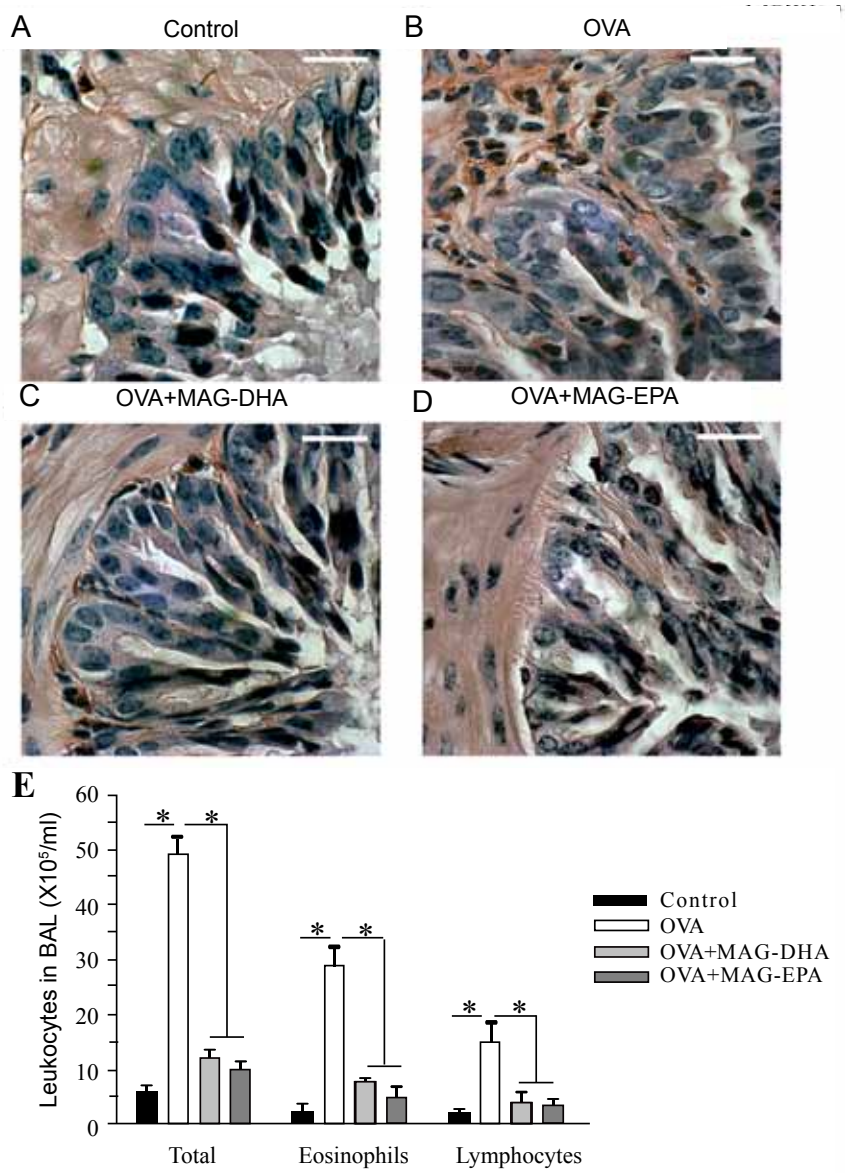


Figure 1: MAG-DHA and MAG-EPA reduce airway inflammation in ovalbumin (OVA)-sensitized lung tissue.
A-D: Representative lung tissue sections obtained from a non-sensitized guinea pig (control) (**A**) as well as from OVA-sensitized (**B**), OVA-sensitized + MAG-DHA-treated (**C**) and OVA-sensitized + MAG-EPA-treated animals (**D**). The degree of inflammation was assessed by determining the extent of inflammatory infiltrates present in lung sections stained with Hematoxylin-Eosin. Scale bar 40 µm. Images are representative of n = 8, 10, 11 and 8 (animals, experiments) per group, respectively. **E**: Total leukocytes, eosinophils (Eos.) and lymphocytes (Lymph.) in BAL fluid were enumerated and identified after Wright-Giemsa staining. Results are expressed as mean ± SEM, (n = 7 per group), * P < 0.05.

were administrated *per os* at pharmacological dose (231 mg/kg) [25]. The extent of inflammation in lung tissues was determined in control and OVA-sensitized guinea pigs treated or not with MAG-DHA and MAG-EPA. Histological analyses performed on lung sections stained with hematoxylin-eosin revealed that animals receiving either MAG-DHA or MAG-EPA had substantially less eosinophils and lymphocytes in the peribronchial regions and airspaces compared to OVA-sensitized animals (Figure 1 A-D). In BAL fluids, MAG-DHA and MAG-EPA treatments led to fewer total leukocytes, namely eosinophils and lymphocytes, when compared to the number of leucocytes found in BAL fluids of untreated OVA-sensitized guinea pigs (Figure 1E).

Effect of oral MAG-DHA and MAG-EPA treatments on mucus production

To determine the effects of *per os* n-3 PUFA monoglyceride treatments on airway mucus production, Alcian blue staining was performed on lung tissue sections derived from control and OVA-sensitized guinea pigs treated or not with either MAG-DHA or MAG-EPA. Histological analyses revealed that MAG-DHA and MAG-EPA reduced goblet cell hyperplasia and airway mucus production in guinea pig lung when compared to OVA-sensitized animals (Figure 2 A-D). Quantitative analysis of lung stained tissue sections derived from OVA-sensitized animals revealed a 2.2 fold increase when compared to the levels obtained in sections derived from non-sensitized control guinea pigs (Figure 2E). Image analyses also revealed that in lung tissue sections derived from OVA-sensitized animals treated with either MAG-DHA or MAG-EPA, significant reductions of 53 and 57 % were observed when compared to the levels obtained in sections derived from OVA-sensitized guinea pigs (Figure 2E). Moreover, no significant difference was quantified between n-3 PUFA-treated and control animals (Figure 2E, grey versus dark bar columns).

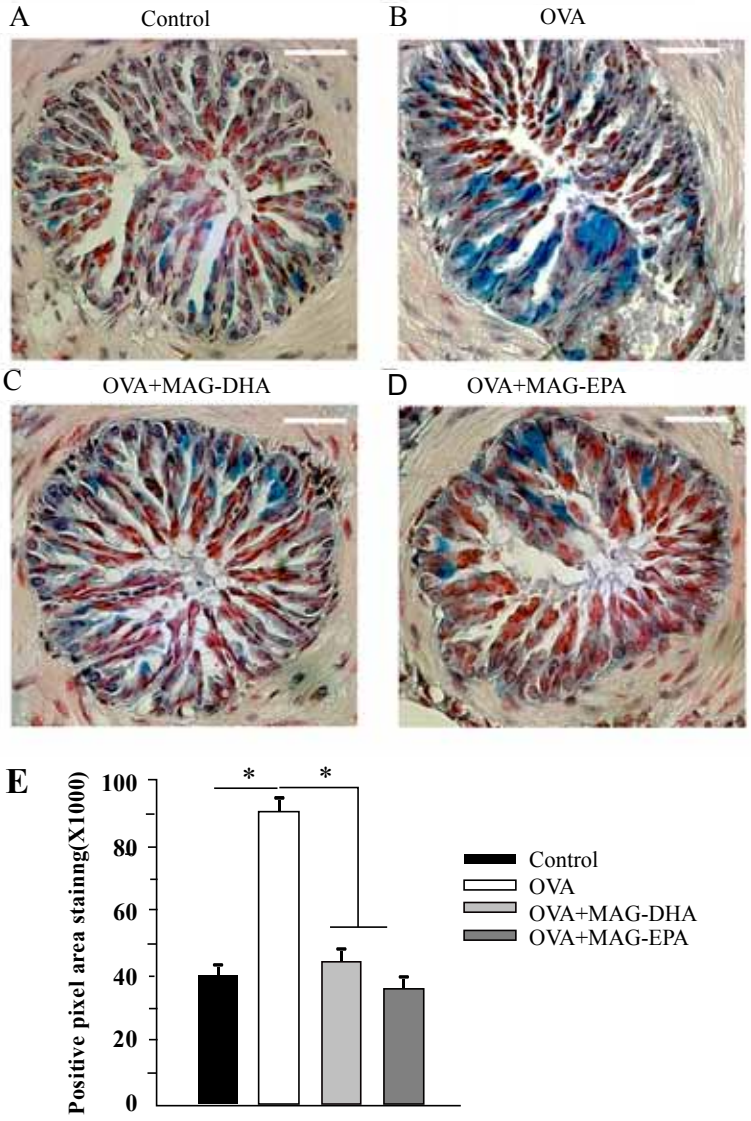


Figure 2: MAG-DHA and MAG-EPA reduce mucus production in OVA-sensitized lung tissue. Lung tissue sections obtained from a non-sensitized guinea pig Control (**A**), as well as from OVA-sensitized (**B**), OVA-sensitized + MAG-DHA-treated (**C**) and OVA-sensitized + MAG-EPA-treated (**D**) animals were stained with Alcian blue. Mucus secretion and goblet cells containing mucus are stained in blue. Scale bar 40 µm. Images are representative of n = 8, 10, 11 and 8 (animals, experiments) per group, respectively (**E**): Quantitative analysis displaying the mean immuno-positive pixel area staining of Alcian blue from lung tissue sections (n = 8-10-11-8 per group, * $P < 0.05$).

Effect of MAG-DHA and MAG-EPA treatments on NFκB pathway

To determine whether n-3 PUFA monoglycerides reduce allergen-induced airway inflammation, the activation of nuclear factor kappa B (NFκB) was investigated. Activation of this factor is usually correlated with phosphorylation of the p65 NFκB subunit as well as a reduction in IκBα due to extensive ubiquitination and proteosomal degradation of this inhibitory subunit, which results in an increased nuclear translocation of the p65 NFκB subunit [28]. Western blot analysis revealed that OVA-sensitized animals resulted in IκBα degradation and an increased phosphorylation of p65 subunit staining in lung homogenate fractions when compared to preparations derived from control guinea pigs (Figure 3A). However, MAG-DHA and MAG-EPA treatments prevented the degradation of IκBα and concomitant phosphorylation of p65 NFκB subunit induced by the allergen when compared to OVA-sensitized guinea pigs. Since increased COX2 activity and expression is related to an inflammatory status in several tissues and that OVA sensitization is able to increase COX2 expression in guinea pig lung [23,29], experiments were therefore designed to evaluate the expression level of COX2 in control and OVA-sensitized guinea pigs treated or not with either MAG-DHA or MAG-EPA. OVA sensitization induced an increased staining level of COX2 protein detection when compared to levels obtained from control animals, whereas MAG-DHA and MAG-EPA prevented the over-expression of COX2 protein induced by OVA in guinea pig lung (Figure 3B).

Effect of n-3 PUFA monoglyceride treatments on OVA-induced AHR

The involvement of the Rho-kinase pathway was assessed using U-46619, a thromboxane A2 analog known to activate the Rho-kinase pathway and to induce airway smooth muscle tone. In order to determine the impact of MAG-DHA and MAG-EPA treatments on U-46619-induced tension in OVA-sensitized guinea pigs, experiments were designed to assess the mechanical properties of bronchial smooth muscle to cumulative concentrations of this pharmacological agonist. Figure 4 displays the cumulative concentration response curves (CCRC) to U-46619 in bronchial preparations derived from control, OVA-sensitized, OVA-sensitized + MAG-DHA-treated, as well as from OVA-sensitized + MAG-EPA-treated animals. Data revealed an over-reactivity of the bronchi from OVA-sensitized guinea pigs to U-46619 with an EC_{50} value of 20 nM, whereas oral MAG-DHA and MAG-EPA administrations prevented the development of AHR to this agonist, with EC_{50} values of 54 and 49 nM, respectively (Figure 4). No difference was quantified between n-3 PUFA-treated animals and control guinea pigs receiving PBS only.

Effects of n-3 PUFA monoglycerides on U-46619 induced Ca^{2+} hypersensitivity and RhoA activation in an OVA-sensitized model of asthma

To assess the effect of MAG-DHA and MAG-EPA treatments on U-46619 induced Ca^{2+} sensitivity, comparative analyses were performed on β-escin-permeabilized guinea pig bronchial rings. Figure

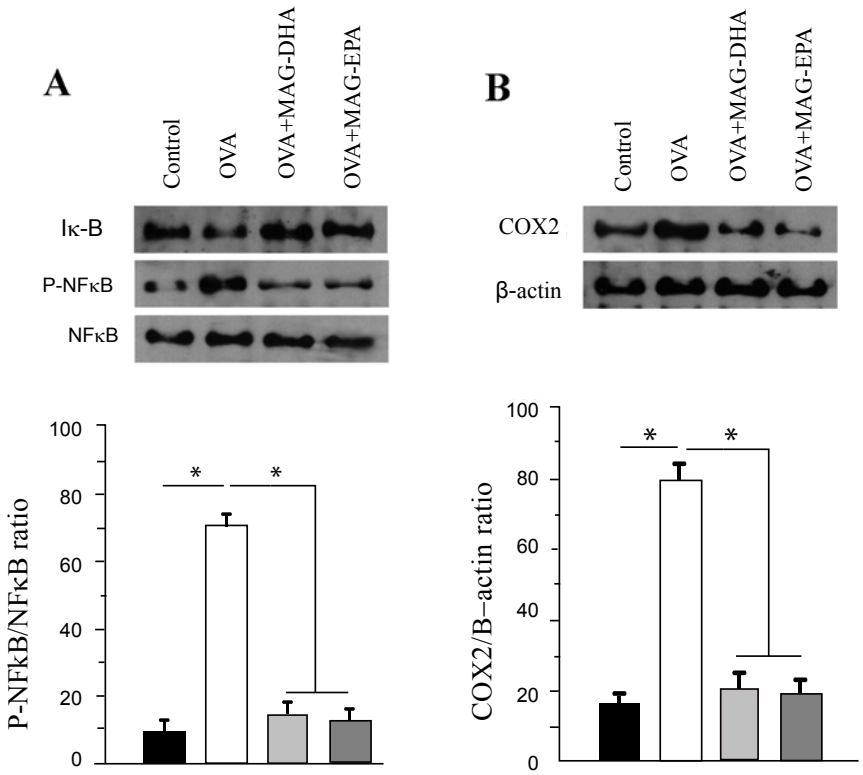


Figure 3: Effect of MAG-DHA and MAG-EPA on airway inflammatory markers in lung tissue. A: Western blot and quantitative analysis of lung homogenate fractions derived from Control, OVA, OVA + MAG-DHA and OVA + MAG-EPA, using specific antibodies against IκBα, p65 NFκB and its phosphorylated form (n = 6). **B.** Western blot and quantitative analysis of COX2 protein detection. Staining densities in the homogenates were expressed as a function of β-actin signals (n=6). * P < 0.05.

5A illustrates CCRC to free Ca^{2+} concentrations in the presence of 20 nM U-46619 on permeabilized bronchial rings obtained from control and OVA-sensitized tissues. When compared to control conditions (closed circles), an enhanced Ca^{2+} sensitivity to pre-calibrated Ca^{2+} step increases in the presence of U-46619 was observed in OVA-sensitized bronchial tissues (Figure 5A, open triangles). However, MAG-DHA and MAG-EPA treatments resulted in a marked inhibitory effect on Ca^{2+}-sensitivity to U-46619 (right shift) developed by OVA-sensitized bronchi. Data analysis demonstrated that MAG-DHA and MAG-EPA treatments induced a shift in EC_{50} values (1.54 ± 0.06 µM and 1.49 ± 0.05 µM, respectively) toward higher Ca^{2+} concentrations when compared to OVA-sensitized untreated tissues challenged with U-46619 (0.38 ± 0.05 µM) (Figure 5A). However, the difference in Ca^{2+} sensitivity between control bronchi (EC_{50} values of 1.54 ± 0.05 µM) and tissues treated with either MAG-DHA or MAG-EPA was not significant (Figure 5A). In order to further investigate the putative processes that would support this negative feedback mechanism induced by n-3 PUFA monoglycerides on the Ca^{2+}-tension relationship induced by U-46619, experiments were performed to assess the activity of RhoA in lung homogenates derived from control and the 3 series of OVA-sensitized animals. RhoA activation assay was performed in homogenized tissue samples by pulldown assays using Rhotekin, the Rho-binding domain of the Rho effector. Data analysis revealed that oral MAG-DHA and MAG-EPA treatments prevented the activation of RhoA, as demonstrated by a lower amount of RhoA coupled to GTP (Figure 5B), thus correlating with the functional measurements described above (Figure 4 and 5A).

Discussion

Anti-inflammatory effects of n-3 PUFA monoacylglyceride derivatives

Asthma prevalence continues to increase in North America despite advancements in treatment options for this pathology [1,3]. Alternative preventive treatment aimed at reducing the dose requirements of pharmacological interventions would henceforth be beneficial, in addition to potentially reducing the public health and socioeconomic burden of this disease [1]. Over the past three decades, there has been significant interest in the therapeutic potential of fish oils rich in *n*-3 PUFA, such as DHA and EPA, for various inflammatory conditions such as asthma, rheumatoid arthritis and inflammatory bowel diseases [11,21,30]. Of interest, DHA levels in the respiratory tract are decreased in asthma as well as in other excess airway inflammation diseases, such as cystic fibrosis [19]. Epidemiological studies suggest that a diet enriched in marine fatty acids (fish oil) may have beneficial

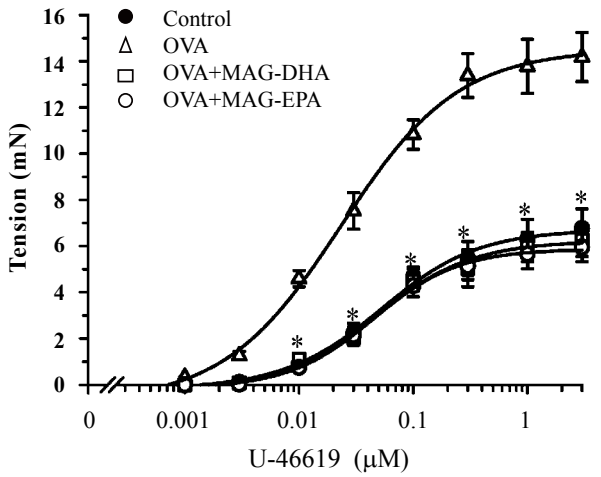


Figure 4: Effect of MAG-DHA and MAG-EPA treatments on airway hyper-responsiveness induced by U-46619 in ovalbumin-sensitized guinea pigs. Cumulative concentration response curves (CCRC) to U-46619 generated in Control (closed circles), OVA sensitized (open triangles), OVA-sensitized + MAG-DHA-treated (open squares) and OVA-sensitized + MAG-EPA-treated (open circles) guinea pigs. Each point represents the mean ± SEM, with n =15 for each experimental condition. * P < 0.05.

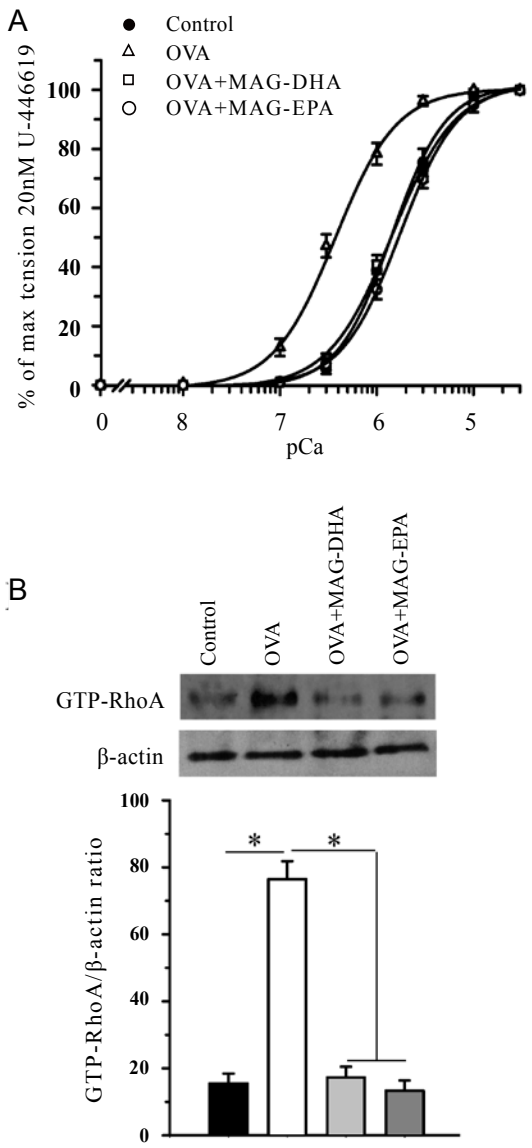


Figure 5: Effect of MAG-DHA and MAG-EPA on U46619-induced Ca^{2+} hypersensitivity and RhoA activation in OVA sensitized guinea pigs. A: CCRC to free [Ca^{2+}] in the presence of 20 nM U-46619 obtained from β-escin permeabilized tissues in Control (closed circle, n = 15), OVA (open triangles, n = 20), OVA + MAG-DHA (open squares, n = 18) and OVA + MAG-EPA (open circles, n = 16). Each point represents the mean ± SEM, * P < 0.05. **B:** Rho activation assay was performed on bronchi homogenates derived from Control (untreated), OVA-sensitized, OVA-sensitized + MAG-DHA-treated and OVA-sensitized + MAG-EPA-treated guinea pigs. Mean bar histogram displaying the quantitative analysis of various GTP-RhoA density ratios. The β-actin staining was obtained from the original homogenates derived from the corresponding preparations, n = 5, * p < 0.05.

effects on inflammatory conditions including asthma [31], and that dietary supplementation with omega-3 fatty acids in children prevents the development of atopic cough, a symptom of allergic airway inflammation [20]. The underlying mechanisms for the beneficial properties of omega-3 fatty acids in asthma remain, however, to be established.

In the present study, we investigated the ability of MAG-DHA and MAG-EPA, newly-synthesized n-3 PUFA monoacylglyceride derivatives, to prevent inflammation and AHR in an *in vivo* model of allergic asthma. Eosinophils, T-cells and mast cell infiltrates with excess mucus secretion are common features of allergic airway inflammation and have been clinically correlated with AHR [32,33]. Herein, a reduced number of eosinophils and lymphocytes were quantified in BAL fluid recovered from OVA-sensitized guinea pigs receiving a daily *per os* dose of MAG-DHA or MAG-EPA. Moreover, airway mucus production was lowered in n-3 PUFA monoacylglyceride-treated animals.

In asthma, there is increased expression of multiple inflammatory proteins in the respiratory tract, including cytokines, adhesion molecules, inflammatory enzymes and receptors. The majority of these inflammatory proteins are regulated at the level of gene transcription [29]. NFκB is the main transcription factor involved in up-regulation of inflammatory cytokines, COX2 genes and adhesion molecules [34]. Since airway macrophages and bronchial epithelial cells from asthmatics exhibit increased NFκB activity compared with cells from healthy individuals [34], it has been suggested that NFκB plays a pivotal role in the pathogenesis of asthma [34]. In the present study, we demonstrate that the activation of NFκB and the over-expression of COX2 inflammatory enzyme are reduced in animals receiving *per os* MAG-DHA or MAG-EPA treatments. Moreover, in a previous study, we demonstrated that MAG-DHA mediates its anti-inflammatory effects through the activation of the nuclear receptor PPARγ resulting in a decrease in NFκB activation and in related pro-inflammatory gene expression such as cytokines and COX2 in an *in vitro* model of TNFα-stimulated human bronchi [23].

Potential usefulness of n-3 PUFA in regulating RhoA-Rho-kinase pathway in asthma

Agonist-induced changes in the Ca^{2+} sensitivity of the contractile apparatus also contribute to pharmaco-mechanical contraction coupling in ASM cells. Evidence has been put forward showing the importance of both PKC and ROCK in this signaling event [7]. Data also suggest that RhoA, a monomeric GTP-binding protein, is a key protein in controlling the tonic response of airway smooth muscles [35]. To explain the profound change seen in AHR which is a hallmark of asthma, there is growing evidence is accumulating for increased activation of the Rho/ROCK signaling pathway following allergen challenge [35]. In a rat model of acute allergen exposure [8], described an increase in acetylcholine-induced Ca^{2+}-independent contractions in rat bronchial smooth muscle which was abolished by treatment with clostridium botulinum C3 exoenzyme (an inhibitor of Rho proteins). Furthermore, molecular biological investigations revealed an upregulation of RhoA and $G_{12,13}$ proteins [36,37], as well as an increase in acetylcholine-induced activation of RhoA [8]. Tumor necrosis factor alpha (a proinflammatory mediator) has been associated with the induction of AHR [23,38,39] and may be responsible for these changes, since it was demonstrated that TNFα enhances the expression and activation of RhoA in ASM [40,41]. Other molecules including IL-13 and IL-4 have been linked to AHR [42]. These evidences suggest that agents able to decrease RhoA activation, could be useful and of clinical interest for prevention and treatment of airway hyperresponsiveness. The usefulness of such agents is supported by the present findings in which newly synthesized n-3 PUFA monoacylglycerides prevented U46619-induced Ca^{2+} hypersensitivity in OVA-sensitized guinea pigs through the inhibition of RhoA activation. The PKC / CPI-17 pathway, another Ca^{2+} sensitizing mechanism, has also been demonstrated to be involved under pathophysiological conditions. Several studies show that CPI-17 activation plays a critical role in bronchial reactivity [43-46]. In a previous study, we demonstrated that pro-inflammatory cytokine treatments were able to activate the PKC / CPI-17 pathway leading to an enhanced Ca^{2+} signaling in human bronchi [43]. Moreover, increase expression and activation of CPI-17 were also observed in lung biopsies derived from asthmatic patients [23]. In addition, we also previously demonstrated in a previous study that oral MAG-DHA administration decreased both activation and expression of CPI-17 leading to decreased Ca^{2+}-sensitivity and AHR in a guinea pig OVA-sensitized model of asthma [23]. Hence it is proposed that, in human lung with pathological conditions, the use of MAG-DHA and MAG-EPA would likely decrease Ca^{2+} hypersensitivity and would be relevant in improving respiratory capacities and recovered pulmonary compliance in asthmatic patients.

Enhanced role of n-3 PUFA in pulmonary pathophysiology

Clinical assessment of dietary supplementation of omega-3 (n-3) polyunsaturated fatty acids (PUFA), including EPA and DHA, has shown their beneficial impact in a wide range of inflammatory diseases [21]. The mode of action of EPA and DHA derivatives through RhoA/Rho-kinase inhibition/inactivation would support these independent observations. An alternative explanation for these beneficial effects would be that n-3 PUFA competes with arachidonic acid (AA) for enzymatic conversion by COX, LOX and CYP450 enzymes [11]. This competition, in turn would lead to reduced formation of AA metabolites while PUFA-metabolites originating from DHA and EPA are increased, thus modifying the circulating and intracellular omega-3 / omega-6 ratio. Recent studies have identified a novel group of DHA-derived trihydroxydocosahexanoic acid mediators termed D-series Resolvins, formed from DHA by a series of reactions involving the 5-lipoxygenase pathway. These mediators appear to exert potent anti-inflammatory actions [16]. Intracellular DHA is metabolized through a series of biochemical steps, several of which involve 5 and 15-lipoxygenases, generating a dihydroxydocosatriene termed ProtectinD1, also a potent anti-inflammatory molecule [47]. Indeed, generation of Resolvins and Protectins could represent important anti-inflammatory compounds in the mode of action of n-3 PUFAs in addition to having a potent protective role against inflammatory disorders [48-52]. Moreover, our group has already demonstrated that MAG-DHA is metabolized by lipoxygenases in order to mediate its anti-inflammatory effects and to reduce AHR triggered by TNFα in human bronchi, as shown by the combined treatments of 5- and 15-lypoxygenases inhibitors [23]. In addition, we demonstrated that ResolvinD1 and ProtectinD1 prevent AHR and Ca^{2+} hypersensitivity primarily through their action in reducing COX2 and CPI-17 over-expression. Moreover, in a murine model of chronic asthma, ProtectinD1 administration prior to aeroallergen challenge was shown to result in a decrease in airway eosinophil and T lymphocyte recruitment as well as in reduced levels of specific pro-inflammatory mediators, including IL-13, cysteinyl leukotrienes and PGD2 [50]. EPA-derived ResolvinE1 also displays anti-inflammatory effects when administrated in a murine model of allergic asthma [49]. Further investigations using high performance liquid chromatography

coupled to tandem mass spectrometry (HPLC/MS/MS) could enable to determine which metabolite would be potentially responsible for the intracellular effects of MAG-DHA and MAG-EPA in lung or other tissues.

The main limitation of metabolites such as ResolvinE1, ResolvinD1 and ProtectinD1, however, is their relative instability, thus further justifying the use of biochemical precursors or stable analogues. We therefore propose that n-3 PUFA monoacylglyceride derivatives, possessing anti-inflammatory properties, may resolve inflammation and provide an interesting new approach for asthma. Fatty acids in monoglyceride form are generally recognized as safe and are widely used as emulsifying agents in the food industry. Furthermore, pharmacokinetic experiments, performed on rats treated with an oral dose of either DHA monoglyceride (MAG-DHA), DHA triglycerides (DHA-TG) or DHA ethyl ester (DHA-EE), demonstrate that MAG-DHA increases the oral bioavailability of DHA compared to DHA-TG and DHA-EE [22]. Moreover, these n-3 PUFA monoacylglycerides prevent U-46619-induced bronchoconstriction through a decrease in RhoA activation thus leading to a reduction in Ca^{2+} sensitivity of smooth muscle cells. Since the activation of RhoA/Rho kinase pathway is associated with both acute pulmonary bronchoconstriction and long term airway remodeling, finding a lipid mediator able to reduce the activation of Rho-kinase is clearly of potential clinical interest. Moreover, animal and clinical studies have demonstrated that Rho-kinase inhibitors could inhibit signal transductions initiated by many broncho-active drugs [9,35]; hence it is possible that MAG-DHA and MAG-EPA may exert broader beneficial effects as compared to single receptor antagonists, largely due to its mode of action on intracellular targets. In this respect, it is proposed that n-3 PUFA monoacylglyceride could lead to the production of bioactive metabolites [23] which represent new and prospective pharmacological compounds of low toxicity and medicinal interest in modulating inflammation and bronchoconstriction in asthma. Hence, the down regulation of RhoA/Rho-kinase pathway by these compounds might explain other systemic effects induced by n-3 PUFA derivatives [23, 24, 27].

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