

Docosahexaenoic Acid Derivative Prevents Inflammation and Hyperreactivity in Lung

Implication of PKC-Potentiated Inhibitory Protein for Heterotrimeric Myosin Light Chain Phosphatase of 17 kD in Asthma

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The effects of a newly synthesized docosahexaenoic acid (DHA) derivative, CRBM-0244, on lung inflammation and airway hyperresponsiveness were determined in an *in vitro* model of TNF- α -stimulated human bronchi and in an *in vivo* model of allergic asthma. Mechanical tension measurements revealed that CRBM-0244 prevented bronchial hyperresponsiveness in TNF- α -pretreated human bronchi. Moreover, treatment with CRBM-0244 resulted in a decrease in NF- κ B activation and cyclooxygenase-2 (COX-2) overexpression triggered by TNF- α . The inhibition of peroxisome proliferator-activated receptor- γ with GW9662 abolished the CRBM-0244-mediated anti-inflammatory effects. CRBM-0244 reduced the Ca²⁺ sensitivity of bronchial smooth muscle through a decrease in the phosphorylation and expression of the PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI-17). Results also revealed an overexpression of CPI-17 protein in lung biopsies derived from patients with asthma. Furthermore, the presence of specialized enzymes such as 5-lipoxygenase and 15-lipoxygenase in the lung may convert CRBM-0244 into active mediators, leading to the resolution of inflammation. The *in vivo* anti-inflammatory properties of CRBM-0244 were also investigated in a guinea pig model of allergic asthma. After oral administration of CRBM-0244, airway leukocyte recruitment, airway mucus, ovalbumin-specific IgE, and proinflammatory markers such as TNF- α and COX-2 were markedly reduced. Hence, CRBM-0244 treatment prevents airway hyperresponsiveness, Ca²⁺ hypersensitivity, and the overexpression of CPI-17 in lung tissue. Together, these findings provide key evidence regarding the mode of action of CRBM-0244 in the lung, and point to new therapeutic strategies for modulating inflammation in patients with asthma.

Keywords: DHA; asthma; inflammation; airway hyperresponsiveness; CPI-17

Asthma is a chronic disorder causing inflammation and reversible airway obstruction. It affects approximately 300 million individuals worldwide. The incidence of asthma has nearly doubled in the past three decades, resulting in higher rates of morbidity, mortality, and healthcare costs (1). Despite the availability of several classes of asthma medications such as β -agonists, leuko-

triene modifiers, and corticosteroids, up to 50% of patients with asthma do not benefit from one or more of these drugs. Asthma is characterized by the airway infiltration of eosinophils, T cells, and mast cells, with an excessive secretion of mucus and, in some cases, airway remodeling with changes in smooth muscle tissue, which together contribute to the clinical hallmark of airflow obstruction (2, 3). Airway hyperresponsiveness (AHR), considered the hallmark of the asthma phenotype, is defined as the ease with which the airways narrow in response to a bronchoconstrictive challenge. AHR manifests as a combination of increased sensitivity and reactivity to a given stimulus (3). Several mechanisms were suggested to explain AHR, such as alterations in the neural control of airway smooth muscle (ASM) (4), inflammatory processes resulting in the release of cytokines and lipid mediators (1), and abnormal calcium handling by ASM cells (5, 6). An eosinophilic or neutrophilic infiltrate is a common feature of allergic airway inflammation, and was correlated clinically with AHR (7). Understanding the mechanisms underlying this response is thus of great importance.

Inflammation underlies and accompanies many prevalent diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease, and cancer (8). Clinical assessments of dietary supplementations with omega-3 (n-3) polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), indicate their beneficial impact on a wide range of human diseases in which unresolved inflammation is suspected as a key component in pathogenesis (9). Several mechanisms to explain the benefits of omega-3 PUFAs are widely held, including (1) substrate competition, preventing the conversion of arachidonic acid into proinflammatory eicosanoids such as prostaglandin (PG) and leukotriene (LT); and (2) actions as an alternative substrate to produce less potent 5-series LTs and 3-series PGs and thromboxanes (10, 11). Systematic metabolomic studies of lipid mediators in the course of acute inflammatory responses revealed that omega-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 (12, 13). These newly identified chemical mediators appear to exert potent anti-inflammatory and pro-resolving actions both *in vitro* and *in vivo* (14).

Our laboratory recently developed an *in vitro* model of bronchial hyperresponsiveness induced by TNF- α , to study the effects of cytochrome P450 epoxygenase arachidonic acid metabolites (15, 16). The present study sought to determine the effects of CRBM-0244, a new DHA monoglyceride derivative (Figure 1A), on lung inflammation and AHR using two approaches, namely, an *in vitro* model of TNF- α -stimulated human bronchi and an *in vivo* model of chronic asthma. Here we report on the first evidence that CRBM-0244 displays anti-inflammatory properties and prevents airway hyperresponsiveness in lung tissue, a finding consistent with the effects of resolvin D1 and protectin D1.

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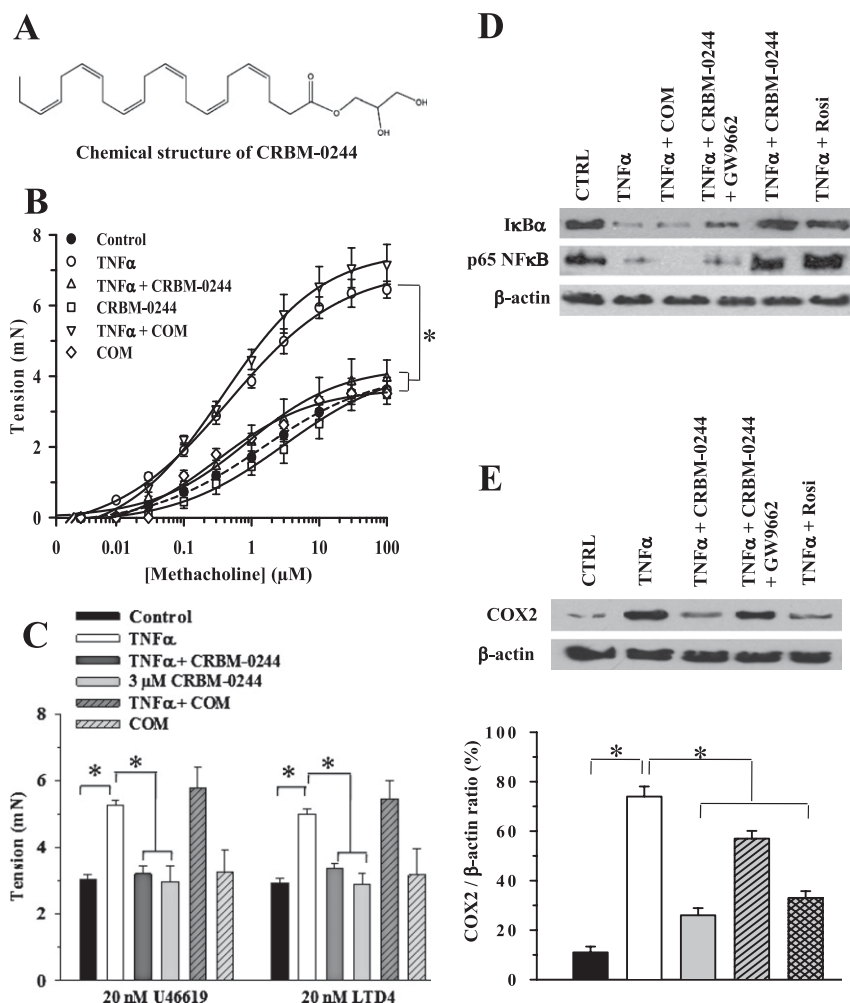


Figure 1. Chemical structure and effects of the docosahexaenoic acid (DHA) monoglyceride derivative CRBM-0244 on overreactivity and inflammation in TNF- α -treated human bronchi. (A) CRBM-0244 was synthesized from highly purified DHA attached to a monoglycerol in the sn-1 position (17). (B) Cumulative concentration response curves (CCRCs) to methacholine (MCh) generated in untreated (CTRL), TNF- α (10 ng/ml)-treated bronchi, TNF- α + 3 μ M CRBM-0244-treated bronchi, TNF- α + 3 μ M corn oil monoglyceride (COM)-treated bronchi, and 3 μ M CRBM-0244-treated bronchi and 3 μ M corn oil monoglyceride (COM)-treated bronchi, alone. Each point represents the mean $\pm$ SEM, with $n = 15$ for each experimental condition. * $P < 0.05$. (C) Bar graph displays the mean contractile responses induced by 20 nM U46619 and 20 nM leukotriene-D4 (LTD-4) in control, TNF- α -pretreated, TNF- α + 3 μ M CRBM-0244, TNF- α + 3 μ M COM, as well as in 3 μ M CRBM-0244-pretreated and 3 μ M COM-pretreated human bronchi, alone ($n = 12$ for each experimental condition). * $P < 0.05$. (D) Western blot analysis of cytosolic protein fractions derived from untreated (control), TNF- α , TNF- α + 3 μ M COM, TNF- α + 3 μ M CRBM-0244, TNF- α + 3 μ M CRBM-0244 + 1 μ M GW9662, and TNF- α + 300 nM Rosiglitazone (Rosi), using specific antibodies against I κ -B α , p65 NF- κ B, and β -actin. Note the high staining level of I κ -B α and p65 NF- κ B in TNF- α + CRBM-0244-treated bronchi as well as in TNF- α + rosiglitazone-treated tissues. Results are representative of six similar experiments. (E) Western blot and quantitative analysis of cyclooxygenase 2 (COX-2) protein detection. Staining densities in the homogenates were expressed as a function of β -actin signals. Results are representative of seven similar experiments. * $P < 0.05$.

MATERIALS AND METHODS

Synthesis of CRBM-0244

CRBM-0244 was synthesized as previously described, using ethyl docosahexaenoate as starting material (17).

Culture of Human Bronchi and Isometric Tension Measurements

This study was approved by the Ethics Committee of the Université de Sherbrooke (Protocol 05-088). Human lung tissue was obtained from 15 patients undergoing surgery for lung carcinoma. Bronchial segments were dissected and cultured in Dulbecco's minimum essential medium (DMEM)-F12 medium (18). Bronchi were untreated (control) or treated (every 24 hours for 2 days) with either CRBM-0244 or 10 ng/ml TNF- α (R&D Systems, Burlington, ON, Canada) or 10 ng/ml in the absence or presence of CRBM-0244, before pharmacologic challenge. The mechanical effects induced by specific agonists were measured as previously described (18). Measurements of the resultant induced myofilament Ca^{2+} sensitivity were performed as previously reported, using a β -escin permeabilization protocol in human bronchi (15) (for further details, *see online supplement*).

Western Blot Analysis

Western blots using specific antibodies against cyclooxygenase-2 (COX-2), p65-NF κ B, the PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kD (CPI-17), its phosphorylated form (anti-P-CPI-17), and β -actin proteins were performed on homogenate fractions, as previously described (16).

Immunofluorescence

Bronchial biopsies from normal subjects and patients with severe asthma were obtained from the Respiratory Health Network Tissue

Bank of the Fonds de la Recherche en Santé du Québec (FRSQ). Tissues were fixed and prepared as previously described (15) (for further details, *see online supplement*).

Sensitization and Challenge Protocols

Male or female albino guinea pigs (Hartley, 200–250 g) were obtained from Charles River Laboratories (Montreal, Quebec, Canada). Guinea pigs were sensitized with intraperitoneal injections of 10 μ g ovalbumin (OVA) (Sigma Chemical, St. Louis, MO). CRBM-0244 was administered orally (231 mg/kg) (19) on Days 0 and 7, 30 minutes before OVA sensitization, and on Days 14–18, before an aerosol challenge containing 1.5% OVA for 10 minutes (*see online supplement*). Measurements of inflammatory mediator were determined in cell-free bronchoalveolar lavage (BAL) fluid according to a specific ELISA for TNF- α (Cusabio Biotech, Newark, DE). Anti-OVA IgE concentrations were measured in serum, using ELISA (Cusabio Biotech).

Data Analysis and Statistics

Results are expressed as means $\pm$ SEM, with n indicating the number of experiments. Statistical analyses were performed using a Student t test or an ANOVA and Dunnett's multiple comparison tests. Differences were considered statistically significant when $P < 0.05$. Data curve fittings were performed using Sigma Plot 11 (SPSS-Science, Chicago, IL) (18).

RESULTS

Effects of CRBM-0244 on Reactivity and Inflammation of TNF- α -Pretreated Bronchi

Our experiments were designed to assess the effects of CRBM-0244 on the hyperreactivity developed by TNF- α -treated

bronchi in response to various pharmacologic challenges. In a previous report, we showed that 10 ng/ml of TNF- α pretreatment induced overreactivity in short-term cultured human bronchi (15). Human distal bronchi were cultured for 2 days in the absence or presence of 3 μ M CRBM-0244, and were challenged with relevant pharmacologic agonists such as methacholine (MCh), U-46619, and leukotriene-D4 (LTD-4). CRBM-0244 was synthesized as previously described (17), and the chemical structure is shown in Figure 1A. The cumulative concentration response curve to CRBM-0244 (0.1–30 μ M) revealed that treatment with 3 μ M for 48 hours was sufficient to completely abolish the hyperreactivity triggered by TNF- α in human bronchi (*see* Figures E1A–E1D and E2 in the online supplement). Figure 1B depicts the cumulative concentration response curve (CCRC) to MCh in control, TNF- α -treated tissue in the absence or presence of 3 μ M CRBM-0244 and in the presence of 3 μ M CRBM-0244-treated bronchi alone. Whereas TNF- α pretreatment consistently induced hyperresponsiveness to MCh with an apparent median effective concentration required to induce a 50% effect (EC_{50}) of 0.46 μ M, treatment with 3 μ M CRBM-0244 significantly reduced this TNF- α -induced reactivity, with an EC_{50} of 0.96 μ M. However, pretreatment with 3 μ M CRBM-0244 exerted no effect on the pharmacologic reactivity of bronchial tissue to MCh, compared with control bronchi. To evaluate the putative nonspecific effects of CRBM-0244 on human bronchi reactivity, a corn oil monoglyceride (COM) of similar structure was synthesized and used as negative control. In the presence of 3 μ M COM, no effect was quantified in the reactivity to MCh, compared with control tissue. Moreover, TNF- α -treated bronchi in the presence of 3 μ M COM displayed a similar reactivity to MCh, compared with TNF- α -treated bronchi alone. These results confirm that CRBM-0244 likely displays specific effects on human bronchi.

The mechanical properties of U46619 (a thromboxane A2 receptor agonist) and LTD-4 were also determined in control, TNF- α -treated bronchi in the absence or presence of CRBM-0244, and after CRBM-0244 pretreatment alone. Figure 1C demonstrates that pretreatment with 3 μ M CRBM-0244 significantly reduced the overreactivity induced by TNF- α to both pharmacologic agonists. No effect on bronchial reactivity to U-46619 and LTD-4 was evident after pretreatment with 3 μ M COM. To determine whether CRBM-0244 reduces TNF- α -triggered hyperresponsiveness through a putative anti-inflammatory effect in TNF- α -treated human bronchi, the activation of NF- κ B was investigated. The activation of this factor is usually reversely correlated with a reduction in I κ -B α because of the extensive ubiquitination and proteosomal degradation of this inhibitory subunit, consequently resulting in the increased nuclear translocation of the p65 NF- κ B subunit (20). Western blot analysis revealed that either TNF- α pretreatment alone or in the presence of 3 μ M COM resulted in the degradation of I κ -B α and a reduction in p65 subunit staining in cytoplasmic fractions, compared with preparations derived from control bronchi (Figure 1D). However, treatment with CRBM-0244 prevented the degradation of I κ -B α and the subsequent nuclear translocation of the p65 NF- κ B subunit triggered by TNF- α , whereas in the presence of 1 μ M GW9662, the opposite effects were evident. The peroxisome proliferator-activated receptor- γ (PPAR- γ) agonist rosiglitazone (50 nM), used as positive control, consistently reduced the degradation of I κ -B α and the nuclear translocation of the p65 subunit triggered by TNF- α (Figure 1D). Because increased COX-2 activity and expression are related to inflammatory status in several tissues, and because TNF- α can increase the expression of COX-2 in the human lung, experiments were designed to evaluate the expression level of COX-2 in control, TNF- α , and TNF- α + CRBM-0244 in the absence or presence of 1 μ M GW9662, as

well as in TNF- α + rosiglitazone-pretreated bronchi. The pretreatment of human bronchi with TNF- α induced an increased staining level of COX-2 protein expression compared with control tissue (Figure 1E). However, CRBM-0244 (3 μ M) prevented the overexpression of COX-2 protein triggered by TNF- α , whereas in the presence of GW9662, expression levels were similar to those observed in TNF- α -treated bronchi alone (Figure 1E). Furthermore, no difference was evident between the level of COX-2 expression obtained in the presence of rosiglitazone (50 nM) and that observed after treatment with CRBM-0244 (Figure 1E). Together, these data further demonstrate that CRBM-0244 pretreatment is able to prevent the bronchial hyperreactivity and inflammation triggered by TNF- α in human bronchi.

Effects of CRBM-0244 on TNF- α -Induced Ca^{2+} Hypersensitivity

Comparative analyses were performed on β -escin permeabilized preparations to assess the effects of CRBM-0244 on Ca^{2+} sensitivity in TNF- α -pretreated bronchi. The CCRCs to free Ca^{2+} concentrations in permeabilized bronchial rings obtained from control, TNF- α -treated bronchi in the absence or presence of 3 μ M CRBM-0244, as well as in 3 μ M CRBM-0244-treated bronchi, are shown in Figure 2A. Data analysis demonstrates that treatment with 3 μ M CRBM-0244 induced a shift in EC_{50} values (1.1 μ M) toward higher Ca^{2+} concentrations, thus reducing the Ca^{2+} hypersensitivity developed in TNF- α -treated bronchi (0.13 μ M). Conversely, the difference in Ca^{2+} sensitivity between tissues treated with TNF- α + 3 μ M CRBM-0244 and control bronchi was not significant, with EC_{50} values of 1.1 and 0.92 μ M, respectively (Figure 2A). Moreover, no significant difference was evident between control and 3 μ M CRBM-0244-treated bronchi alone. COM treatments exerted no effects on the Ca^{2+} sensitivity developed by β -escin permeabilized human bronchi in the absence or presence of TNF- α . To investigate further the putative processes that would support this negative feedback mechanism induced by CRBM-0244 on the Ca^{2+} -tension relationship, Western blot analyses were performed to assess the status of the regulatory protein CPI-17 in homogenates derived from control and TNF- α -treated bronchi in the absence or presence of CRBM-0244. Figure 2B demonstrates that CPI-17 was present in all tested fractions, although treatment with TNF- α increased the density of the phosphorylated active form as well as the total expression of CPI-17. In contrast, phosphorylated and total CPI-17 bands were reduced upon treatment with 1 and 3 μ M CRBM-0244. Control β -actin staining was relatively constant from one preparation to the next (Figure 2B). As shown in Figure 2B, 48-hour pretreatment of bronchial explants with CRBM-0244 largely reduced the P-CPI-17/ β -actin staining density ratio, compared with the ratio in TNF- α -pretreated bronchi (Figure 2B). No difference was evident between control and TNF- α + 3 μ M CRBM-0244-treated tissues. These data suggest that the mode of action of CRBM-0244 results in the reduce expression of CPI-17 protein, which in turn decreases the Ca^{2+} sensitivity of bronchial smooth muscle stimulated with TNF- α . Moreover, the involvement of the Rho-kinase pathway was evaluated, and complementary experiments demonstrated that treatment with 3 μ M CRBM-0244 reduced the responses to U-46619 (a Rho-kinase activator) in TNF- α -treated human bronchi (*see* Figure E3 in the online supplement).

Immunohistochemical Detection of CPI-17 in Inflammatory Conditions

To extend these results to human pathophysiologic conditions, the distribution of total and P-CPI-17 were identified through

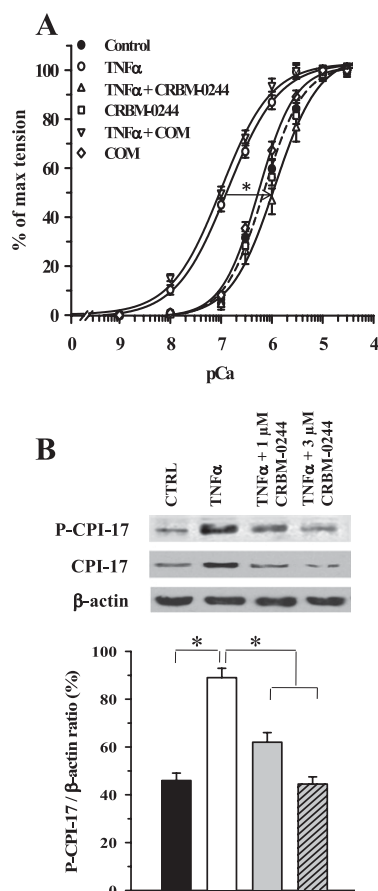


Figure 2. Effects of CRBM-0244 on Ca^{2+} hypersensitivity triggered in TNF- α -treated human bronchi. (A) CCRCs to free $[\text{Ca}^{2+}]$ obtained from β -escin permeabilized tissues in control (solid circles), TNF- α (open circles), TNF- α + 3 μM CRBM-0244 (open triangles), 3 μM CRBM-0244 (open squares), TNF- α + 3 μM corn oil monoglyceride (COM), and 3 μM COM. Each point represents the mean $\pm$ SEM, where $n = 15$ for each experimental condition. $*P < 0.05$. (B) Western blot and quantitative analysis of PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kD (CPI-17) and phosphorylated CPI-17 (P-CPI-17) protein detection. Note the reduced staining of P-CPI-17 bands in TNF- α + CRBM-0244-treated tissues, as opposed to increased staining in treatment with TNF- α alone. Staining densities of P-CPI-17 in the homogenates were expressed as a function of β -actin signals. Significant differences were evident between control and TNF- α alone, and in TNF- α alone versus TNF- α + CRBM-0244-treated tissues. Results are representative of six similar experiments. $*P < 0.05$.

immunofluorescence staining on thin sections of bronchial biopsies derived from normal subjects and patients with severe asthma (Figure 3). Image analyses demonstrated that the P-CPI-17 was largely increased in biopsies obtained from patients with asthma compared with biopsies derived from normal subjects (Figures 3A and 3B). As shown in Figure 3D, increased total CPI-17 staining was evident in patients with asthma, predominantly at the smooth muscle level, compared with biopsies obtained from normal subjects (Figure 3C). In bronchial biopsies derived from patients with asthma, quantitative analysis revealed a 92% and 62% increase in P-CPI-17 and CPI-17 staining, respectively, compared with levels obtained in sections from normal subjects (Figure 3E). Image analyses also revealed that the P-CPI-17 represents 80% of the total CPI-17 detected in biopsies of patients with asthma. However, in

biopsies from normal subjects, P-CPI-17 staining represented 15% of the total CPI-17 detected. These analyses reveal for the first time the presence and overexpression of CPI-17 in patient with severe asthma, suggesting that CPI-17 represents an inflammatory marker that may play a critical role in AHR.

Effects of Lipoxygenase Inhibitors on CRBM-0244-Mediated Anti-Inflammatory Properties

Further experiments were performed to determine whether CRBM-0244 is metabolized or not, to mediate the anti-inflammatory effects in TNF- α -stimulated human bronchi. The lipoxygenase (LOX) pathways were investigated using two specific inhibitors, namely, the 5-LOX inhibitor MK886 and the 15-LOX inhibitor luteolin, in combination with CRBM-0244 treatment in TNF- α -treated bronchi. Known metabolites produced by

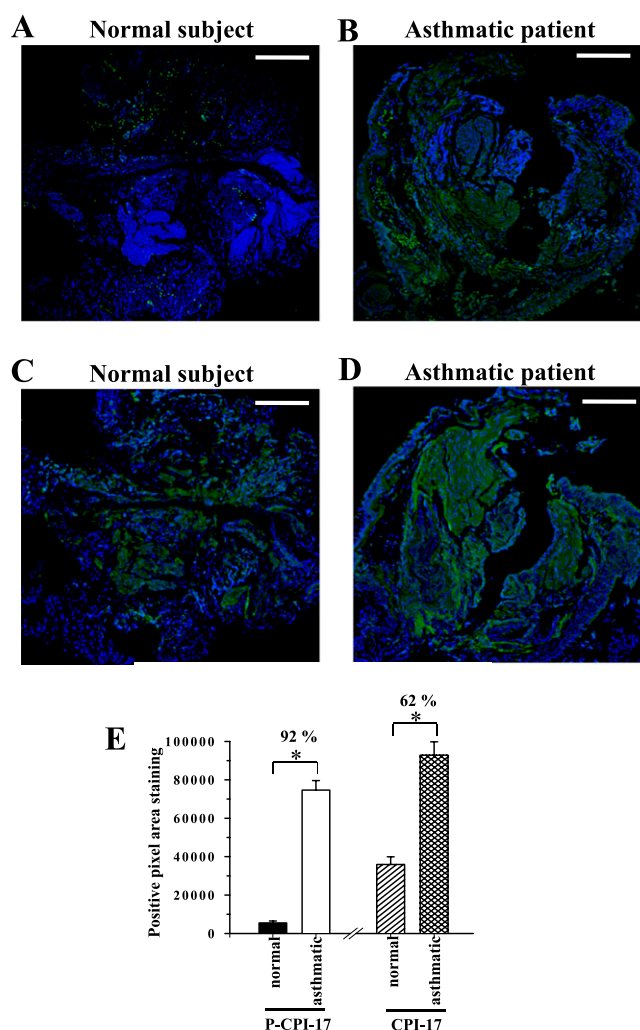


Figure 3. Detection of CPI-17 by immunofluorescence microscopy in contiguous thin sections derived from human bronchial biopsies. (A and B) Detection of P-CPI-17 protein (green) with DAPI nuclear counterstaining (blue), performed on bronchial biopsies derived from a normal subject and a patient with severe asthma, respectively. (C and D) Staining of the total CPI-17 (green) in bronchial biopsies derived from a normal subject and a patient with severe asthma, respectively. Illustrations are representative of eight biopsies for each group (bar, 100 μm). (E) Quantitative analysis displays the mean immunopositive pixel area staining of P-CPI-17 (left) and total CPI-17 (right) from bronchial biopsies of normal subjects and patients with asthma ($n = 8$ for each experimental condition). $*P < 0.05$.

these pathways, such as resolvin-D1 and protectin-D1, were evaluated in terms of both their mechanical properties and the inflammation triggered by TNF- α in bronchi. Figure 4A demonstrates that combined treatment with 1 μ M MK886 and 0.6 μ M luteolin in the presence of 3 μ M CRBM-0244 incurred an increase in tension induced by 1 μ M MCh, similar to that of TNF- α -treated bronchi. However, treatment with 3 μ M CRBM-0244, 300 nM resolvin-D1, or 300 nM protectin-D1 significantly reduced the tension induced by MCh, compared with TNF- α -treated bronchi alone. Moreover, no differences were evident between CRBM-0244, resolvin-D1, and protectin-D1 treatments on MCh-induced tension in TNF- α -treated bronchi. The Ca²⁺ sensitivity of myofilaments was also evaluated in five series of TNF- α -treated bronchi permeabilized with β -escin, as illustrated in CCRC to free [Ca²⁺] concentrations (Figure 4B). The combined 5-LOX and 15-LOX inhibitors prevented the effect induced by 3 μ M CRBM-0244 on the Ca²⁺ sensitivity of airway smooth muscles in TNF- α -treated bronchi. In contrast, the use of CRBM-0244, resolvin-D1, or protectin-D1 resulted in a significant reduction in the Ca²⁺ hypersensitivity triggered by TNF- α . Complementary experiments were designed to assess the independent effects of 5-LOX and 15-LOX inhibitors in the presence of CRBM-0244 and TNF- α treatment on MCh-induced bronchial reactivity and Ca²⁺ sensitivity. Both pathway inhibitions induced a partial decrease in the effects of CRBM-0244 on TNF- α -treated bronchi (see Figure E4). These results were corroborated in terms of the decrease in CPI-17 expression induced by either CRBM-0244, resolvin-D1, or protectin-D1 treatment, compared with the level of CPI-17 expression detected in TNF- α -treated bronchi alone (Figure 4C). However, combined treatment with

MK886 and luteolin in the presence of 3 μ M CRBM-0244 prevented this reduction in the expression level of CPI-17, compared with its expression level in TNF- α + CRBM-0244-treated tissue. No difference was evident between CPI-17 expression levels detected in TNF- α + CRBM-0244-treated bronchi in the presence of combined LOX inhibitor treatments and TNF- α -treated bronchi alone. The effect of combined LOX inhibitors was also assessed according to COX-2 protein expression in Western blots derived from different preparations of bronchi homogenate. Figure 4D demonstrates that COX-2 expression levels were reduced in TNF- α + CRBM-0244, TNF- α + resolvin-D1, and TNF- α + protectin-D1-treated bronchi, whereas TNF- α treatment alone induced an increase in COX-2 expression. Combined treatment with MK886 and luteolin prevented the reduction of COX-2 protein expression compared with TNF- α + CRBM-0244-treated tissues. Together, these results show that CRBM-0244 is likely metabolized by lipoxygenases in mediating these anti-inflammatory effects and the reduction of MCh-induced AHR, as well as the Ca²⁺ oversensitivity triggered by TNF- α in human bronchi.

Effects of Oral CRBM-0244 Administration on Allergen-Induced Lung Inflammation

An *in vivo* model of allergic asthma was used to determine the impact of CRBM-0244 on lung inflammation and AHR. The experimental protocol was designed to investigate both the anti-allergic and anti-inflammatory properties induced by the oral administration of CRBM-0244 in OVA-sensitized guinea pigs. CRBM-0244 was administered orally at a pharmacologic dose (231 mg/kg), based on the recommended daily dose of DHA for humans, following the dose translation of human to guinea pig

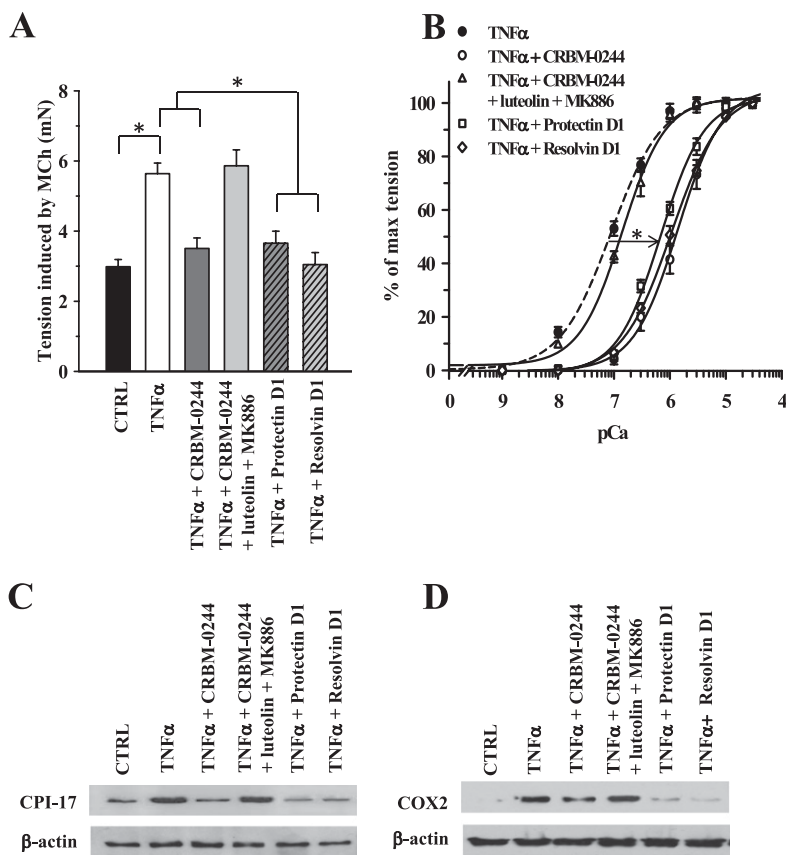


Figure 4. Combined inhibition of 5-lipoxygenase and 15-lipoxygenase by MK886 and luteolin reverses the anti-inflammatory effects of CRBM-0244 in TNF- α -treated human bronchi. (A) Bar histogram displays mean responses induced by 1 μ M methacholine (MCh) in control and treated human bronchi. Significant decreases in tension were quantified in CRBM-0244, resolvin-D1, and protectin-D1-treated bronchi, whereas in the presence of CRBM-0244 and lipoxygenase inhibitors, the contractile response was similar to the mean response observed in TNF- α -treated bronchi alone ($n = 12$ for each experimental condition). (B) CCRC to free [Ca²⁺] obtained in β -escin-permeabilized tissues in TNF- α (solid circles, $n = 18$), TNF- α + 3 μ M CRBM-0244 (open circles, $n = 20$), TNF- α + 3 μ M CRBM-0244 in the presence of 1 μ M MK886 and 0.6 μ M luteolin (open triangles, $n = 23$), and TNF- α -treated bronchi in the presence of 300 nM protectin-D1 (open squares, $n = 21$) and 300 nM resolvin-D1 (open diamonds, $n = 21$). Each point represents the mean $\pm$ SEM, * $P < 0.05$. (C) Western blot analysis of CPI-17 and β -actin proteins detected in homogenate fractions derived from control bronchi and on five series of TNF- α -treated human bronchi. Note the low staining level of the CPI-17 immunoreactive band after treatment with CRBM-0244, resolvin-D1, and protectin-D1, whereas a marked staining of these enzymes was evident in TNF- α -treated bronchi alone and after combined treatment with MK886 and luteolin. The results are representative of six similar experiments. Resolvin-D1 and protectin-D1 as well as CRBM-0244 treatment largely decreased the COX-2 immunoreactive band. (D) Proteins from the corresponding homogenates (as described in C) were stained using specific antibodies against COX-2 and β -actin. Results are representative of eight similar experiments.

via the equation described by Reagan-Shaw and colleagues (19). Pulmonary inflammation is characterized by leukocytic infiltrates in the airway after an allergic response to an allergen, in which the predominant leukocyte is the eosinophil, in addition to lymphocytes, primarily of the Th2 phenotype (21). Hence, the extent of inflammation in lung tissue was determined in control and OVA-sensitized guinea pigs, treated or not treated with an oral dose of CRBM-0244. Histologic analyses performed on lung sections stained with hematoxylin–eosin revealed that animals receiving CRBM-0244 had substantially fewer eosinophils and lymphocytes in the peribronchial regions and airspaces compared with OVA-sensitized animals (Figures 5A–5C). CRBM-0244 also reduced goblet-cell hyperplasia and the production of airway mucus, as determined by alcian blue staining (Figures 5D–5F).

In BAL fluid, treatment with CRBM-0244 decreased total leukocytes, namely eosinophils and lymphocytes, compared with the number of leukocytes found in the BAL fluid of untreated, OVA-sensitized guinea pigs (Figure 6A). No difference was evident between OVA + CRBM-0244-treated animals and control animals receiving vehicle only (Figure 6A). The level of TNF- α , known to play a pivotal role in airway inflammation and hyperresponsiveness (22), was determined by specific ELISA in the three animal groups. CRBM-0244 blocked the allergen-induced increase in TNF- α level found in BAL fluids, compared with the level detected in the OVA group (Figure 6B). Moreover, no significant difference was quantified between CRBM-0244-treated and control animals (Figure 6B). In conjunction with decreased airway inflammation, concentrations of COX-2 were lower in the presence of CRBM-0244, as shown by Western blot analysis (Figure 6C) and by immunofluorescence microscopy on thin sections of bronchi (*see* Figure E5). The concentrations of OVA IgE in serum derived from control, OVA, and OVA + CRBM-0244-treated guinea pigs were also evaluated using specific ELISAs. As shown in Figure 6D, treatment with CRBM-0244 significantly decreased the concentration of OVA IgE in sera by approximately 39%, compared with the concentration detected in the sera of OVA-sensitized animals. No behavioral or physical signs of toxicity with CRBM-0244 treatment were observed. Together, these results indicate that CRBM-0244 significantly reduces allergen-induced pulmonary inflammation and displays potent antiallergenic properties by decreasing the serum concentration of IgE, suggesting that its mechanism of action may be relevant in the prevention of allergic asthma.

Effects of CRBM-0244 Treatment on Allergen-Induced AHR

AHR to specific agonists and Ca²⁺ hypersensitivity are important hallmarks of allergic asthma (5). To determine the impact of CRBM-0244 treatment on AHR developed in OVA-sensitized guinea pigs, experiments were designed to assess the mechanical properties of bronchial smooth muscle in response to several pharmacologic agonists and to free [Ca²⁺] concentrations in β -escin permeabilized bronchial rings. Figure 7A displays the reactivity induced by MCh and histamine in bronchial preparations derived from control, OVA-sensitized guinea pigs, and from OVA-sensitized + CRBM-0244-treated animals. The data reveal an overreactivity of OVA-sensitized guinea pigs to muscarinic and histaminergic agonists, whereas the oral administration of CRBM-0244 prevented the development of AHR to these stimuli (Figure 7A). The responsiveness of bronchial tissues was also assessed in the presence of U-46619 and LTD-4 in control and OVA-sensitized guinea pigs, either untreated or treated with CRBM-0244. Figure 7B demonstrates that treatment with CRBM-0244 abolished the hyperreactivity to the thromboxane agonist (U-46619) and to LTD-4. Measure-

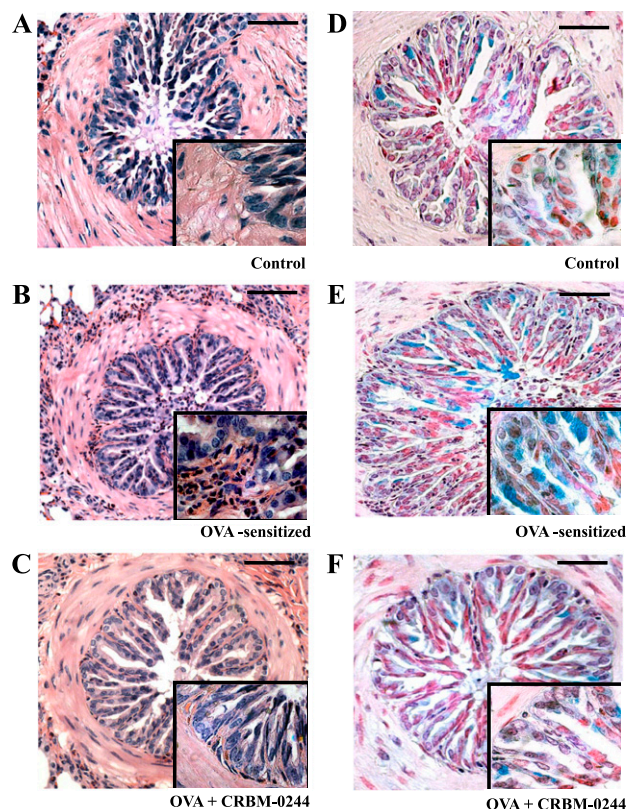


Figure 5. CRBM-0244 reduces airway inflammation in ovalbumin (OVA)-sensitized lung tissue. (A–C) Sections of lung tissue obtained from a nonsensitized guinea pig (control) (A), and from OVA-sensitized (B) and OVA-sensitized + CRBM-0244-treated animals (C). The degree of inflammation was assessed by determining the extent of inflammatory infiltrates in lung sections stained with hematoxylin–eosin (*bar*, 40 μ m; $n = 8, 10$, and 11 per respective group). (D–F) Representative tissue sections, obtained from a nonsensitized guinea pig (control, D), and from OVA-sensitized (E) and OVA-sensitized + CRBM-0244-treated (F) animals, were stained with alcian blue (*bar*, 40 μ m; $n = 8, 10$, and 11 per respective group). Mucus secretion and goblet cells containing mucus were stained in blue. *Insets* represent $\times 100$ zoom view of the corresponding images.

ments of Ca²⁺ sensitivity revealed that OVA-sensitized guinea pigs developed an increased reactivity to free [Ca²⁺] concentrations compared with control animals (Figure 7C), with EC₅₀ values of 0.37 μ M for OVA-sensitized guinea pigs and 1.31 μ M for control animals. However, in the presence of CRBM-0244, the Ca²⁺ sensitivity of bronchi was significantly decreased, with an apparent shift in EC₅₀ value toward higher concentrations (1.59 μ M). No difference was quantified between CRBM-0244-treated animals and control guinea pigs receiving vehicle only. To investigate whether this increase in Ca²⁺ sensitivity was correlated with a change in expression of the CPI-17 regulatory protein, the status of this protein was assessed in lung homogenates derived from control and OVA-sensitized guinea pigs, treated or not treated with CRBM-0244. Western blot analysis showed that allergen sensitization significantly increased the expression of CPI-17, whereas treatment with CRBM-0244 largely reduced the expression of CPI-17 in lung tissue (Figure 7D). Together, these results demonstrate that treatment with CRBM-0244 effectively abolished the development of AHR to several agonists and reduced the Ca²⁺ sensitivity of ASM, which is correlated with a decrease in the expression of CPI-17 protein.

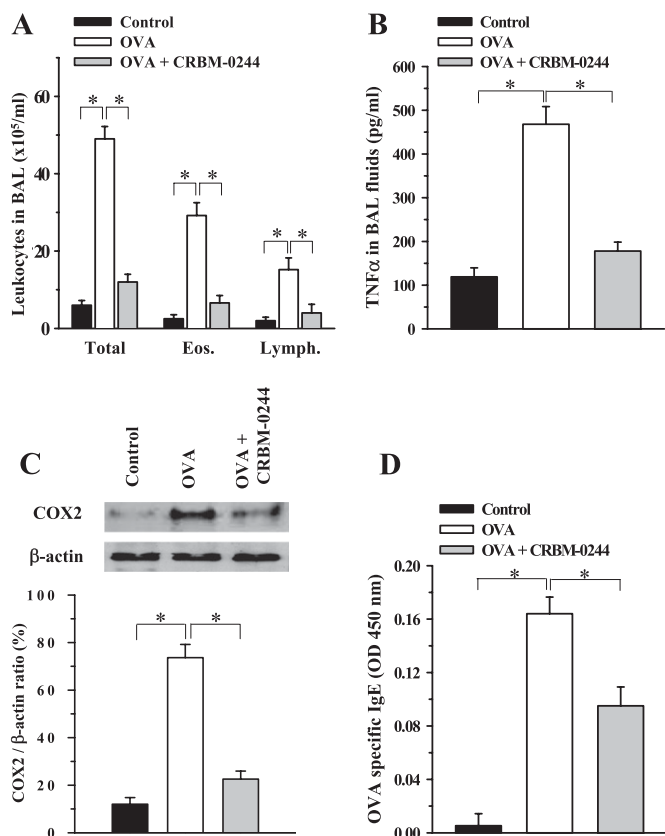


Figure 6. Protective effects of CRBM-0244 on airway inflammation in lung tissue. (A) Total leukocytes, eosinophils (Eos.), and lymphocytes (Lymph.) in bronchoalveolar lavage (BAL) fluid were enumerated and identified after Wright-Giemsa staining. Results are expressed as mean $\pm$ SEM ($n = 7$). $*P < 0.05$. (B) BAL fluid TNF- α concentrations were assessed using ELISA, as described in MATERIALS AND METHODS. CRBM-0244 significantly decreased BAL fluid TNF- α concentrations, compared with the OVA-sensitized group. Results are expressed as means $\pm$ SEM of seven animals for each group. $*P < 0.05$. (C) Western blot and quantitative analysis of COX-2 protein detection in lung homogenate fractions obtained from nonsensitized guinea pigs (control), and from OVA-sensitized and OVA-sensitized + CRBM-0244-treated animals. Staining densities in the homogenates for COX-2 were expressed as a function of the β -actin signal. Note the lower staining level of COX-2 in bronchi from OVA + CRBM-0244 guinea pigs, compared with OVA-sensitized guinea pig bronchi. This result is representative of six similar experiments. (D) OVA-specific IgE concentrations were determined using ELISA. CRBM-0244 significantly decreased serum OVA-specific IgE concentrations, compared with OVA-sensitized guinea pigs. Results are expressed as means $\pm$ SEM of 12–13 animals for each group. $*P < 0.05$.

DISCUSSION

CRBM-0244 Mediated Anti-Inflammatory Effects in TNF- α -Stimulated Bronchi

Bronchial inflammation plays a key role in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD) (3). Pharmacologic evidence also points to an important role of TNF- α in ASM cell hyperresponsiveness (22). TNF- α is also released from airway macrophages in patients with asthma after an allergen challenge (23), and may exert an important amplifying effect in asthmatic inflammation (24). Over the past three decades, significant interest has arisen 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 disease (25). Here, we investigated the ability of CRBM-0244, a newly synthesized DHA monoglyceride derivative, to reduce inflammation and AHR in the lung. Our data revealed that low concentrations of CRBM-0244 prevented the human bronchial hyperreactivity triggered by TNF- α to methacholine, U46619 (thromboxane agonist), and LTD-4. Such anti-inflammatory properties of DHA were also reported in several tissues (25, 26). For example, Komatsu and colleagues (27) showed that 60 μ M of DHA inhibited the activity of NF- κ B in interferon- γ and LPS-stimulated mouse leukemic monocyte macrophage cell line (RAW macrophages) macrophages, whereas Weldon and colleagues (28) demonstrated that DHA inhibits the expression of p65 and increases the expression of I κ B- α in LPS-stimulated human acute monocytic leukemia cell line (THP-1 monocytes) monocytes. The inhibition of NF- κ B activity by DHA is especially important, because airway macrophages and bronchial epithelial cells from patients with asthma exhibit increased NF- κ B activity compared with cells from healthy individuals (20). Therefore, NF- κ B was suggested to play a pivotal role in the pathogenesis of asthma (20). The activation of transcription factors such as NF- κ B and PPAR- γ was also investigated here. NF- κ B is the principal transcription factor involved in the upregulation of inflammatory cytokines, COX-2 genes, and adhesion molecules (29), whereas the nuclear receptor PPAR- γ was shown to exert potent anti-inflammatory activity, mainly through its ability to down-regulate the expression of proinflammatory genes (30). In the present study, treatment with CRBM-0244 prevented the degradation of I κ B- α and the nuclear translocation of p65 NF- κ B triggered by TNF- α , whereas in the presence of the PPAR- γ antagonist GW9662, these effects were abolished. Moreover, CRBM-0244 prevented the overexpression of COX-2 protein induced by treatment with TNF- α , whereas in the presence of GW9662, the detected expression levels were similar to those observed in TNF- α -treated bronchi alone. Together, our data indicate that CRBM-0244 mediates its anti-inflammatory effects through the activation of PPAR- γ , resulting in a decrease in the activation of NF- κ B and in the related expression of proinflammatory genes such as cytokines and COX-2. Such results suggest that the activation of PPAR- γ by CRBM-0244 or one of its metabolites may be one of the underlying mechanisms of these beneficial effects. Hence PPAR- γ was shown to have an allosteric binding site for fatty acids, with a higher affinity for PUFAs (26, 29).

CRBM-0244 Decreases Ca²⁺ Hypersensitivity and the Expression of CPI-17 in TNF- α -Treated Bronchi

Several studies demonstrated that Ca²⁺ sensitizing mechanisms may also be primed under pathophysiologic conditions by various cytokine and lipid mediators, and that these processes of Ca²⁺ sensitization are involved in AHR (31, 32). Therefore, potential clinical interest arose in finding a specific agent that would significantly oppose the shift in Ca²⁺ sensitivity induced by proinflammatory cytokines such as TNF- α . Our data reveal that 48 hours of pretreatment with CRBM-0244 reduced the Ca²⁺ hypersensitivity developed by TNF- α -stimulated bronchi. Similarly, TNF- α , IL-13, and IL-1 β were shown to regulate agonist-associated Ca²⁺ signaling directly, using primary cultures of ASM cells (6, 33). Western blot analysis performed on TNF- α -treated bronchi homogenates attest that treatment with CRBM-0244 was able to decrease CPI-17 phosphorylation levels and protein expression. Sakai and colleagues (32) also demonstrated an increase in the expression and activation of CPI-17 in hyperresponsive bronchial smooth muscle in rodents, which in turn may be responsible for the enhanced muscarinic responses of bronchial contraction associated with AHR (32). These results suggest that CRBM-0244 targets CPI-17 protein

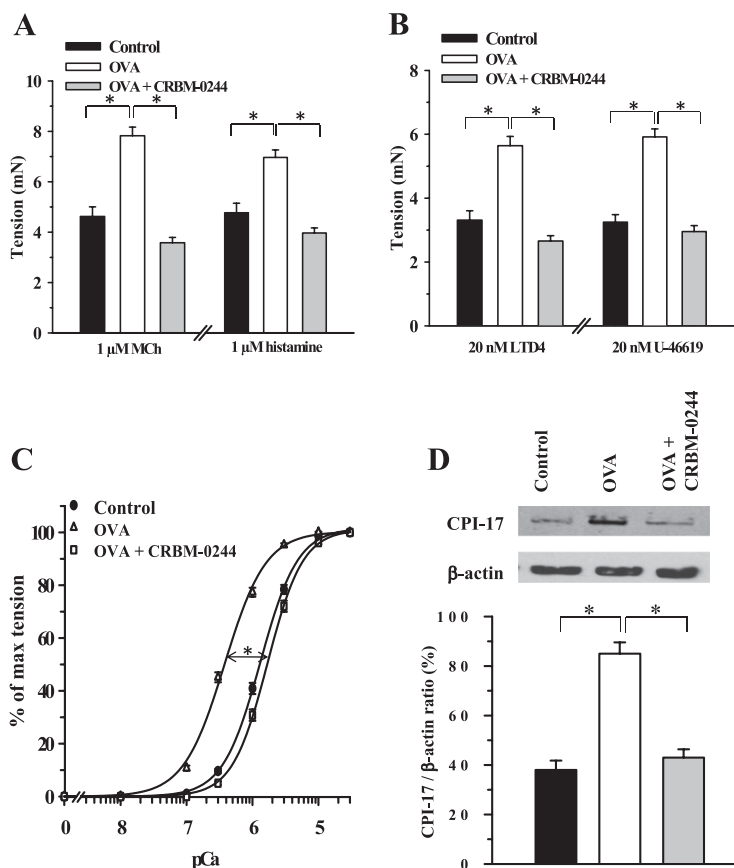


Figure 7. Effects of CRBM-0244 treatment on airway hyperresponsiveness developed in OVA-sensitized guinea pigs. (A) Bar histogram displays mean responses induced by 1 μ M MCh and 1 μ M histamine in nonsensitized (control), OVA-sensitized, and OVA-sensitized + CRBM-0244-treated guinea pigs. (B) Bar histogram illustrates mean contractile responses induced by 20 nM LTD-4 and 20 nM U46619 in control, OVA-sensitized, and OVA-sensitized + CRBM-0244-treated guinea pigs. For each pharmacologic challenge, $n = 8$ and $n = 48$ for nonsensitized (control) guinea pigs, $n = 10$ and $n = 54$ for OVA-sensitized guinea pigs, and $n = 11$ and $n = 66$ for OVA-sensitized + CRBM-0244-treated guinea pigs. $*P < 0.05$. (C) CCRC to free $[Ca^{2+}]$ obtained from β -escin permeabilized tissues in control (solid circles, $n = 30$), OVA (open triangles, $n = 40$), and OVA + CRBM-0244 (open squares, $n = 48$). Each point represents the mean $\pm$ SEM. $*P < 0.05$. (D) Western blot analysis of homogenate protein fractions derived from control, OVA-sensitized, and OVA-sensitized + CRBM-0244-treated guinea pigs, using specific antibodies against CPI-17 and β -actin. Staining densities in the homogenates for CPI-17 were expressed as a function of the β -actin signal. Results are representative of six similar experiments. $*P < 0.05$.

to decrease the Ca^{2+} sensitivity of bronchial smooth muscle. In light of these observations, the status of CPI-17 protein in lung tissues from normal and inflammatory conditions was assessed. Our data demonstrated an upregulation of CPI-17 protein expression in TNF- α -stimulated bronchi, whereas treatment with CRBM-0244 reduced the overexpression induced by TNF- α (data not shown). In addition, increases in CPI-17 staining and in its phosphorylated form were consistently detected in biopsies derived from patients with severe asthma, compared with biopsies from normal subjects. These data suggest that CPI-17 represents both a " Ca^{2+} rheostat" and a proinflammatory protein that could play a critical role in the AHR and remodeling occurring in asthma. Moreover, our group recently demonstrated that the expression of CPI-17 was under the control of PPAR- γ activation in this *in vitro* human bronchial inflammatory model (16). In human lungs with pathologic conditions, the use of CRBM-0244 is suspected to decrease the expression level of CPI-17, which could be relevant in improving the respiratory capacities of patients with inflammatory respiratory diseases such as asthma and COPD.

The Rho-kinase pathway, another Ca^{2+} sensitizing mechanism, is involved in pathologic conditions. Several studies show that the activation of Rho-kinase plays a critical role in bronchial reactivity (5, 31, 34). Treatment with proinflammatory cytokines was shown to activate the Rho-kinase pathway directly, leading to enhanced Ca^{2+} signaling in primary cultures of ASM cells (31). Moreover, the increased expression and activation of Rho-A in hyperresponsive bronchial smooth muscle were also demonstrated in rodents, and may be responsible for the enhanced reactivity of bronchial responses (35, 36). Results obtained in the presence of a Rho-kinase activator such as U46619 suggest that CRBM-0244 interacts with this pathway to reduced bronchial hyperreactivity trigger via TNF- α . Complementary experiments

were performed to assess the possibility that CRBM-0244 interacts with the Rho-kinase pathway to reduced Ca^{2+} sensitivity in TNF- α -treated human bronchi. Our data demonstrate that treatment with CRBM-0244 reduced the U46619-induced Ca^{2+} sensitivity of TNF- α -treated human bronchi (see Figure E3).

CRBM-0244 Is Metabolized by Lipoxygenase Pathways in TNF- α -Treated Bronchi

Recent studies identified a novel group of DHA-derived trihydroxydicosahexanoic 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 (37), although no data are available to describe their respective effects and roles in the lung. The metabolism of DHA via a series of steps, several of which involve 5-lipoxygenase and 15-lipoxygenase, generates a dihydroxydicosatriene termed protectin-D1, another potent anti-inflammatory molecule (37). Indeed, the generation of resolvins and protectins could represent important anti-inflammatory compounds in the mode of action of n-3 PUFAs. Such mediators may also play a potent protective role in asthmatic disorders. Our data strongly suggest that CRBM-0244 is metabolized by lipoxygenases to mediate these anti-inflammatory effects and to reduce the AHR triggered by TNF- α in human bronchi, as shown by combined treatments with 5-lipoxygenase and 15-lipoxygenase inhibitors. In addition, we demonstrate for the first time, to the best of our knowledge, that resolvin-D1 (RD1) and protectin-D1 (PD1) prevent bronchial hyperresponsiveness and Ca^{2+} hypersensitivity, primarily through their action in reducing the overexpression of COX-2 and CPI-17, suggesting that these metabolites are involved in the resolution of lung inflammation in this *in vitro* model of human bronchial inflammation. Moreover, in a murine model of chronic asthma,

the administration of PD1 before aeroallergen challenge resulted in a decrease in airway eosinophil and T-lymphocyte recruitment, as well as in reduced concentrations of specific pro-inflammatory mediators, including IL-13, cysteinyl leukotrienes, and prostaglandin D-2 (38). The EPA-derived resolvin-E1 also displays anti-inflammatory effects when administered in a murine model of allergic asthma (39). Further investigations using high performance liquid chromatography coupled to tandem mass spectrometry would be required to determine which metabolite is responsible for the effects of CRBM-0244 in human bronchi.

CRBM-0244 Displays Anti-Inflammatory Effects in an Allergic Model of Asthma

The prevalence of asthma continues to increase, despite advances in treatment options. Alternative preventive treatments aimed at reducing the dose requirements of pharmacologic interventions would be beneficial, and may reduce the public health burden of this disease (2). Of interest, concentrations of DHA in the respiratory tract are decreased in asthma and other diseases of excess airway inflammation, such as cystic fibrosis (40). Epidemiologic studies suggest that a diet high in marine fatty acids (fish oil) may exert beneficial effects on inflammatory conditions, including asthma (41), and that dietary supplementation with omega-3 fatty acids in children prevents the development of atopic cough, a symptom of allergic airway inflammation (42). The underlying mechanisms for the beneficial properties of omega-3 fatty acids in asthma remain to be established. An *in vitro* model of allergic asthma was used here to determine the impact of CRBM-0244 treatment on airway inflammation and hyperresponsiveness. Pulmonary inflammation is characterized by the infiltration of leukocytes, such as eosinophils and lymphocytes (21). Here, reduced numbers of eosinophils and leukocytes were quantified in BAL fluid recovered from OVA-sensitized guinea pigs receiving oral doses of CRBM-0244. Moreover, the production of airway mucus was normalized in CRBM-0244-treated animals. In asthma, the expression of multiple inflammatory proteins is increased 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 (20). Our data demonstrate that treatment with CRBM-0244 largely decreased concentrations of TNF- α in the BAL fluid of OVA-sensitized guinea pigs. Hence the overexpression of COX-2 inflammatory enzyme was prevented in animals receiving an oral administration of CRBM-0244. Altogether, these results indicate that CRBM-0244 exerts potent anti-inflammatory properties and prevents allergen-induced pulmonary inflammation. Furthermore, our data attest that treatment with CRBM-0244 significantly reduces serum concentrations of OVA-IgE, demonstrating that this new DHA monoglyceride derivative is able to reduce allergen sensitization. Such data could strengthen the relevance of using CRBM-0244 as a preventive and therapeutic agent in treating allergic asthma.

This study also demonstrated that the oral of CRBM-0244 in guinea pig resulted in a marked reduction of AHR to several agonists. Pharmacologic concentrations of this compound could exert direct effects on ASM responses to allergen challenge. We also established that treatment with CRBM-0244 reduces the Ca²⁺ sensitivity of ASM, an important characteristic of the asthmatic airway. To our knowledge, this is the first study to demonstrate that the oral administration of CRBM-0244 to OVA-sensitized guinea pigs reduces the expression of a proinflammatory CPI-17 protein implicated in AHR, suggesting a relevant role for CRBM-0244 in treating asthma. The results of a recent clinical trial in patients with asthma challenged long-held views regarding the relationship between airway inflammation and bronchial hyperresponsiveness (43). However, the results to date are in-

sufficient for developing methods to prevent both of these phenotypes. Consequently, the use of CRBM-0244 may be of clinical interest in preventing both lung inflammation and AHR through interactions with NF- κ B and PPAR- γ transcription factor in the lung, without resorting to corticosteroid compounds.

In conclusion, the present data provide relevant evidence regarding the mode of action of CRBM-0244 in human airways and in a model of allergic asthma. This DHA monoglyceride may represent a new and prospective nutraceutical compound of medicinal interest in modulating the inflammation of patients with asthma.

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