UNIVERSITAT DE
BARCELONA

Study of the expression and regulation of the autocrine loop components of the cyclooxygenase pathway and their implication in aspirin exacerbated respiratory disease

Estudio de la expresión y regulación de los componentes del bucle de retroalimentación autocrina de la vía de la ciclooxigenasa y su implicación en la enfermedad respiratoria exacerbada por antiinflamatorios no esteroides

Liliana Sofia Machado de Carvalho

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Tesis presentada por

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Para obtención del título de doctor por la Universidad de Barcelona

Dirigida por

Dr. César Picado i Vallés **Dr. Jordi Roca Ferrer**

Programa de Doctorado en Biomedicina
Facultad de Medicina, Universidad de Barcelona
2015

Aos meus seres mais queridos

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Trabajo realizado en el Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)

ACKNOWLEDGEMENTS

A meta aproxima-se e chega o momento em que finalmente entendes os agradecimentos de uma tese de doutoramento. É nesta fase que paras e te apercebes da importância de todas as pessoas que estiveram ao "teu lado" (embora nem sempre fisicamente) ao longo deste percurso. Por isso, queria dedicar a todas essas pessoas estes agradecimentos. É verdade que nem sempre há palavras para descrever sentimentos. E palavras, muitas vezes, não passam de palavras… Mas, neste caso, e para as pessoas que me conhecem, sabem que são de coração!

Em primeiro lugar, quero agradecer ao César, o meu diretor de tese, por me ter acolhido neste grupo de maneira tão carinhosa, pela confiança que depositou em mim desde o princípio (sem nem sequer me conhecer), pela oportunidade de trabalhar num projeto e de ganhar experiência na investigação, pela orientação, pela dedicação e por ter estado sempre presente ao longo destes cinco anos, obrigada. Aproveitando as tuas palavras; também acho que formámos uma boa equipa!

Também quero agradecer ao meu segundo diretor de tese, Jordi, por me teres ensinado a confiar mais nas minhas capacidades científicas e por me teres tornado uma pessoa mais independente no mundo da investigação!

Quero agradecer à Laura e ao Quim pela disponibilidade demonstrada na hora de resolver qualquer dúvida ou problema e por terem contribuído na minha formação científica. Ao Isam já que sem a matéria-prima fornecida por ele, este trabalho nunca poderia ter sido possível! Quero agradecer-te especialmente pelos teus conselhos e ajuda médica porque a elaboração de uma tese (e não só) com amigdalites constantes estava-se a tornar um pesadelo! Também quero agradecer à enfermeira Sílvia pela amabilidade e hospitalidade! Agradecer ao serviço de pneumologia e alergia (Joan, Jaime, Rosa, Maria del Carmen, Adriana) por toda a vossa ajuda, carinho e apoio!

Um agradecimento especial a duas grandes investigadoras, Rosa Torres e Marga, que tornaram possível a elaboração e publicação de trabalhos científicos importantes. Obrigada pelo vosso apoio, pelas vossas palavras, pela dedicação e disponibilidade que sempre demonstraram! Aprendi muito com a vossa experiência. Obrigada por me ajudarem a crescer como cientista!

Também tenho que agradecer esta tese a todos os meus companheiros (sendo alguns deles grandes amigos e a família que não tinha aqui) de laboratório, os que atualmente ainda estão presentes e os que já passaram por aqui e deixaram uma grande marca. Tendo sendo a última a chegar (e sendo de fora) pude ter o privilégio de aprender muito com todos eles. Obrigada por me terem ensinado tanto e sem muitos de vocês o estar aqui teria sido muito duro! Por isso e por tantas outras coisas quero agradecer especialmente ao Borja, à Mireya e à Marta! Também quero agradecer à Laura F. pela tua boa disposição e tranquilidade, à Mercê pela tua amizade e por teres sido sempre uma pessoa afável e finalmente à Maria pela paciência que tiveste comigo no início! Obrigada a todos.

Ao longo deste doutoramento tive a oportunidade de partilhar o meu local de trabalho com pessoas fantásticas! Quero agradecer muito carinhosamente a cada membro do grupo UTR: Dani, Natália, Valeria, Mari, Marta, Victor, Jordi, Elisenda. Obrigada por me terem incluído tão carinhosamente no vosso grupo. Com alguns de vocês tive a oportunidade de desenvolver uma relação de amizade à qual dou muito valor e que nunca irei esquecer! Obrigada por me ouvirem e obrigada por confiarem em mim…obrigada pelos vossos conselhos…obrigada pelos almoços, saídas e gargalhadas! Obrigada por tudo! Neste bom ambiente, também quero incluir a Eli e a Anna, pelas longas conversas e desabafos, pelo carinho e amizade que demonstraram, por terem sempre uma palavra calorosa nos momentos mais delicados! Obrigada meninas! Quero agradecer também ao grupo de cardiologia (Cira, Nádia, Manel, Quim, Montserrat) que durante tanto tempo foram os nossos vizinhos de laboratório! Obrigada pelo companheirismo e pela amizade que desenvolvi com alguns de vocês.

Fora do ambiente de trabalho, quero agradecer especialmente a várias pessoas. Quero agradecer-te Sandra por teres sido a amiga que és e por teres estado a meu lado sempre! Obrigada pela tua amizade e carinho! Obrigada Mari, sem dúvida és uma mulher de armas pela qual tenho uma grande admiração, a tua presença faz-me bem. Obrigada por tudo e levo-te no meu coração! Obrigada Cira por tudo e mais alguma coisa, tornaste-te uma pessoa indispensável na minha vida, acho que sabes disso. Sempre presente para tudo…o meu anjo da guarda! És uma pessoa muito especial pela qual tenho um carinho imensurável! Marta agradeço-te os bons momentos que passámos juntas, a tua amizade, a partilha e o teu carinho! Recordações que ficarão sempre no meu coração. Mireya, a minha confidente, o meu porto seguro em muitas ocasiões. Foste, és e serás sempre uma grande amiga para mim! Admiro a tua força!

Na verdade, não sei nem como agradecer tudo o que fizeste por mim… Mas acho que não é nem preciso… Tu já sabes! Lucía, apesar de te conhecer há pouco tempo, agradeço-te tudo o que partilhamos! Obrigada pelo teu apoio e amizade! Xavi, sem ti esta tese não teria cara! Por isso e pela boa disposição que transmites, um muito obrigada! Também não me posso esquecer “del equipo”! Obrigada pelo companheirismo, foram sem dúvidas bons momentos para sempre recordar (apesar das derrotas)!

Quero agradecer a toda a minha família (Nuno, já és da família por isso também estás incluído), aos mais ou menos presentes, todo o apoio, toda a paciência e todo o carinho que sempre demonstraram por mim! Um agradecimento muito especial aos meus pais e à minha irmã que estiveram sempre a meu lado apesar da distância, pela vossa dedicação, pelo vosso amor, pelo vosso apoio, pela vossa paciência, por me ajudarem a ser uma pessoa mais forte, por me motivarem, por serem como são, obrigada de todo o meu coração! Bruno, quero agradecer-te tudo o que fizeste por mim, por nós, ao longo destes anos. Sei que nem sempre foi fácil, mas sempre me deste apoio, carinho, amor, e força para não desistir e continuar a lutar por este objetivo. Obrigada pela paciência e obrigada por fazeres parte da minha vida!

Quem disse que escrever os agradecimentos era a parte mais fácil bem me enganou! Espero não me ter esquecido de ninguém! Mas se me esqueci, esse esquecimento apenas se reflete no papel, não no coração!

<<Even the largest avalanche is triggered by small things>>

Vernor Vinge

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ABBREVIATIONS

ABBREVIATIONS

AA: arachidonic acid

AC: adenyl cyclase

AERD: aspirin exacerbated respiratory disease

ASA: acetylsalicylic acid

α-sma: α-smooth muscle actin

BSA: bovine serum albumin

cAMP: cyclic adenosine monophosphate

CF: cystic fibrosis

cPGES: cytosolic prostaglandin E synthase

cPLA2: cytosolic phospholipase A2

COPD: chronic obstructive pulmonary disease

COX: cyclooxygenase

CRS: chronic rhinosinusitis

CRSsNP: chronic rhinosinusitis without nasal polyps

CRSwNP: chronic rhinosinusitis with nasal polyps

CT: computerized tomography

cys-LT: cysteinyl leukotriene

DMEM: Dulbecco's Modified Eagle Media

DTT: dithiothreitol

ECP: eosinophilic cationic protein

EdU: 5-ethynyl-2'-deoxyuridine

ELISA: enzyme-linked immunosorbent assay

EMEM: Eagle's Minimal Essential Media

EP: E-prostanoid

Escherichia coli: *E. coli*

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

GM-CSF: granulocyte-macrophage colony-stimulating factor

GPCR: G-protein-coupled membrane receptor

IBMX: 3-isobutyl-1-methylxanthine

IFN: interferon

Ig: immunoglobulin

IkB: inhibitor of kappa B

IKK: I kappa B kinase

IL: interleukin

IL-1R: interleukin-1 receptor

IL-1Ra: interleukin-1 receptor antagonist

IL-1RAcP: interleukin-1 receptor accessory protein

IL-1RI: interleukin-1 receptor type I

IL-1RII: interleukin-1 receptor type II

IRAK: interleukin-1 receptor-associated kinase

kDa: kilodalton

LB: Luria-Bertani

LO: lipoxygenase

LT: leukotriene

MCP: monocytes chemotactic protein

mPGES: microsomal prostaglandin E synthase

MyD88: myeloid differentiation primary response gene 88

NF-kB: nuclear factor-kappa B

NM: nasal mucosa

NM-C: nasal mucosa of control subjects

NP: nasal polyp

NP-AERD: nasal polyps of patients with aspirin exacerbated respiratory disease

NSAID: non-steroidal anti-inflammatory drug

PBS: phosphate-buffered saline

PDE: phosphodiesterase

PG: prostaglandin

PGES: prostaglandin E synthase

PKA: protein kinase A

RANTES: regulated upon activation in normal T cells expressed and secreted

SDS: sodium dodecyl sulphate

SFM: serum free media

siRNA: small interfering RNA

SNP: single-nucleotide polymorphism

Staphylococcus aureus: *S. aureus*

Th: T helper

TIR: toll-Interleukin-1

TLR: toll-like receptor

TNF: tumour necrosis factor

T-PBS: Tween®20 in PBS

INTRODUCTION

CHAPTER I: THE RESPIRATORY SYSTEM

1. The Airways

1.1. Anatomy of the respiratory tract

The main and primary function of the respiratory apparatus is gas exchange between inhaled air and the circulatory system, i.e. oxygen from the external environment is transferred into our bloodstream, while carbon dioxide is expelled into the outside air. Anatomically, the respiratory tree is divided into two parts (**Figure 1**): the upper respiratory tract and the lower respiratory tract; the larynx is the portion that separates the two areas. The upper respiratory tract comprises the organs located outside of the chest cavity area, i.e. it begins with the nostrils and the mouth and ends with the larynx. In turn, the lower respiratory tract includes the trachea, the bronchi, the bronchiole, the alveolar duct, and the alveoli (Tu *et al.*, 2013).

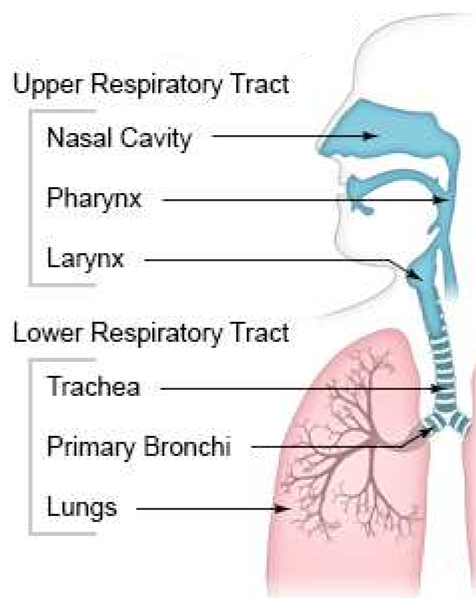


Figure 1. Schematic representation of the respiratory tree (adapted from: http://training.seer.cancer.gov/anatomy/respiratory/passages/; accessed July 28, 2015).

The nasal cavity is limited by bones and is divided into two separate compartments by the nasal septum, a vertical wall that offers support to the nose. A visible part, the vestibule, and an inner part, which represents the main portion of the nasal cavity, form the nose. In the main nasal cavity, three projections (superior, middle, and inferior) form the nasal turbinates (or *conchae*) and they increase the inner surface of the nose, while reducing the lumen at the same time (Jones, 2001). This conformation allows a close contact between the inhaled air and the nasal mucosa, resulting in humidification and heating of the air, which maintains the normal function of the lungs (Sahin-Yilmaz *et al.*, 2011; Jones, 2001; Jankowski, 2011). Additionally, the turbulent flow of the air allows the deposition of smallest particles, which are entrapped by the nasal mucosa (NM) of turbinates and nasal septum and transported to the nasopharynx by the mucociliary system. Thus, this area of the respiratory tract has an action of filtration as well. The nasal sinuses, which communicate with the nasal cavity, are involved in thermal regulation of the inhaled air (Ciprandi *et al.*, 2012).

After the nasal cavity, the pharynx communicates with the larynx, which is the organ of phonation, but it is also a valve that protects the airways and the lungs from a possible passage of foreign bodies (Baroody, 1999). The trachea, formed by regular cartilaginous rings without a back cartilaginous wall, is the area where the pharynx ends. This particular configuration allows the trachea to prevent an abnormal rise of pressure that occurs in some situations. Distally, the trachea is divided into two bronchi (right and left), which divide into bronchioles. After the terminal bronchioles, there are the respiratory bronchioles and the air cells that constitute the area of the lungs with the function of gas exchange (Ciprandi *et al.*, 2012).

1.2. United airways

The upper and lower respiratory airways form a *continuum* and share anatomical and histological properties. In fact, the surface of the whole respiratory tract is protected by the airway mucosa (Gaga *et al.*, 2001). This structure is composed of a respiratory epithelium with an underlying submucosa layer, equipped with a variety of innate and acquired immune defence mechanisms, being the first barrier against external foreign agents. In both upper and lower respiratory tracts, the mucosa is lined with a pseudostratified columnar epithelium which contains ciliated and non-ciliated columnar cells, secretory cells, and basal cells. The bronchioles are lined with a cuboidal simple epithelium (Ciprandi *et al.*, 2012; Sahin-Yilmaz *et al.*, 2011).

The nose is a very complex organ with multiple functions that include not only olfaction, but also the conditioning (e.g., humidifying, warming, and filtering) of inhaled air to maintain homeostasis in the lower airways (Harkema *et al.*, 2006). In pathological conditions involving the upper respiratory tract, the impairment of the regular functions of the nose can affect the lower airways, leading to the increase exposure of this portion to allergens with the consequent inflammation of the airways (**Figure 2**) (Braunstahl, 2009; Braunstahl, 2011).

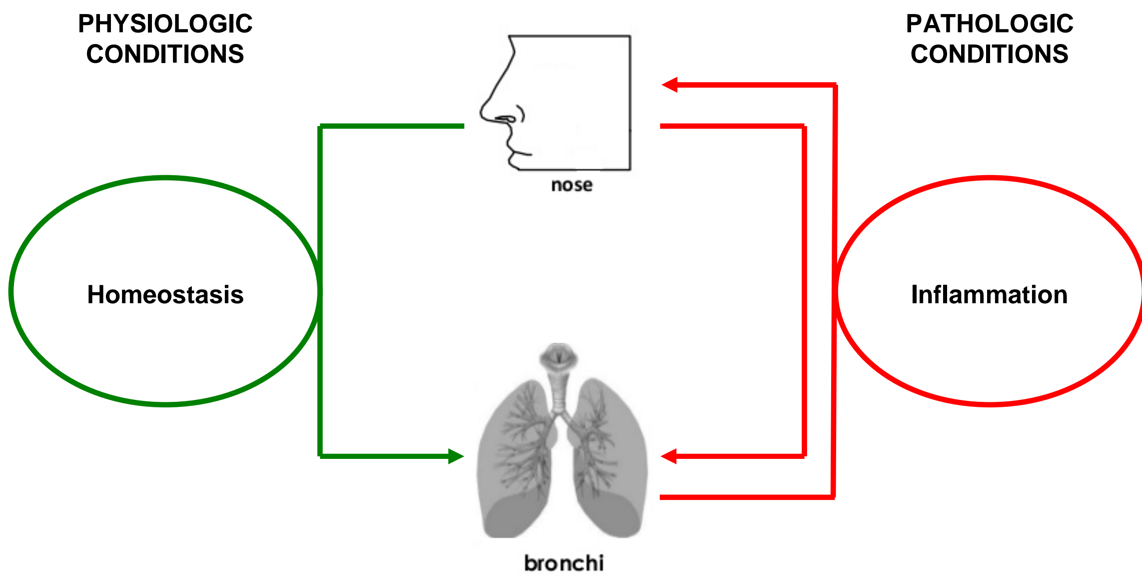


Figure 2. Schematic representation of the association between upper and lower respiratory tracts in physiologic and pathologic conditions.

The association between upper and lower respiratory tracts has been described in numerous studies and the concepts of “one airway disease” or “united airways disease” have gained strength over the past years (Bousquet *et al.*, 2003; Bousquet *et al.*, 2008; Fokkens *et al.*, 2012; Ciprandi *et al.*, 2012). In fact, epidemiological data and clinical observations demonstrate a strong interaction between upper (allergic rhinitis, chronic rhinosinusitis (CRS), and nasal polyps (NPs)) and lower (asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis) airway diseases (**Figure 3**) (Bousquet *et al.*, 2008; Fokkens *et al.*, 2012). Thus, and despite the knowledge concerning this link is still limited and incomplete, the evidence that support the unity of the respiratory tract are the pillars for the diagnosis, clinical evaluation and, management of airway inflammation.

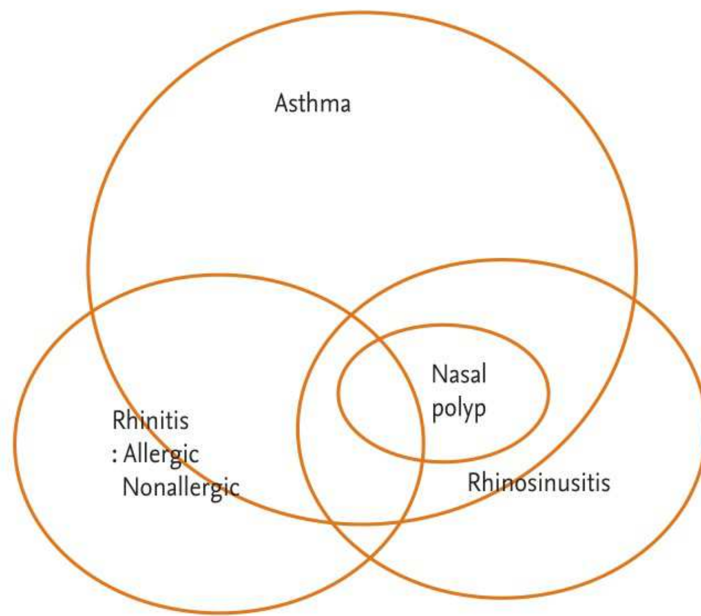


Figure 3. Global airway disease (Jang, 2013).

CHAPTER II: AIRWAY INFLAMMATORY DISEASES

1. Asthma

1.1. Introduction

Asthma is a multifactorial and one of the most common chronic inflammatory diseases of the airways affecting 1 to 18% of the population in different countries. The knowledge that asthma is an inflammatory disorder has become a core fundamental in the definition of this disorder. Asthma is characterized by variable symptoms including wheeze, shortness of breath, chest tightness and/or cough, and by variable expiratory airflow limitation. Both symptoms and airflow limitation characteristically vary over the time and in intensity. These variations are often triggered by factors such as exercise, allergen or irritant exposure, change in weather, or viral respiratory infections (Global Initiative for Asthma 2015; Kim *et al.*, 2011; Myers *et al.*, 2011).

Symptoms and airflow limitation may resolve spontaneously or in response to medication, and may sometimes be absent for weeks or months at a time. In turn, patients can experience episodic exacerbations of asthma that may be life-threatening and carry a significant burden to patients and the community. Asthma is usually associated with airway hyperresponsiveness to direct or indirect stimuli, and with chronic airway inflammation. These characteristics frequently persist, even when symptoms are absent or lung function is normal, but may normalize with treatment (Global Initiative for Asthma 2015).

Over the past 30–40 years, asthma prevalence has increased within numerous industrialized countries; however, recent statistics suggest that a plateau has been reached in the frequency of the disease. Besides the remarkable advancements made in asthma pathogenesis field, the aetiology and basis of airway inflammation still remain somewhat obscure (Myers *et al.*, 2011).

1.2. Epidemiology

Epidemiologically, asthma is a heterogeneous chronic respiratory disease that affects over 300 million of people worldwide, who will probably become more than 400 million by 2020 (Pelaia *et al.*, 2015). Many adults with asthma will have experienced symptoms for the first time in childhood. Age-specific incidence rates are not as commonly reported as prevalence estimates. Furthermore, because asthma is a disorder that has a relapse and remitting clinical course, it may be difficult for study individuals to accurately recall the age when symptoms began for the very first time (Jarvis, 2014). Asthma affects all ages and has been considered as a serious challenge to public health, since its direct and indirect costs are high. Poor asthma control contributes to unnecessary morbidity, limitations to daily activities and impairments in overall quality of life. Although asthma is often believed to be a disorder localized to the lungs, current evidence indicates that it may represent a component of systemic airway disease involving the entire respiratory tract (Kim *et al.*, 2011).

1.3. Asthma phenotypes

Recognizable clusters of demographic, clinical, and/or pathophysiological characteristics are often called “asthma phenotypes”. Many phenotypes have been identified and are represented in **Figure 4** (Bel, 2004; Moore *et al.*, 2012; Wenzel, 2012).

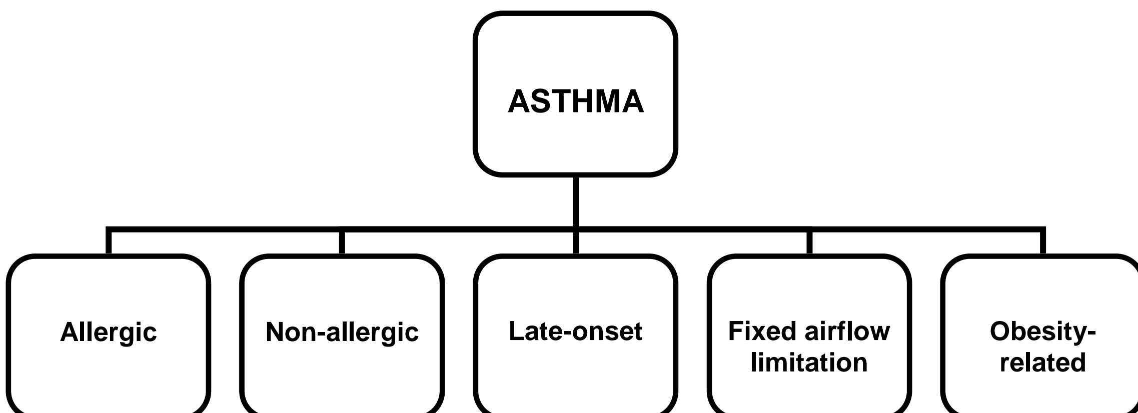


Figure 4. Diagram representation of “asthma phenotypes”.

1.4. Diagnosis

The diagnosis of asthma involves a thorough medical history, physical examination, and objective assessments of lung function (spirometry preferred) to confirm the diagnosis. Bronchoprovocation challenge testing and assessing for markers of airway inflammation may also be helpful for diagnosing the disease (Levy *et al.*, 2009). The different steps in the diagnosis of asthma in adults (Reddel *et al.*, 1999; Miller *et al.*, 2005; Kaplan *et al.*, 2009; Lougheed *et al.*, 2010; Global Initiative for Asthma 2015) are shown in **Figure 5**.

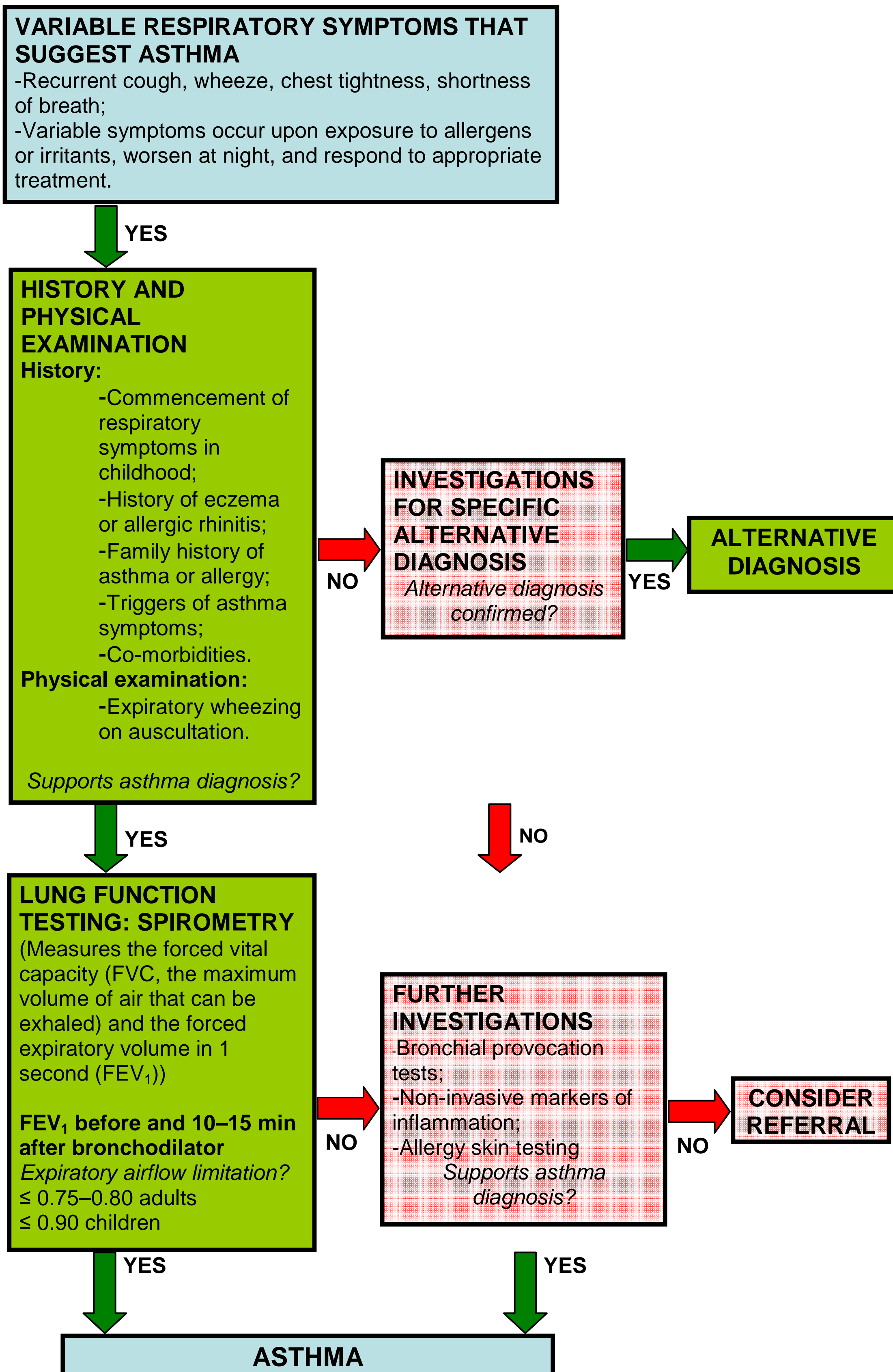

VARIABLE RESPIRATORY SYMPTOMS THAT SUGGEST ASTHMA
-Recurrent cough, wheeze, chest tightness, shortness of breath;
-Variable symptoms occur upon exposure to allergens or irritants, worsen at night, and respond to appropriate treatment.
YES
HISTORY AND PHYSICAL EXAMINATION
History:
-Commencement of respiratory symptoms in childhood;
-History of eczema or allergic rhinitis;
-Family history of asthma or allergy;
-Triggers of asthma symptoms;
-Co-morbidities.
Physical examination:
-Expiratory wheezing on auscultation.
Supports asthma diagnosis?
NO
INVESTIGATIONS FOR SPECIFIC ALTERNATIVE DIAGNOSIS
Alternative diagnosis confirmed?
YES
ALTERNATIVE DIAGNOSIS
YES
NO
LUNG FUNCTION TESTING: SPIROMETRY
(Measures the forced vital capacity (FVC, the maximum volume of air that can be exhaled) and the forced expiratory volume in 1 second (FEV_1))
FEV_1 before and 10–15 min after bronchodilator
Expiratory airflow limitation?
≤ 0.75–0.80 adults
≤ 0.90 children
NO
FURTHER INVESTIGATIONS
-Bronchial provocation tests;
-Non-invasive markers of inflammation;
-Allergy skin testing
Supports asthma diagnosis?
NO
CONSIDER REFERRAL
YES
YES
ASTHMA

Figure 5. Steps in the diagnosis of asthma in adults (Reddel *et al*., 1999; Miller *et al*., 2005; Kaplan *et al*., 2009; Lougheed *et al*., 2010; Global Initiative for Asthma 2015).

1.5. Pathogenesis and pathophysiology of asthma

In asthma, the limitation of the airflow is recurrent and is caused by a variety of changes in the airway. These include:

- **Bronchoconstriction**

The main physiological event that leads to clinical symptoms in asthma is airway narrowing, which interferes with normal airflow. The bronchoconstriction is a process that occurs quickly to narrow the airways in response to a variety of stimuli (allergens or irritants). It involves the contraction of bronchial smooth muscle. Allergen-induced bronchoconstriction results from an immunoglobulin (Ig)E-dependent release of mediators from mast cells that directly contract airway smooth muscle (Busse *et al*., 2001).

- **Airway edema**

The persistent and progressive inflammation that accompanies the disease contributes to limit the airflow through many factors. These include edema, inflammation and the formation and hypersecretion of mucus, as well as structural changes including hypertrophy and hyperplasia of the airway smooth muscle (Barnes, 1996).

- **Airway hyperresponsiveness**

This feature is an exaggerated bronchoconstrictor response to a wide variety of stimuli. The multiple mechanisms influencing airway hyperresponsiveness include inflammation, dysfunctional neuroregulation, and structural changes (Barnes, 1996; Rees, 2011).

- **Airway remodeling**

In some asthma patients, airflow limitation may be only partially reversible. Effectively, permanent changes at a structural level can occur in the airway, contributing to the

progressive loss of lung function. This whole process involves the activation of many structural cells, with consequent permanent changes in the airway increasing airflow obstruction and airway responsiveness. At this level, structural changes consist of thickening of the sub-basement membrane, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia and hypersecretion (Rees, 2011).

1.6. Pathophysiologic mechanisms in the development of airway inflammation

The process of airway inflammation that plays a central role in the pathophysiology of asthma (**Figure 6**) involves an interaction of many cell types and multiple mediators with the airways that eventually results in the characteristic features of the disease: bronchial inflammation and airflow limitation which lead to recurrent episodes of cough, wheeze, and shortness of breath. The pattern of airway inflammation presents in asthma does not necessarily vary depending upon disease severity, persistence, and duration of disease (Myers *et al.*, 2011).

1.6.1. Cellular profile

- **Lymphocytes**

T lymphocytes subpopulations, which include T helper (Th) 1 and Th2 cells, play a major role on airway inflammation. In human asthma, a shift toward the Th2-cytokine profile results in eosinophilic inflammation (Cohn *et al.*, 2004). The activation of this response leads to the production of specific cytokines such as interleukin (IL)-4, IL-5, and IL-13 which could contribute to explain the overproduction of IgE, presence of eosinophils, and development of airway hyperresponsiveness. The reduced number of regulatory T cells, which normally inhibit Th2 cells, and the increment in natural killer cells, that release large amounts of Th1 and Th2 cytokines, are also observed in asthma (Akbari *et al.*, 2006; Larché *et al.*, 2003). T lymphocytes also can determine the development and degree of airway remodeling.

- **Mast cells**

Activation of mast cells, which are very abundant in the airways, is the process responsible to the release of bronchoconstrictor mediators, such as histamine, cysteinyl leukotrienes (cys-LTs), prostaglandin (PG) D_2, and platelet-activating factor (Boyce, 2003; Galli *et al.*, 2005). Mast cells may be activated either through high-affinity IgE receptors or by osmotic stimuli to account for exercise-induced bronchospasm. High numbers of mast cells in airway smooth muscle may contribute to airway responsiveness (Brightling *et al.*, 2002). Moreover, the activation of these cells also can release a large number of cytokines to change the airway environment and promote inflammation.

- **Eosinophils**

Several patients who suffer from asthma show increased numbers of eosinophils in the airways (Sampson, 2000; Williams, 2004). Eosinophilic inflammation can be associated with the whole spectrum of asthma severity, ranging from mild-to-moderate to severe uncontrolled disease (Pelaia *et al.*, 2015). Eosinophils contain inflammatory enzymes, generate leukotrienes (LTs), and express a variety of pro-inflammatory cytokines.

- **Neutrophils**

Neutrophils are increased in the airways and sputum of some patients who have severe asthma, during acute exacerbations, and in the presence of smoking (Fahy *et al.*, 1995). Neutrophilic airway inflammation is triggered by Th1 and especially Th17 lymphocytes (Pelaia *et al.*, 2015).

- **Dendritic cells**

Dendritic cells play critical roles in initiating and directing immune responses, serving as sentinels at the mucosal surfaces, where they constantly sample the antigens at the interface between the external and internal environment (Gill, 2012). In fact, these cells are professional antigen-presenting cells that have the capacity to capture foreign antigen, migrate to the draining lymph nodes, present antigen to naïve T cells and initiate an immune response (Kuipers *et al.*, 2004; Van Helden *et al.*, 2013).

- **Macrophages**

Macrophages can be activated by allergens through low-affinity IgE receptors to release inflammatory mediators and cytokines amplifying the inflammatory response (Peters-Golden, 2004).

- **Epithelial cells**

The generation of inflammatory mediators, recruitment and activation of inflammatory cells, and infection by viruses can induce epithelial cells to produce more inflammatory mediators or to injure the epithelium itself. The abnormal process of repair after epithelium injury frequently observed in asthma can promote the obstructive lesions that occur in this disorder (Polito *et al.*, 1998).

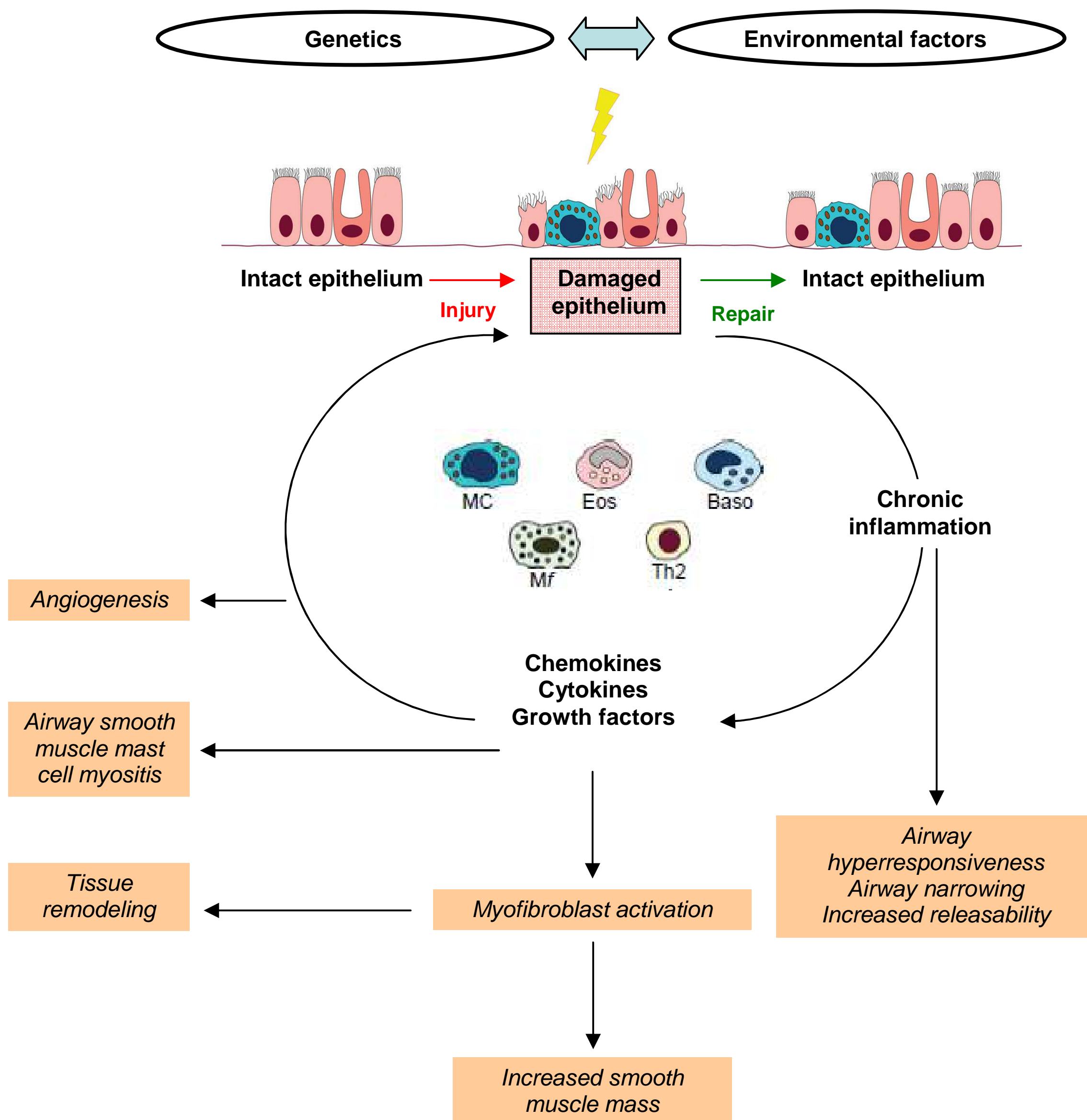


Figure 6. Pathogenic mechanisms of asthma (**Adapted from: Kroegel, 2009**). In genetically predisposed individuals, environmental factors such as allergens, infections or irritants may induce epithelial damage that leads to a deregulated immune response. Several cells including T lymphocytes (Th2 cells), mast cells (MC), eosinophils (Eos), basophils (Baso) and macrophages (Mf) are activated in the airways of asthmatics and secrete mediators responsible for persisting inflammation, bronchoconstriction and airway remodeling.

1.6.2. Inflammatory mediators

- **Chemokines**

Mainly expressed by epithelial cells, these mediators are important in the recruitment of inflammatory cells into the airways. Chemokines are potent leukocyte chemoattractants, cellular activating factors, and histamine-releasing factors, which makes them particularly important in the pathogenesis of allergic inflammation. In particular, the eotaxin subfamily of chemokines and their receptor CC chemokine receptor 3 have emerged as central regulators of the asthmatic response (Zimmermann *et al.*, 2003).

- **Cytokines**

These mediators play an important role in the coordination and persistence of inflammation in asthma, determining its severity (Mahajan *et al.*, 2006). **Table 1** summarizes the effects of the lymphokines (mainly produced by T lymphocytes) and **Table 2** the pro-inflammatory cytokines in asthma.

Table 1. Summary of cellular effects of lymphokines in asthma (Mahajan *et al.*, 2006).

Limphokines	Effects
IL-2	• Eosinophilia *in vivo* • T cell growth and differentiation
IL-3	• Eosinophilia *in vivo* • Pluripotential hematopoietic factor
IL-4	• Eosinophil growth ↑ • Th2 cells ↑; Th1 cells ↓; IgE ↑
IL-5	• Eosinophil: maturation ↑, apoptosis ↓ • IgE ↑; Th2 cells ↑ • Broncho-hyperresponsiveness
IL-13	• Eosinophil: activation ↑, apoptosis ↓ • IgE ↑
IL-15	• Eosinophilia *in vivo* • T cell growth and differentiation
IL-16	• Eosinophil migration ↑ • T cell growth factor and chemotaxis
IL-17	• T cell proliferation • Epithelial cell activation • Fibroblast activation

Table 2. Summary of cellular effects of pro-inflammatory cytokines in asthma (Mahajan *et al.*, 2006).

Pro-inflammatory cytokines	Effects
IL-1	• Adhesion to vascular endothelium ↑ • Th2 cell growth factor • B cell growth factor • Neutrophil chemoattractant • T cell and epithelial cell activation ↑ • Eosinophil accumulation *in vivo* • Broncho-hyperresponsiveness ↑
TNF-α	• Epithelium activation ↑ • Broncho-hyperresponsiveness ↑

IL-6	• T cell growth factor • B cell growth factor • IgE ↑
IL-11	• B cell growth factor • Fibroblast ↑ • Broncho-hyperresponsiveness ↑
GM-CSF	• Eosinophil: activation ↑, apoptosis ↓ • LTs release ↑ • Hematopoietic cell proliferation and maturation ↑ • Endothelial cell migration ↑

- **Cysteinyl leukotrienes**

Potent bronchoconstrictors derived mainly from mast cells, being that their inhibition is associated with lung function and asthma symptoms improvement (Busse, 1996; Leff, 2001).

- **Immunoglobulin E**

IgE is responsible for the activation of allergic responses and for the development/persistence of inflammation. It binds to cell surfaces via a specific receptor and contributes to the release of a wide variety of mediators from activated cells to initiate bronchospasm and also to release pro-inflammatory cytokines to perpetuate inflammation (Boyce, 2003b).

1.7. Management and therapies

There are two long-term goals of asthma management: *(1)* to achieve good control of symptoms and maintain normal activity levels and *(2)* to minimize future risk of exacerbations, fixed airflow limitation and side-effects. The accomplishment of these main points is only possible if the person with asthma (or the caregiver) develop a partnership with health care providers. Therefore, the education of the patient, i.e. the acquisition of knowledge, confidence, and skills to assume a major role in the management of its own condition, is crucial and contributes to the reduction of asthma morbidity in both children and adults. In most patients with asthma, control can be

achieved through the use of both avoidance measures (avoidance to relevant allergens/irritants) and pharmacological interventions (Kim *et al.*, 2011).

The pharmacological agents commonly used for the treatment of asthma can be classified as (Lougheed *et al.*, 2010; Kaplan *et al.*, 2009):

- **Controllers**

Medication that need to be taken daily on a long-term basis to reduce airway inflammation, control symptoms, and reduce future risks, such as exacerbations and decline in lung function. They include inhaled corticosteroids, LT receptor antagonists, long-acting beta$_2$-agonists in combination with an inhaled corticosteroids, and anti-IgE therapy.

- **Relievers**

Medication used on as-needed relief of bronchoconstriction and symptoms. They include rapid-acting inhaled beta$_2$-agonists and inhaled anticholinergics.

2. Chronic rhinosinusitis and nasal polyposis

2.1. Introduction

CRS is a heterogeneous disease that is frequently divided into two groups based on the presence or absence of NPs: CRS with NPs (CRSwNPs) and CRS without NPs (CRSsNPs). Both forms are characterized by local and persistent inflammation of the airway mucosa of the nose and paranasal sinuses (Kato, 2015). CRS is a public health problem that has a significant socio-economic impact. However and despite being a common condition encountered in medicine, the etiology and pathogenesis of this disease still remain unknown (Bachert *et al.*, 2014).

CRS in adults is, in most guidelines, defined as an inflammatory condition of the nose and the paranasal sinuses characterized by at least 8–12 weeks of at least 2 symptoms: *(1)* nasal blockage/obstruction/congestion or nasal discharge (anterior/posterior nasal drip), *(2)* facial pain/pressure, *(3)* reduction or loss of smell (Fokkens *et al.*, 2012).

2.2. Epidemiology

Estimates of the prevalence of CRS worldwide vary significantly, in parte because of differences in the diagnostic criteria used. In the United States, the International Classification of Diseases-9 diagnosis code and the National Health Interview Survey estimate ranged from 2 to 16 percent of the population (Shashy *et al.*, 2004), while in European countries, the prevalence based on a postal survey ranges from 7 to 27 percent with an average of 10.9 percent (Hastan *et al.*, 2011).

CRS is a condition that occurs in both children and adults, although is typically diagnosed in young or middle-aged adults (Lusk, 2007). Some studies have described that women were disproportionately affected, although this finding is not found in some studies (Shashy *et al.*, 2004; Pilan *et al.*, 2012).

Economically, CRS is a chronic disorder with a high economic burden resulting from the costs of diagnostic tests, medical and surgical therapies, lost and reduced school and work productivity, and detrimental impact on physical and emotional health (Bhattacharyya, 2011).

2.3. Risk factors and associated conditions

CRSwNPs is frequently associated with other inflammatory disorders from both upper and lower airways (Fokkens *et al.*, 2012):

- **Asthma**

CRSwNPs and asthma are frequently associated in the same patients. According to a cross-sectional study in multiple European centres, there is a strong association between the presence of asthma and the presence of CRS. Wheezing and respiratory discomfort are present in 31% to 42% of patients with CRSwNPs, and asthma is reported by 26% of patients with CRSwNPs, compared to 6% of controls. Alternatively, 7% of asthmatic patients have NPs, with a prevalence of 13% in non-atopic asthma and 5% in atopic asthma. Women that have NPs are 1.6 times more likely to be asthmatic.

Asthmatic patients with CRSwNPs have more nasal symptoms. Alobid and co-workers (Alobid *et al.*, 2011) showed that patients with CRSwNPs have an impaired sense of

smell, that asthma has a further impact on the sense of smell, and that loss of smell may be used as a clinical tool to identify the severity of both nasal polyposis and asthma.

- **Aspirin exacerbated respiratory disease**

The combination of asthma, CRSwNPs, and aspirin sensitivity is called aspirin exacerbated respiratory disease (AERD). In patients who have aspirin sensitivity, 36 to 96% have CRSwNPs and up to 96% have radiographic changes affecting their paranasal sinuses. These patients are usually non-atopic and the prevalence increases over the age of 40 years (Fokkens *et al.*, 2012). AERD affects women more frequently than men; the onset of symptoms in women is often earlier, and the disease is more severe (Stevenson *et al.*, 2006).

- **Allergic rhinitis**

The most common type of chronic rhinitis is allergic rhinitis. An estimated 10% to 25% of the population has allergic rhinitis and its prevalence continues to increase worldwide (Dykewicz *et al.*, 2010). Allergic rhinitis is the result of an IgE-mediated, type I hypersensitivity allergic reaction in response to specific allergens (Bousquet *et al.*, 2001; Brozek *et al.*, 2010; Bousquet *et al.*, 2012).

Caplin and co-workers (Caplin *et al.*, 1971) examined 3000 consecutive atopic patients and found that only 0.5% had polyps. Bunnag and collaborators (Bunnag *et al.*, 1983) reported a 4.5% incidence of NPs in 300 patients with allergic rhinitis. Settipane *et al.* (Settipane *et al.*, 1977) found that only 0.1% of paediatric patients attending an allergy clinic had NPs. Thus, the prevalence of NPs in allergic patients is low, usually under 5%, which is similar to that of the general population.

- **Cystic fibrosis**

In patients with CF, there is an inability of the cilia to transport the viscous mucus causing ciliary malfunction and consequently CRS. NPs are present in about 40% of patients with CF. The composition of these polyps are generally more neutrophilic than eosinophilic (Fokkens *et al.*, 2012).

- **Chronic obstructive respiratory disease**

Several pro-inflammatory mediators have been found in nasal lavages of COPD patients and nasal symptoms corresponded with the overall impairment of the quality of life. Recent findings have shown that several patients with bronchiectasis also present rhinosinusitis symptoms, radiologic abnormalities on computerized tomography (CT) scans and a reduced smell capacity (Guilemany *et al.*, 2011; Fokkens *et al.*, 2012).

2.4. Diagnosis

The diagnosis of CRS is based on the presence of suggestive symptoms, in combination with objective evidence of mucosal inflammation. In fact, the presence of at least two of the four cardinal signs/symptoms is indicative of CRS: (a) anterior and/or posterior nasal mucopurulent drainage, (b) nasal obstruction/nasal blockage/congestion, (c) facial pain, pressure, and/or fullness, and (d) reduction or loss of sense of smell. Moreover, the cardinal signs/symptoms should be present for 12 weeks or longer despite attempts at medical management (Fokkens *et al.*, 2012).

The signs/symptoms (**Figure 7**) should be supported by an objective evidence of sinus mucosal disease, either on direct endoscopic examination or sinus CT imaging. Endoscopic signs include NPs, and/or mucopurulent discharge primarily from middle meatus, and/or edema/mucosal obstruction primarily in middle meatus. On the other hand, CT changes include mucosal changes within the ostiomeatal complex and/or sinuses (Benninger *et al.*, 2003; Fokkens *et al.*, 2012).

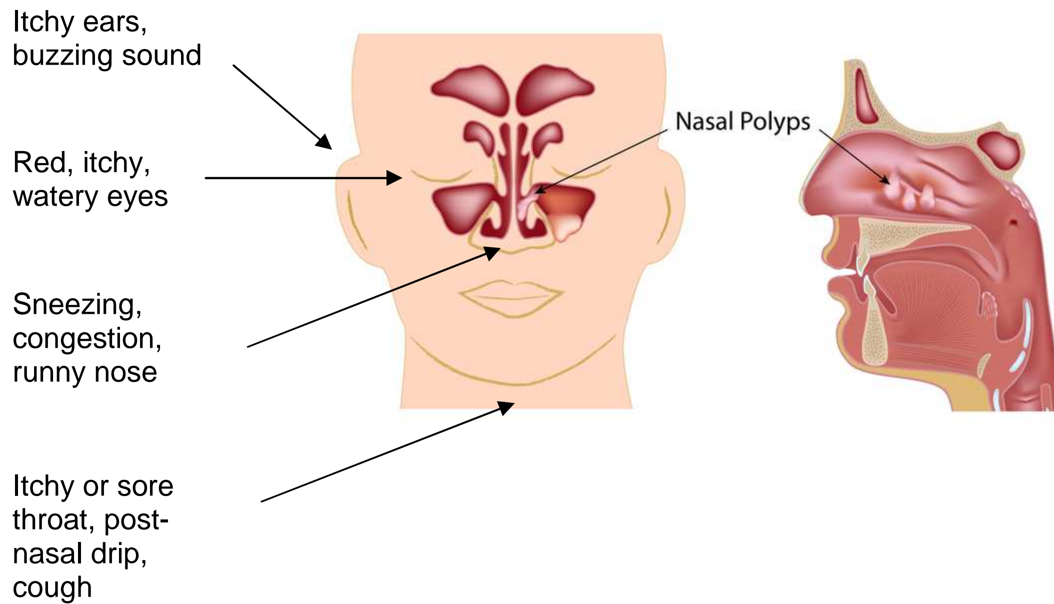


Figure 7. Illustration of NP localization and symptoms of nasal polyposis. NP: nasal polyp.

2.5. Pathogenesis and pathophysiology of chronic rhinosinusitis with nasal polyps

Histomorphological characterization of polyp tissue (**Figure 8**) reveals frequent epithelial damage, a thickened basement membrane, and edematous to sometimes fibrotic stromal tissue, with a reduced number of vessels and glands, and no visible neural structures (Van Crombruggen *et al.* 2011). NPs contain a great amount of inflammatory cells, especially mast cells, eosinophils, and T lymphocytes. Inflammatory cells together with structural cells as fibroblasts and epithelial cells release and express molecules such as histamine, cytokines, chemokines, transcription factors, and eicosanoids that act as inflammatory mediators playing a crucial role in the persistent chronic eosinophilc inflammation (Tomassen *et al.*, 2011). Structural cells are also responsible of tissue remodeling and fibrogenic processes, which are characteristic of NP formation.

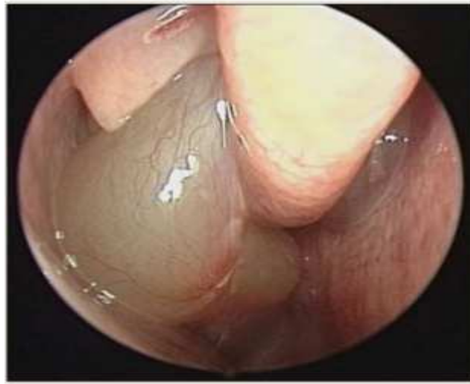


Figure 8. NP by endoscopy. NP: nasal polyp.

2.5.1 Inflammatory triggers

The sinonasal epithelium is in constant contact with the outside environment and serves as the first line of defence against inhaled pathogens and particulates (Lee *et al.*, 2011). This interaction depends on a set of innate and adaptive immune pathways at the mucosal surface driving inflammatory responses, protecting the host from infection (Ramanathan *et al.*, 2007).

The cause of NP formation is still unclear, although it has been regarded to be of inflammatory origin. Microbial elements are frequently observed in association with CRS and for that reason it is widely speculated that infection plays a role as an initiator of inflammation, or at least contributes to its persistence. The transient presence of microorganisms and particulates is not necessarily pathologic. However, it remains unclear whether pre-existing inflammation with impairment of mucociliary clearance is responsible for retention of microbial and environmental materials in CRS, or whether these agents in fact stimulate inflammation (Lee *et al.*, 2011). One pathogen that has been intensively investigated is *Staphylococcus aureus* (*S. aureus*), a gram-positive bacterium that can be frequently cultivated from the nasal vestibule. It seems that a pathogenic role for *S. aureus* in CRS appears possible as several studies demonstrated increased incidence of *S. aureus* in patients with CRS (Nadel *et al.*, 1998; Ozcan *et al.*, 2002; Araujo *et al.*, 2007; Sachse *et al.*, 2010). Pathogenic impact of *S. aureus* in CRS has been mainly attributed to virulence factors secreted by *S. aureus* such as staphylococcal enterotoxins. In CRSwNPs, IgE antibodies against staphylococcal enterotoxins have been associated with local superantigen activation of T cells, tissue eosinophilia, and a Th2-dominated cytokine pattern (Zhang *et al.*, 2005;

Bachert *et al.*, 2008). Bacterial biofilms have also been implicated in NPs aetiology and pathogenesis. Fungi, allergens, and viruses can also be related to inflammation and NP formation (Jang *et al.*, 2006). Finally, exposure of toxins (tobacco smoke, ozone, sulphur dioxide and particulate air pollutants) seems to contribute as potential triggers to damage the epithelium (Fokkens *et al.*, 2012).

2.5.2. Cellular profile

- **Epithelial cells**

Besides their important presence as component of the physical barrier, epithelial cells play an active role in both innate and acquired immune response (Schleimer *et al.*, 2007), producing a variety of inflammatory mediators (tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxins, regulated upon activation in normal T cells expressed and secreted (RANTES), IL-6, and CXCL8).

- **Fibroblasts**

Fibroblasts are the major and nearly exclusive producers of extracellular matrix proteins, such as collagens, fibronectin, and proteoglycans in airways. These cells also release enzymes and a variety of pro-inflammatory mediators, including growth factors, cytokines, and chemokines, which perpetuate the chronic inflammation condition characteristic of CRSwNP (Smith *et al.*, 1997; Vancheri *et al.*, 2005; Watelet *et al.*, 2006).

- **Mast cells**

Activated mast cells suffer a process of degranulation in which they release histamine, serotonin, platelet activating factor, LTs, and PGs. Mast cells also produce cytokines such as IL-1β, TNF-α, interferon (IFN)-γ, IL-4, IL-5, and IL-6 that activate adhesion molecules, induce eosinophilic infiltration, and perpetuate inflammation.

- **Eosinophils**

NPs are usually characterized by a massive infiltration of eosinophils. This process occurs due to several reasons: *(1)* increased production of eosinophils in the bone

marrow induced by growth factors, *(2)* eosinophils chemotaxis induced by cytokines, adhesion molecules, and chemoattractants, *(3)* eosinophil activation, and *(4)* increased eosinophil survival (Jankowski *et al.*, 1989; Stoop *et al.*, 1993).

- **T cells**

In Western populations, CRSwNP is a skewed Th2 disorder with relatively higher levels of IL-5 (Van Zele *et al.*, 2006), whereas in Asia, there is a Th1/Th17 cytokine bias, with less IL-5 than Caucasian polyps, consistent with the higher neutrophilic inflammation (Zhang *et al.*, 2006; Cao *et al.*, 2009).

- **B cells**

These cells play an important role in the production of IgE, which is involved in the early allergic reaction through activation and degranulation of mast cells.

2.5.3. Inflammatory mediators

- **Histamine**

Inflammatory mediator released by activated mast cells. Histamine has strong effects on smooth muscle constriction and increases vascular permeability and edema. The total histamine content of polyps is much higher than other tissues, and large quantities of free histamine can be measured in the edema fluid (Drake-Lee *et al.*, 1982).

- **Cytokines**

Promote the induction of intracellular signaling by activation of membrane specific receptors, resulting in cellular proliferation, differentiation, chemotaxis, growing, and Ig secretion modulation (Singh *et al.*, 2003). Several cytokines are upregulated in nasal polyposis: IL-1β, IL-6, TNF-α, IFN-γ and GM-CSF (Fokkens *et al.*, 2012).

- **Chemokines**

Recruit leukocyte populations during inflammation. It has been demonstrated that RANTES, eotaxin, macrophage inflammatory protein, monocytes chemotactic protein

(MCP), and myeloid progenitor inhibitory factor increase eosinophil chemotaxis. The main chemokines found in NPs are RANTES and eotaxin. RANTES is produced by T lymphocytes and fibroblasts and in addition to chemotaxis this mediator also induces transendothelial migration of eosinophils and leads to activation of eosinophils, resulting in the release of cytotoxic agents such as superoxide and eosinophilic cationic protein (ECP) (Ebisawa *et al.*, 1994; Kapp *et al.*, 1994). Eotaxin also activates eosinophils, but unlike RANTES and other chemokines, eotaxin is particularly selective for eosinophils (Ponath *et al.*, 1996). CXCL8 is produced by several cell types including epithelial cells, fibroblasts, macrophages, T cells, endothelial cells and neutrophils. It is mainly chemotactic for neutrophils, but also stimulates the migration of T cells and basophils (Miller *et al.*, 1992).

- **Eicosanoids**

Molecules with immunologic and inflammatory properties generated by the arachidonic acid (AA) metabolism and include two mediator families named LTs and prostanoids. The role of these mediators is discussed in more detail on Chapter III.

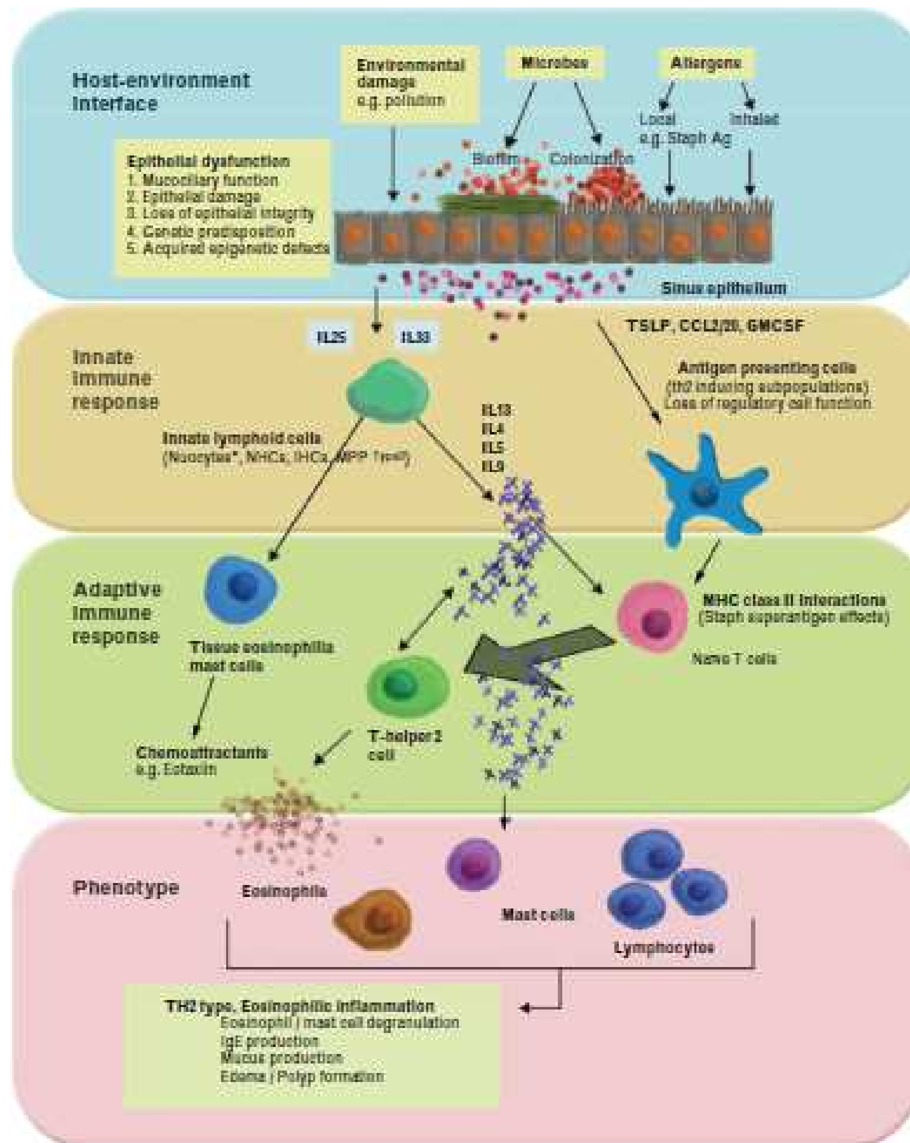


Figure 9. The current understanding of epithelial mediation of the Th2 immune response and local tissue eosinophilia (Chin *et al.*, 2013)**.** Even though there may be sinus lumen antigens (fungus, staph, biofilms, etc) that might be implicated in the process. **Th2:** T helper type 2.

2.6. Management and therapies

The objectives of CRSwNP management are to eradicate NPs from nasal and sinusal cavities, eliminate symptoms, and prevent recurrences. Many patients undergo extensive medical management and multiple surgeries and still experience continuous relapses. Most authors agree on the fact that management of nasal polyposis should

be based primarily on a medical approach to be addressed by surgical procedures only in the case of drug failure (Alobid *et al.*, 2012).

The advent of topically administered corticosteroids has improved the treatment of upper (NP and rhinitis) and lower (asthma, chronic obstructive airways disease) airway diseases (Fokkens, 2007; Newton *et al.*, 2008).Their clinical efficacy is based in a combination of anti-inflammatory effects along with their ability to reduce airway eosinophilic infiltration by preventing their increased viability and activation (Burgel *et al.*, 2004). Both topical and systemic corticosteroids may affect eosinophilic function through the direct reduction of eosinophils viability and activation and the indirect reduction of chemotactic cytokines secretion by epithelial cells (Roca-Ferrer *et al.*, 1997).

Multiple medical treatments are used in the management of CRS and the best approach varies according to the types of CRS that are present:

- Topical corticosteroids
- Oral steroids
- Oral antibiotics (short-term or long-term)
- Nasal decongestants
- Antihistamines
- Antifungal therapy
- Anti-LTs
- Aspirin desensitization
- Antibodies
- Immunosuppressants
- Saline douching

Intranasal corticosteroids and short courses of oral steroids are the most effective drugs for treating CRSwNP constituting the first line of treatment. Nasal saline irrigation and antihistamine (in allergic conditions) also are effective treatments. However, some of the above mentioned treatments have not shown real efficiency, and others need to be more investigated. Sinus surgery is recommended when medical treatment fails. Follow-up and medical treatments are essential after the procedure.

CHAPTER III: ARACHIDONIC ACID PATHWAY

1. Arachidonic acid metabolism

AA is a 20-carbon polyunsaturated fatty acid and is the main precursor of eicosanoids, mediating important functions in homeostasis, inflammation, and immunoregulation (Chilton *et al.*, 2008). Under normal conditions, AA is not freely available and its concentration within the cell is very low. The availability of free AA is essential for the biosynthesis of eicosanoids. Upon activation of the cell and by the action of various phospholipases (preferentially cytosolic phospholipase A2 (cPLA2), AA is released from the membrane phospholipids (Ghosh *et al.*, 2006). Therefore, as shown in **Figure 10**, once released, AA is rapidly metabolized through three enzymatic pathways namely cyclooxygenase (COX), lipoxygenase (LO), and cytochrome oxidases (hydrolase, epoxygenase) and one non-enzymatic pathway (Folco *et al.*, 2006).

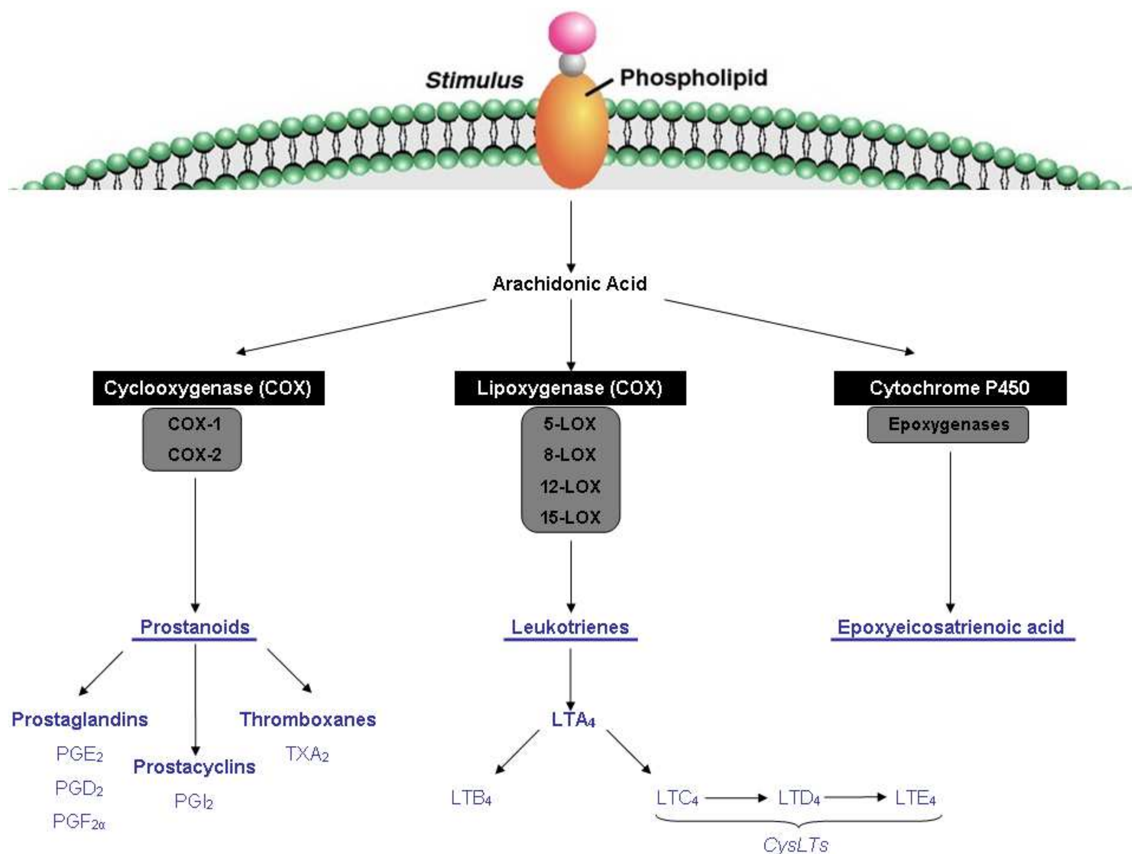


Figure 10. AA metabolism (Adapted from: Panigrahy *et al.*, 2010). AA: arachidonic acid.

1.1. Lipoxygenase pathway

AA, which is esterified on plasma membrane phospholipids, is released and converted into LTA_4 through 5-LO activity. LTA_4 is subsequently converted by LTA_4 hydrolase into LTB_4 and by LTC_4 synthase, which conjugates reduced glutathione into LTC_4. This product is metabolized into LTD_4, which is then metabolized into LTE_4. LTC_4, LTD_4 and LTE_4 are designated as cys-LTs. LTs are synthesized upon cellular activation and the intracellular expression and distribution of 5-LO varies considering the cell type. In the airways, 5-LO is present in several types of leukocytes and becomes activated during allergic inflammation. Cys-LTs activate three receptors with differential selectivity (cys-LT_1, cys-LT_2 and GPR17) and the stimulation of these receptors, principally cys-LT_1, account for most of their effects (Montuschi *et al.*, 2007). This receptor is expressed in a large variety of cells which include monocytes, macrophages, eosinophils, basophils, mast cells, neutrophils, T cells, B lymphocytes, pluripotent hematopoietic cells, interstitial cells of the NM, airway smooth muscle cells, bronchial fibroblasts and vascular endothelial cells (Montuschi, 2008) and its activation contributes to most of the effects of cys-LTs that are relevant to the pathophysiological changes observed in patients with asthma (Montuschi *et al.*, 2007).

1.2. Cyclooxygenase pathway

AA can be metabolized through the COX pathway by the action of the COX enzymes. COX is a bifunctional enzyme with two active sites, one with COX activity that catalyzes the reduction of AA to form PGG_2 and the other with peroxidase activity involved in the reduction of peroxidase group in PGG_2 to hydroxyl group forming PGH_2. These enzymes catalyze the reactions responsible for the production of several bioactive prostanoids, such as PGs (PGE_2, PGD_2, and $PGF_{2\alpha}$), prostacyclins and thromboxane A_2 (Smith *et al.*, 2000). Cellular specificity in the profiles of eicosanoid generation exists. Both leukocytes and structural cells are capable of prostanoid production. Studies show the presence of two isoforms of COX enzymes, namely COX-1 and COX-2. COX-1, the dominant source of prostanoids that serves a number of physiologic “housekeeping” functions, presents a uniform expression in almost all tissue and is generally considered constitutive (Simmons *et al.*, 2004). COX-2 is described, in diverse studies, as highly induced and only expressed in response to certain inflammatory stimuli (Siegle *et al.*, 1998; Lohinai *et al.*, 2001; Roca-Ferrer *et al.*,

2006; Xaubet *et al*. 2004; Togo *et al.* 2008). Furthermore, COX-2 is the most important source of prostanoid formation in inflammatory processes and proliferative diseases (Ricciotti *et al.*, 2011). Both isoforms are located in the endoplasmatic reticulum and nuclear envelope, COX-2 is more highly concentrated in the nuclear envelope (Morita *et al.*, 1995).

1.2.1. Prostaglandin E_2

PGE_2 is the major prostanoid product of epithelial cells, fibroblasts, and smooth muscle cells. The production of PGE_2 depends on three PGE synthases (PGES), termed cytosolic PGES (cPGES) and microsomal PGES (mPGES)-1 and -2. mPGES-1 expression is upregulated simultaneously with COX-2, permitting increased PGE_2 generation during inflammatory responses (Liu *et al.*, 2013).

PGE_2 exhibits pleiotropic and contrasting effects in different cell types and organs. According to Vancheri and collaborators (Vancheri *et al.*, 2004), the lung is considered a privileged site for the action of PGE_2. Although in this organ PGE_2 can exert anti-inflammatory, anti-fibrotic and immune restrictive actions, it can also mediate pro-inflammatory responses (Vancheri *et al.*, 2004; Bauman *et al.*, 2010).

1.2.1.1. Prostaglandin E_2 receptors

The activity of PGE_2 is mediated by a group of rhodopsin-type G-protein-coupled membrane receptors (GPCRs) denominated E-prostanoid (EP) receptors (Sugimoto *et al.*, 2007). There are four GPCR subtypes: EP_1, EP_2, EP_3 and EP_4. The physiological and contrasting effects of PGE_2 depend on the expression or the co-expression of more than one receptor or isoform (Rocca, 2006). Additionally, each receptor may be differentially expressed in tissues. EP receptors differ in terms of intracellular signaling (Narumiya, 1994; Narumiya *et al.*, 1999) and could be classified according to their intracellular signaling and second messenger (Norel *et al.*, 1999). The EP_1 receptor signals via Ca^{2+} mobilization with slight phosphatidylinositol activity (Watabe *et al.*, 1993; Woodward *et al.*, 2011). Distribution of this receptor in human tissues and cells is restricted and has been demonstrated in the myometrium, pulmonary veins, mast cells, colonic longitudinal muscle and keratinocytes. EP_1 receptor exerts mostly constrictive functions, however and compared with other prostanoid receptors, it seems to be less

studied (Woodward *et al.*, 2011). EP_2 and EP_4 receptors increase intracellular cyclic adenosine monophosphate (cAMP) through activation of adenyl cyclase (AC) (Regan *et al.*, 1994; Vancheri *et al.*, 2004). Functional studies have demonstrated that the EP_2 receptor is widely distributed (Coleman *et al.*, 1994) and it seems to be involved in processes of relaxation such as bronchodilation (Kay *et al.*, 2006) and anti-inflammation (Takayama *et al.*, 2002). On the other hand, EP_4 is also widely distributed (Narumiya *et al.*, 1999). In a direct comparison with EP_2 receptor subtype signaling, the EP_4 receptor demonstrated a less efficient functional coupling to cAMP (Fujino *et al.*, 2002; Fujino *et al.*, 2005). Effectively, studies have reported several cAMP-independent signaling pathways for EP_4 receptor activation (Fiebich *et al.*, 2001; Pozzi *et al.*, 2004; Mendez *et al.*, 2005; Frias *et al.*, 2007; George *et al.*, 2007; Rao *et al.*, 2007). EP_4 mediates vasorelaxation of pulmonary arterial veins and also promotes anti-inflammatory effects (Foudi *et al.*, 2008). Consistent with its bronchoprotective action, PGE_2 inhibited proliferation and migration of bronchial smooth muscle cells through the action of the EP_4 receptor (Aso *et al.*, 2013). Studies have revealed that the EP_3 receptor shows a wide distribution in almost all tissues and consists of multiple isoforms generated by numerous alternative splicing of the C-terminal (Sugimoto *et al.*, 1993; Schmid *et al.*, 1995; Kotelevets *et al.*, 2007). Signaling through this receptor reduces the levels of cAMP and increases intracellular Ca^{2+} (Narumiya *et al.*, 1999). EP_3 exerts mainly contractile functions such as the constriction of human pulmonary artery in the lung (Qian *et al.*, 1994). Moreover, this receptor has been implicated in inflammation, pain and cough (Woodward *et al.*, 2011; Maher *et al.*, 2009).

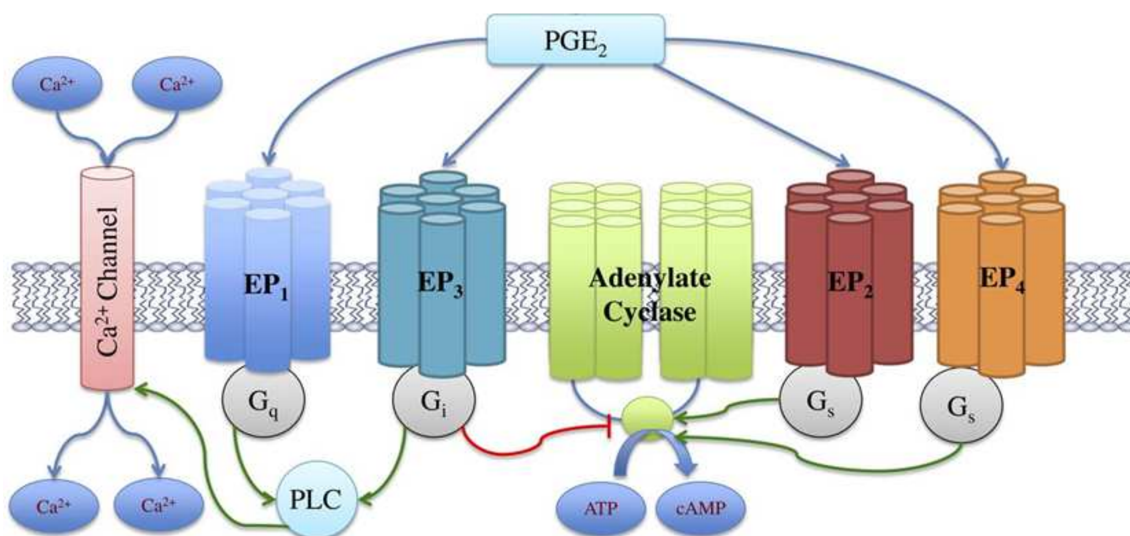


Figure 11. PGE_2 receptors: types and signaling (Adapted from: Nasrallah *et al.*, 2014). PGE_2: Prostaglandin E_2.

CHAPTER IV: ARACHIDONIC ACID PATHWAY AND INFLAMMATION

1. Inflammation

1.1. Definition

Inflammation is defined as the protective response of vascularised tissue to injury (physical damage or infection by microorganisms) in order to repair/wound healing, restore and, if necessary, remodel the injured tissue. Inflammation is composed by an initial acute phase characterized by several changes in molecular, cellular and physiological patterns. The key sings of acute inflammation are redness, swelling, heat, pain, and loss or altered function. Besides of macroscopically features, microscopically changes as alterations in vascular calibre and blood flow (as a result of vascular permeability changes) and leukocytes migration may also be observed. Edema is a typical characteristic of this phase and is due to the massive infiltration of inflammatory cells such as neutrophils, monocytes, and macrophages. Concluded the set of short-duration responses to injury, infection, or allergen, inflammation is resolved and the restoration of the inflamed tissue, as well as homeostasis is re-established. However, if the injury is not well resolved or is severe, the process of inflammation will persist and will become chronic which may lead to an abnormal tissue remodeling with excess tissue damage (**Figure 12**) (Levine, 1995; Ricciotti *et al.*, 2011).

Diseases characterized by airway inflammation, excessive airway secretion, and airway obstruction affect a great proportion of the worldwide population. These disorders are characterized by an excessive airway production of cytokines, chemokines, and growth factors in response to irritants, infectious agents, and inflammatory mediators contributing to the modulation of acute and chronic airway inflammation (Levine, 1995).

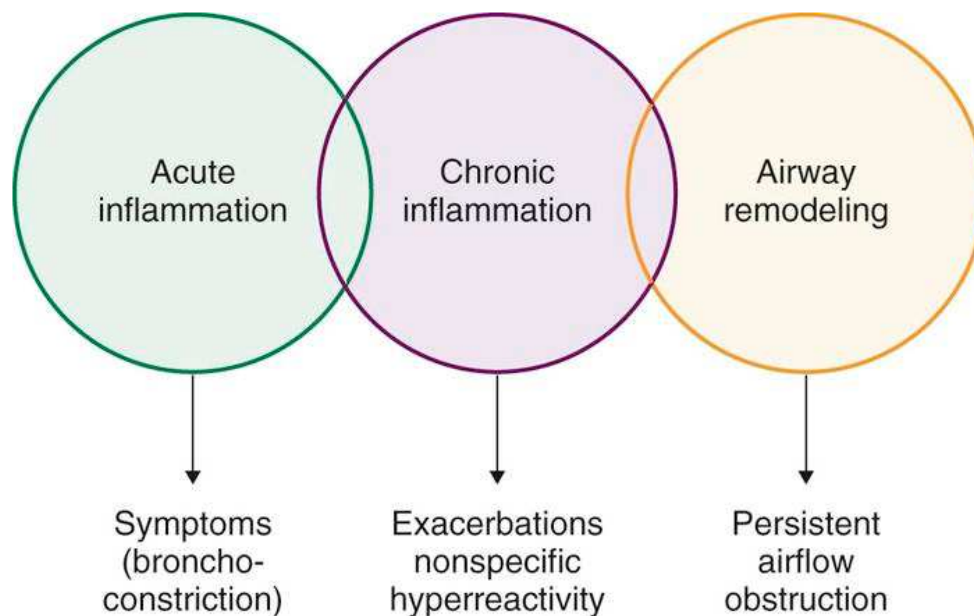


Figure 12. Phases of airway inflammation (Bousquet *et al.*, 2000).

1.2. Interleukin-1β

IL-1β is a potent pro-inflammatory cytokine produced and secreted by a variety of cell types such as blood monocytes, tissue macrophages, and dendritic cells and, to a lesser extent, B lymphocytes and natural killer cells. It belongs to the IL-1 family and it maintains a central function in defending the organism against infections with pathologic microorganisms including bacteria, fungi and viruses (Dinarello, 1996; Krause *et al.*, 2012). Moreover, the activation of IL-1β also occurs by a variety of host-derived or environmental cellular stressors (Krause *et al.*, 2012). Besides its well known physiologic role in host protection, this cytokine is crucial in a number of severe inflammatory diseases. IL-1β is produced as an inactive precursor termed pro-IL-1β, which through the activation of the pro-inflammatory protease caspase-1 results in active IL-1β (Thornberry *et al.*, 1992). Mature IL-1β leaves the cell and activates target cells through the interaction with its functional receptor. These events are responsible to the expression of a large range of genes involved in the regulation of inflammation (Martin *et al.*, 2002; Weber *et al.*, 2010).

1.2.1. Interleukin-1 receptor

IL-1 receptor (IL-1R) belong, together with Toll-like receptors (TLRs), to a superfamily of conserved proteins involved in the activation of innate immunity, the early non-specific defence response against pathogenic microorganisms and other insults (Boraschi *et al.*, 2013; Garlanda *et al.*, 2013). The common characteristic of the members of this family is the presence of a domain in the cytoplasmatic region, called Toll-Interleukin-1 (TIR) domain, which initiates the signaling cascade. In non-pathological conditions, receptors of the TIR superfamily and their respective ligands play an important role on tissue homeostasis maintenance. Nevertheless, receptors activation initiates an inflammatory response that involves the production of inflammatory cytokines and other mediators involves in inflammation (Lopetuso *et al.*, 2013).

TIR receptors can be divided into two main subgroups based on the extracellular domains, those containing Ig-like domain, and those characterized by a leucine-rich repeat motif (Boraschi *et al.*, 2006). The ten members of IL-1R subfamily contain Ig domains.

1.2.1.1. Interleukin-1 receptor family

As previously described, members of IL-1R subfamily are usually characterized by an extracellular Ig domain and an intracellular TIR domain, with the exception of IL-1 receptor type II (IL-1RII) (**Figure 13**) (Boraschi *et al.*, 2006).

IL-1 receptor type I, IL-1R1 (IL-1RI): the IL-1 binding chain. It binds to IL-1α and IL-1β (agonist ligands) and to IL-1R antagonist (IL-1Ra) (antagonist ligand).

IL-1R2 (IL-1RII): the IL-1 decoy receptor, lacking the intracellular TIR domain. It binds to IL-1α, IL-1β, and IL-1Ra.

IL-1R accessory protein, IL-R3 (IL-1RAcP): the accessory receptor chain for IL-1.

IL-1R4 – IL-1R10: these members include the IL-33, IL-18 and IL-36 binding chain as well as two orphan receptors.

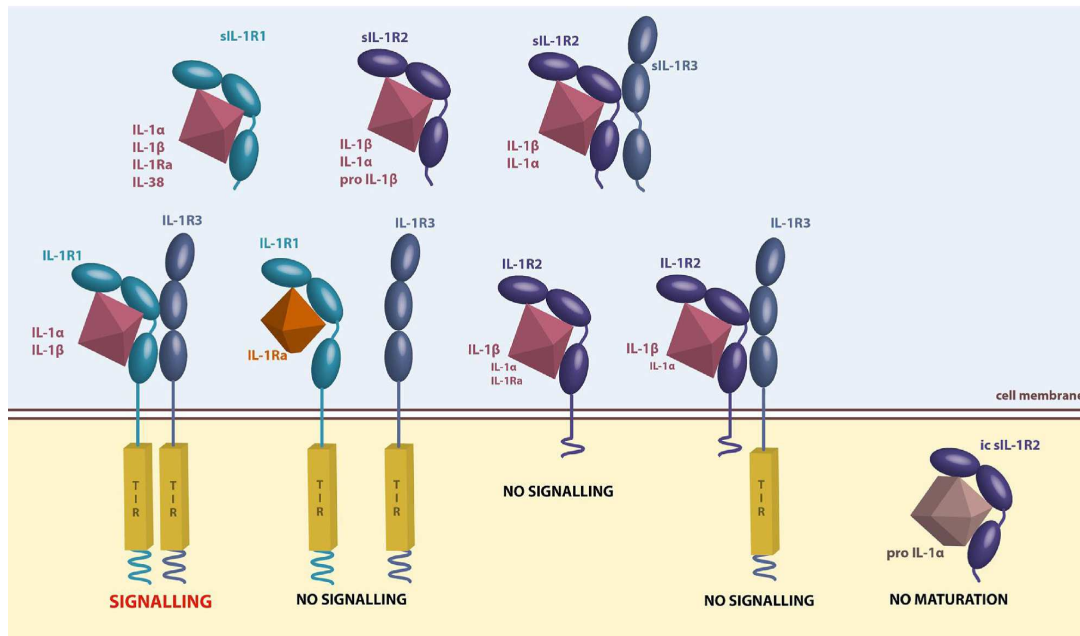


Figure 13. The IL-1R complexes (Boraschi *et al.*, 2013). IL-1R: interleukin-1 receptor.

1.2.1.2. Activation and regulation of interleukin-1 receptor type I

The agonist ligands of IL-1RI are IL-1α and IL-1β (Dinarello, 1998). The third ligand is the IL-1Ra, a molecule that resembles IL-1α and IL-1β in its amino acid sequence, three dimensional folding pattern and gene structure (Eisenberg *et al.*, 1990; Nicklin *et al.*, 1994). However, contrary to IL-1α or IL-1β, IL-1Ra binds to IL-1RI but does not initiate signal transduction (Greenfeder *et al.*, 1995). Thus, initiation of activation signaling only occurs when an agonist IL-1RI binds to its specific ligand binding TIR-containing receptor and IL-1RAcP is recruited into the complex. The signaling process begins with the approximation of the TIR-containing intracellular domains of the two chains (IL-1RI and IL-1RAcP), which formed a heterodimeric complex. Once formed, the myeloid differentiation primary response gene 88 (MyD88) and the IL-1 receptor-associated kinase (IRAK) are recruited to the complex, followed by interaction with the TNF receptor-associated factor 6. Activation of I kappa B kinase (IKK) causes degradation of the inhibitor of kappa B (IkB) and subsequent activation of the nuclear factor-kappa B (NF-kB) (Boraschi *et al.*, 2013; Garlanda *et al.*, 2013).

1.2.1.3. Downregulation mechanisms of receptor-mediated activation in the interleukin-1 receptor family

There are several mechanisms responsible to the regulation of IL-1R family receptor functions (Boraschi *et al*., 2013b; Garlanda *et al*., 2013):

- **Receptor antagonism**

IL-1Ra, a molecule that resembles at the structural level with IL-1, can bind to IL-1RI, but does not allow engagement of the IL-1RAcP. It sequesters the activating receptor into an inactive ligand-receptor complex.

- **Decoy receptors**

IL-1RII is able to bind to the agonists IL-1; however, since it lacks the intracellular TIR domain it results into a non-active ligand-receptor complex that blocks the signal transduction and impedes the binding of agonists to the functional IL-1RI.

- **Soluble decoys**

Several members of IL-1R family are present in soluble form, which results from alternative splicing or cleavage of the cellular membrane. These forms can capture the ligang avoiding its union to the active membrane receptors.

- **Inhibition of ligand maturation**

The precursor form of IL-1 cytokines needs to be matured by proteases to become biologically active. For instance, IL-1β precursor can be bound by IL-1RII, avoiding its proteolytic maturation.

- **Co-receptor competition**

IL-1RII can binds to accessory chain IL-1RAcP forming a non-active complex by availability limitation of the accessory chain for engaging receptor complexes and activate the signal transduction.

1.3. Interleukin-1: activation of arachidonic acid metabolism

As previously mentioned, after cell stimulation, the cPLA2 enzyme mediates the reaction by which AA is released from the membrane phospholipids. This reaction is of particular significance because it serves as the rate-limiting precursor for PGs and LTs. cPLA2 is secreted from certain cell types in response to pro-inflammatory cytokines such as IL-1 (Scott *et al.*, 1991). Previous studies have shown that in human endothelial cells and human dermal fibroblasts, IL-1 induced COX activity and therefore stimulates the conversion of AA into PGs (Maier *et al.*, 1990; Raz *et al.*, 1989). In fact, IL-1 has been shown to increase PG production (Elias *et al.,* 1987; Dinarello, 1988). Lin and collaborators (Lin *et al.*, 1992) demonstrated in a human lung fibroblast line that cell treatment with IL-1 resulted in an 8- to 10-fold increase in the production of PGE_2 and this effect was closely related with an increase in the activity of cPLA2. Additionally, COX-2 and PGES-1 protein expression are concordantly induced by IL-1β. It has been demonstrated that both the coregulaton and upregulation of these enzymes are crucial to stimulate the synthesis of PGE_2 (**Figure 14**) (Park *et al.*, 2006). Indeed, in studies performed with HEK293 cells, the cotransfection of human mPGES-1 and COX-2 and the subsequent stimulation of the cells with IL-1β resulted in a significant increase in PGE_2 production. However, in cells cotransfected with COX-1 and mPGES-1, the stimulation with IL-1β resulted in a smaller increase in PGE_2 production. The authors conclude that mPGES-1 preferentially couples with COX-2 activity to increase the production of PGE_2 (Murakami *et al.*, 2003).

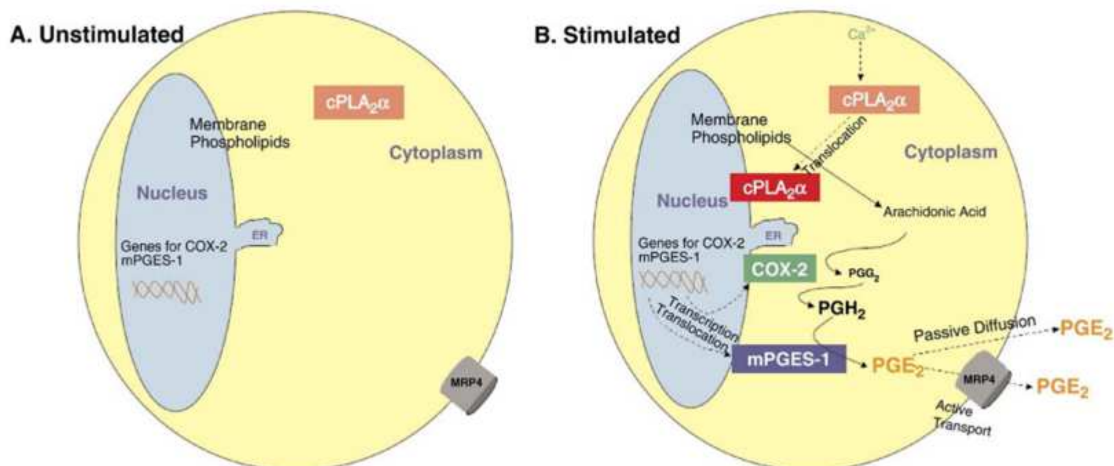


Figure 14. Coordinate production of PGE_2 by cPLA2, COX-2, and mPGES-1 (Park *et al.*, 2006). (A) Unstimulated cell. cPLA2 is constitutively present in the cytoplasm. In unstimulated cells, COX-2 and mPGES-1 are not expressed. **(B)** Stimulated cell. Inflammatory stimulation results in calcium influx which leads to the translocation of cPLA2 from the cytosol to the nuclear membrane where it enzymatically hydrolyzes membrane phospholipids to release AA. Inflammatory stimuli also induce the transcription and protein expression of both COX-2 and mPGES-1 at the nuclear membrane and endoplasmic reticulum. COX-2 transforms AA to PGG_2 which is subsequently converted to PGH_2. mPGES-1 may then act on PGH_2 to generate PGE_2. PGE_2 may exit the cell by simple diffusion or by active transport via the MRP4 transporter. **AA:** arachidonic acid; **COX-2:** cyclooxygenase-2; **cPLA2:** cytosolic phospholipase A2; **mPGES-1:** microsomal prostaglandin E synthase-1; **MRP4:** multidrug resistance protein 4; **PGE_2:** prostaglandin E_2.

The signal transduction of IL-1 depends on the expression of IL-1RI (Sims *et al.*, 1993). Parker and co-workers (Parker *et al.*, 2002), using cultures derived from IL-1RI$^{-/-}$ and wild type mice, described that IL-1β failed to induce release of PGE_2 or activate NFκB in glial cells form mice lacking IL-1RI, confirming that loss of IL-1RI is responsible for resistance to IL-1β. Moreover, there is an important role for IL-1-induced PGE_2 in regulation of IL-1 receptors. PGE_2 itself is able to upregulate gene and cell surface expression of IL-1RI in human fibroblasts via induction of PGE_2 (Akahoshi *et al.*, 1988; Takii *et al.*, 1992), which suggests a potential mechanism for self-augmentation of responses to IL-1.

CHAPTER V: ASPIRIN EXACERBATED RESPIRATORY DISEASE

1. Aspirin exacerbated respiratory disease

1.1. Introduction

AERD is a clinical syndrome characterized by the combination of NPs, chronic hypertrophic eosinophilic sinusitis, asthma and sensitivity to any medication that inhibits COX-1 enzymes, namely aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) (Szczeklik *et al.*, 2009; Stevenson, 2009). Indeed, ingestion of aspirin, and most NSAIDs, results in a spectrum of upper and/or lower respiratory reactions, to include rhinitis, conjunctivitis, laryngospasm and bronchospasm (Szczeklik *et al.*, 2009; Stevenson, 2009). The combination of the diseases mentioned above and the intake of NSAIDs constitutes a fatal combination for some patients, which may be difficult to treat with standard medical and surgical procedures.

AERD is subdivided, based on physical reactions, to *(1)* NSAID-induced rhinitis and asthma, *(2)* NSAID-induced urticaria/angioedema, *(3)* multiple-drug-induced urticaria/angioedema, *(4)* single-drug-induced anaphylaxis, and *(5)* single-drug- or NSAID-induced blended reaction (Graefe *et al.*, 2012).

AERD is characterized by intense inflammation of the respiratory mucosa, with large numbers of activated tissue eosinophils, mast cells, monocytes, and neutrophils (Laidlaw *et al.*, 2014). The persistent inflammation likely contributes to the recurrent development of severe NPs, with the soluble products of eosinophils and mast cells causing edema and fibroproliferation.

1.2. Epidemiology

AERD has been estimated to affect 0.3 to 2.5% of the general population (Graefe *et al.*, 2012). The frequency of symptoms associated with AERD is 10–20% with bronchial asthma, 5–30% with NPs, 10% with rhinitis, and 5–10% with

urticaria/angioedema. The estimation of AERD prevalence varies depending on the determination through questionnaire (11–20%), medical record (approximately 3%), or oral provocation test (21%). AERD appears typically during the third decade and is more commonly reported in females. In this clinical syndrome, when asthma is present, it often becomes severe and is associated with aggressive airway remodeling (Mascia *et al.*, 2005). Similarly, CRS present in patients who suffer from AERD, is often severe and associated with complete or near complete sinus opacification (Mascia *et al.*, 2005b).

1.3. Clinical presentation

AERD has been described as "*Samter's triad*", aspirin induced asthma, aspirin sensitive asthma and aspirin hypersensitivity (Szczeklik *et al.*, 2009; Samter *et al.,* 1968).

The clinical picture and course of AERD is well established and starts usually with nasal congestion and anosmia, progressing to CRSwNP that frequently relapses rapidly after surgery. Asthma and hypersensitivity to aspirin or other NSAIDs develop subsequently (**Figure 15**). About 50% of the patients demonstrate chronic, severe, corticoid-dependent asthma. Following ingestion of aspirin or NSAIDs, an acute asthma attack develops within minutes to 1–2 h. It is generally accompanied by rhinorrhoea, nasal obstruction, conjunctival irritation, and scarlet flush of head and neck. Moreover, in some cases it may progress to severe bronchospasm, shock, loss of consciousness, and respiratory arrest (Stevenson *et al.*, 2006).

The formation of NPs in patients suffering from AERD follows an aggressive course filling the nasal cavity, often protruding anteriorly in the face or posteriorly into the nasopharynx (Kowalskin 2000; Picado 2002). A strong positive correlation has also been found between the number of polypectomies and the peripheral blood eosinophil count (Min *et al.*, 2000; Mascia *et al.*, 2005b).

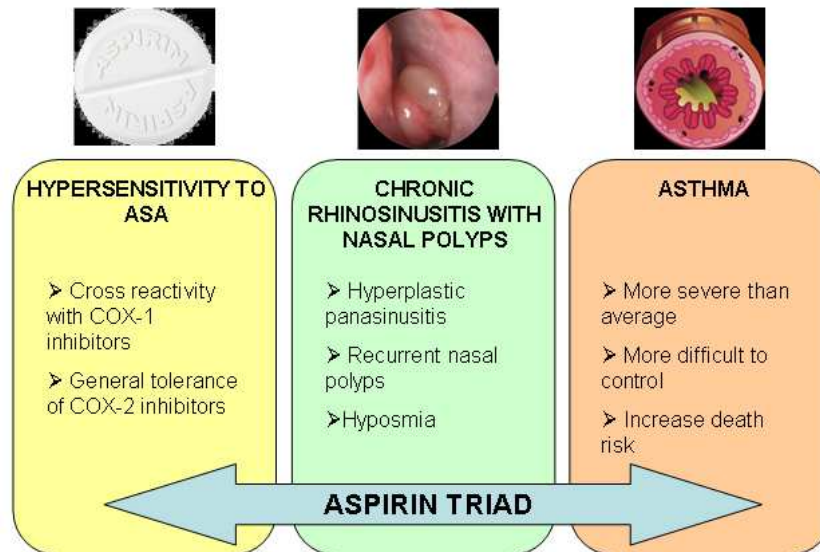


Figure 15. Clinical characteristics of AERD (Adapted from: Kroegel, 2009). **AERD:** aspirin exacerbated respiratory disease.

1.4. Diagnosis approaches

The diagnosis of AERD can often be performed when all three of the conditions that characterize AERD are present: asthma, visible nasal polyposis (or a history of nasal polypectomy), and a history of a typical reaction to NSAIDs. Moreover, this will always be supported in preposition by imaging techniques, including CT or endoscopy. CT or plain radiographs of the sinuses reveal complete opacification in nearly all AERD patients (Stevenson, 2009; Mascia *et al.*, 2005b). Normal imagining of the sinuses essentially rules out the diagnosis of AERD.

Some patients have a definitive history of adverse reactions to NSAIDs. However, some patients had not experienced AERD-associated symptoms, suggesting that challenge tests are critically for diagnosis. Aspirin challenge consists of increased dose of aspirin intake during the challenges. There are four routes of provocation challenge: *(1)* oral, *(2)* bronchial inhalation, *(3)* nasal inhalation, *(4)* and intravenous (Graefe *et al.*, 2012).

1.5. Pathogenesis and pathophysiology of aspirin exacerbated respiratory disease

1.5.1. Cytokine expression in aspirin exacerbated respiratory disease

Numerous observations suggest that in contrast to asthmatic or eosinophilic sinusitis patients who tolerate aspirin, patients who suffer from AERD present a mixed Th2- and Th1-like milieu with prominent expression of IFN-γ. Studies performed in NP tissue from patients with AERD showed that IFN-γ mRNA transcripts and protein were overexpressed compared with aspirin-tolerant and control subjects, being that eosinophils were the major source of this cytokine (Steinke *et al.*, 2015). Eosinophils secrete several cytokines and chemokines, which are stored and released upon cell activation. IL-4, IL-5, IL-6, IL-13, GM-CSF, eotaxin, MCP3/CCL7, RANTES/CCL5 are all shown to be increased in AERD (Narayanankutty *et al.*, 2013).

1.5.2. Cysteinyl leukotriene overproduction/overresponsiveness and prostaglandin E_2/prostaglandin E_2 receptors deregulation in aspirin exacerbated respiratory disease

The pathogenesis of AERD is not fully understood and many questions remain unsolved. However, several studies have reported that the pathogenic mechanisms of this disorder may be due, at least in part, to a marked imbalance in eicosanoid metabolism, possibly increasing and perpetuating the process of inflammation (**Figure 16**). A central feature of AERD is its association with profound overproduction and overresponsiveness to cys-LTs occurring concomitantly with a marked underproduction and underresponsiveness to PGE_2, which result in a disproportion between pro-inflammatory and anti-inflammatory mediators (Steinke *et al.*, 2015). Cys-LTs have important pro-inflammatory and pro-fibrotic effects, contributing to asthma severity and to the extensive hyperplastic sinusitis and nasal polyposis. On the other hand, the downregulation of PG pathways impedes its mediators to participate in the attenuation of pro-inflammatory responses.

Overproduction of cys-LTs in AERD is due, partially, to the increased expression of both 5-LO and LTC_4 synthase, their primary synthesis enzymes. Upregulated levels of

these enzymes are found in the lungs, sinuses, and NPs of patients with AERD, and are localized, in large part, in the infiltrating eosinophils and resident mast cells (Stevenson *et al.*, 2006). In AERD, there is a dependency on COX products to modulate and maintain homeostasis over 5-LO activity. Therefore, the inhibition of COX pathway by aspirin or other NSAIDs in those patients results in the overproduction of cys-LTs (Feng *et al.*, 2006; Luo *et al.*, 2004). Several studies have described the biological effects and the contribution of these lipid mediators in AERD. After challenging AERD patients with oral, intravenous and intranasal aspirin treatments, the levels of cys-LTs increased significantly. Moreover, it was demonstrated that patients with AERD excrete higher levels of LTE_4 in their urine when compared with asthmatic patients without aspirin intolerance (Smith *et al.*, 1992; Higashi *et al.*, 2012). Pérez-Novo and collaborators (Pérez-Novo *et al.*, 2005) reported that the nasal tissue of patients with CRSwNP shows elevated levels of cys-LTs when compared with NM from aspirin-tolerant asthmatic patients and this increased production is associated with the elevated expression of LTC_4 synthase. Besides the overproduction of cys-LTs, AERD subjects also show an increased sensitivity to cys-LTs, which was attributed to the upregulation of their receptors (Steinke *et al.*, 2015).

PGE_2 may be the most essential PG for maintaining homeostasis of inflammatory responses in the airways. At this level, PGE_2 is unique for its bronchoprotective and anti-inflammatory effects (Vancheri *et al.*, 2004). In fact, PGE_2 acting through anti-inflammatory EP_2 receptors has the capacity to block eosinophil migration, activation and survival, and mast cell degranulation (Steinke *et al.*, 2015). PGE_2 also uses the same signaling mechanism to induce phosphorylation and prevent translocation of 5-LO to the nuclear envelope, controlling then the generation of cys-LTs (Sestini *et al.*, 1996). The importance of this process is supported by the fact that inhaled PGE_2 prevents both the airway obstruction and the increase in urinary LTE_4 occurring in response to aspirin challenge in AERD. Besides its effects on hematopoietic cells, PGE_2 dampens the proliferation of fibroblasts and their production of collagen, acting thus in structural cells also (Huang *et al.*, 2008).

In patients who suffer from AERD the levels of PGE_2 are abnormally low, which could contribute to attenuate its anti-inflammatory properties. Effectively, NPs from patients who suffer from AERD contain markedly lower levels of PGE_2 than do sinonasal tissues from aspirin-tolerant individuals. Additionally, peripheral blood leukocytes isolated from AERD patients also generated less PGE_2 than aspirin-tolerant, and both nasal epithelial cells and cultured fibroblasts from NP from AERD patients produce less PGE_2

in vitro than do cells from aspirin-tolerant asthmatic patients. Considering that, several studies investigated the mechanisms behind the reduced levels of PGE_2 in AERD.

As previously described, PGE_2 is generated primarily by COX-2 and a partner terminal synthetic enzyme, mPGES-1. These two enzymes are coexpressed by epithelial cells, fibroblasts, and macrophages and are frequently upregulated in tandem during inflammatory responses (Uematsu *et al.*, 2002). There is substantial evidence that the functions of COX-2/mPGES-1 are impaired in the respiratory tissues in AERD. Although there are no studies reporting the expression of mPGES-1 in AERD, the gene encoding this enzyme (*PTGES*) is hypermethylated in NPs from patients with AERD (NP-AERD) (Cheong *et al.*, 2011). On the other hand, several studies have addressed the expression of COX-2 in AERD. Compared with NM of control subjects (NM-C) and aspirin-tolerant patients, the expression of COX-2 mRNA is diminished in NP-AERD. Moreover, it is well-known that the stimulation with IL-1β poorly induces COX-2 mRNA and protein expression in cultured fibroblasts from NP-AERD when compared with aspirin-tolerant asthmatic patients and control subjects (Laidlaw *et al.*, 2013). Therefore, the impaired functions of COX-2/mPGES-1 that results in reduced levels of PGE_2 contributed to the severity of inflammation observed in AERD and accentuated the sensitivity of these individuals to the inhibition of PGE_2 synthesis associated with aspirin and other NSAIDs. A predicted outcome of the downregulation of COX-2 is a dependency on COX-1 to provide the residual PGE_2 that is necessary to restrain mast cell and eosinophil activation, and to block 5-LO activity. Consequently, the dependency on COX-1 to generate PGE_2 could contribute to explain the fact that most of AERD subjects tolerate selective COX-2, but not COX-1 inhibitors.

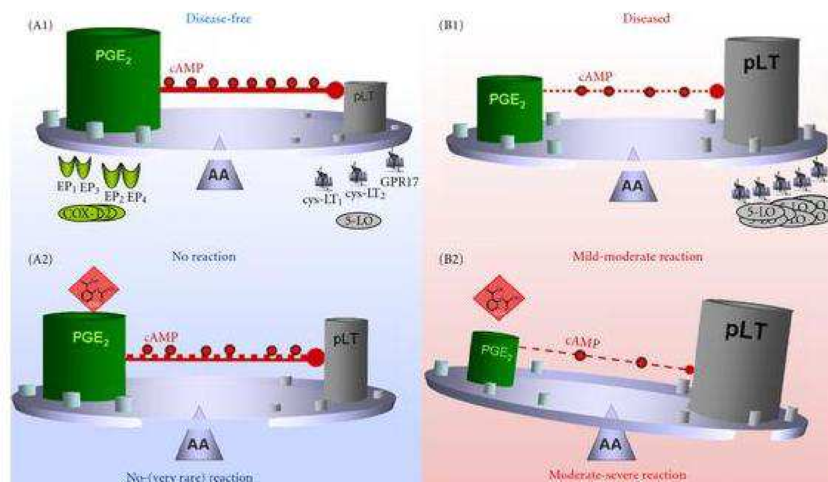


Figure 16. Causal concept of NSAIDs-triggered eicosanoid imbalance for *in vitro* diagnosis of AERD (Schäfer *et al.*, 2012). The causal concept of NSAID-trigger eicosanoid imbalance for *in vitro* diagnosis of AERD is best allegorised as a tray balancing all parameters (which might be relevant for the pathway) on a needle. ***Disease-free individuals:*** **(A1)** housekeeping PGE_2 balances synthesis of pLTs (e.g., by induction of endogenous cAMP, which inhibits synthesis of pLTs). **(A2)** Upon exposure to NSAIDs the PGE_2 level is diminished but remains high enough ensuring “uncritical” levels of pLTs (even though cAMP might by diminished). ***Patients with AERD:*** **(B1)** synthesis of housekeeping PGE_2 is diminished, but still balances synthesis of pLTs (e.g., by reduced endogenous cAMP); expression of enzymes (upregulation of LTC_4-synthase) or receptors (upregulation of cys-LTs) can be mutated in some cases. **(B2)** Exposure to NSAIDs/aspirin blocks the COX-pathway causing reduced synthesis of PGE_2 (and consequently further reduced cAMP level), and consequently the metabolism of AA is shifted to the 5LO-pathway provoking elevated synthesis of pLTs; expression of enzymes and/or receptors may by altered. **AA:** arachidonic acid; **cAMP:** cyclic adenosine monophosphate; **COX:** cyclooxygenase; **cys-LTs:** cysteinyl leukotrienes; **5-LO:** 5-lipoxygenase; **LT:** leukotriene; **NSAID:** non-steroidal anti-inflammatory drug; **PGE_2**: prostaglandin E_2; **pLT:** peptidoleukotrienes;

Alterations in the expression of PGE_2 receptors, particularly in the EP_2 receptor subtype also seem to be involved in the pathology of AERD (Machado-Carvalho *et al.*, 2014). The percentages of neutrophils, mast cells, eosinophils, and T cells expressing EP_2 receptor are lower in NM biopsies from patients with AERD compared with those in biopsies from aspirin-tolerant asthmatic patients. Similar results were reported for bronchial biopsies. In addition, fibroblast cell cultures isolated from NP-AERD showed low levels of EP_2 protein receptor expression under inflammatory conditions when compared with cultured fibroblasts isolated from NM-C (Roca-Ferrer *et al.*, 2011). Several single-nucleotide polymorphisms (SNPs) in the promoter region of the EP_2 gene have been found and associated with AERD, and the most significantly related functional SNP confers decreased transcription levels of the receptor *in vitro* (Jinnai *et al.*, 2004; Kim *et al.*, 2006). Besides these mechanisms, Cahill and co-workers (Cahill *et al.*, 2015), in a study performed in fibroblasts isolated from NP-AERD, showed that epigenetic factors could contribute to down-regulate the expression of the EP_2 receptor in those patients.

In summary, the complex set of disturbances in the homeostasis of inflammation and lipid mediator production that characterize the basal state of the disease may hinge on

a deficient synthesis of PGE_2 or on a diminished ability for it to signal through EP_2 receptor. Separately or even together, these alterations would likely promote persistent inflammation and ongoing tissue remodeling, which are both strong characteristics of AERD (**Figure 17**).

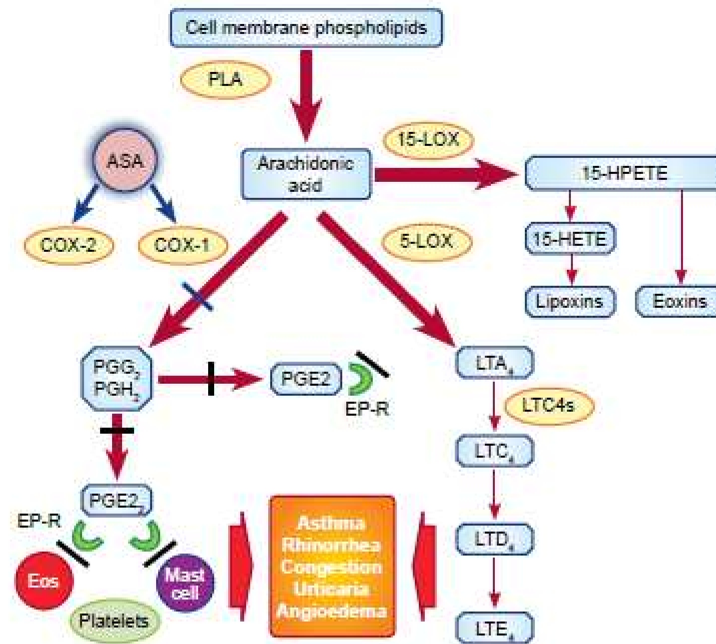


Figure 17. Pathomechanism of aspirin induced hypersensitivity reactions in patients suffering from AERD (Kroegel, 2009). AERD: aspirin exacerbated respiratory disease.

1.6. Management and therapies

The management of AERD involves the previously discussed guideline-based treatment of the patient's with asthma and CRS, in addition to suppression of the consequences of abnormal LT metabolism (LT-modifying agents). Patients must avoid all COX-1 inhibiting NSAIDs or, in selected cases, undergo aspirin desensitization followed by daily NSAID therapy, which seems to reduce IL-4-induced expression of LTs (Graefe *et al.*, 2012).

HYPOTHESIS & OBJECTIVES

HYPOTHESIS

In this thesis, we hypothesized that the characteristics of the inflammatory airway milieu (cytokine profile) in aspirin exacerbated respiratory disease is responsible for the deficient expression of E-prostanoid 2 receptor, modifying its capacity to respond to prostaglandin E_2, which results in an altered regulation of the autocrine positive loop of the cyclooxygenase pathway contributing then to the exacerbated inflammation and remodeling processes usually present in aspirin exacerbated respiratory disease.

SPECIFIC OBJECTIVES

Study 1

1. To investigate the expression of prostaglandin E_2 receptors and their impact on intracellular signaling (cyclic adenosine monophosphate production) after coupling to prostaglandin E_2 or specific E-prostanoid receptor agonists in aspirin exacerbated respiratory disease.

2. To assess the capacity of prostaglandin E_2 to mediate anti-inflammatory and anti-proliferative effects in aspirin exacerbated respiratory disease and to identify the receptor(s) involved in those processes.

Study 2

To determine the mechanisms that linked the deficient expression of E-prostanoid 2 receptor and the altered regulation of the cyclooxygenase pathway in aspirin exacerbated respiratory disease.

Study 3

To evaluate the effect of the inflammatory milieu present in aspirin exacerbated respiratory disease on the regulation of the cyclooxygenase pathway.

MATERIAL & METHODS

1. Material

1.1. Study population

We obtained NM samples from fifteen non-asthmatic control subjects (NM-C) who had septal deviations, turbinate hypertrophy, or both and underwent corrective surgery. Clinical dosages of aspirin or other NSAIDs were taken by all the control subjects without any adverse reaction (asthma and/or rhinitis, urticaria, angioedema or anaphylaxis). On the other hand, human NP specimens were collected from fifteen patients with AERD (NP-AERD) who had undergone functional endoscopic sinus surgery. Patients were selected based on established criteria, such as severe nasal polyposis, reversible bronchial obstruction, and history of asthma attacks precipitated by NSAIDs. AERD was confirmed by means of lysine-aspirin nasal challenge as previously described (Casadevall, 2000). None of the subjects had any oral or intranasal corticosteroids treatment for at least 2 weeks before the surgery. All patients gave informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our institution. Demographic and clinical characteristics of the study population are shown in **Table 3.**

Table 3. Demographic and clinical characteristics of the study population.

Characteristics	NM-C	NP-AERD
Samples, n	15	15
Age, years (mean ± SD)	50.0 ± 21.9	55.8 ± 14.9
Female sex, n	7	9
Moderate / severe asthma, n	0	9 / 6
Aspirin intolerance, n	0	15
Skin prick test positivity, n	2	4
Blood eosinophilia, cells/µL (mean ± SD)	300.0 ± 170.6	773.3 ± 484.7
Intranasal corticosteroids (budesonide equivalent), n (µg/day, mean ± SD)	0 (0)	15 (286.7 ± 106.0)
Inhaled corticosteroids (budesonide equivalent), n (µg/day, mean ± SD)	0 (0)	15 (949.3 ± 215.7)
Previous polypectomies, mean (range)	0 (0)	1.50 (1 – 3)

2. Methods

2.1. Tissue handling and cell culture

At the time of surgery, and for further whole tissue analysis, NM-C and NP-AERD specimens were cut into small fragments and snap-frozen in liquid nitrogen and stored at -80 ºC.

To prepare fibroblast cultures, NM-C and NP-AERD fresh samples were cut into small fragments (approximately 3x3 mm), in sterile conditions, and placed in 6-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco's modified Eagle's media (DMEM) (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Cölbe, Germany), 100 IU/mL penicillin, 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 2 µg/mL amphotericin B (Sigma, St. Louis, MO, USA). Plates were kept in a humidified atmosphere at 37°C and 5% CO_2.

Fibroblasts were isolated from the tissue fragments through adhesion and migration on the plastic surface. Once fibroblast cultures reached 80% confluence (approximately 3 weeks), the tissue fragments were removed, and the first passage was performed. Cells were detached from the plastic surface through the addition of 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (Invitrogen) for 5 min and the reaction was stopped using 10% FBS-supplemented DMEM. Fibroblasts were then centrifuged (400x*g*, 5 min) and seeded in 150-cm^2 flasks (NUNC, Wiesbaden, Germany) in order to obtain a greater number of cells to perform the different experimental protocols and to avoid contaminations by other cell types or frozen to maintain phenotypic stability (**Figure 18**).

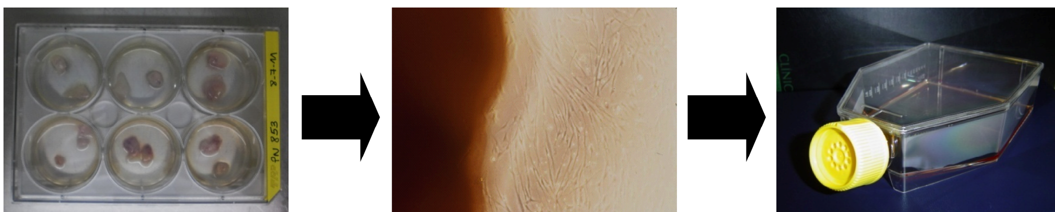


Figure 18. Tissue handling and cell culture.

2.1.1. Cryopreservation and storage

For long-term storage, cells were frozen in liquid nitrogen. Briefly, cells were trypsinized and centrifuged using the same protocol explained before (**see section 2.1**). Cells were counted in an automated cell counter (Luna^{TM} automated cell counter, Logos Biosystems) using the trypan blue die exclusion staining (Sigma) to determine the number of viable cells present in the cell suspension. This technique is based on the principle that live cells possess intact cell membranes that exclude dyes, such as trypan blue, whereas dead cells do not (Strober, 2001). Approximately two millions of cells were counted and distributed in each cryovials. Cell pellet was resuspended in Eagle's Minimal Essential Media (EMEM) (Lonza) supplemented with 20% FBS and in a solution of cryoprotective media (Basal Eagle's Media with Hank's and 15% dimethyl sulfoxide without L-glutamine) (Lonza). All the process was performed under sterile conditions. Cells transferred in cryovials were frozen by slow cooling using a freezing container (Nalgene, VWR) for approximately 24 h at -80°C. This container provides the critical, repeatable, 1°C/min cooling rate required for successful cryopreservation of cells. After this time, cells were stored under liquid nitrogen. When required cells were thaw and seeded with fresh culture media in order to perform new experimental protocols.

2.1.2. Culture characterization

Although the culture media used is selective to fibroblast growth, other structural cells (e.g. epithelial cells) could grow in these conditions. Additionally, fibroblasts may differentiate into myofibroblasts. For those reasons, the purity of fibroblast cultures was assessed by immunofluorescence of characteristic markers for mesenchymal and epithelial cells. The differential expression of structural proteins such as α-smooth muscle actin (α-sma), vimentin, and cytokeratins has been proved to be efficient markers in differentiating myofibroblasts, fibroblasts, and epithelial cells, respectively. α-sma is a contractile protein found in the cytoplasm of myofibroblasts, but not in fibroblasts (Adegboyega *et al.*, 2002). Vimentin is present in myofibroblasts and fibroblasts phenotype, but not in epithelial cells. Finally, cytokeratins are found in epithelial cells, but not in fibroblasts.

Fibroblasts were grown until they reached sub-confluence. After this time, immunofluorescence (see detailed protocol in Methods section) of vimentin (1:100;

V5255, Sigma, St. Louis, MO, USA), α-sma (1:500; M0851, DAKO, Glostrup, Denmark) and pan-cytokeratin (1:200; C2562, recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19, Sigma) was performed. The percentage of positive cells was quantified using fluorescence microscopy.

As shown in **Figure 19**, all cultured cells were vimentin positive **(A)** and cytokeratin-negative **(B)**, confirming the fibroblast phenotype and the total absence of epithelial cells. The myofibroblast percentage, determined through α-sma, was very low in the fibroblast cultures **(C)** and no significant differences were observed between the groups (NM-C = 1.6% (1.3 – 3.3) and NP-AERD = 4.5% (2.2 – 5.7)).

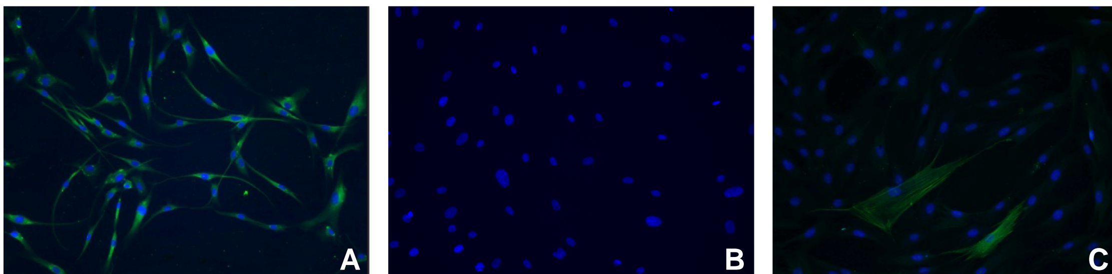


Figure 19. Characterization of cultured cells.

2.2. Study designs

To carry out the experiments and in order to obtain the desired experimental conditions, different study designs were performed.

2.2.1. Study 1

The set of experiments to carry out this study is based on two models:

1- Serum starvation model: the addition of serum free media (SFM), 24 h before the stimulation, arrested cell proliferation and synchronized them into a non-dividing G0-phase to cell cycle. In these conditions, the response of cells to external stimuli is higher and most homogeneous.

2- FBS model: the presence of serum represents a fundamental source of nutrients, cytokines, and adhesive molecules necessary for *in vitro* cell growth, metabolism and proliferation. Depending on the experiment, FBS was added to the cells 24 h after serum starvation.

- **E-prostanoid receptor expression in whole tissue and cultured fibroblasts from nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease**

The expression of EP receptors was assessed in whole tissue and in fibroblasts isolated from NM-C and NP-AERD. The expression in the whole tissue was assessed in samples frozen at the time of surgery. To assess the expression in cultured fibroblasts, cells were plated on 90-cm^2 and six-well culture dishes in DMEM supplemented with 10% FBS. When the cells reached approximately 80% confluence, FBS-supplemented media was switched to SFM for 24 h. After this time, protein and RNA extraction protocols were performed.

- **Effect of prostaglandin E_2 and E-prostanoid receptor agonists on intracellular cyclic adenosine monophosphate production in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To assess intracellular cAMP production, cultured NM-C and NP-AERD fibroblasts were plated into six-well plates in DMEM supplemented with 10% FBS at $2x10^5$ cells/well and allowed to growth to subconfluence. The cells were then serum starved in DMEM for 24 h at 37°C. Cyclic nucleotide phosphodiesterases (PDEs) regulate the cellular levels of cAMP and participate in its degradation. In order to measure the cellular concentration of cAMP, cultured fibroblasts were treated with 3-Isobutyl-1-methylxanthine (IBMX) (13347, Cayman), a PDE inhibitor, for 1 h at 1 mM before the stimulation with different concentrations of PGE_2, or the specific EP_2 receptor agonist butaprost free-acid (13741, Cayman) and EP_4 receptor agonist CAY10598 (13281, Cayman) or forskolin (F3917, Sigma), an AC activator, for 15 min at 37°C. The concentration of intracellular cAMP levels produced by the EP_3 receptor stimulation was evaluated by incubating the cells with both forskolin (20 µM) and sulprostone (14765, Cayman), an EP_3 receptor agonist, at different concentrations for 15 min (**Figure 20**).

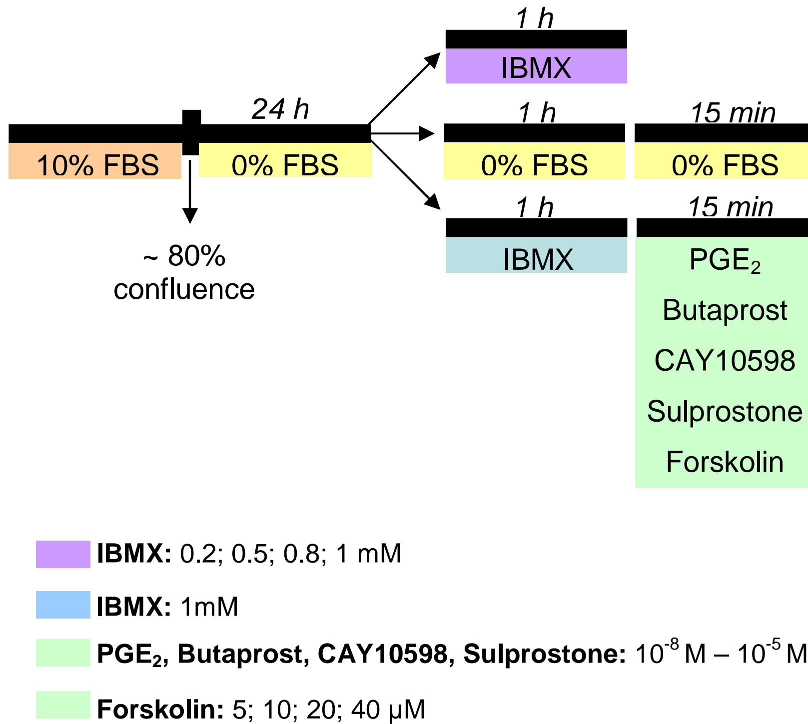
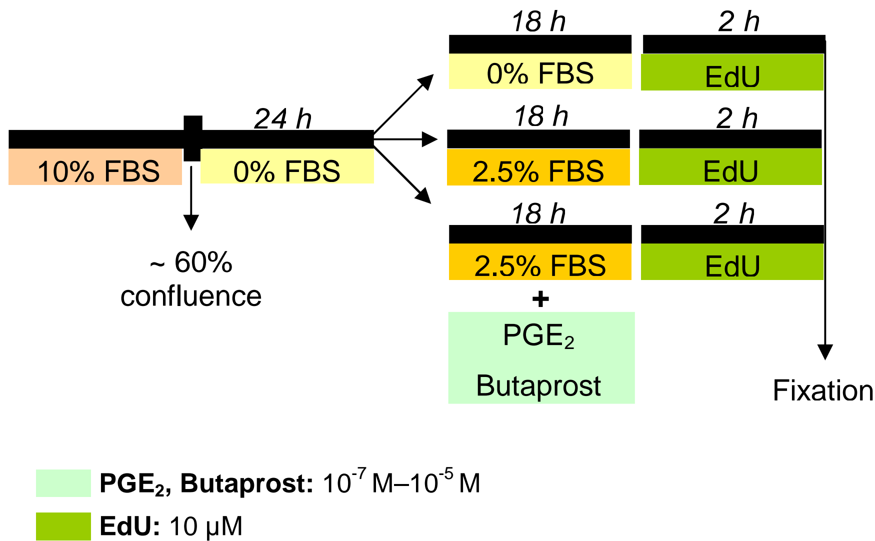


Figure 20. Study design to assess intracellular cAMP production in cultured NM-C and NP-AERD fibroblasts. cAMP: cyclic adenosine monophosphate; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

- **Effect of prostaglandin E_2 and E-prostanoid 2 receptor agonist on nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblast proliferation**

The effect of PGE_2 and EP_2 receptor agonist on cell proliferation was assessed in NM-C and NP-AERD fibroblasts. Briefly, fibroblasts ($1.5x10^5$ cells/plate) were plated on 60-mm petri dishes and allowed to recover overnight. Cells were deprived of serum for the next 24 h, switched to media with 2.5% FBS and treated with or without PGE_2 (10^{-7} M–10^{-5} M) or butaprost (10^{-7} M–10^{-5} M) for 18 h. As negative control, cells were incubated with SFM. After that, cells were incubated for an additional 2 h with 10 µM 5-ethynyl-2’-deoxyuridine (EdU) (**Figure 21**).



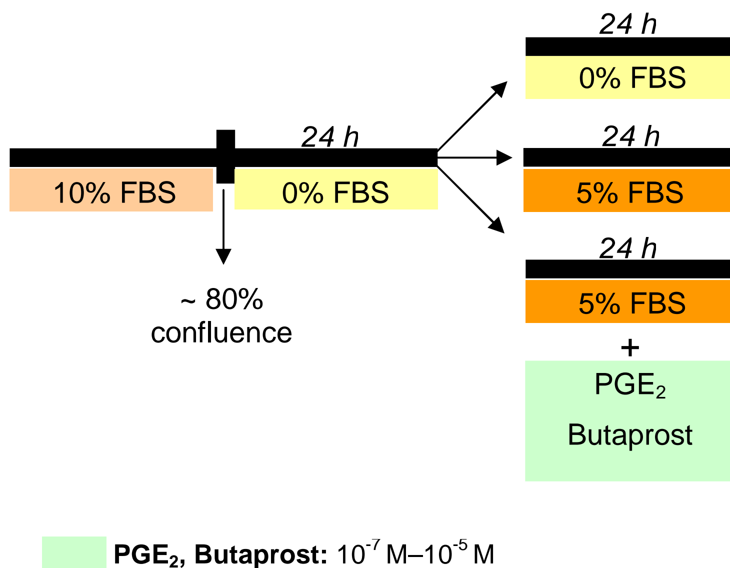
PGE_2, Butaprost: 10^{-7} M–10^{-5} M

EdU: 10 µM

Figure 21. Study design to assess NM-C and NP-AERD fibroblast proliferation. **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

- **Effect of prostaglandin E_2 and E-prostanoid 2 receptor agonist on ganulocyte-macrophage colony-stimulating factor release in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To measure GM-CSF release in cell culture supernatants, fibroblasts from NM-C and NP-AERD were seeded in 24-well plates and allowed to attach for 24 h. Cells were washed with phosphate-buffered saline (PBS), and cultures were incubated with SFM prior to treatment. Fibroblasts were incubated with different concentrations of PGE_2 (10^{-7}M–10^{-5}M) or butaprost (10^{-7}M–10^{-5}M) in DMEM containing 5% FBS for 24 h (**Figure 22**).



PGE_2, Butaprost: 10^{-7} M–10^{-5} M

Figure 22. Study design to assess GM-CSF release in supernatants of cultured NM-C and NP-AERD fibroblasts. GM-CSF: Granulocyte-macrophage colony-stimulating factor; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.2.2. Study 2

To perform this study, we used the serum starvation model for 24 h before specific stimuli treatment.

- **Basal and interleukin-1β-induced interleukin 1 receptor family, cyclooxygenase-2, and microsomal prostaglandin synthase-1 expression in whole tissue and cultured fibroblasts from nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease**

Cultured fibroblasts were treated with SFM and with or without IL-1β (10 ng/mL, R&D Systems, Minneapolis, USA) for 24 h. After this time, the expression of COX-2, mPGES-1, IL-1RI, IL-1RII and IL-1RAcP was measured. The expression of IL-1RI and COX-2 was also assessed in whole tissue in samples frozen at the time of surgery.

- **Correlation between the expression of interleukin 1 receptor type I and cyclooxygenase-2 and interleukin 1 receptor type I and microsomal prostaglandin E synthase-1 in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To confirm the correlation between the expression of IL-1RI and COX-2 and IL-1RI and mPGES-1, cultured NM-C fibroblasts were transiently transfected with IL-1RI small interfering RNA (siRNA) before the stimulation with or without 10 ng/mL IL-1β (**Figure 23**).

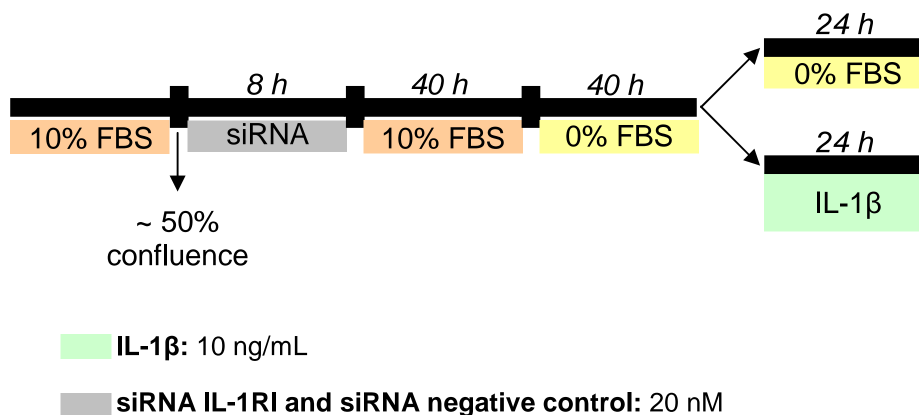


Figure 23. Study design to correlate IL-1β-induced expression of IL-1RI and IL-1β-induced expression of COX-2 and mPGES-1. IL-1β: interleukin-1β; **IL-1RI:** interleukin-1 receptor type 1; **COX-2:** cyclooxygenase-2; **mPGES-1:** microsomal prostaglandin E synthase-1.

- **Effect of prostaglandin E_2 and acetylsalicylic acid on interleukin 1 receptor type 1 expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To assess the effect of PGE_2 on IL-1RI expression, cultured NM-C and NP-AERD fibroblasts were incubated with or without PGE_2 (10^{-7} M–10^{-5} M) for 24 h. Additionally, NM-C fibroblasts were pre-treated with 10^{-5} M or 10^{-3} M acetylsalicylic acid (ASA) (A5376, Sigma-Aldrich Chemical, St. Louis, MO, USA) for 1 h before the addition of 10 ng/mL IL-1β for 24 h. After this period, the expression of IL-1RI was measured.

- **Effect of interleukin-1β on intracellular cyclic adenosine monophosphate production in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To assess intracellular cAMP production, NM-C and NP-AERD fibroblasts were cultivated as previously described and treated with 1 mM IBMX before the stimulation with 10 ng/mL IL-1β for 1 h.

- **E-prostanoid receptor involved in the regulation of interleukin-1 receptor type I in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To evaluate the role of PGE_2/EP/cAMP axis activation in the regulation of IL-1R, cultured fibroblasts from NM-C and NP-AERD were incubated with or without different concentrations (10^{-7} M–10^{-5} M) of butaprost or CAY10598 or 20 µM forskolin in the presence or absence of different concentrations (10^{-7} M–10^{-5} M) of sulprostone for 24 h. After this time, the expression of IL-1RI was measured.

- **Effect of E-prostanoid 2 receptor transfection in cultured nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts**

To evaluate the effect of EP_2 receptor transfection on NP-AERD, NP-AERD fibroblasts expressing expression plasmids encoding green fluorescent protein (GFP) or GFP chimeras of EP_2 receptor were treated with or without PGE_2 at 10^{-7} M–10^{-5} M and with or without 10 ng/mL of IL-1β for 24 h. After this time, the expression of IL-1RI, COX-2 and mPGES-1 was measured.

2.2.3. Study 3

To perform these experiments, we used the serum starvation model for 24 h before specific stimuli treatment.

- **Effect of interleukin-4 and interferon-γ on the expression of E-prostanoid receptors, interleukin 1 receptor type 1, cyclooxygenase-2, and microsomal prostaglandin E synthase-1 in cultured fibroblasts from nasal mucosa of control subjects**

To evaluate the role of IL-4 (10 ng/mL, R&D Systems, Minneapolis, USA) and IFN-γ (10 ng/mL, R&D Systems, Minneapolis, USA) on COX pathway, NM-C fibroblasts were incubated with or without IL-1β (10 ng/mL) in the presence or absence of IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h. After this period, the expression of EP_2, EP_3, EP_4, IL-1RI, COX-2, and mPGES-1 was measured by using western blot.

2.3. Western blot

2.3.1. Protein extraction

Whole tissue

Total protein extraction from whole tissue samples was carried out by adding 200 µL of an ice-cold lysis buffer containing Complete™ protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) in 0.05 M Hepes buffer solution (Life Technologies, Carlsbad, CA, USA), 0.5% v/v Triton X-100 (Sigma), and 625 mM phenylmethylsulfonyl fluoride (Pefabloc) (Roche Diagnostics, Mannheim, Germany) directly to the tissue fragments. Samples were kept on ice for 20 min and sonicated twice for 15 s in a sonifier (Branson, Danbury, CT, USA) to obtain homogenized mixture. The lysates were centrifuged at 12,000x*g* for 10 min at 4°C. Supernatants containing protein lysates were collected into a new tube to discard cellular debris. Lysates were stored at -80°C until use.

Fibroblasts

Fibroblasts plated on 90-cm^2 dishes were washed twice with ice-cold PBS and lysed in 200 µL of ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% sodium dodecyl sulphate (SDS), 1% nonidet P-40 [Igepal], 1 µg/mL leupeptin, 1 µg/mL aprotinin, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM dithiothreitol (DTT), 0.5 mg/mL Pefabloc, and 5 mg/mL sodium deoxycholate (Sigma, St. Louis, MO, USA). Samples were sonicated and centrifuged as previously described.

2.3.2. Determination of protein concentration

To ensure an equal loading in western blot assay, the amount of protein in cell lysates was quantified, using a kit based on the Lowry method. The kit measures the total protein presents in each sample using for that known bovine serum albumin (BSA) concentrations as standard.

Briefly, the standards were prepared by diluting Protein Standard Solution (Sigma) in deionized water to a final volume of 250 µL. From the maximum concentration (400 µg/mL), serial dilutions were performed until reached the minimum concentration of 50 µg/mL. The blank was prepared by adding 250 µL of water. Samples were analyzed at 1:20 dilution in water.

First, Lowry reagent (Sigma) was added to each sample, vortexed and allowed to stand for 20 min. After this time, a second reagent - Folin & Ciocalteau's Phenol Reagent Working Solution (Sigma) – was added to each tube and vortexed. To allow full colour development, tubes were allowed to stand for 30 min. The content of each tube was then transferred to a 96-well plate (NUNC, Wiesbaden, Germany) and all samples were read in duplicate. The absorbance was measured at 630 nm using a microplate spectrophotometer (Thermo Multiskan EX).

2.3.3. Protein electrophoresis

Under denaturing conditions, protein samples can be separated according to their size on acrylamide gels. Once the migration of proteins with similar molecular weight may vary due to differences in secondary, tertiary or quaternary structures, SDS, an anionic detergent, is used to reduce proteins to their primary structure, giving nearly a uniform negative charge along the length of the polypeptide. The applied voltage during the electrophoresis process allows the migration of the anions and the proteins are separated according to their molecular weight.

Twenty microgram of protein were denatured (100°C, 5 min) in a thermocycler (MJ Research PTC-100, USA) in the presence of a loading buffer (1:4 ratio) (NuPAGE lithium dodecyl sulphate sample buffer) and NuPAGE sample reducing agent (1:10 ratio), which contains DTT. The proteins were then loaded in 7% TRIS-Acetate or 12% Bis-Tris gels (Invitrogen) in SDS running buffer (Novex TRIS-acetate SDS running buffer 20x or Novex MOPS SDS running buffer 20x), set in a vertical electrophoresis

chamber (Novex XCell II Mini-Cell; Invitrogen, San Diego, CA, USA). To maintain proteins in a reduced state during the process of electrophoresis, 500 µL of an antioxidant (NuPAGE) which contains N,N-dimethylformamide was added to the running buffer.

The known molecular weight marker (SeeBlue Plus2 Pre-Stained Standard) was loaded on the gel as reference. This marker facilitates the identification of the band, since it indicates the molecular weight in kilodaltons (kDa) of the protein and allows a faster evaluation of the transfer efficiency. Proteins were separated according to their molecular weight during electrophoresis (90 min, 125 V) using a power source (BioRad Power Pac 1000, BioRad Laboratories Ltd. Hemel Hemstead, UK).

2.3.4. Transfer

The proteins were transferred using a dry blotting system (iBlot® Dry Blotting System, Invitrogen). This system efficiently and reliably blots proteins from polyacrylamide gels in 7 min without the need of additional buffers. It includes an integrated 0.2 µm nitrocellulose membrane, in which the proteins are transferred. The order of layers in the transfer system was from bottom to top: anode stack (includes the nitrocellulose membrane), acrylamide gel, filter paper, cathode stack and finally disposable sponge.

2.3.5. Blocking

Blocking of all potential non-specific binding sites is achieved by placing the nitrocellulose membrane in a blocking buffer that contains 5% non-fat dry milk and 0.1% Tween®20 in 10 nmol/L PBS (T-PBS) for 1 h at room temperature with gentle shaking in an orbital shaker (Stuart Scientific SO3 Orbital Shaker, UK).

2.3.6. Immunodetection and densitometric analysis

After blocking, membranes are incubated with a dilute solution of primary antibody (**Table 4**) in blocking buffer under gentle agitation overnight at 4°C.

Table 4. Antibodies used to perform immunodetection.

Antibodies	Company	Molecular weight (kDa)	Source	Dilution
Anti EP_1	Cayman	~ 42	Rabbit	1:500
Anti EP_2 (C-terminal)	Cayman	~ 52	Rabbit	1:1000
Anti EP_2 (N-terminal)	Origene	~ 53	Rabbit	1:1000
Anti EP_3	Cayman	~ 53	Rabbit	1:1000
Anti EP_4	Abcam	~ 53	Goat	1:1000
Anti COX-2	Cayman	~ 72	Mouse	1:1000
Anti mPGES-1	Cayman	~16	Rabbit	1:200
Anti IL-1RI	Santa Cruz	~ 80	Rabbit	1:1000
Anti IL-1RII	Santa Cruz	~ 46	Mouse	1:1000
Anti IL-1RAcP	Santa Cruz	~ 66	Mouse	1:1000
Anti GFP	Origene	~ 26	Mouse	1:2000
Anti β-actin	Sigma	~ 42	Mouse	1:10000

After rinsing the blots with 0.1% T-PBS to remove unbound primary antibody, the incubation with a secondary antibody (horseradish peroxidase-labeled) (Santa Cruz Biotechnology) directed at a species-specific portion of the primary antibody was performed. Specific secondary antibodies were diluted 1:3000 (except the secondary antibody for β-actin detection whose was diluted 1:10000) in blocking buffer and incubated with the membrane for approximately 2 h at room temperature.

After repeating the washes, membranes were incubated for 5 min with an enhanced chemiluminescent substrate (Supersignal West Pico Chemiluminescent Substrate, Rockford, IL, USA) and light emissions were detected using a CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge v.4.0 Software. Band intensities were normalized to the intensity of control β-actin assessed in the same sample.

2.4. RNA isolation and quantitative real-time PCR

2.4.1. RNA extraction

Whole tissue

The extraction of total RNA from whole tissues was performed using a TRIzol® reagent (Invitrogen) according to the manufacturer's protocol with the exception of minor modifications. Briefly, tissue samples were homogenized using a power homogenizer in 1 mL of TRIzol® reagent. After incubation of the homogenized samples for 5 min at room temperature to allow for cell membrane dissociation, 0.2 mL chloroform per mL of TRIzol was added to the mixture. The samples were mixed vigorously and centrifuged at 12,000x*g* for 15 min at 4°C. As shown in **Figure 24**, the homogenate is allowed to separate into a clear upper aqueous layer (containing the RNA), an interphase, and a red lower organic layer (containing the DNA and the proteins).

The aqueous upper phase was recovered carefully and 0.5 mL of 100% isopropanol was added to precipitate RNA. Tubes were then mixed and incubated overnight at -20°C. After this time and in order to pellet RNA in the bottom of the tubes, they were centrifuged at 12,000x*g* for 10 min at 4°C. The pellet was washed once with ice cold 75% ethanol, centrifuged again at 7,500x*g* for 5 min at 4°C, air-dried and dissolved in 20 µL of RNase-free water. Total RNA was quantified with a spectrophotometer (NanoDrop 2000, Thermo Scientific, Milan, Italy) and stored at -80°C.

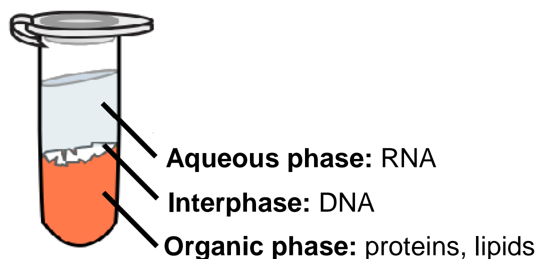


Figure 24. Illustration of a sample after the first separation in the TRIzol extraction protocol.

Fibroblasts

Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Valencia, CA), following the protocol suggested by the manufacturer. The RNeasy procedure is based on the selective binding of RNA longer than 200 to a silica membrane.

Fibroblasts were first lysed and homogenized with 600 µL of a highly denaturing buffer (RLT), which immediately inactivates RNases to ensure purification of intact RNA. Then, the same volume of ethanol was added to provide appropriate binding conditions to the RNeasy spin column. The next steps included multiple washes and centrifugations to remove contaminants and to allow the binding of RNA to the membrane. Finally, RNA was eluted in 30 µL of RNase-free water. Total RNA was quantified as described before.

2.4.2. Reverse transcription

1 µg of total RNA was converted to cDNA by reverse transcription using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Foster City, CA).

First, the required volume of each sample was determined in order to obtain 1 µg of total RNA and RNase-free water was added to reach a final volume of 10 µL. The dilution was performed in siliconized tubes (Clear-view™ Snap-Cap microtubes, Sigma). After that, the master mix with the necessary components to perform reverse transcription was prepared according to **Table 5**.

Table 5. Components used to prepare master mix for reverse transcription.

Components	µl per reaction
10x RT buffer	2.0
25x dNTP mix	0.8
10x RT random primers	2.0
Multiscribe™ reverse transcriptase	1.0
RNase inhibitor	1.0
Nuclease-free H_2O	3.2

To avoid sample evaporation during reverse transcription, 40 µL of mineral oil was added to each tube. The process was performed in a thermocycler (MJ Research PTC-100, USA) using the conditions below:

- ✓ Step 1: 25°C, 10 min
- ✓ Step 2: 37°C, 120 min
- ✓ Step 3: 85°C, 5 min
- ✓ Step 4: 4°C, ∞

Samples were stored at 4°C in case of short term storage or -20°C for long term storage.

2.4.3. Quantitative real-time PCR

From the cDNA obtained by reverse transcription, quantitative real-time PCR was performed using a TaqMan® Gene Expression Assays (Applied Biosystems, Life Technologies), which target to specific genes. The genes analyzed through this procedure are represented below.

- ✓ COX-2: Hs00153133_m1; *PTGS2*
- ✓ EP_1: Hs00168752_m1; *PTGER1*
- ✓ EP_2: Hs00168754_m1; *PTGER2*
- ✓ EP_3: Hs00168755_m1; *PTGER3*
- ✓ EP_4: Hs00168761_m1; *PTGER4*
- ✓ IL-1RI: Hs00991002_m1; *IL1R1*
- ✓ IL-1RII: Hs01030384_m1; *IL1R2*
- ✓ IL-1RacP: Hs00895050_m1; *IL1RAP*
- ✓ Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Hs02758991; *GAPDH*

Target mRNA expression was normalized to GAPDH, and the data were calculated using the ΔΔCt method.

2.5. Immunofluorescence

Fibroblasts grown in 4-well CultureSlides® (BD Biosciences, USA) were washed with ice-cold PBS, fixed with cold 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 30 min, and blocked with 1% BSA-PBS for 1 h. Cells were incubated with an antibody against IL-1RI (Santa Cruz Biotechnologies) diluted 1:500 in 1% BSA-PBS for 1 h at 37°C. Then, cells were washed and incubated with Cy3-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:500 for 1 h at 37°C. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) diluted 1:10000 and were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). The slides were analyzed by fluorescent microscopy (Axiovert 200M, Zeiss) and the images were visualized using the imaging software ImageJ (Bethesda, Maryland, USA).

2.6. Cyclic adenosine monophosphate measurement

Intracellular cAMP levels were determined using a commercial enzyme-linked immunosorbent assay (ELISA) (Arbor Assays, Ann Arbor), according to the manufacturer's instructions. Briefly, fibroblasts were grown in 6-well plates until sub-confluence and, after perform the experimental protocols to measure cAMP production and in order to stop the reaction, culture media was aspirated and cells were washed with ice-cold PBS. Then, the fibroblasts were treated directly with an acidic Sample Diluent provided in the kit for 10 min at room temperature and scraped to detach them from the surface of the wells. This reagent contains additives and stabilizers to allow the efficient measurement of cAMP levels. Cell lysates were centrifuged at 10,000x*g* at 4°C for 15 min and the supernatants were collected into a new tube and frozen at -80°C for further analysis.

For cAMP measurement all samples and standards were diluted into a Sample Diluent. The standard curve ranging from 0.617 to 150 pmol/mL was prepared using the cAMP standard provided in the kit. After several steps, which include incubations and washes, the reactions were stopped and the intensity of the generated colour was measured at 450 nm wavelength using a microplate spectrophotometer (Thermo Multiskan EX). The total protein content of each sample was determined as described in 2.3.2 section and used as loading control. The concentration of cAMP was expressed as pmol of cAMP/µg of total protein.

2.7. Analysis of DNA replication

To assess cell proliferation, a commercial kit, Click-iT® EdU (5-ethynyl-2´-deoxyuridine) Flow Cytometry Assay Kits (Invitrogen), was used. EdU is a nucleoside analog to thymidine and is incorporated into DNA during active DNA synthesis. Detection is based in a click reaction: a cooper catalysed covalent reaction between an azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the azide is coupled to Alexa Fluor® 488 dye, which emits green fluorescence. The advantage of Click-iT® EdU labeling is that the small size of the dye azide allows for efficient detection of the incorporated EdU using mild conditions. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT® detection reagent to gain access to the DNA, without the need of DNA denaturation.

The volumes of Click-iT® components required to the preparation of the reaction cocktail for a desired number of reactions were calculated according to **Table 6**.

Table 6. Reaction components of Click-iT cocktail.

Reaction components	µL per reaction
1x Click-iT reaction buffer	438
$CuSO_4$	10
Fluorescent dye azide	2.5
1x Reaction buffer additive	50

Once prepared, the cocktail reaction must be protected from the light and used within 15 min.

2.7.1. Cell proliferation

For flow cytometry analysis, cells were grown in 90-cm^2 culture dishes. After their respective treatment, cells were incubated for 2 h with 10 µM EdU. In this time, EdU is incorporated in the cells and this process allows to visualize the percentage of S-phase cells in the total population. Thus, the result is a clear separation of proliferating cells which have incorporated EdU and non-proliferating cells which have not. After metabolic labeling with EdU, cells were trypsinized and fixed cold 70% ethanol. Ethanol

was added drop wise to cell pellet with gentle agitation with a vortex. Samples were kept in ethanol and stored at -20°C for at least 24 h. After that, cell pellets were washed twice with 1% BSA-PBS and permeabilized for 15 min with saponin diluted 1:10 in 1% BSA-PBS.

Posteriorly, the components required for cocktail reaction were mixed and added to each reaction tube. The reaction mixture was incubated for 30 min at room temperature and protected from the light. After this time, cell pellets were washed with 1% BSA-PBS and resuspended in 1% BSA-PBS for flow cytometry analysis. Samples were analyzed by a BD FACSCanto™ II flow cytometer (BD Biosciences) and the results were analyzed with FACSDiva Software. Over 10,000 events were collected per sample and the results indicated the percentage of cells that have incorporated the modified nucleoside EdU in the total population. Auto-fluorescence controls and negative controls were also included in the analysis to measure the basal fluorescence of cells without EdU and to establish a positive marker of the technique in the absence of the reaction cocktail respectively.

2.8. Granulocyte-macrophage colony-stimulating factor measurement

Human GM-CSF levels in supernatants were determined by a commercially available ELISA kit (R&D Systems, Abingdon, UK). After perform the experimental protocols, cell culture supernatants were collected, filtered through a 0.22 µg filter (BD Biosciences), and stored at -80°C for further analysis.

Before starting, all reagents were brought to room temperature and allowed to sit for a minimum of 15 min with gentle agitation after initial reconstitution. The working solutions were prepared and used immediately. Briefly, plates were coated overnight with 100 µL of 2 µg/ml mouse anti-human GM-CSF dissolved in PBS. On the next day, each well was aspirated and washed three times with Wash Buffer (0.05% T-PBS [pH 7.2–7.4]). After the last wash, any remaining Wash Buffer was removed and plates were blocked by adding Reagent Diluent (1% BSA-PBS [pH 7.2–7.4], 0.2 µm filtered) to each well and incubated at room temperature for 1 h. After this time, the aspiration/wash previously described was repeated. Plates ready for sample addition were incubated for 2 h at room temperature with 100 µL of samples or standards in Reagent Diluent. Samples and standards were tested in duplicate. Next, plates were aspirated, washed again and incubated with 100 µL of the Detection Antibody

(biotinylated mouse anti-human GM-CSF), diluted in Reagent Diluent for an additional 2 h at room temperature. After aspiration and washes, plates were incubated with 100 µL of the working solution of Streptavidin-HRP for 20 min at room temperature and protected from the light. Wells were aspirated and washed and incubated with 100 µL of Substrate Solution for 20 min at room temperature and protected from the light. Substrate Solution detects HRP activity, yielding a blue colour that changes to yellow upon addition of 50 µL sulphuric acid stop solution. The optical density of each well was determined immediately, using a microplate spectrophotometer set to 450 nm.

The standard curve ranged from 15.6 to 1000 pg/mL and cytokine production was normalized by cell number using the colorimetric Cell Proliferation Kit II (XTT, Roche Diagnostics GmbH, Mannheim, Germany).

Cell Viability

Metabolic active cells reduce the tetrazolium salt (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) to orange coloured compounds of formazan. The dye formed is water soluble and the dye intensity can be read at a give wavelength with a spectrophotometer. The intensity of the dye is proportional to the number of metabolic active cells.

At the time of cell culture supernatant collection, the XTT labeling mixture was prepared under sterile conditions and protected from the light. The mixture was obtained by mixing XTT labeling reagent and electron-coupling reagent according to the instructions of the supplier. 24-well plates were washed twice with sterile PBS and the XTT labeling mixture was added to each well. The plates were incubated at 37°C and the absorbance was measured after 3 h at 490 nm using a microplate spectrophotometer.

2.9. Gene silencing with small interfering RNA

The forward transfection method was used for gene silencing with siRNA. For that, cells were plated in culture dishes, and the transfection mix was prepared and added the next day. The number of cells seeded was calculated such that the cells would be 50% confluent at the time of transfection. To prepare the transfection mix, Lipofectamine RNAiMAX Reagent (Life Technologies) was diluted in Opti-MEM® I Reduced Serum Media (Life Technologies) that consists of a modification of EMEM,

buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements, and growth factors, ideal for use during cationic lipid transfections. In different tubes, IL-1RI Silencer Select pre-designed siRNA (Life Technologies) and Silencer Select negative control siRNA (Life Technologies) were also diluted in Opti-MEM® I Reduced Serum Media. The three different reaction tubes were incubated 5 min at room temperature and diluted Lipofectamine RNAiMAX Reagent was added to diluted siRNA (1:1 ratio). The tubes were allow to stand for 20 min at room temperature. After that, siRNA-lipid complexes were added to cells in order to obtain a final concentration of 20 nM siRNA. All the steps performed in the process of transfection were done in antibiotic-free media and after transfection cells were kept at 37°C. Approximately 8 h after transfection, media was changed to DMEM supplemented with 10% FBS and cells were incubated for an additional 40 h.

2.10. Bacterial culture

2.10.1. Bacterial growth media

LB (Luria-Bertani) Broth media preparation

To prepare LB Broth media (CONDA laboratories, Spain), 20 g of LB Broth were resuspended in 1 L of distilled water, mixed well and dissolved by heating with frequent agitation. The mixture was boiled for one minute until complete dissolution and was dispensed into appropriate containers and sterilized in autoclave at 121°C for 15 min.

LB-agar plates

To prepare LB-agar plates, 20 g of Agar LB (CONDA laboratories, Spain) were resuspended in 500 mL of distilled water, mixed well and dissolved by heating with frequent agitation. The mixture was boiled as for one minute until complete dissolution. After that, the solution was sterilized in autoclave at 121ºC for 15 minutes and cooled to 45–50°C, before adding ampicillin (Sigma) to reach a final concentration of 100 µg/mL. Ths solution was dispensed into plates and after 30 to 60 min stored at 4°C.

2.10.2. Transformation of chemically competent *Escherichia coli*

100 µL of chemically competent *Escherichia coli* (*E. coli*) were thawed on ice, mixed gently with 100 ng plasmid DNA in a sterile tube and incubated on ice for 30 min. Cells were heat-shocked in a water bath at 42°C for 60 s and immediately cooled on ice for 5 min. After that, 900 µL of LB media was added. To express the resistance, bacteria were incubated for 1 h at 37°C with shaking and 100 µL of the transformation were plated and incubated overnight at 37°C. On the next day, one single isolated colony was selected, picked with a sterile toothpick, and grown in 200 mL LB media supplemented with ampicillin overnight at 37°C with shaking (220 rpm) to reach an OD_{600} of 2. Cells were pelleted by centrifugation (4500x*g*, 10 min, 4°C) and supernatant was discarded.

Plasmid DNA was purified using a NucleoBond® Xtra Midi kit (Macherey-Nagel GmbH, Düren, Germany). Basically, cell pellet was resuspended in Resuspension Buffer RES + RNase A by pipetting the cells up and down. Then, Lysis Buffer LYS was added gently to the suspension and mixture was incubated 5 min at room temperature. Meanwhile, the NucleoBond® Xtra Column was equilibrated together with the inserted column filter with Equilibration Buffer EQU by applying the buffer onto the rim of the column filter and allowed to empty by gravity flow. On the other hand, Neutralization Buffer NEU was added to the suspension and the lysate was immediately mixed until blue samples turned colourless. The homogenous suspension obtained was loaded onto the equilibrated column and was allowed to empty by gravity flow. After that, column and filter were washed with buffer EQU, filter was removed and column was washed once again with Wash Buffer WASH. Then, plasmid DNA was eluted with Elution Buffer ELU, the eluate was collected in a centrifuge tube and isopropanol at room temperature was added to precipitate eluted plasmid DNA. Samples were vortexed and centrifuged at 15000x*g* for 30 min at 4°C. Supernatant was discarded and pellet was washed with 70% ethanol. After centrifugation (15000x*g*, 5 min, room temperature), ethanol was carefully removed and pellet was allowed to dry at room temperature. Once dried, the DNA pellet was dissolved in 250 µL of sterile water and tubes were placed in a shaker at constant spinning (550 rpm) for 30 min at 38°C for a better dissolution of the pellet.

Plasmid yield was determined by using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Milan, Italy) and kept frozen at -20°C.

2.11. Transient transfection of E-prostanoid 2 receptor

Cells were transiently transfected with human *PTGER2* cDNA plasmid using Lipofectamine 2000 and Opti-MEM® I Reduced Serum Media (Life Technologies). The expression vector pCMV6-AC-GFP (Origene Technologies, Inc., Rockville, MD) was used for expression of *PTGER2* gene (NCBI accession: NM_000956). The vector plasmid pCMV6-AC-GFP (Origene Technologies, Inc.) without the insert was used as control. Plasmids DNA were amplified and purified as described in 2.10.

To perform transient transfection protocol, cells were seeded in 90-cm^2 culture dishes and incubated until reaching 80–90% confluence. After that, cells were transiently transfected with 10 µg pCMV6-AC following the same protocol that was described for gene silencing with siRNA. After approximately 8 h, transfection media was switched to fresh media without antibiotics. The expression of EP_2 receptor-GFP chimeras or GFP only was checked out in real-time using an Axiovert 200M (Zeiss) inverted fluorescence microscope.

2.12. Statistical analysis

All statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). The data are expressed as the median and 25th–75th percentile. The non-parametric statistical Mann-Whitney U-test was used for NM-C and NP-AERD group comparisons and the Wilcoxon test was used for paired comparisons. Pearson's correlation was used to establish a correlation between the expression of IL-1RI and COX-2 and IL-1RI and mPGES-1. Statistical significance was established as *p* value below 0.05.

Results

RESULTS

1. Study 1

1.1. E-prostanoid receptor expression in nasal samples from nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease

The baseline protein and mRNA expression of EP receptors was assessed by western blot and quantitative real-time PCR techniques, respectively. The expression of EP_1 receptor protein and mRNA was undetectable in nasal samples from both NM-C and NP-AERD, while EP_2, EP_3, and EP_4 receptors protein and mRNA were detected in both groups. Protein (**Figure 25A**) and mRNA (**Figure 25C**) expression of EP_2 receptor was significantly lower in NP-AERD when compared with NM-C samples. No differences were found in protein and mRNA levels of EP_3 receptor between NM-C and NP-AERD (**Figure 26A and 26B**). On the other hand, protein (**Figure 25B**) and mRNA (**Figure 25D**) expression of EP_4 receptor was higher in NP-AERD than in NM-C.

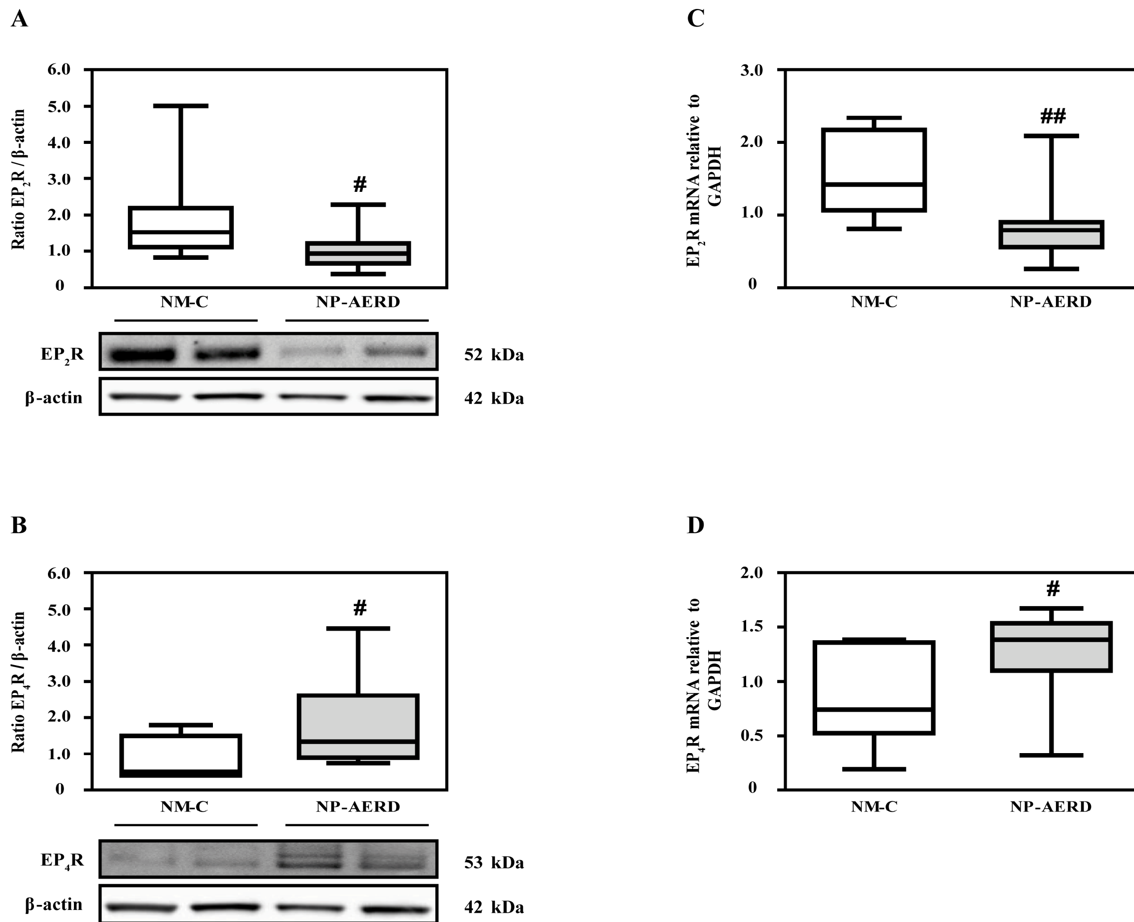


Figure 25. Basal expression of EP_2 and EP_4 receptors in nasal samples from NM-C and NP-AERD. Densitometric analysis and representative western blot of **(A)** EP_2 and **(B)** EP_4 protein expression normalized to β-actin in nasal samples from NM-C (n=9) and NP-AERD (n=9). **(C)** EP_2 and **(D)** EP_4 mRNA expression in nasal samples from NM-C (n=9) and NP-AERD (n=9) was analyzed by quantitative real-time PCR and normalized to the GAPDH constitutive gene. # $p<0.05$, ## $p<0.01$ compared with NM-C. **EP:** E-prostanoid; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

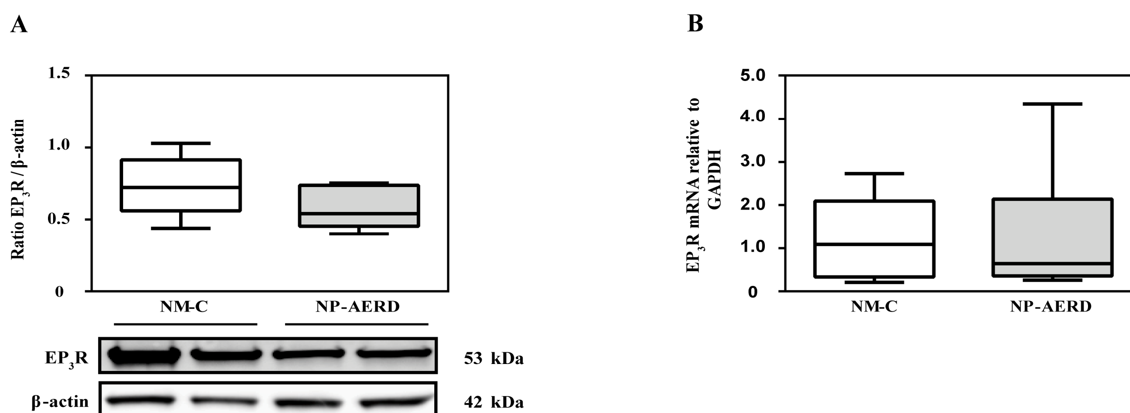


Figure 26. Basal expression of EP_3 receptor in nasal samples from NM-C and NP-AERD. (A) Densitometric analysis and representative western blot of EP_3 expression normalized to β-actin in nasal samples from NM-C and NP-AERD (n=9 each). **(B)** EP_3 mRNA expression in nasal samples from NM-C and NP-AERD (n=9 each) was analyzed by quantitative real-time PCR and normalized to the GAPDH constitutive gene. **EP:** E-prostanoid; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

1.2. E-prostanoid receptor expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The baseline expression of EP receptors in cultured NM-C and NP-AERD fibroblasts was also measured. After 24 h in SFM, the expression of EP receptors was assessed by both western blot and quantitative real-time PCR. As observed for the whole tissue, the results showed no presence of EP_1 receptor in cultured NM-C and NP-AERD fibroblasts while EP_2, EP_3, and EP_4 protein and mRNA levels were detected in both NM-C and NP-AERD fibroblast. EP_2 receptor protein expression was significantly lower in cultured NP-AERD fibroblasts (p<0.01) compared with NM-C fibroblasts (**Figure 27A**). Likewise, mRNA levels were also lower in NP-AERD fibroblasts (**Figure 27C**) than in the NM-C group (p<0.001). Protein and mRNA levels of EP_3 receptor were similar between cultured NM-C and NP-AERD fibroblasts (**Figure 28A and 28B**). Finally, we found upregulated protein (p<0.001) and mRNA (p<0.01) expression of EP_4

receptor in cultured NP-AERD fibroblasts compared with NM-C fibroblasts (**Figure 27B and 27D**).

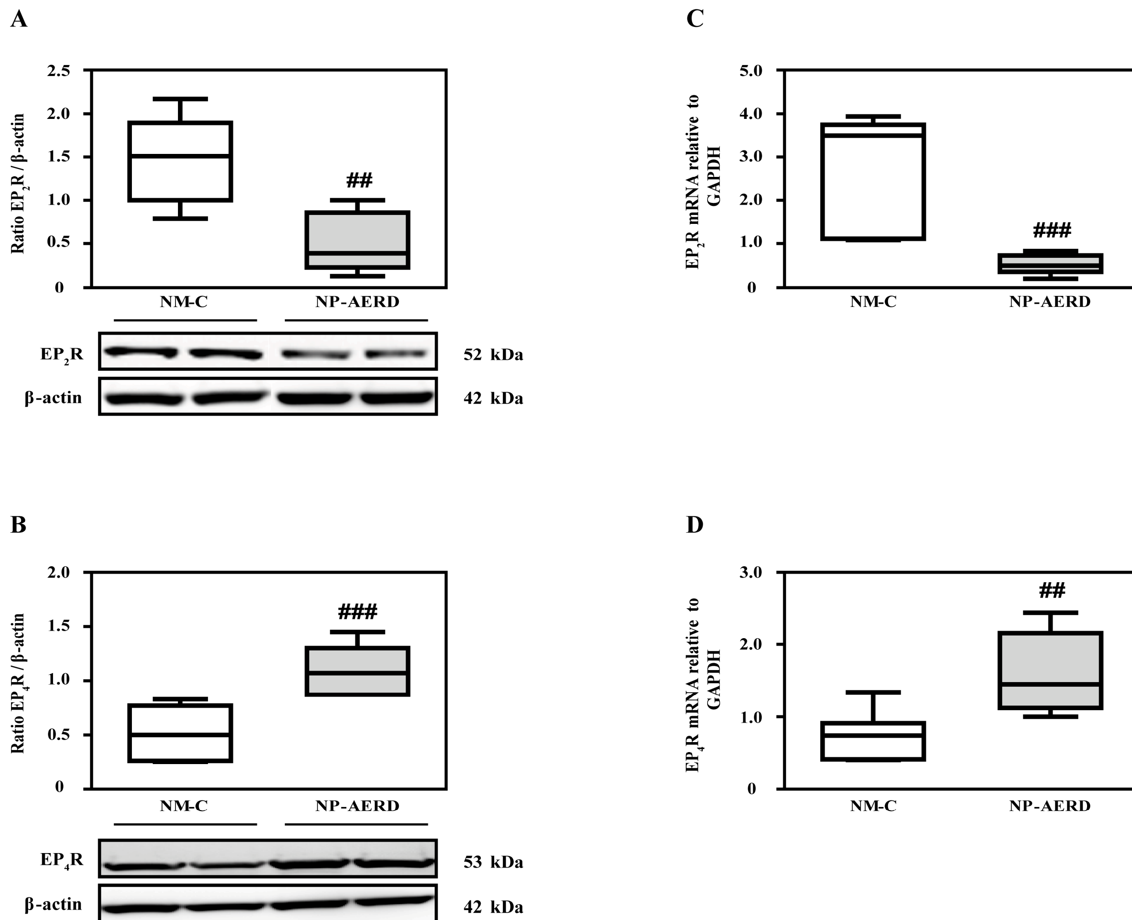


Figure 27. Basal expression of EP_2 and EP_4 receptors in cultured NM-C and NP-AERD fibroblasts. Quiescent fibroblasts from NM-C and NP-AERD (n=7 each) were incubated for 24 h with culture media without FBS. Densitometric analysis and representative western blot of **(A)** EP_2 and **(B)** EP_4 protein expression normalized to β-actin. Quantitative real-time PCR analysis of **(C)** EP_2 and **(D)** EP_4 mRNA expression normalized to the GAPDH constitutive gene. ## p<0.01, ### p<0.001 compared with NM-C fibroblasts. **EP:** E-prostanoid; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

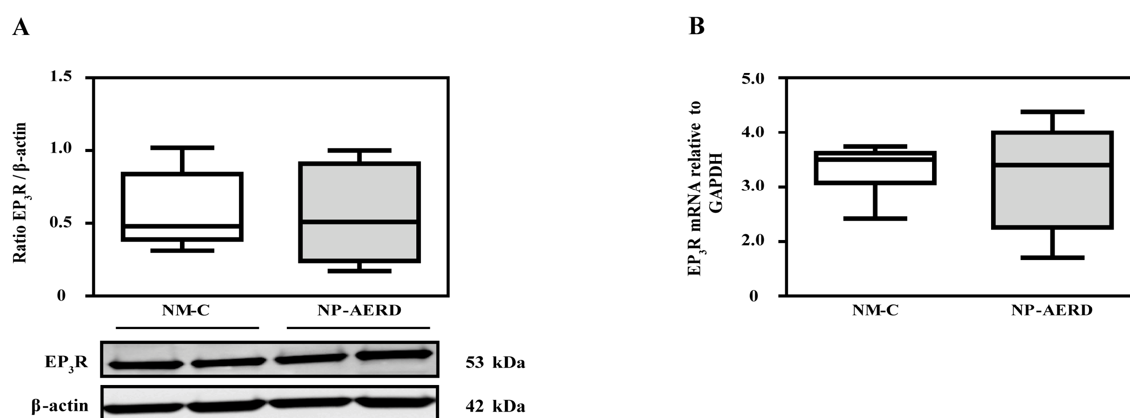


Figure 28. Basal expression of EP_3 receptor in cultured NM-C and NP-AERD fibroblasts. Quiescent fibroblasts from NM-C and NP-AERD (n=7 each) were incubated for 24 h with cell media without FBS. **(A)** Densitometric analysis and representative western blot of EP_3 expression normalized to β-actin. **(B)** Quantitative real-time PCR analysis of EP_3 mRNA expression normalized to the GAPDH constitutive gene. **EP:** E-prostanoid; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

1.3. Intracellular cyclic adenosine monophosphate production in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibrobalsts upon E-prostanoid receptor activation

To test whether PGE_2 receptors expression correlates with the amount of intracellular cAMP released upon their activation cultured NM-C and NP-AERD fibroblasts were treated with PGE_2 or specific EP receptor agonists.

After 24 h in SFM, the concentration of intracellular cAMP was almost undetectable. Incubation of fibroblasts with 1 mM IBMX significantly increased the levels of intracellular cAMP in both NM-C and NP-AERD fibroblasts compared with the respective non-treated cells. In these conditions, no differences were found among cultured fibroblasts from NM-C and NP-AERD (**Figure 29**).

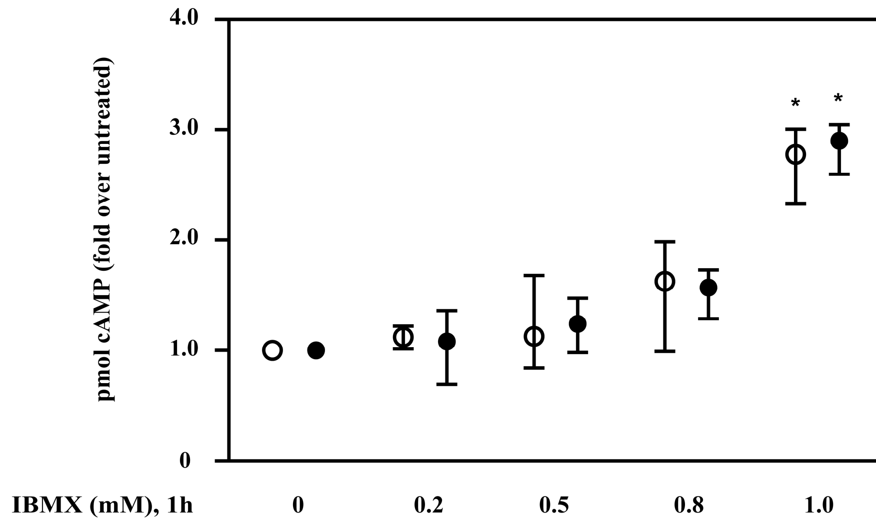


Figure 29. IBMX effect on intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were incubated with or without IBMX at different concentrations (0.2, 0.5, 0.8, and 1 mM) for 1 h. Intracellular levels of cAMP were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. White circle: NM-C fibroblasts; Black circle: NP-AERD fibroblasts. * $p<0.05$ compared with respective untreated cells. **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **IBMX:** 3-isobutyl-1-methylxanthine; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

Incubation with exogenous PGE_2 induced a dose-dependent increase in intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts compared with respective controls (IBMX, 1 mM). Nevertheless, significant differences were found between cultured NM-C and NP-AERD fibroblasts. We found that intracellular cAMP levels were significantly lower in NP-AERD fibroblasts compared with NM-C fibroblasts at PGE_2 concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M. No differences were found between NM-C fibroblasts and NP-AERD fibroblasts at 10^{-8} M PGE_2 (**Figure 30**).

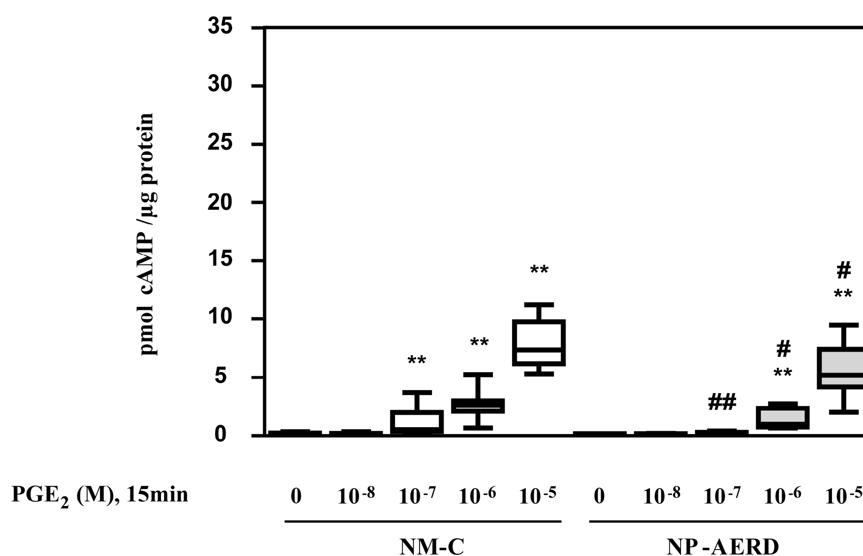


Figure 30. PGE_2 effect on intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. Fibroblasts from NM-C and NP-AERD (n=7 each) were preincubated with 1 mM IBMX for 1 h and treated for 15 min with PGE_2 from 10^{-8} M–10^{-5} M. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. ** $p<0.01$ compared with respective control (IBMX, 1mM); # $p<0.05$, ## $p<0.01$ compared with NM-C fibroblasts under the same conditions. **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **IBMX:** 3-isobutyl-1-methylxanthine; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **PGE_2:** prostaglandin E_2.

Stimulation with the EP_2 receptor agonist butaprost also induced a dose-dependent increase in intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. However, butaprost-induced intracellular cAMP levels were significantly lower in NP-AERD compared with NM-C fibroblasts. Significant differences were found at 10^{-7} M, 10^{-6} M, and 10^{-5} M of butaprost (**Figure 31A**).

On the other hand, CAY10598 (EP_4 receptor agonist) caused a dose-dependent increase in intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. CAY10598-stimulated intracellular cAMP levels were significantly higher in NP-AERD fibroblasts than in NM-C fibroblasts (**Figure 31B**).

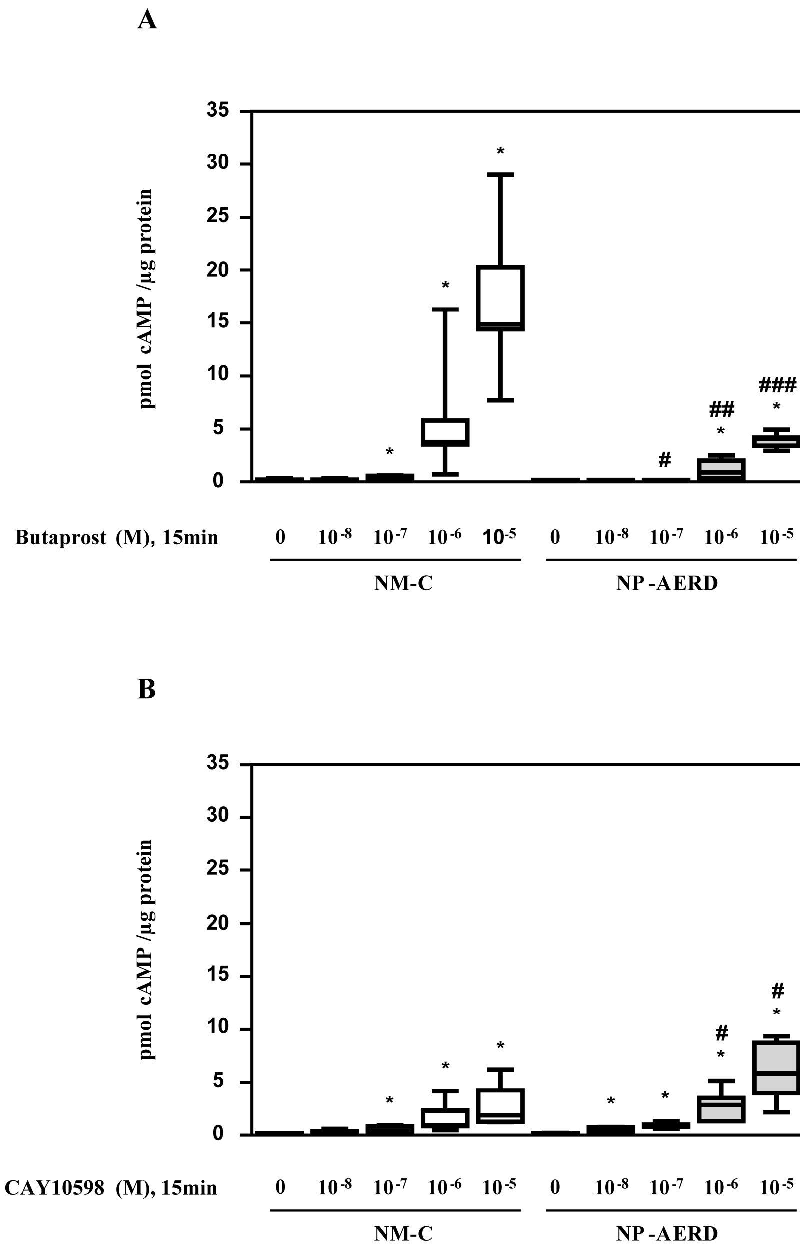


Figure 31. Butaprost and CAY10598 effects on intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=7 each) were preincubated with 1 mM IBMX for 1 h and treated for 15 min with 10^{-8} M–10^{-5} M **(A)** butaprost (EP_2 receptor selective agonist) or **(B)** CAY10598 (EP_4 receptor selective agonist). Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. * p<0.05 compared with respective control (IBMX, 1mM); # p<0.05, ## p<0.01, ### p<0.001 compared with NM-C fibroblasts under the same conditions. **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **EP:** E-prostanoid; **IBMX:** 3-isobutyl-1-methylxanthine; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

Therefore, we also assessed the additive effects of butaprost and CAY10598 using the results obtained from the individual agonist stimulation produced in cultured fibroblasts. The sum of EP_2 and EP_4 receptor agonists induced significantly lower levels of intracellular cAMP in NP-AERD fibroblasts at 10^{-6} M and 10^{-5} M compared with NM-C fibroblasts under the same conditions (**Figure 32**).

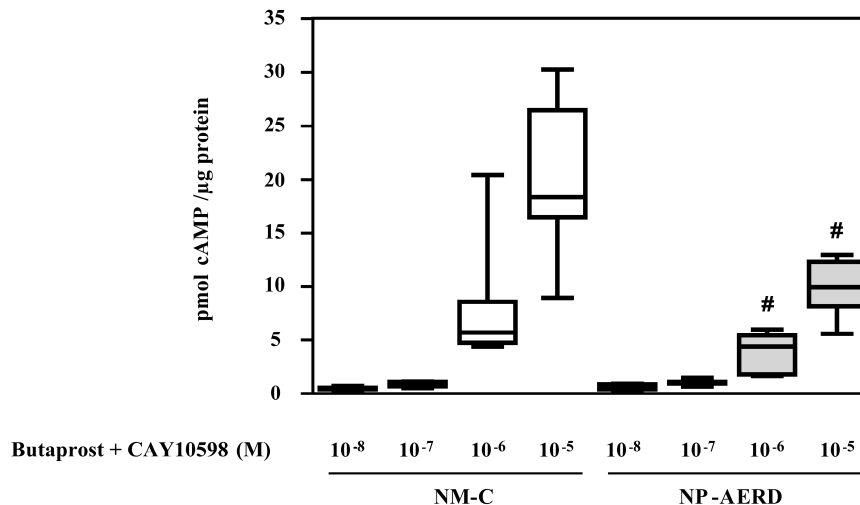


Figure 32. Additive effects of butaprost and CAY10598 on intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. The values of intracellular cAMP concentration obtained through the single stimulation of fibroblasts from NM-C and NP-AERD with different concentrations of butaprost and CAY10598 were summed. # $p<0.05$ compared with NM-C fibroblasts under the same conditions. **cAMP:** cyclic adenosine monophosphate; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

Differences observed in the amount of intracellular cAMP production upon EP_2 and EP_4 receptor activation between NM-C and NP-AERD fibroblasts could be related to the different expression of the aforementioned receptors. Cultured NM-C and NP-AERD fibroblasts were stimulated with or without different concentrations of forskolin (5 µM–40 µM) and intracellular cAMP levels were measured. The results showed that forskolin significantly and similarly stimulated intracellular cAMP production in NM-C and NP-AERD fibroblasts compared with their respective controls (**Figure 33**).

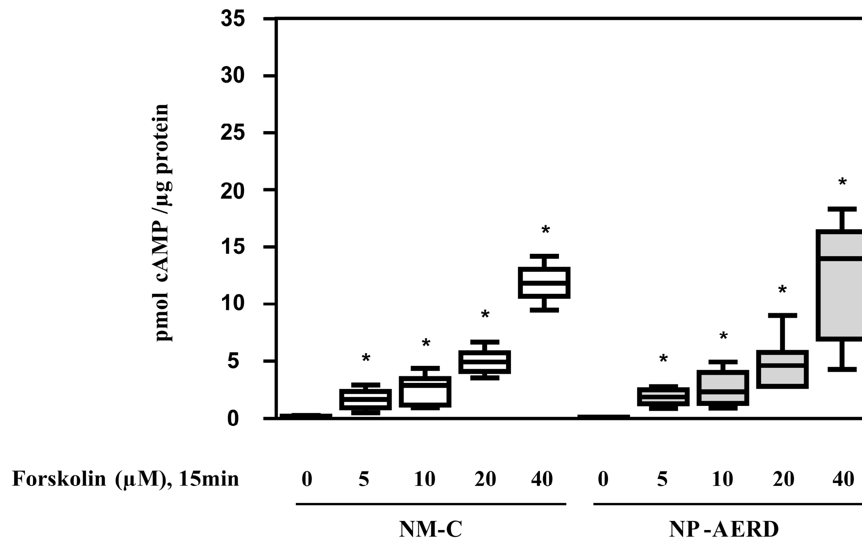


Figure 33. Forskolin effect on intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=7 each) were preincubated with 1 mM IBMX for 1 h and treated for 15 min with 5 µM to 40 µM forskolin. Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. * $p<0.05$ compared with respective control (IBMX, 1 mM). **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **IBMX:** 3-isobutyl-1-methylxanthine; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

To determine the effect of selective EP_3 activation on intracellular cAMP concentration, cultured fibroblasts were treated with or without forskolin (20 µM), to induce the production of intracellular cAMP, and with or without sulprostone (EP_3 receptor agonist) at different concentrations for 15 min. Forskolin-induced increases in intracellular cAMP levels were significantly and similarly reduced by sulprostone in cultured fibroblasts isolated from both NM-C and NP-AERD (**Figure 34**).

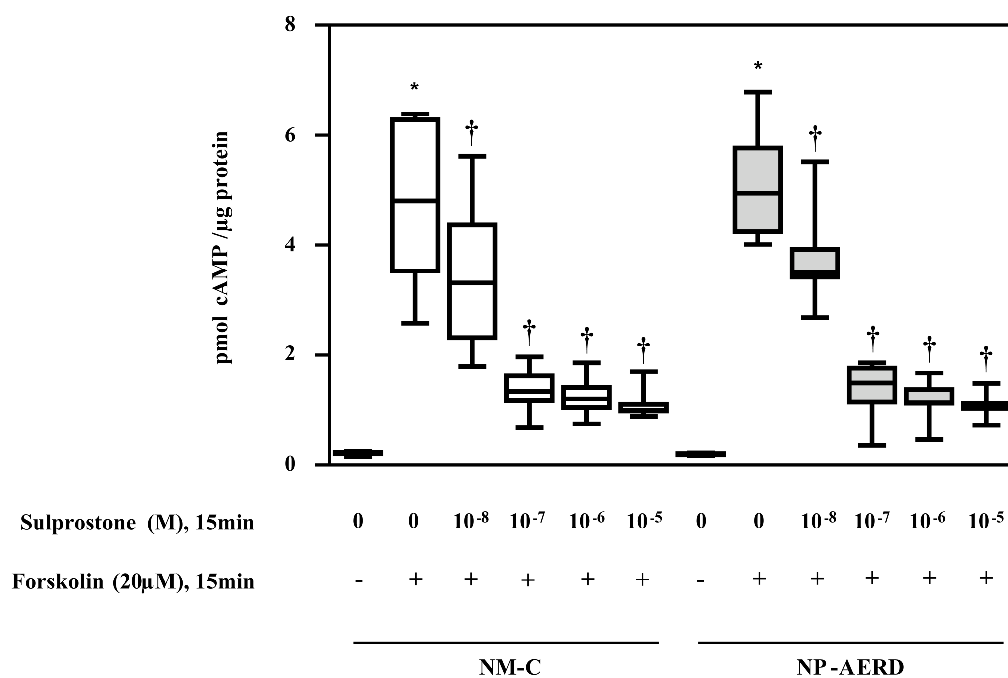


Figure 34. Sulprostone effect on forskolin-stimulated intracellular cAMP concentration in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=7 each) were preincubated with 1 mM IBMX for 1 h and treated for 15 min with or without 20 µM forskolin and with or without 10^{-8} M–10^{-5} M sulprostone (EP_3 agonist). Intracellular cAMP concentration was measured by ELISA. Results were normalized to the total protein concentration measured in each sample. * $p<0.05$ compared with respective control (IBMX, 1 mM); † $p<0.05$ compared with respective cells treated with forskolin at 20 µM. **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **EP:** E-prostanoid; **IBMX:** 3-isobutyl-1-methylxanthine; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

1.4. Effect of prostaglandin E_2 and butaprost on nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts proliferation

To evaluate whether the diminished intracellular cAMP production derived from the low expression of EP_2 receptor in NP-AERD has an effect on cell proliferation, cultured NM-C and NP-AERD fibroblasts were incubated with exogenous PGE_2 or butaprost, and the percentage of proliferating cells was measured by setting control groups as 100%.

Incubation with exogenous PGE_2 provoked a dose-dependent inhibition of cell proliferation in both NM-C and NP-AERD fibroblasts (**Figure 35A**). However, the anti-proliferative effect mediated by PGE_2 was significantly lower in NP-AERD cultured fibroblasts when compared with NM-C. As observed for PGE_2, the EP_2 receptor agonist butaprost also produced a significant inhibition of cell proliferation in fibroblasts from NM-C and NP-AERD (**Figure 35B**). Nevertheless, the capacity of butaprost to inhibit cell proliferation was significantly lower in NP-AERD cultured fibroblasts compared with NM-C. In control groups (cells incubated with DMEM 2.5% FBS), we found no significant differences between NM-C and NP-AERD cultured fibroblasts.

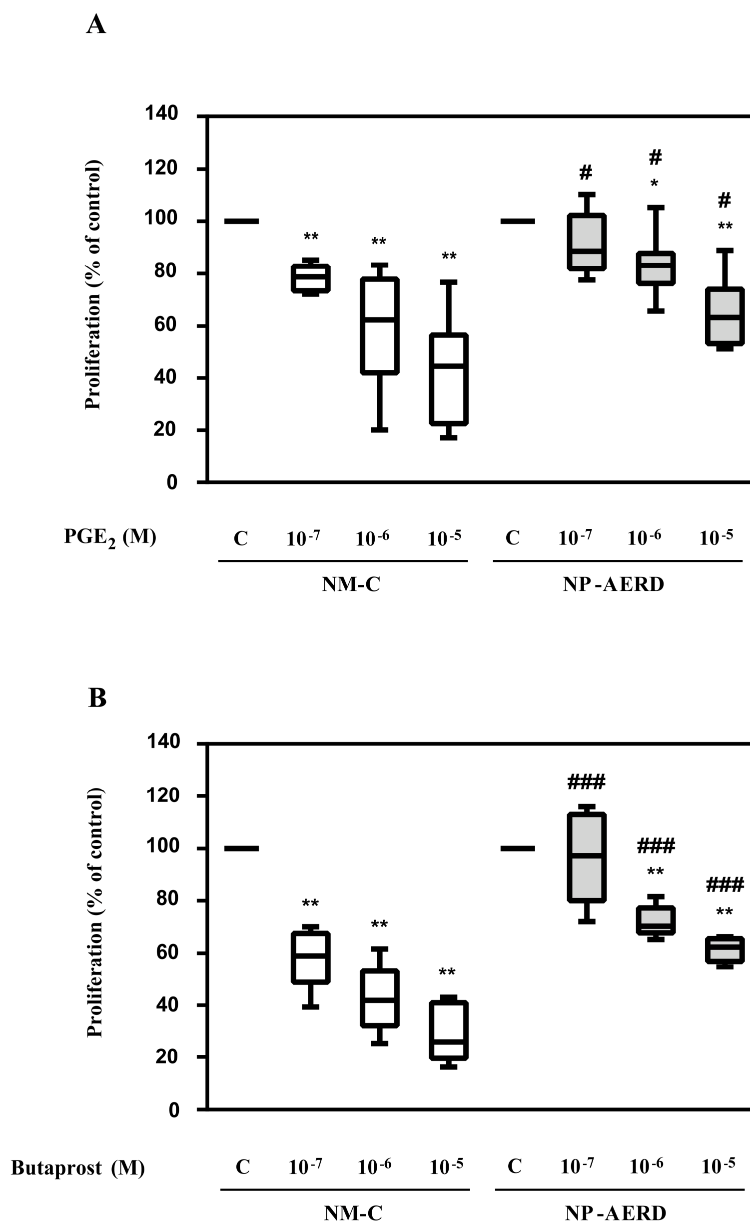


Figure 35. PGE_2 and butaprost effect on NM-C and NP-AERD fibroblasts proliferation. Fibroblasts from NM-C and NP-AERD (n=8 each) were incubated with **(A)** PGE_2 or **(B)** butaprost from 10^{-7} M–10^{-5} M for 18 h. Proliferation of fibroblasts was assessed by analyzing the proportion of cells that incorporated EdU using flow cytometry. Samples were normalized to the respective control group (cells incubated with DMEM 2.5% FBS) which was set as 100. * $p<0.05$, ** $p<0.01$ compared to control group; # $p<0.05$, ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **DMEM:** Dulbecco's Modified Eagle's media; **EdU:** 5-ethynyl-2´-deoxyuridine; **FBS:** fetal bovine serum; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with exacerbated respiratory disease; **PGE_2:** prostaglandin E_2.

1.5. Effect of prostaglandin E_2 and butaprost on granulocyte-macrophage colony-stimulating factor release in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

We also evaluated whether the low expression of EP_2 receptor and its low capacity to stimulate the production of intracellular cAMP upon its stimulation (through PGE_2 and butaprost) have an effect on GM-CSF release. Thus and as previously described, cultured NM-C and NP-AERD fibroblasts were incubated with exogenous PGE_2 or butaprost, and the percentage of GM-CSF was measured by setting control groups as 100%.

As shown in **Figure 36**, the stimulation of fibroblasts for 24 h with 10^{-6} M and 10^{-5} M exogenous PGE_2 (**Figure 36A**) or butaprost (**Figure 36B**) inhibited FBS-induced increases GM-CSF release in NM-C cultured fibroblasts. In turn, in cultured fibroblasts from NP-AERD, the inhibition of GM-CSF release was only observed at 10^{-5} M PGE_2 or butaprost. The inhibitory effect of both PGE_2 and butaprost on FBS-induced GM-CSF release was significantly lower in NP-AERD cultured fibroblasts when compared with NM-C. In control groups (cells incubated with DMEM 5% FBS), we found no significant differences in GM-CSF release between NM-C and NP-AERD cultured fibroblasts.

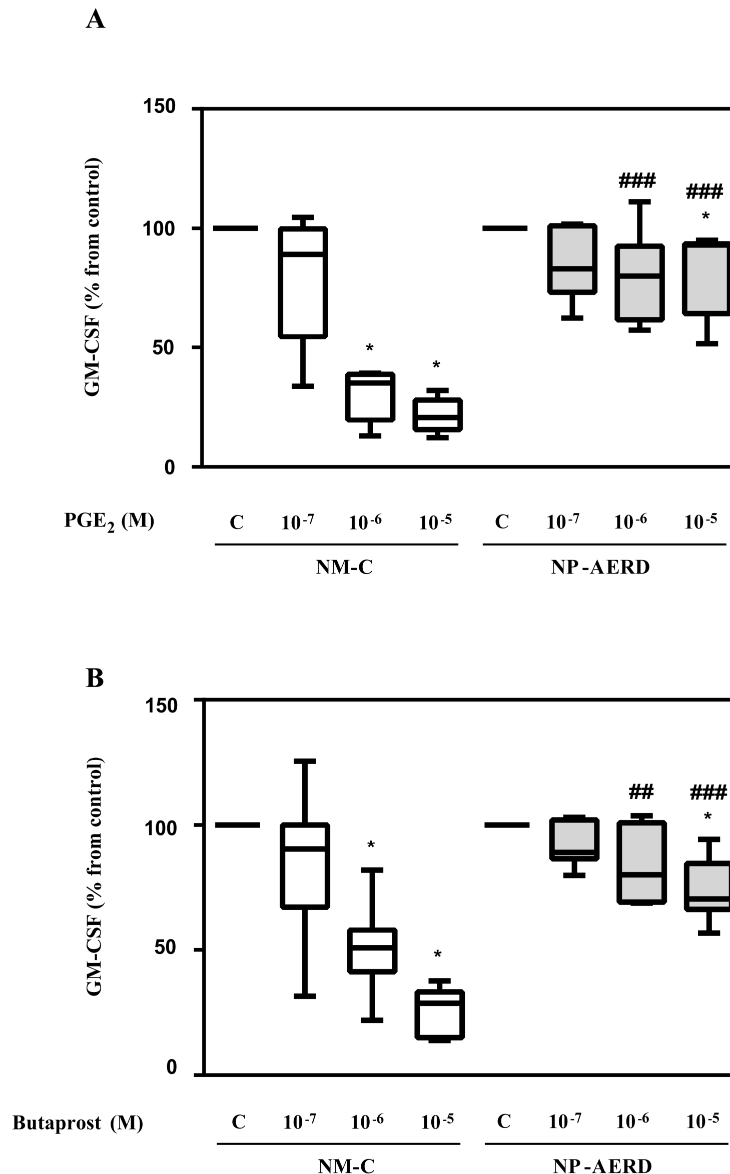


Figure 36. PGE_2 and butaprost effect on GM-CSF release in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=7 each) were incubated with or without 10^{-7} M–10^{-5} M **(A)** PGE_2 or **(B)** butaprost for 24 h in media containing 5% FBS. GM-CSF release in cell culture supernatants was measured by ELISA and cytokine production was corrected by cell number using XTT assay. Samples were normalized to the respective control group (cells incubated with DMEM 5% FBS) which was set as 100. * $p<0.05$ compared to control group; ## $p<0.01$, ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **DMEM:** Dulbecco's Modified Eagle's media; **ELISA:** enzyme-linked immunosorbent assay; **FBS:** fetal bovine serum; **GM-CSF:** granulocyte-macrophage colony-stimulating factor; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with exacerbated respiratory disease; **PGE_2:** prostaglandin E_2.

2. Study 2

2.1. Basal and interleukin-1β-induced interleukin-1 receptor type I expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The baseline protein and mRNA expression levels of IL-1RI in cultured NM-C and NP-AERD fibroblasts were assessed 24 h after cell incubation with SFM by western blot and quantitative real-time PCR techniques, respectively. Both IL-1RI protein (**Figure 37A**) and mRNA (**Figure 37B**) levels were significantly lower ($p<0.001$) in NP-AERD fibroblasts compared to NM-C fibroblasts.

To analyze the effect of IL-1β on IL-1RI expression, cultured NM-C and NP-AERD fibroblasts were incubated with or without IL-1β (10 ng/mL) for 24 h. The expression of IL-1RI was measured by both western blot (**Figure 37C**) and quantitative real-time PCR (**Figure 37D**). The incubation with IL-1β significantly increased IL-1RI expression in both NM-C and NP-AERD fibroblasts. However, the levels of IL-1RI induced by IL-1β were significantly lower in NP-AERD fibroblasts compared with NM-C fibroblasts.

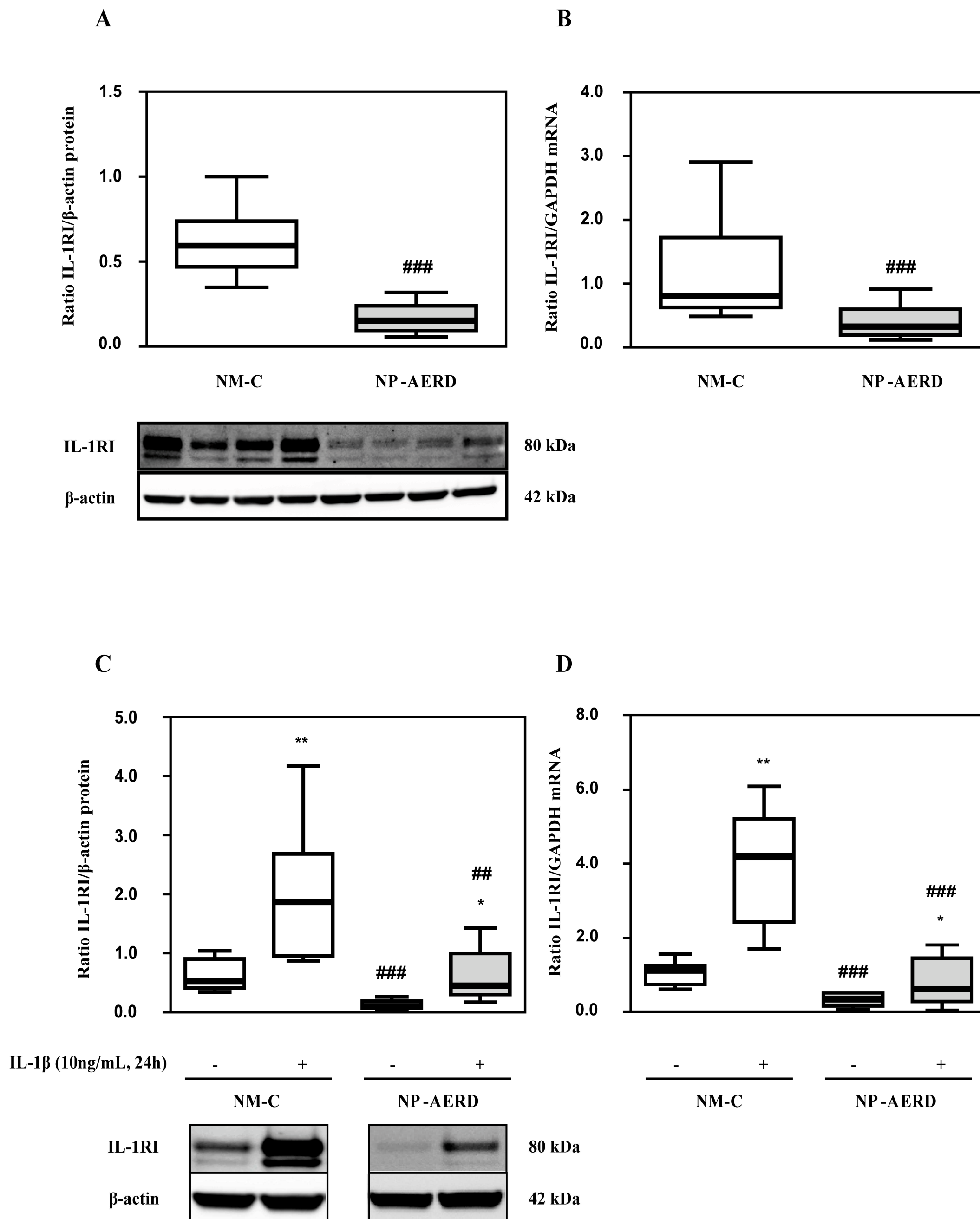


Figure 37. Basal and IL-1β-induced IL-1RI expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with SFM for 24 h and with or without IL-1β (10 ng/mL) for additional 24 h. IL-1RI levels were measured by western blot and quantitative real-time PCR. (**A, C**) Densitometric analysis and representative western blot of IL-1RI protein expression normalized to β-actin. (**B, D**) IL-1RI mRNA expression was analyzed by quantitative real-time PCR and normalized to GAPDH. * p<0.05, ** p<0.01 compared with respective untreated cells; ## p<0.01, ### p<0.001 compared with NM-C fibroblasts under the same conditions. **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **NM-C:**

nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **SFM:** serum-free media.

To confirm the results obtained by western blot and quantitative real-time PCR, we performed a qualitative analysis of the expression of IL-1RI using immunofluorescence staining. **Figure 38** depicts IL-1RI expression in cultured fibroblasts from NM-C and NP-AERD in the absence or presence of IL-1β (10 ng/mL) for 24 h. We observed a lower IL-1RI immunoreactivity in media-treated NP-AERD fibroblasts compared with media-treated NM-C fibroblasts. Despite the increase in IL-1RI immunoreactivity after incubation with IL-1β, the staining was less intense in NP-AERD fibroblasts compared with NM-C fibroblasts under the same conditions.

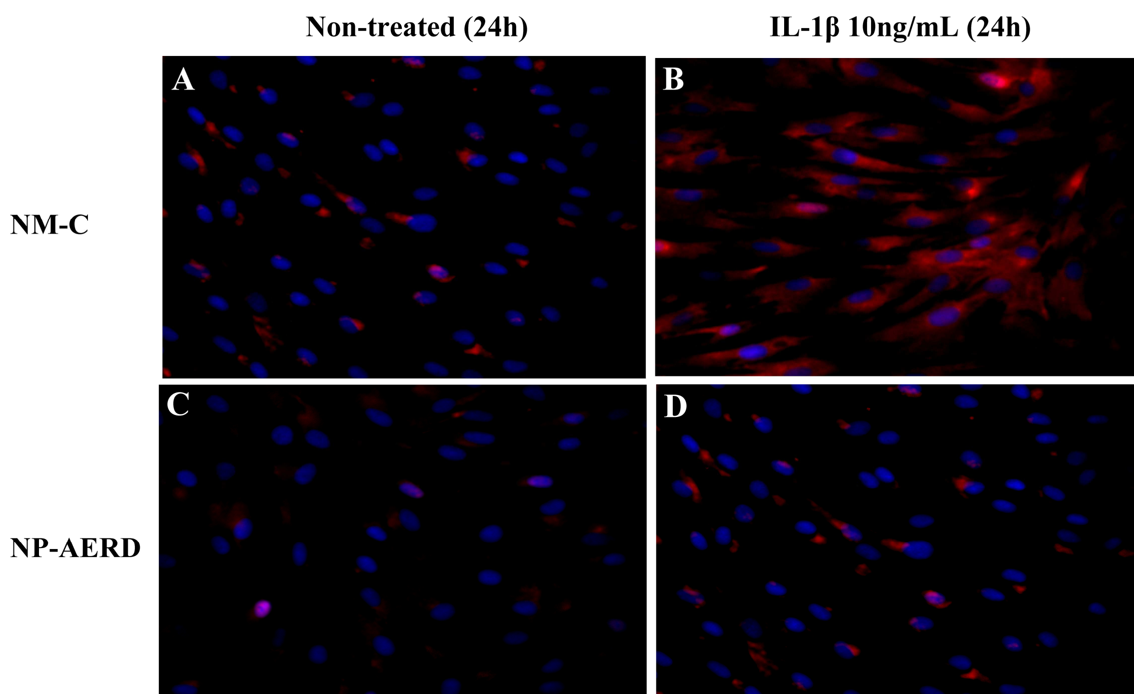


Figure 38. Immunofluorescence of IL-1RI in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with media (**A, C**) or with 10 ng/mL IL-1β (**B, D**) for 24 h and immunofluorescence staining of IL-1RI (red) was performed. Nuclei (blue) were stained using DAPI. x20 magnification. **DAPI:** 4',6-diamidino-2-phenylindole; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.2. Basal and interleukin-1β-induced interleukin-1 receptor accessory protein expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The baseline protein and mRNA expression of IL-1RAcP in cultured fibroblasts from NM-C and NP-AERD was assessed 24 h after cell incubation with SFM by western blot and quantitative real-time PCR techniques respectively. No differences were found in IL-1RAcP protein (**Figure 39A**) and mRNA (**Figure 39B**) expression between cultured NM-C and NP-AERD fibroblasts.

To analyze the effect of IL-1β on IL-1RAcP expression, cultured fibroblasts from NM-C and NP-AERD were incubated with or without IL-1β (10 ng/mL) for 24 h. The expression of IL-1RAcP was measured by both western blot (**Figure 39C**) and quantitative real-time PCR (**Figure 39D**). The incubation with IL-1β significantly increased IL-1RAcP mRNA expression in both NM-C and NP-AERD fibroblasts. However, no differences were found using western blot technique.

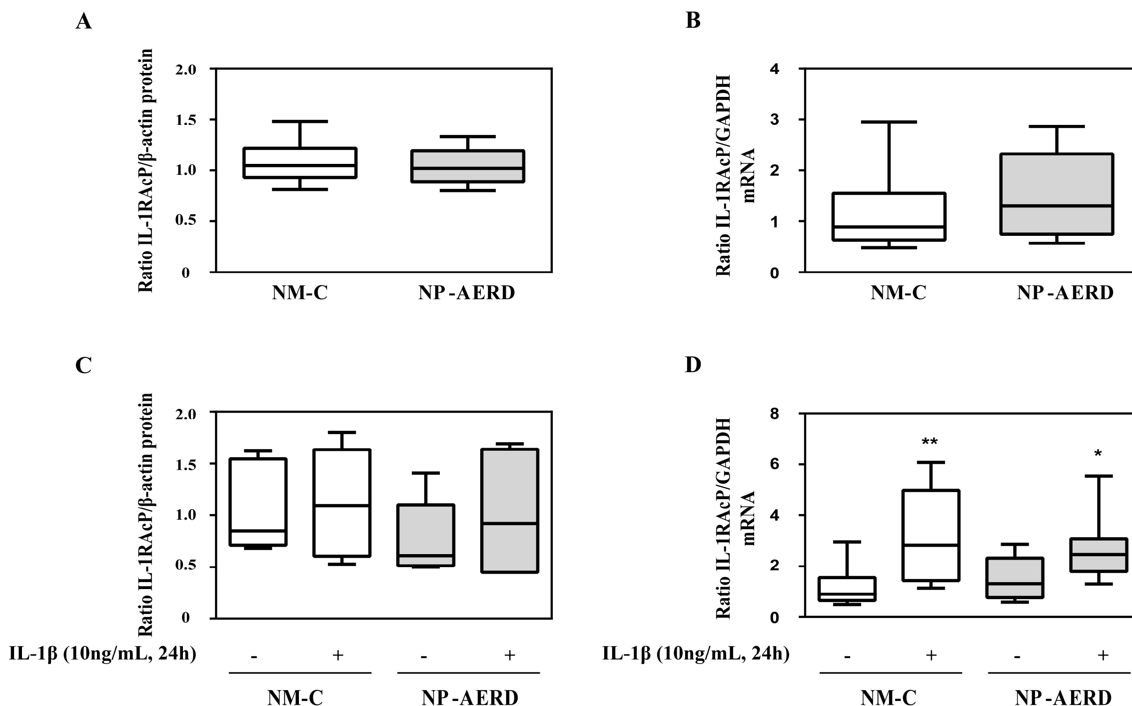


Figure 39. Basal and IL-1β-induced IL-1RAcP expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with SFM for 24h and with or without IL-1β (10 ng/mL) for additional 24 h. IL-1RAcP levels were measured by western blot and quantitative real-time PCR. **(A, C)** Densitometric analysis of IL-1RAcP

protein expression normalized to β-actin. **(B, D)** IL-1RAcP mRNA expression was analyzed by quantitative real-time PCR and normalized to GAPDH. * $p<0.05$, ** $p<0.01$ compared with respective untreated cells. **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **IL-1β:** interleukin-1β; **IL-1RAcP:** interleukin-1 receptor accessory protein; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **SFM:** serum-free media.

2.3. Basal and interleukin-1β-induced interleukin-1 receptor type II expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The baseline protein and mRNA expression of IL-1RII in cultured fibroblasts from NM-C and NP-AERD was assessed 24 h after cell incubation with SFM by western blot and quantitative real-time PCR techniques respectively. No differences were found in IL-1RII protein (**Figure 40A**) and mRNA (**Figure 40B**) expression between cultured NM-C and NP-AERD fibroblasts.

To analyze the effect of IL-1β on IL-1RII expression, cultured fibroblasts from NM-C and NP-AERD were treated with or without IL-1β (10 ng/mL) for 24 h. The expression of IL-1RII was detected and measured by both western blot (**Figure 40C**) and quantitative real-time PCR (**Figure 40D**). The incubation of cells with IL-1β significantly increased IL-1RII mRNA expression in both NM-C and NP-AERD cultured fibroblasts. However, and as seen for IL-1RAcP expression, no differences were found using western blot technique.

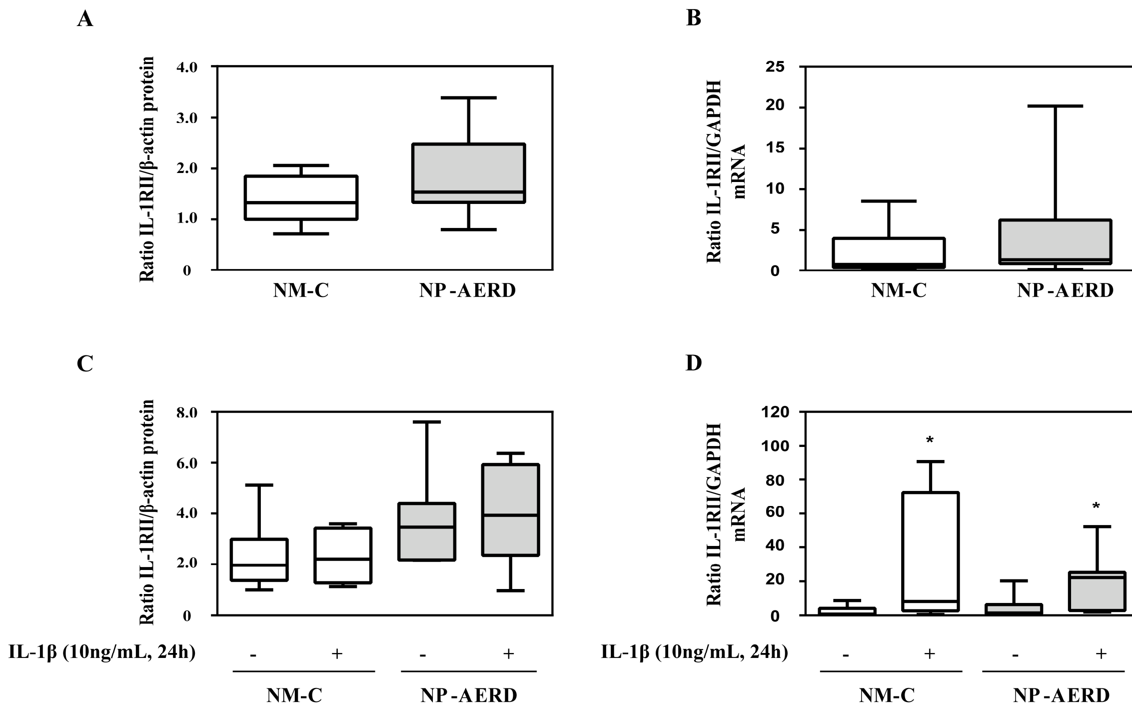


Figure 40. Basal and IL-1β-induced IL-1RII expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with SFM for 24h and with or without IL-1β (10 ng/mL) for additional 24 h. IL-1RII levels were measured by western blot and quantitative real-time PCR. **(A, C)** Densitometric analysis of IL-1RII protein expression normalized to β-actin. **(B, D)** IL-1RII mRNA expression was analyzed by quantitative real-time PCR and normalized to GAPDH. * $p<0.05$ compared with respective untreated cells. **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **IL-1β:** interleukin-1β; **IL-1RII:** interleukin-1 receptor type II; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **SFM:** serum-free media.

2.4. Non-induced and interleukin-1β-induced interleukin-1 receptor antagonist levels in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The effect of IL-1β on the expression of intracellular IL-1Ra was measured by ELISA in cultured fibroblasts from NM-C and NP-AERD. As shown in **Figure 41**, we found reduced levels of intracellular IL-1Ra in media-treated condition, in cultured fibroblasts from NP-AERD when compared with NM-C. Moreover, the incubation with IL-1β was

able to significantly increase intracellular IL-1Ra expression in cultured fibroblasts from NM-C but not in NP-AERD. Under these conditions, intracellular IL-1Ra expression was significantly lower in NP-AERD compared to NM-C fibroblasts.

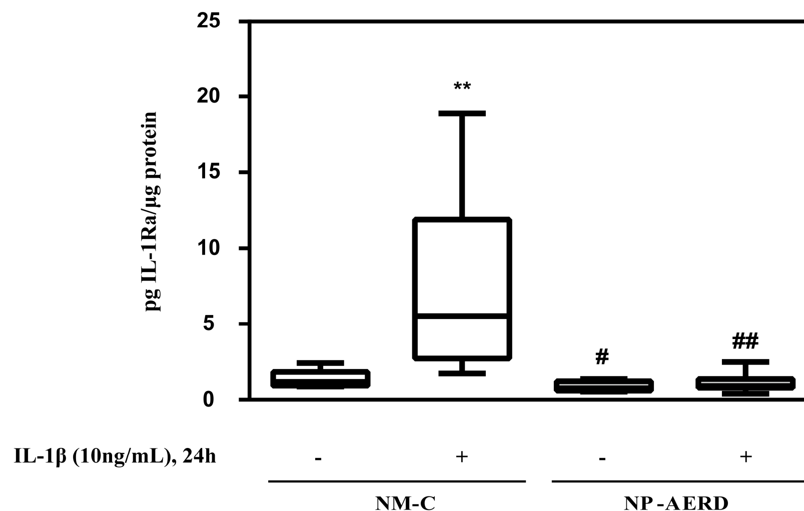


Figure 41. Non-induced and IL-1β-induced IL-1Ra levels in cultured NM-C and NP-AERD fibroblasts. Cultured fibroblasts (n=8 each) were incubated with or without IL-1β (10 ng/mL) for 24 h. IL-1Ra levels on cell lysates were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. ** $p<0.01$ compared with respective control; # $p<0.05$; ## $p<0.01$ compared with NM-C fibroblasts under the same conditions. **ELISA:** enzyme-linked immunosorbent assay; **IL-1β:** interleukin-1β; **IL-1Ra:** interleukin-1 receptor antagonist; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.5. Non-induced and interleukin-1β-induced cyclooxygenase-2 expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

The effect of IL-1β on the expression of COX-2 was measured in cultured NM-C and NP-AERD fibroblasts by both western blot and quantitative real-time PCR. As described elsewhere (Roca-Ferrer *et al.*, 2011), western blot results showed diminished induction of COX-2 protein expression in response to IL-1β in NP-AERD fibroblasts compared to NM-C fibroblasts (**Figure 42A**). Moreover, quantitative real-

time PCR analysis of IL-1β-induced COX-2 mRNA expression demonstrated that the levels of COX-2 were significantly lower in NP-AERD fibroblasts compared to NM-C fibroblasts (**Figure 42B**).

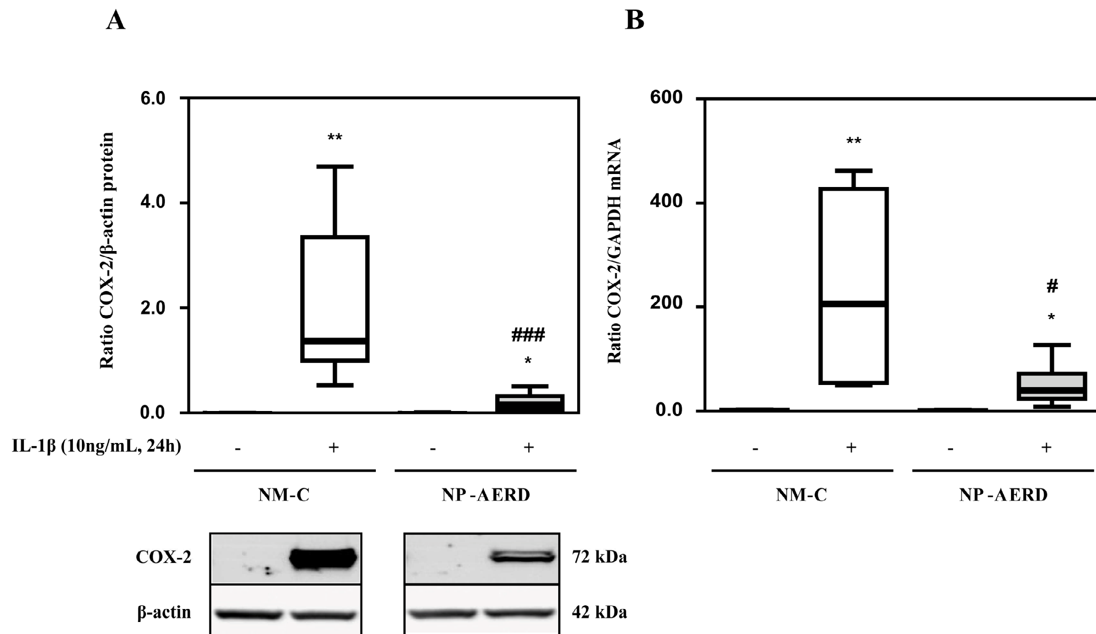


Figure 42. Non-induced and IL-1β-induced COX-2 expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with or without IL-1β (10 ng/mL, 24 h) and COX-2 levels were measured by western blot and quantitative real-time PCR. **(A)** Densitometric analysis and representative western blot of COX-2 protein expression normalized to β-actin. **(B)** COX-2 mRNA expression was analyzed by quantitative real-time PCR and normalized to GAPDH. * $p<0.05$, ** $p<0.01$ compared with respective untreated cells; # $p<0.05$, ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **COX-2:** cyclooxygenase-2; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.6. Non-induced and interleukin-1β-induced microsomal prostaglandin E synthase-1 expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

To evaluate the effect of IL-1β on mPGES-1 expression, cultured NM-C and NP-AERD fibroblasts were treated with or without IL-1β (10 ng/mL) for 24 h. After this time, mPGES-1 expression was measured by western blot. The incubation with IL-1β significantly increased the expression of mPGES-1 in cultured NM-C and NP-AERD fibroblasts. However, the IL-1β-induced expression of mPGES-1 was significantly lower in NP-AERD fibroblasts compared with NM-C fibroblasts (Figure 43).

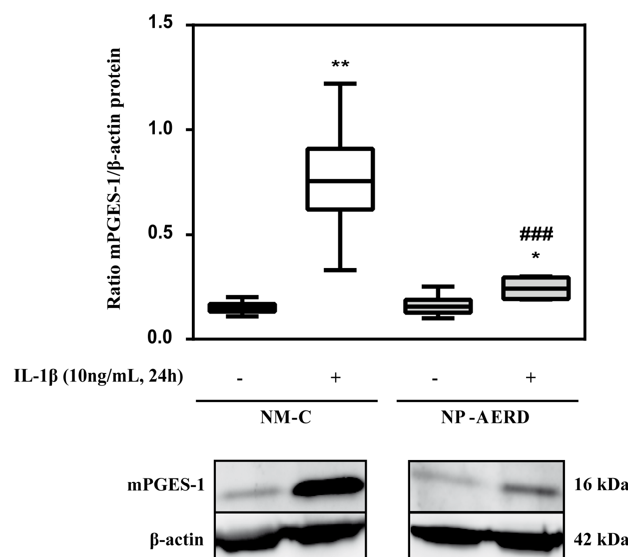


Figure 43. Non-induced and IL-1β-induced mPGES-1 expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (N=8 each) were incubated with or without IL-1β (10 ng/mL, 24 h) and mPGES-1 levels were measured by western blot. Densitometric analysis and representative western blot of mPGES-1 protein expression normalized to β-actin. * $p<0.05$, ** $p<0.01$ compared with respective untreated cells; ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **IL-1β:** interleukin-1β; **mPGES-1:** microsomal prostaglandin E synthase-1; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.7. Interleukin-1 receptor type I and cyclooxygenase-2 expression in nasal samples obtained from nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease

We also evaluated whether the alterations observed in the expression of IL-1RI and COX-2 in cultured NP-AERD fibroblasts were also present in whole tissue. As demonstrated for cultured fibroblasts, we found that the expression of both IL-1RI (**Figure 44A**) and COX-2 (**Figure 44B**) was significantly lower in NP-AERD compared with NM-C.

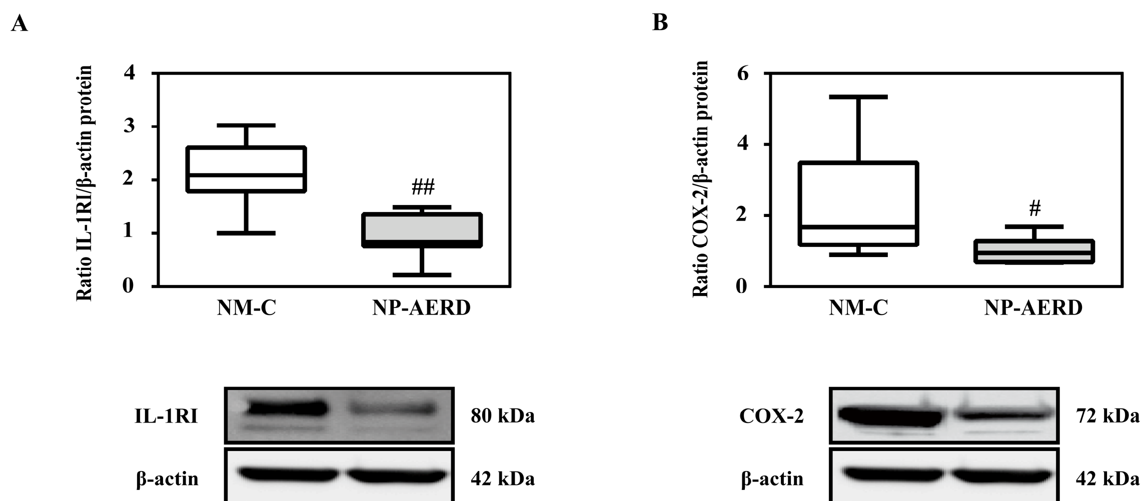


Figure 44. Expression of IL-1RI and COX-2 in nasal samples obtained from NM-C and NP-AERD. Densitometric analysis and representative western blot of **(A)** IL-1RI and **(B)** COX-2 protein expression normalized to β-actin in nasal samples from NM-C (n=7) and NP-AERD (n=9). # p<0.05, ## p<0.01 compared with NM-C. **COX-2:** cyclooxygenase-2; **GAPDH:** glyceraldehyde 3-phosphate dehydrogenase; **IL-1RI:** interleukin-1 receptor type I; **NM:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.8. Correlation between interleukin-1β-induced expression of interleukin-1 receptor type I and interleukin-1β-induced expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

As shown in **Figure 45A and 45B**, respectively, significant correlations between IL-1RI and COX-2 expression (Pearson correlation coefficient 0.8181; p=0.0002, two tailed) and between IL-1RI and mPGES-1 expression (Pearson correlation coefficient 0.9313; p<0.0001, two tailed) were observed.

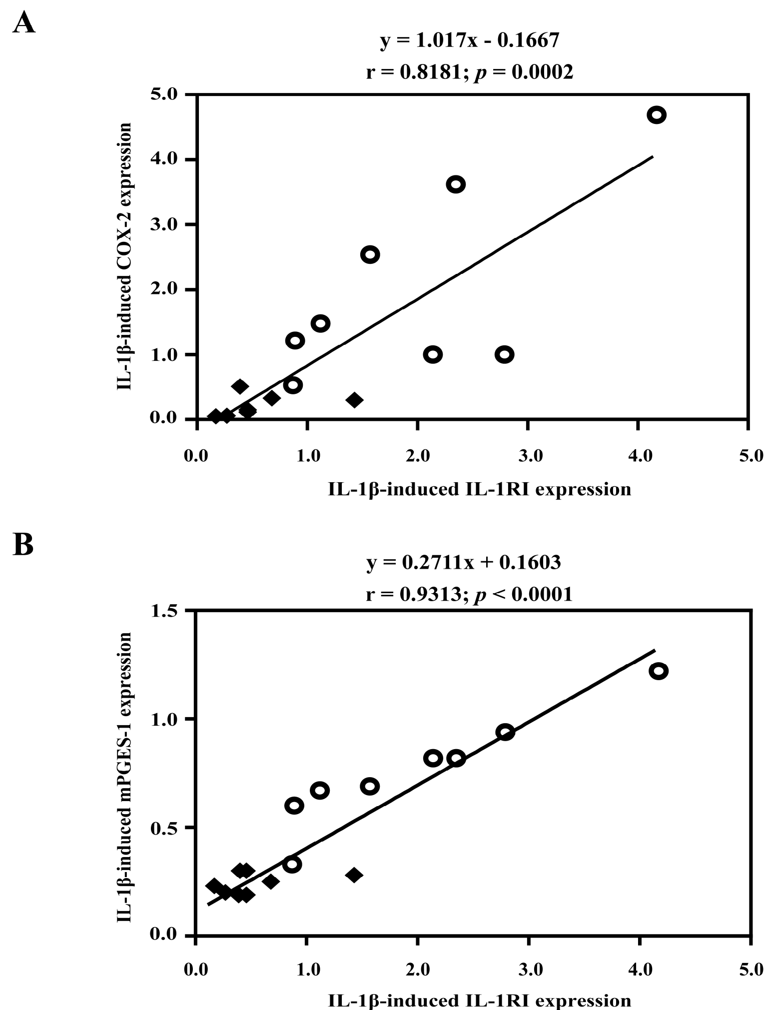


Figure 45. Correlation between IL-1β-induced expression of IL-1RI and IL-1β-induced expression of COX-2 and mPGES-1 in cultured fibroblasts from NM-C and NP-AERD. The fitting line equation, Pearson's correlation coefficient, and its

associated *p* value are shown at the upper region of the graph. Correlation between IL-1β-induced expression of IL-1RI and IL-1β-induced expression of **(A)** COX-2 and **(B)** mPGES-1. White circles: NM-C fibroblasts (N=8); Black diamond: NP-AERD fibroblasts (N=8). **COX-2:** cyclooxygenase-2; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **mPGES-1:** microsomal prostaglandin E synthase-1; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

To confirm these results, NM-C fibroblasts were transfected with a specific siRNA against IL-1RI, and we measured the expression of COX-2 and mPGES-1 after stimulation with IL-1β. Under optimized experimental conditions, IL-1RI expression was completely inhibited by a specific siRNA against IL-1RI. In this set of experiments, we used a non-target siRNA (siRNA-Neg) to check and demonstrate the effectiveness and specificity of our siRNA (**Figure 46**). In cells treated with a specific siRNA against IL-1RI, incubation with IL-1β was not able to increase the expression of either COX-2 or mPGES-1, suggesting that the IL-1β-induced expression of COX-2 and mPGES-1 is directly dependent on IL-1RI expression.

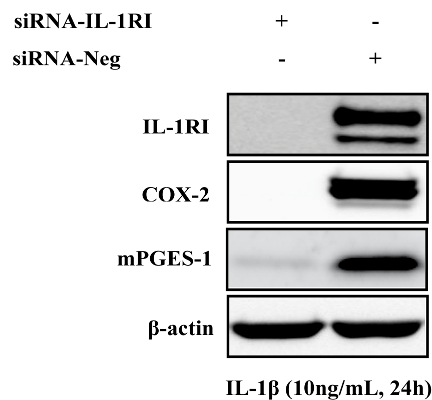


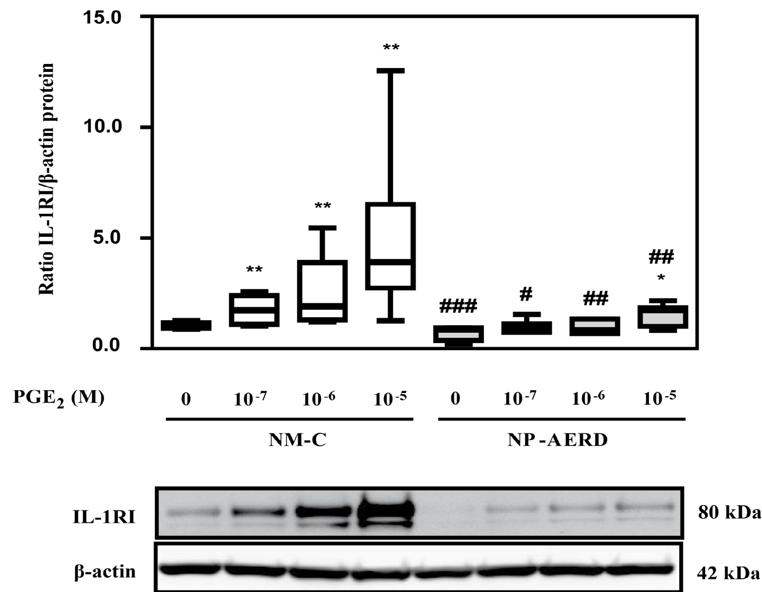
Figure 46. Effect of IL-1RI gene silencing by siRNA on COX-2 and mPGES-1 expression in cultured NM-C fibroblasts. NM-C fibroblasts (N=8) were transfected with 20 nM of IL-1RI siRNA (siRNA-IL-1RI) or with negative control siRNA (siRNA-Neg) using Lipofectamine RNAiMAX. Representative western blot of IL-1RI, COX-2 and mPGES-1 expression normalized to β-actin after cell stimulation with IL-1β (10 ng/mL, 24 h). **COX-2:** cyclooxygenase-2; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **mPGES-1:** microsomal prostaglandin E synthase-1; **NM-C:** nasal mucosa of control subjects; **siRNA:** small interfering RNA.

2.9. Effect of prostaglandin E_2 and acetylsalicylic acid on interleukin-1 receptor type I expression in cultured nasal mucosa of control subjects and nasal polyps of patients with exacerbated respiratory disease fibroblasts

To analyze the mechanism by which IL-1β induces its own receptor, cultured NM-C and NP-AERD fibroblasts were incubated with or without PGE_2 at different concentrations and the levels of IL-1RI were measured by western blot. Incubation with exogenous PGE_2 (**Figure 47A**) caused a dose-dependent increase in IL-1RI protein expression in NM-C fibroblasts. In NP-AERD fibroblasts, incubation with exogenous PGE_2 only caused an increase in IL-1RI protein levels at 10^{-5} M PGE_2. Moreover, the expression of IL-1RI was significantly lower in NP-AERD fibroblasts compared with NM-C fibroblasts at PGE_2 concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M.

To confirm that PGE_2 is able to increase IL-1RI expression, NM-C fibroblasts were incubated with IL-1β in the absence or presence of ASA (10^{-5} M and 10^{-3} M), a drug used to block the production of PGE_2. Incubation of NM-C fibroblasts with IL-1β increased IL-1RI expression as described previously. As shown in **Figure 47B**, ASA inhibited the IL-1β-induced expression of IL-1RI in NM-C fibroblasts, indicating that PGE_2 mediates the induction of IL-1RI expression by IL-1β.

A



B

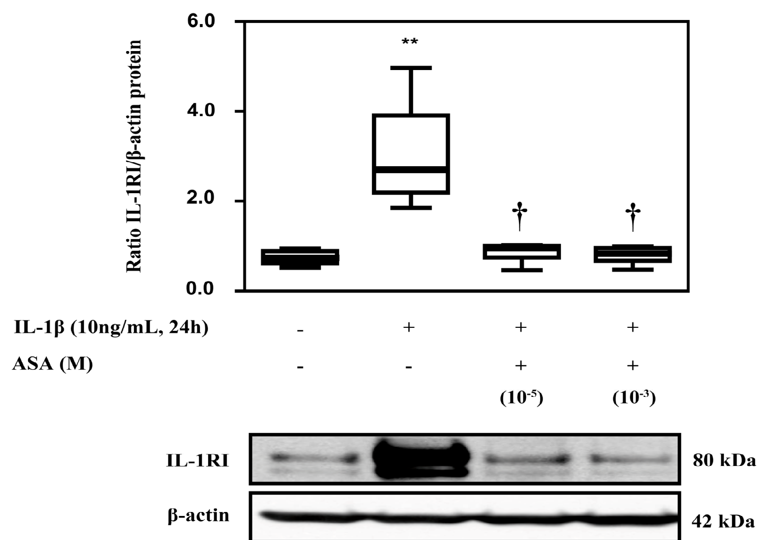


Figure 47. Effect of exogenous PGE_2 on the expression of IL-1RI in cultured NM-C and NP-AERD fibroblasts. Densitometric analysis and representative western blot of **(A)** IL-1RI expression normalized to β-actin after cell stimulation with PGE_2 (10^{-7} M–10^{-5} M) for 24 h (N=8) and **(B)** IL-1RI expression normalized to β-actin after NM-C fibroblasts (N=8) stimulation with or without IL-1β (10 ng/mL) for 24 h in the presence or absence of ASA (10^{-5} M and 10^{-3} M). * p<0.05, ** p<0.01 compared with respective untreated cells; # p<0.05, ## p<0.01, ### p<0.001 compared with NM-C fibroblasts under the same conditions; † p<0.05 compared with respective cells treated with IL-1β (10 ng/mL). **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of

control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **PGE_2:** prostaglandin E_2.

2.10. Non-induced and interleukin-1β-induced intracellular cyclic adenosine monophosphate levels in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

To evaluate the effect of IL-1β on intracellular cAMP production, cultured fibroblasts isolated from NM-C and NP-AERD were preincubated with IBMX and treated in the presence or absence of IL-1β for 1 h. After this time, intracellular cAMP production was assessed by ELISA. As shown in **Figure 48**, the stimulation with IL-1β in the presence of IBMX induced a significant increase in intracellular levels of cAMP in cultured fibroblasts isolated from both NM-C and NP-AERD. However, IL-1β-induced intracellular cAMP levels were significantly lower in NP-AERD fibroblasts when compared with NM-C under the same condition.

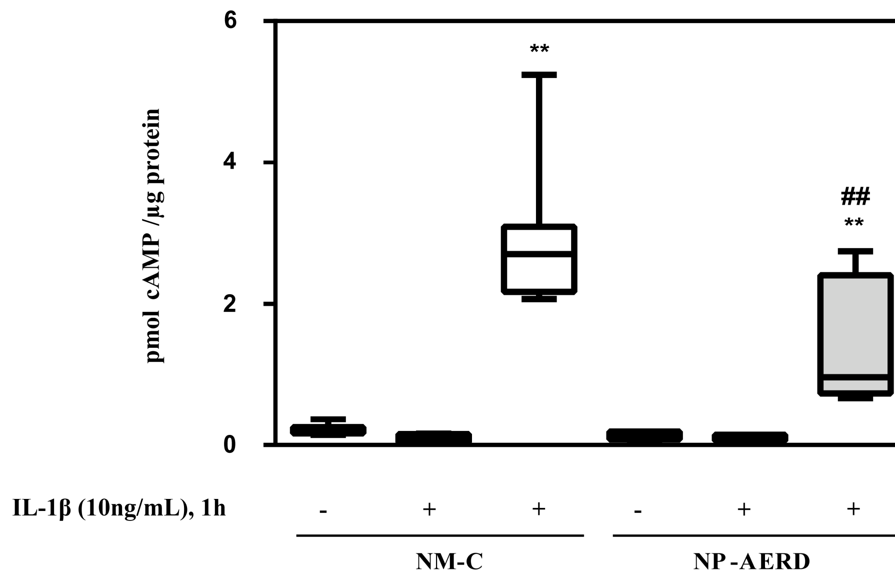


Figure 48. Non-induced and IL-1β-induced intracellular cAMP levels in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=8 each) were preincubated with 1 mM IBMX for 1 h and treated for 1 h with or without IL-1β (10 ng/mL). Intracellular cAMP levels were measured by ELISA. Results were normalized to the total protein concentration measured in each sample. ** *p*<0.01 compared with respective control

(IBMX, 1 mM); ## *p*<0.01 compared with NM-C fibroblasts under the same conditions. **cAMP:** cyclic adenosine monophosphate; **ELISA:** enzyme-linked immunosorbent assay; **IBMX:** 3-isobutyl-1-methylxanthine; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

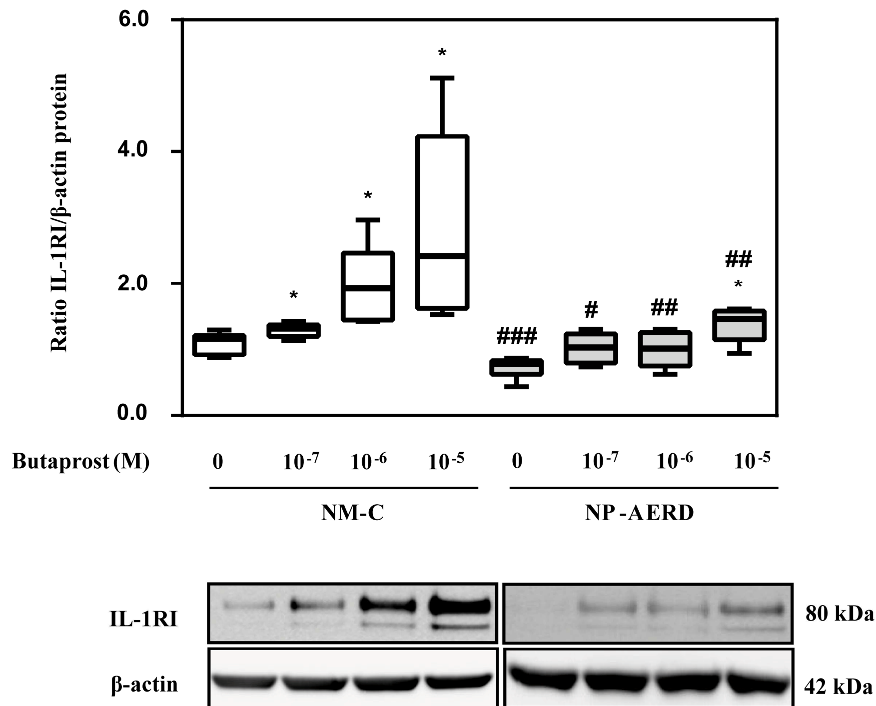
2.11. Effect of E-prostanoid receptor agonists on interleukin-1 receptor type I expression in cultured nasal mucosa of control subjects and nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

To evaluate the effect of EP_2, EP_3 and EP_4 receptors activation on IL-1RI expression, cultured NM-C and NP-AERD fibroblasts were treated with or without EP_2 (butaprost), EP_3 (sulprostone) or EP_4 (CAY10598) receptor-specific agonists and IL-1RI levels were detected and measured by western blot. Taking into account the potential role of intracellular cAMP on IL-1RI expression regulation and in order to assess the effect of EP_3 receptor activation, intracellular cAMP production was firstly induced by adding forskolin, and sulprostone was added subsequently.

The EP_2 receptor agonist butaprost increased IL-1RI protein levels in a dose-dependent manner in cultured NM-C fibroblasts. In contrast, as observed for PGE_2, butaprost increased IL-1RI levels in NP-AERD fibroblasts only at a concentration of 10^{-5} M. Moreover, significant differences between NM-C and NP-AERD fibroblasts were found at all butaprost concentrations (**Figure 49A**), which suggests that EP_2 receptor is likely responsible for the deficient induction of IL-1RI expression by PGE_2.

The EP_4 receptor selective agonist CAY10598 also caused a significant increase in IL-1RI levels in cultured NM-C and NP-AERD fibroblasts. However, we found no differences in IL-1RI induction between NM-C and NP-AERD fibroblasts at this level (**Figure 49B**).

A



B

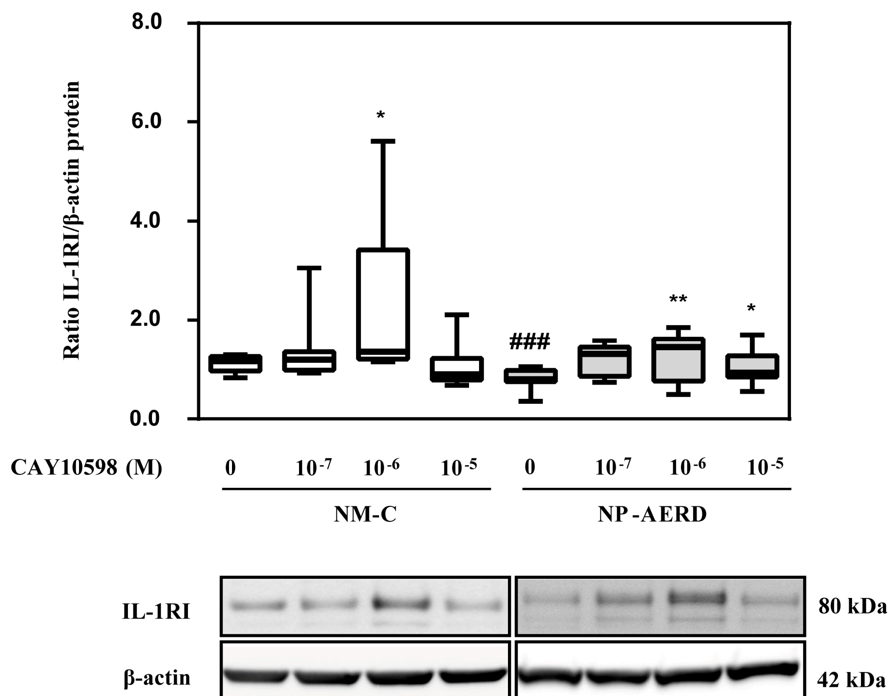


Figure 49. Effects of EP_2 and EP_4 receptor agonists on IL-1RI expression in cultured NM-C and NP-AERD fibroblasts. Densitometric analysis and representative western blot of IL-1RI expression normalized to β-actin after cell stimulation with or without **(A)** butaprost (10^{-7} M–10^{-5} M) or **(B)** CAY10598 (10^{-7} M–10^{-5} M) for 24 h (N=8

each). * $p<0.05$, ** $p<0.01$ compared with respective untreated cells; # $p<0.05$, ## $p<0.01$, ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **EP:** E-prostanoid; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

Forskolin was used to determine whether the second messenger cAMP was involved in IL-1RI expression. Forskolin significantly increased the expression of IL-1RI on cultured NM-C and NP-AERD fibroblasts (**Figure 50**), suggesting that the deficient upregulation of IL-1RI levels in NP-AERD fibroblasts upon PGE_2 stimulation is probably due to the previously described low levels of EP_2 receptor in these cells.

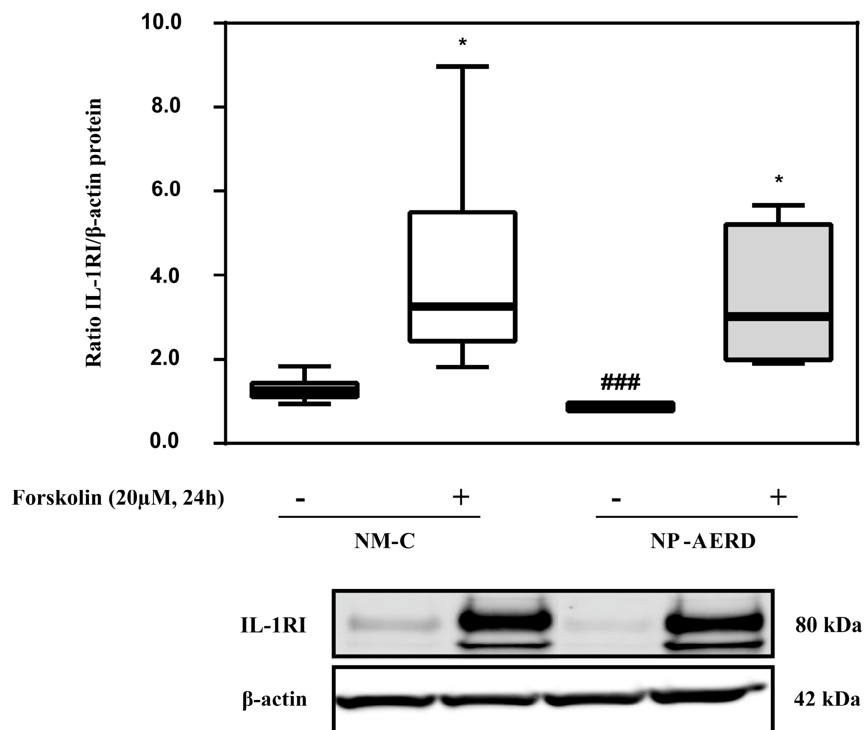


Figure 50. Effect of forskolin on IL-1RI expression in cultured NM-C and NP-AERD fibroblasts. Densitometric analysis and representative western blot of IL-1RI expression normalized to β-actin in cells treated with or without forskolin (20 µM) for 24 h (N=8 each). * $p<0.05$ compared with respective untreated cells; ### $p<0.001$ compared with NM-C fibroblasts under the same conditions. **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

Finally, to determine the effect of specific EP_3 receptor activation on IL-1RI expression, cultured fibroblasts were treated with or without forskolin (20 µM) and with or without

sulprostone (EP_3 agonist) at different concentrations for 24 h. Forskolin-induced IL-1RI expression was significantly and equally reduced in a dose-dependent manner by sulprostone in both cultured NM-C and NP-AERD fibroblasts (**Figure 51**).

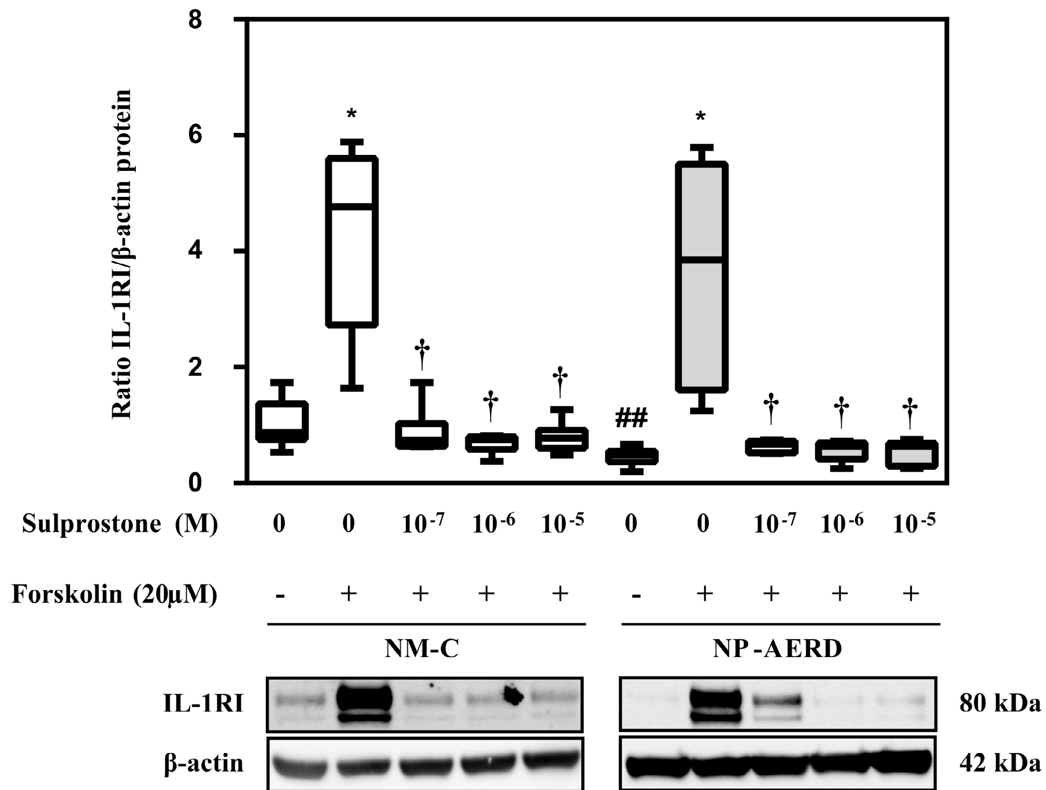
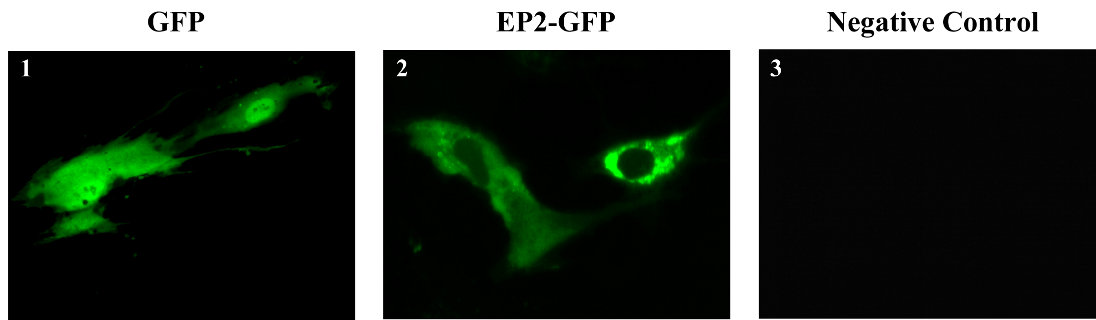


Figure 51. Sulprostone effect on forskolin-induced IL-1RI expression in cultured NM-C and NP-AERD fibroblasts. Fibroblasts (n=8 each) were treated with or without 20 μM forskolin in the presence or absence of 10^{-7} M–10^{-5} M sulprostone (EP_3 receptor agonist). Densitometric analysis and representative western blot of IL-1RI expression normalized to β-actin. * $p<0.05$ compared with respective untreated cells; ## $p<0.01$ compared with NM-C fibroblasts under the same conditions; † $p<0.05$ compared with respective cells treated with 20 μM forskolin. **EP:** E-prostanoid; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

2.12. Effect of E-prostanoid 2 receptor transient transfection on interleukin-1 receptor type I expression in cultured nasal polyps of patients with aspirin exacerbated respiratory disease fibroblasts

According to our hypothesis, the observed differences in PGE_2-induced IL-1RI expression between NM-C and NP-AERD fibroblasts could be related to the deficient expression of EP_2 receptor in the latter. Therefore, cultured NP-AERD fibroblasts were transiently transfected with expression plasmids encoding GFP as control or GFP chimeras of EP_2 receptor (**Figure 52A; 1 and 2**). Moreover, as a negative control of the transfection process, we incubated fibroblasts with media alone (**Figure 52A; 3**). To confirm the results obtained by fluorescence microscopy, we performed western blot for the detection of both GFP and EP_2 protein expression. As shown in **Figure 52B**, we observed immunoreactive bands for EP_2 receptor conjugated with GFP and for GFP alone, therefore confirming the efficacy of the transient transfection technique. Moreover, we also demonstrated high EP_2-GFP expression relative to the low endogenous EP_2 receptor levels.

A



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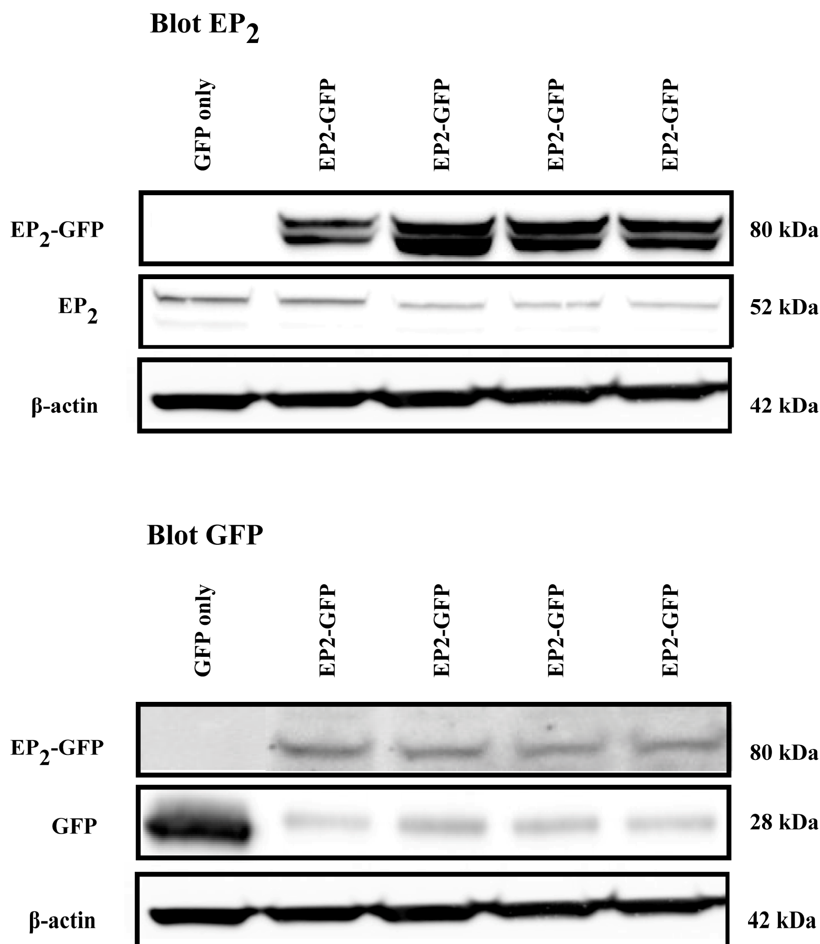


Figure 52. Transient transfection of EP_2 receptor in cultured NP-AERD fibroblasts. NP-AERD fibroblasts (N=8) were transiently transfected with expression plasmids encoding GFP as control or with GFP chimeras of EP_2 receptor. **(A)** Representative fluorescence images of **(1)** GFP plasmid, **(2)** EP_2-GFP plasmid, and **(3)** negative control taken 24 h post-transfection with an Axiovert 200M (Zeiss) inverted

fluorescence microscope (x40 magnification). **(B)** Representative blots of EP_2, GFP and β-actin expression. **EP:** E-prostanoid; **GFP:** green fluorescent protein; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

As demonstrated in **Figure 53**, NP-AERD fibroblasts that were transiently transfected with EP_2 receptor exhibited a significant increase in IL-1RI expression upon stimulation with PGE_2 in a dose-dependent manner. Moreover, we found no differences between the induction of IL-1RI by PGE_2 in EP_2-transfected NP-AERD fibroblasts and non-transfected NM-C fibroblasts treated with the same concentrations of exogenous PGE_2.

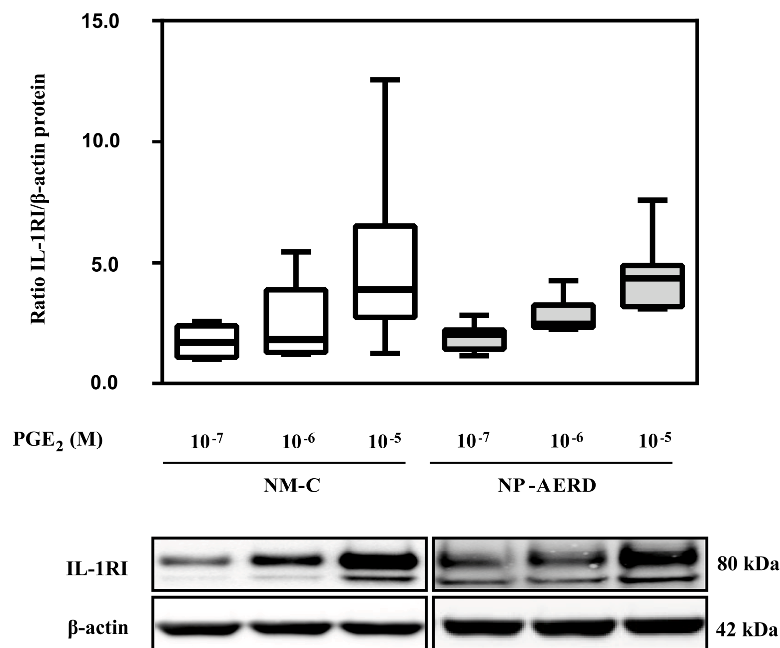


Figure 53. Effect of transient transfection of EP_2 receptor on PGE_2-induced IL-1RI expression in cultured NP-AERD fibroblasts. Fibroblasts (N=8) were transiently transfected with expression plasmids encoding GFP chimeras of EP_2 receptor using Lipofectamine 2000 reagent and incubated with PGE_2 (10^{-7} M–10^{-5} M) for 24 h. Densitometric analysis and representative western blot of IL-1RI expression normalized to β-actin in non-transfected NM-C and transfected NP-AERD fibroblasts. **EP:** E-prostanoid; **GFP:** green fluorescent protein; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease; **PGE_2:** prostaglandin E_2.

Using the same optimized transfection conditions, we also evaluated whether IL-1β-induced expression of COX-2 (**Figure 54A**) and mPGES-1 (**Figure 54B**) was affected. We found that EP_2-transfected fibroblasts expressed more COX-2 and mPGES-1 in the

presence of IL-1β compared with non-transfected cells under the same conditions. Moreover, the induced expression of both COX-2 and mPGES-1 did not differ between EP_2-transfected NP-AERD fibroblasts and non-transfected NM-C fibroblasts. These results indicate that the reestablishment of the EP_2 receptor on NP-AERD fibroblasts is able to restore IL-1RI levels (induced by PGE_2) and COX-2 and mPGES-1 expression (induced by IL-1β).

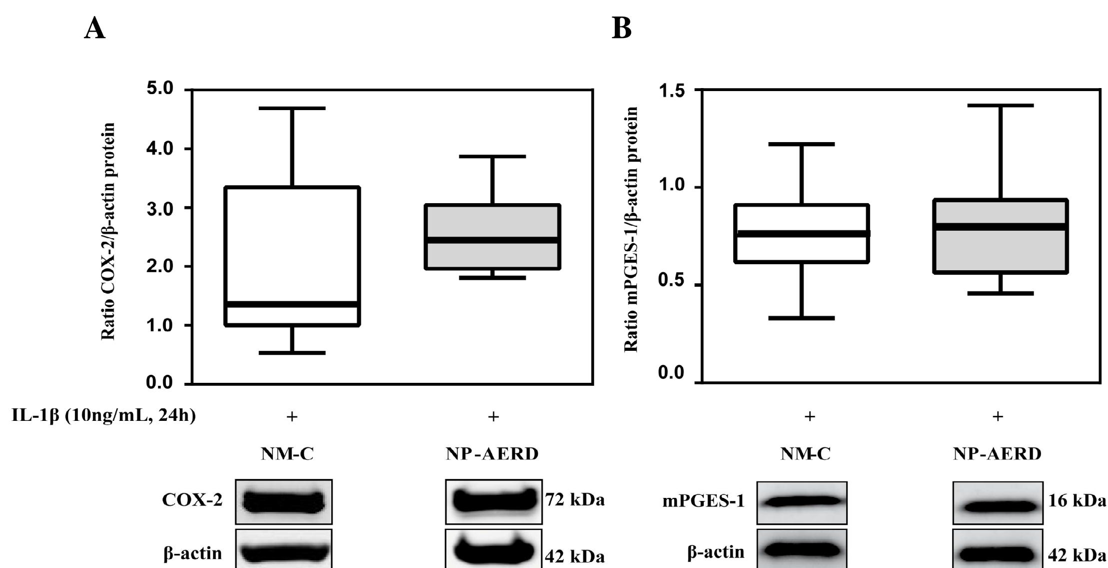


Figure 54. Effect of transient transfection of EP_2 receptor on IL-1β-induced COX-2 and mPGES-1 expression in cultured NP-AERD fibroblasts. Fibroblasts (N=8) were transiently transfected with expression plasmids encoding GFP chimeras of EP_2 receptor using Lipofectamine 2000 reagent and incubated with IL-1β (10 ng/mL) for 24 h. Densitometric analysis and representative western blot of **(A)** COX-2 and **(B)** mPGES-1 expression normalized to β-actin in non-transfected NM-C fibroblasts and transfected NP-AERD fibroblasts. **COX-2:** cyclooxygenase-2; **EP:** E-prostanoid; **GFP:** green fluorescent protein; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **mPGES-1:** microsomal prostaglandin E synthase; **NM-C:** nasal mucosa of control subjects; **NP-AERD:** nasal polyps of patients with aspirin exacerbated respiratory disease.

3. Study 3

3.1. Effect of interleukin-4 and interferon-γ on E-prostanoid receptor expression in cultured nasal mucosa of control subjects fibroblasts

To evaluate the effect of IL-4 and/or IFN-γ on non-induced and IL-1β-induced EP_2, EP_3 and EP_4 receptors expression, cultured NM-C fibroblasts were treated with or without IL-4 and/or IFN-γ in the presence or absence of IL-1β for 48 h. After this time, protein expression of EP_2, EP_3 and EP_4 receptors was detected and measured by western blot.

Compared to media-treated cells, the incubation with IL-4, IFN-γ or the combination of both was not able to modify the expression of EP_2, EP_3 or EP_4 receptors. Stimulation of NM-C fibroblasts with IL-1β at 10 ng/mL significantly increased EP_2 receptor expression (**Figure 55**). Nevertheless, the expression of EP_3 (**Figure 56**) or EP_4 receptors (**Figure 57**) was not modified upon incubation of NM-C fibroblasts with IL-1β. The stimulation of NM-C fibroblasts with IL-4 or IFN-γ in the presence of pro-inflammatory IL-1β did not alter the expression of any EP receptors compared to respective IL-1β alone. We observed that the co-treatment with IL-4 and IFN-γ at 10 ng/mL for 48 h was able to significantly inhibit IL-1β-induced EP_2 receptor protein expression and, in these conditions, EP_2 receptor levels were not different to the measured in fibroblasts treated with media only (**Figure 55**).

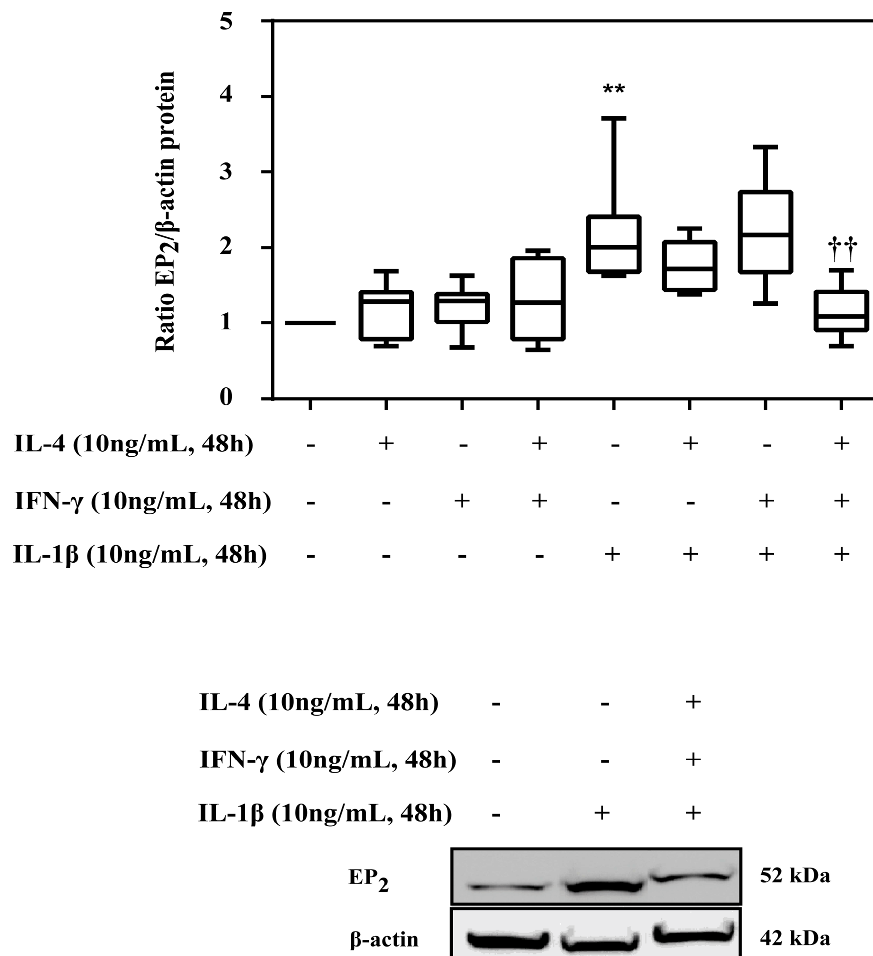


Figure 55. Effect of IL-4 and IFN-γ on non-induced and IL-1β-induced EP_2 receptor expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of EP_2 receptor expression corrected to β-actin. Samples were normalized to the control group (media-treated fibroblasts) which was set as 1. ** $p<0.01$ compared to control group; †† $p<0.01$ compared to IL-1β-treated fibroblasts. **EP:** E-prostanoid; **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects.

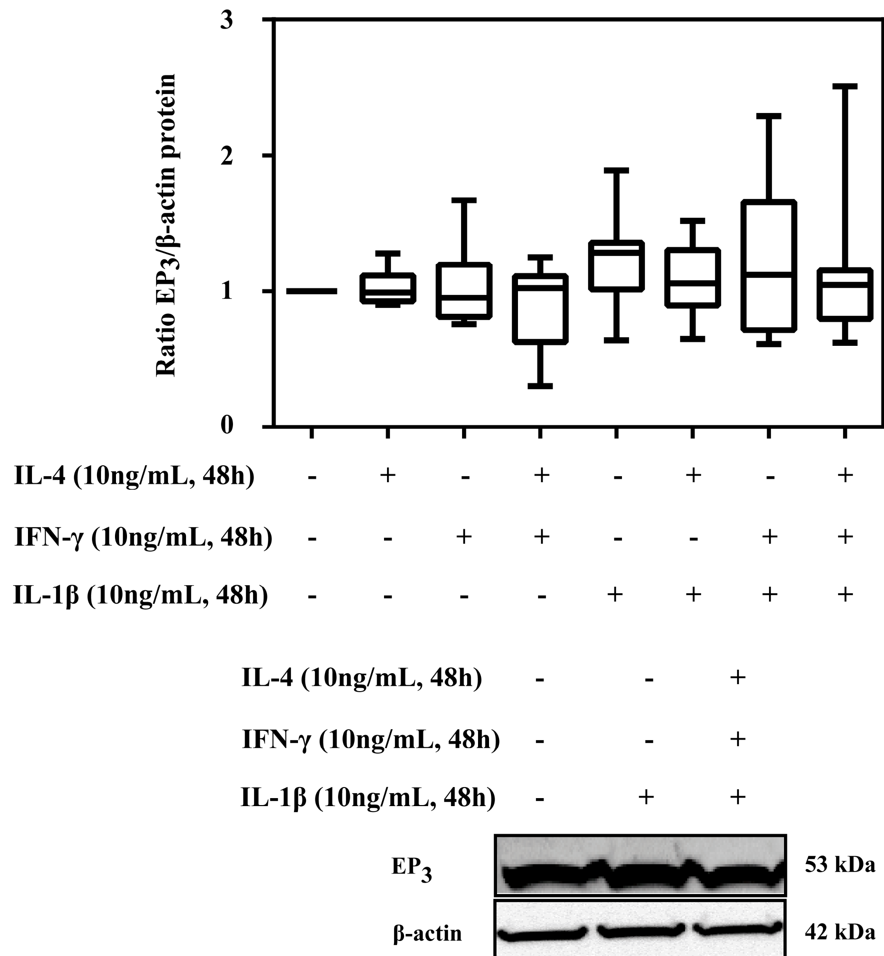


Figure 56. Effect of IL-4 and IFN-γ on non-induced and IL-1β-induced EP_3 receptor expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of EP_3 expression corrected to β-actin. Samples were normalized to the control group (media-treated fibroblasts) which was set as 1. **EP:** E-prostanoid; **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects.

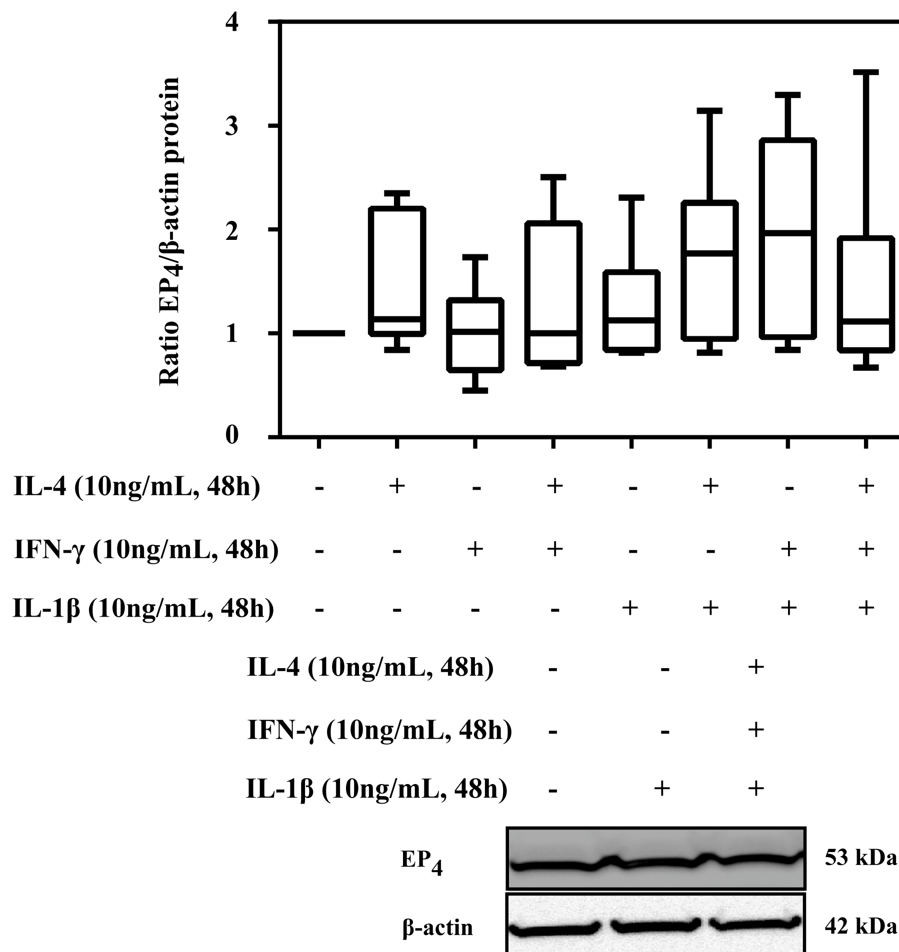


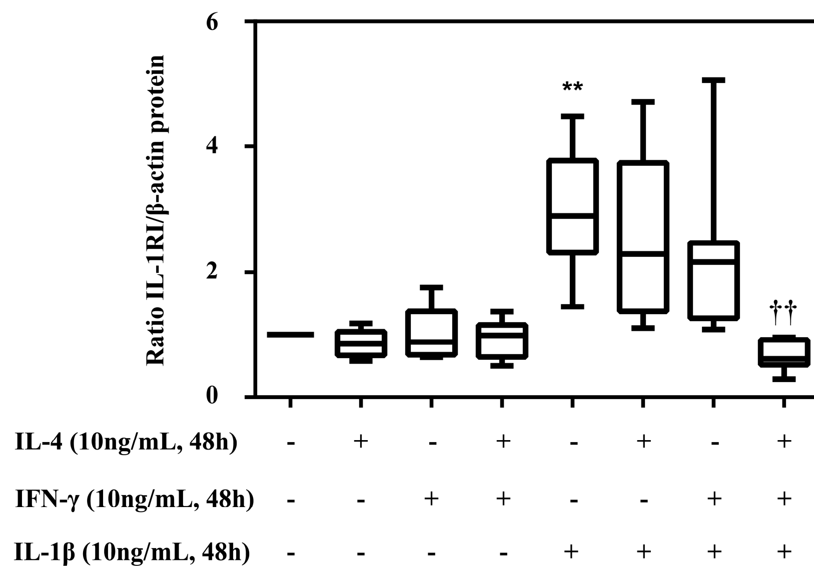
Figure 57. Effect of IL-4 and IFN-γ on non-induced and IL-1β-induced EP_4 receptor expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of EP_4 expression corrected to β-actin. Samples were normalized to the control group (media-treated fibroblasts) which was set as 1. **EP:** E-prostanoid; **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects.

3.2. Effect of interleukin-4 and interferon-γ on interleukin-1 receptor type I, cyclooxygenase-2 and microsomal prostaglandin E synthase-1 expression in cultured nasal mucosa of control subjects fibroblasts

Considering the results obtained in study 2, in which we described that altered EP_2 receptor expression affects several components of the COX pathway in AERD, we also evaluated the effect of IL-4 and/or IFN-γ on non-induced and IL-1β-induced IL-1RI

protein expression (**Figure 58**), on IL-1β-induced COX-2 protein expression (**Figure 59**), and on non-induced and IL-1β-induced mPGES-1 protein expression (**Figure 60**). As described before, cultured fibroblasts from NM-C were treated with or without IL-4 and/or IFN-γ at 10 ng/mL in the presence or absence of IL-1β (10 ng/mL) for 48 h. After this time, the expression of IL-1RI, COX-2, and mPGES-1 was detected and measured by western blot.

Compared to media-treated fibroblasts, the incubation with IL-4, IFN-γ or the combination of both was not able to modify the expression of either IL-1RI or mPGES-1. As observed in study 2, when NM-C fibroblasts were stimulated with IL-1β at 10 ng/mL, we detected a significant increase in IL-1RI, COX-2, and mPGES-1 protein expression. The incubation with IL-4 or IFN-γ in the presence of IL-1β did not change the expression of IL-1RI, COX-2 or mPGES-1. However, we observed that the co-treatment with IL-4 and IFN-γ was able to significantly inhibit IL-1β-induced IL-1RI, COX-2 and mPGES-1 protein expression.



IL-4 (10ng/mL, 48h) - - +

IFN-γ (10ng/mL, 48h) - - +

IL-1β (10ng/mL, 48h) - + +

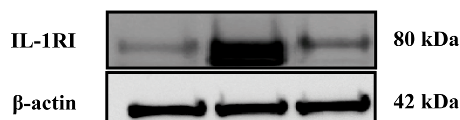


Figure 58. Effect of IL-4 and IFN-γ on non-induced and IL-1β-induced IL-1RI receptor expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of IL-1RI expression corrected to β-actin. Samples were normalized to the control group (media-treated fibroblasts) which was set as 1. ** p<0.01 compared to control group; †† p<0.01 compared to IL-1β-treated fibroblasts. **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **NM-C:** nasal mucosa of control subjects.

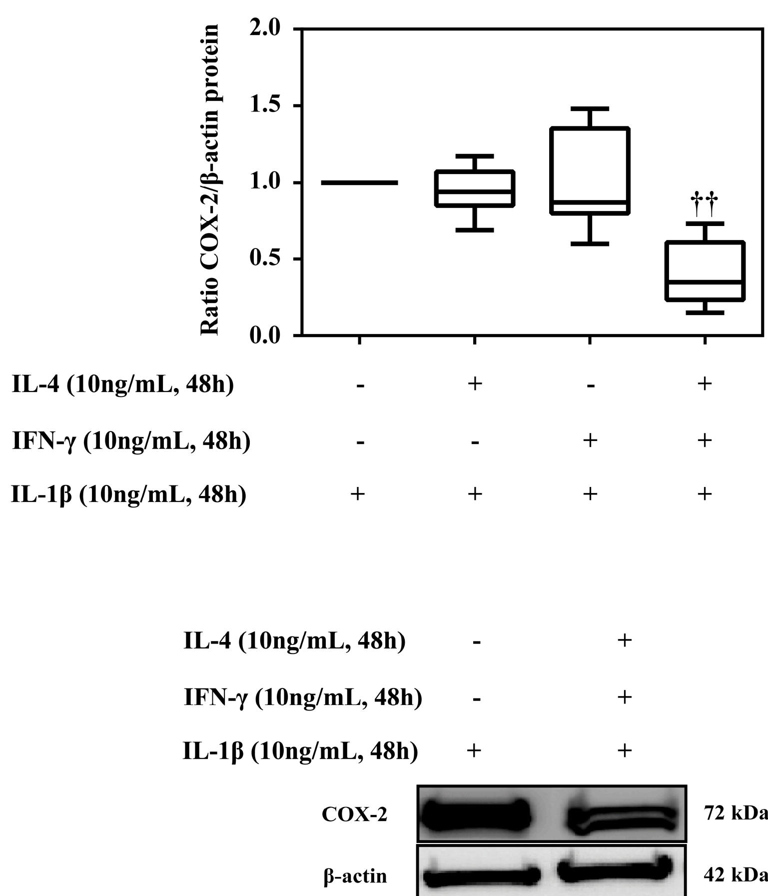


Figure 59. Effect of IL-4 and IFN-γ on IL-1β-induced COX-2 expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of COX-2 expression corrected to β-actin. Samples were normalized to the control group (IL-1β-treated fibroblasts) which was set as 1. †† p<0.01 compared to IL-1β-treated fibroblasts. **COX-2:** cyclooxygenase-2; **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **NM-C:** nasal mucosa of control subjects.

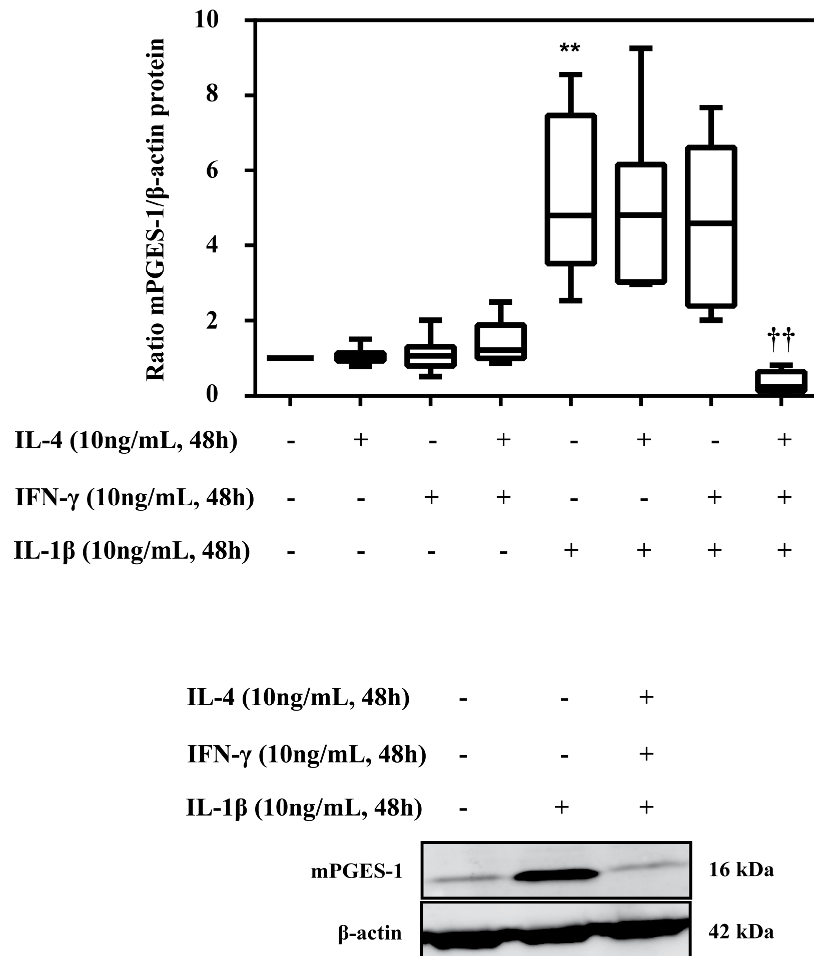


Figure 60. Effect of IL-4 and IFN-γ on non-induced and IL-1β-induced mPGES-1 receptor expression in cultured NM-C fibroblasts. Fibroblasts (N=8) were incubated with IL-4 (10 ng/mL) and/or IFN-γ (10 ng/mL) for 48 h in the presence or absence of IL-1β (10 ng/mL). Densitometric analysis and representative western blot of mPGES-1 expression corrected to β-actin. Samples were normalized to the control group (media-treated fibroblasts) which was set as 1. ** $p<0.01$ compared to control group; †† $p<0.01$ compared to IL-1β-treated fibroblasts. **IFN-γ:** interferon-γ; **IL-4:** interleukin-4; **IL-1β:** interleukin-1β; **mPGES-1:** microsomal prostaglandin E synthase; **NM-C:** nasal mucosa of control subjects.

DISCUSSION

DISCUSSION

Altered expression and signaling of prostaglandin E receptor E-prostanoid 2 in nasal polyps of aspirin exacerbated respiratory disease patients: role in inflammation and remodeling

In study 1 we aimed to investigate the expression and functionality of EP receptors in AERD and their role in PGE_2 signaling. The main findings of this research work were the following: (i) at baseline, there is decreased EP_2 receptor and increased EP_4 receptor expression in NP tissue samples and in cultured fibroblasts obtained from NP-AERD; (ii) stimulation with PGE_2, specific EP_2 and EP_4 receptors agonists differentially increased cAMP levels in cultured fibroblasts from NM-C and NP-AERD; (iii) intracellular cAMP levels increased similarly between cultured fibroblasts from NM-C and NP-AERD when stimulated with a strong cAMP activator; (iv) stimulation with PGE_2 and EP_2 receptor agonist differentially inhibited cell proliferation in cultured fibroblasts from NM-C and NP-AERD; and finally (v) stimulation with PGE_2 and EP_2 receptor agonist differentially inhibited GM-CSF release in cultured fibroblasts from NM-C and NP-AERD.

We first confirmed the baseline expression of each EP receptor in whole tissue and cultured fibroblasts isolated from NM-C and NP-AERD. Using two different techniques (western blot and quantitative real-time PCR) we found reduced expression of EP_2 receptor and enhanced expression of EP_4 receptor in both nasal tissue and cultured fibroblasts obtained from NP-AERD. Moreover, we observed no expression of EP_1 receptor and no differences in EP_3 receptor expression in both nasal tissue and cultured fibroblasts obtained from NM-C and NP-AERD.

EP_1 receptor expression has been reported by some previous studies in NP of AERD and of NSAID-tolerant asthmatic patients (Machado-Carvalho *et al.*, 2014) but was not found in other studies (Torres-Atencio *et al.*, 2014; Serra-Pages *et al.*, 2012). Differences in the methodology (immunohistochemistry *vs* PCR and western blot) and in the quality and affinity of EP_1 receptor antibodies used probably account for these differences.

Our results are in accordance with previous studies that show low expression of EP_2 receptor in a variety of samples from both lower (Corrigan *et al.*, 2012) and upper

(Roca-Ferrer *et al.*, 2011; Ying *et al.*, 2006; Adamusiak *et al.*, 2012) airways of patients with AERD. Additionally, several polymorphisms of the EP_2 receptor gene have been associated with risk of AERD (Jinnai *et al.*, 2004; Kim *et al.*, 2006). Jinnai and co-workers (Jinnai *et al.*, 2004), using a genetic screening of a candidate gene for AERD, concluded that variants in the promoter of the EP_2 receptor gene are strongly and significantly associated with AERD, and are functional by reducing transcriptional activity of the EP_2 gene, which result in a reduced expression of EP_2 receptor.

The molecular basis of reduced EP_2 receptor expression is still under investigation. In a recent study, Cahill and co-workers (Cahill *et al.*, 2015), by using cultured fibroblasts isolated from NP-AERD, aimed to confirm the impairment of EP_2 protein expression in these cells, examining a potential epigenetic basis. The authors showed that the histone H3 acetylated at lysine 27 at the EP_2 receptor promoter strongly correlated with baseline EP_2 mRNA expression, suggesting that the EP_2 promoter is under epigenetic control. Thus, these observations suggest that the reduction of EP_2 receptor expression observed in AERD may be related to a mechanism involving epigenetic modifications.

The reduced expression of E-prostanoid 2 receptor in aspirin exacerbated respiratory disease has downstream consequences upon receptor activation

Cultured fibroblasts and inflammatory cells from NP-AERD show reduced EP_2 receptor expression compared to aspirin-tolerant asthma patients and control subjects (Roca-Ferrer *et al.*, 2011; Ying *et al.*, 2006). Although such deficiency could have substantial consequences at a pathophysiologic level, the repercussions of decreased EP_2 receptor on its functionality were not entirely elucidated. In a study performed with peripheral blood cells, Corrigan and co-workers (Corrigan *et al.*, 2012) hypothesized that the decreased expression of EP_2 receptor on AERD patients has functional effects, resulting in a reduced anti-inflammatory activity of PGE_2. Nevertheless, the authors were not able to report any differences in the global mRNA expression of EP_2 receptor in peripheral blood cells obtained from AERD patients when compared with non-AERD asthma patients. In consequence, the use of a specific EP_2 receptor agonist did not result in any significant difference in their inhibitory effects between subjects. The results presented in this study suggest that the abnormal regulation of EP_2 receptor takes place in the inflamed airway milieu of AERD but is not present in peripheral blood cells that cannot be used as surrogates of cells or tissues collected from either the upper or the lower airways.

Based on the concept that EP_2 receptor drives signaling messages by increasing intracellular cAMP production (Vancheri *et al.*, 2004), we investigated the effects of an EP_2 agonist on intracellular cAMP production in cultured fibroblasts from NM-C and NP-AERD. Our results show that the low expression of EP_2 receptor in AERD is associated with a significant reduction in the levels of intracellular cAMP, a finding that supports that the altered expression of this receptor has cognizable downstream consequences.

Airway remodeling and chronic airway inflammation are features frequently associated to AERD. PGE_2 is known as a potent inhibitor of both fibroblast proliferation and collagen production (Vancheri *et al.*, 2004), primarily by stimulating intracellular cAMP accumulation and exchange protein activated by cAMP through EP_2 receptors. On the other hand, PGE_2 also participates in the modulation of the production of several cytokines involved in reparative processes and inflammation. Previous studies have demonstrated that PGE_2 acts as a “braking mechanism” to limit the secretion of pro-inflammatory cytokines, such as GM-CSF and this process involves increases in intracellular cAMP production (Lazzeri *et al.*, 2001).

In our study we demonstrated that PGE_2 via EP_2 receptor is able to inhibit cell proliferation in cultured fibroblasts from both NM-C and NP-AERD. However, inhibition in NP-AERD fibroblasts was significantly lower when compared with NM-C, probably due to the low levels of EP_2 receptor on these cells. According with our results, Huang and co-workers (Huang *et al.*, 2008) described that in control lung fibroblasts, PGE_2 suppression of proliferation occurs through ligation of the EP_2 receptor, resulting in the activation of AC, increase of intracellular cAMP production, and activation of cAMP-dependent signaling pathways. Our findings are also in accordance with those performed by Cahill and co-workers (Cahill *et al.*, 2015). The authors reported that reduced EP_2 receptor expression leads in resistance to the anti-proliferative effects mediated by PGE_2 in NP-AERD fibroblasts, attributing also an important role of EP_2 signaling in the aberrant growth of NP tissue.

Our study also demonstrated that PGE_2 through EP_2 signaling inhibits GM-CSF release induced by FBS on NM-C fibroblasts. In cultured fibroblasts from NP-AERD the percentage of GM-CSF inhibition was significantly lower when compared with NM-C. AERD patients frequently exhibit a massive eosinophilic inflammation, resulting from an increase migration/infiltration, activation, and survival of eosinophils into tissue (Vignola *et al.*, 2003). Effectively, several pro-inflammatory cytokines such as GM-CSF sustained these processes. The results presented by us are consistent with those

obtained by Clarke and collaborators (Clarke *et al.*, 2004) which showed that the EP_2 receptor subtype mediates the inhibitory effect of PGE_2 on GM-CSF release from human airway smooth muscle cells. In cultured fibroblasts from NP-AERD, we observed that the inhibition of GM-CSF release by PGE_2 is lower than that verified in NM-C. These data emphasizes that the low expression of EP_2 receptor in these cells could contribute to the perpetuation of the chronic eosinophilic inflammation characteristic of AERD patients.

E-prostanoid 4 receptor upregulation in aspirin exacerbated respiratory disease does not completely compensate the deficient expression of E-prostanoid 2 receptor

In our experiments we found augmented baseline expression of EP_4 receptor in cultured fibroblasts from NP-AERD when compared with NM-C. As expected, we observed an increased capacity of an EP_4 agonist to induce intracellular cAMP production in AERD. In agreement with our findings, high immunoreactivity has been reported for EP_4 receptor on induced sputum macrophages of patients with asthma (Ying *et al.*, 2004), despite that they also reported increased levels of EP_2 receptor on these cells.

Considering the convergent signaling cascade of EP_2 and EP_4 receptors, it is possible that the high levels of EP_4 receptor found in NP-AERD in this study resulted from a compensatory upregulation mechanism to counteract the deficient expression of EP_2 receptor. To investigate whether the upregulated EP_4 receptor can compensate the deficient regulation of EP_2 receptor we compared the effects of PGE_2 and the additive effects of EP_2 and EP_4 agonists on intracellular cAMP biosynthesis. We observed that the capacity of cultured fibroblasts from NP-AERD to increase intracellular cAMP levels in the presence of PGE_2 or both EP_2 and EP_4 agonists is significantly lower when compared with NM-C. These results suggest that the high levels of EP_4 receptor in cultured fibroblasts from NP-AERD, and the consequent high capacity of the selective EP_4 agonist to increase the production of intracellular cAMP, may be not sufficient to compensate for the low expression of EP_2 receptors in these cells. This is in accordance with previous studies that compared signaling through EP_2 and EP_4 receptors and demonstrated that EP_4 has less efficient functional coupling to cAMP than EP_2 receptor (Fujino *et al.*, 2002).

Biosynthesis of intracellular cyclic adenosine monophosphate in aspirin exacerbated respiratory disease through an E-prostanoid receptor-independent pathway

Since we reported that the unbalanced expression of EP receptors is responsible to the impaired production of intracellular cAMP in AERD, we studied the effect of a potent cAMP-elevating agent on intracellular cAMP production in cultured fibroblasts from both NM-C and NP-AERD. Forskolin acts via a pathway which does not involve either EP_2 or EP_4 receptors. We demonstrated no differences in intracellular cAMP production between fibroblast cultures when stimulated with forskolin. Stumm and co-workers (Stumm *et al.*, 2011) studied responsiveness to PGE_2 in lung fibroblasts isolated from asthmatic mice and reported that responses to forskolin mirrored those to PGE_2. The authors attributed these results to the unaltered expression of EP_2 receptor, which preserves responsiveness to PGE_2. According to these data and since the amount of intracellular cAMP production was recovered in NP-AERD fibroblasts after forskolin stimulation, we corroborated that the differences observed between NM-C and NP-AERD are probably due to the alterations aforementioned for the expression of EP receptors and their coupling with the cAMP biosynthesis machinery.

Similar expression of E-prostanoid 3 receptor in aspirin exacerbated respiratory disease patients and control subjects

It is well-known that EP_3 receptor activation normally reduces intracellular cAMP production. We have not detected any difference in the expression of EP_3 receptor between NM-C and NP-AERD. In keeping with this observation we found that the activation of EP_3 receptor through its agonist sulprostone inhibits forskolin-induced cAMP levels in cultured fibroblasts and this effect was similar in NM-C and NP-AERD. With these results we confirmed previous studies that described the capacity of EP_3 receptor activation to decrease intracellular cAMP production *in vitro* (Israel *et al.*, 2009; White *et al.*, 2008) and we excluded the potential involvement of inhibitory EP_3 receptor in the differences observed in intracellular cAMP production between NM-C and NP-AERD when stimulated with PGE_2.

The use of NM as control group could be taken as a potential limitation of this study. However, we sought to highlight the functional repercussion of the low expression of

EP_2 receptor in AERD, more than to establish a comparison between the phenotypes of NPs that has already been reported (Roca-Ferrer *et al.*, 2011).

In summary, the altered expression of PGE_2 receptors on AERD has downstream functional repercussions. These events associated with the impaired production of PGE_2 reported in AERD could contribute to perpetuating the chronic inflammatory and remodeling processes usually present in upper and lower airways of these patients. In clinical practice, EP receptor agonists represent potential new therapies for inflammatory airways diseases. A reduced expression of EP_2 receptor and the limited capacity of an enhanced EP_4 receptor to counteract the deficient EP_2, suggests that a combination of EP_2 and EP_4 agonists, might prove more helpful that isolated agonists for the treatment of asthma variants such as AERD, in which airway PGE_2-mediated homeostasis is deficient.

Low E-prostanoid 2 receptor and deficient induction of interleukin-1β/interleukin-1 receptor type I/cyclooxygenase-2 pathway: vicious circle in aspirin exacerbated respiratory disease

Although the mechanisms responsible for AERD are not fully understood, part of the explanation appears to lie in the reduced production of PGE_2, which is concomitantly associated with diminished expression of EP_2 receptor (Roca-Ferrer *et al.*, 2011; Steinke *et al.*, 2015; Laidlaw *et al.*, 2013; Pérez-Novo *et al.*, 2005; Picado *et al.*, 1999; Gosepath *et al.*, 2005; Steinke *et al.*, 2009). In this study, we hypothesized that the two anomalies are related.

Because all the actions of IL-1β identified to date appear to be mediated through its only known functional receptor, IL-1RI (Roca-Ferrer *et al.*, 2011; Dinarello *et al.*, 2011; Ricciotti *et al.*, 2011) and considering the importance of this cytokine in the upregulation of COX-2 and mPGES-1 to increase PGE_2 production, we examined the relationship between IL-1RI expression and COX-2, mPGES-1 and EP_2 receptor expression in AERD.

We first demonstrated that cultured fibroblasts isolated from NP-AERD showed reduced baseline levels of IL-1RI mRNA and protein compared with NM-C fibroblasts. The deficient expression of IL-1RI could contribute to impeding the induction of COX-2 and mPGES-1 expression upon cellular stimulation with IL-1β. Indeed, when cultured fibroblasts were treated with IL-1β, we observed increases in IL-1RI protein and mRNA levels in both NM-C and NP-AERD fibroblasts. However, IL-1RI induction was significantly lower in NP-AERD fibroblasts than in NM-C fibroblasts. Our results are in accordance with previous studies performed in human lung (Takii *et al.*, 1992; Takii *et al.*, 1994) and dermal (Akahoshi *et al.*, 1988) fibroblast cell lines, in which the authors established positive feedback for IL-1β on the expression of its own receptor.

The induction of COX-2 expression by IL-1β is a well-described phenomenon and results from the translocation of the transcription factor NF-κB to the nucleus (Martin *et al.*, 2002; Weber *et al.*, 2003). Using quantitative real-time PCR and western blot techniques, we observed reduced levels of COX-2 in NP-AERD fibroblasts compared with NM-C fibroblasts upon stimulation with IL-1β (Roca-Ferrer *et al.*, 2011). We were also able to establish a correlation between the levels of IL-1RI and the expression of COX-2 in cultured NM-C and NP-AERD fibroblasts. In our experiments, and in

accordance with the literature, we found that the stimulation of NM-C and NP-AERD fibroblasts with IL-1β demonstrated a positive correlation between the levels of IL-1RI and COX-2 expression. Moreover, using gene silencing approaches, we corroborated that in the absence of IL-1RI, which is the only functional receptor through which pro-inflammatory IL-1β signals, stimulation of NM-C fibroblasts with IL-1β has a null effect on COX-2 protein expression. These results demonstrate that the deficient induction of COX-2 in NP-AERD is essentially due to the low levels of IL-1RI expressed on these cells.

Several reports describe that the functions of COX-2/mPGES-1 are altered in the respiratory tissues in AERD. Cheong and collaborators (Cheong *et al.*, 2011) reported that the gene encoding PGES enzyme is hypermethylated in NPs from patients with AERD. Effectively, the presence of this epigenetic mechanism could be part of the explanation for the impairment in inflammation-induced PGE_2 synthesis via PGES in AERD. Considering the capacity of IL-1β to modulate the expression of mPGES-1 (Cahill *et al.*, 2015), we also investigated whether the reported alterations in IL-1RI expression in NP-AERD fibroblasts affect the expression of mPGES-1. We found that IL-1β-induced mPGES-1 expression is significantly diminished in NP-AERD fibroblasts compared with NM-C fibroblasts. As demonstrated for COX-2 protein expression, we were also able to establish a positive correlation between the expression of IL-1RI and mPGES-1 in cultured NM-C and NP-AERD fibroblasts. These results are in agreement with previous studies that describe a coregulatory mechanism of COX-2 and mPGES-1 by IL-1β (Park *et al.*, 2006), in which IL-1RI seems to play a central role.

In order to assess if the alterations observed in cultured fibroblasts from NP-AERD were also present in whole tissue, we measured the expression of both IL-1RI and COX-2 in whole tissue isolated from NM-C and NP-AERD. The results showed that IL-1RI protein expression was significantly lower in NP-AERD when compared to NM-C. Moreover, we also observed reduced levels of COX-2 in NP-AERD comparing with NM-C. Our findings are in accordance with those reported by Picado and co-workers (Picado *et al.*, 1999). The authors reported that COX-2 mRNA expression in NPs of patients suffering from aspirin-intolerant asthma/rhinitis is markedly and significantly reduced than in polyps from the aspirin-tolerant asthma and rhinitis group and in healthy mucosa.

Besides the alterations observed in the expression of IL-1RI, and considering that all members of IL-1R subfamily participate in the regulation of IL-1 signaling, we also evaluated potential alterations in other components of the family in cultured fibroblasts

from NP-AERD comparing with NM-C. As previously described, IL-1RAcP is the accessory receptor chain for IL-1 and forms a heterodimeric complex with IL-1RI to mediate signal transduction (Boraschi *et al.*, 2006). Many observations have demonstrated that IL-1RAcP is an absolutely necessary constituent of the multimeric IL-1RI signal transduction complex (Wesche *et al.*, 1992). Taking into account the low levels of IL-1RI in cultured fibroblasts from NP-AERD, we also could expect a diminished expression of IL-1RAcP in these cells. However, in non-stimulated conditions, we observed no differences in the expression of IL-1RAcP between cultured fibroblasts from NM-C and NP-AERD. On the other hand, the treatment with IL-1β was able to induce IL-1RAcP mRNA expression in cultured fibroblasts from NM-C and NP-AERD. This mechanism could be related to the increased levels of IL-1RI expression observed in these same conditions, functioning as a positive feedback to equalize the expression of IL-1RI and making possible the signal transduction of IL-1β.

IL-1RII is able to bind to IL-1β; however since it lacks the intracellular TIR domain it results into a non-active ligand-receptor complex that blocks signal transduction (Boraschi *et al.*, 2013). At baseline we also found no differences in the expression of IL-1RII between cultured fibroblasts from NM-C and NP-AERD. Nevertheless, IL-1β was able to significantly induce IL-1RII mRNA expression in cultured fibroblasts from both NM-C and NP-AERD. This regulation of IL-1RII by IL-1β could be the result of a negative feedback mechanism in order to control its effect, maintaining the balance of IL-1 signaling.

Finally, we also evaluated the effect of IL-1β on IL-1Ra expression in lysates of cultured fibroblasts from NM-C and NP-AERD. IL-1Ra can bind to IL-1RI, but does not allow engagement of the accessory protein, blocking thus IL-1 signaling (Boraschi *et al.*, 2013). We observed that, in non-stimulated cells, the expression of IL-1Ra is reduced in both NM-C and NP-AERD fibroblasts; yet IL-1Ra expression was significantly diminished in cultured fibroblasts from NP-AERD when compared with NM-C. After cell stimulation with IL-1β, the expression of IL-1Ra increased significantly in cultured fibroblasts from NM-C but not in NP-AERD. Moreover, the expression of IL-1Ra under this condition was significantly lower in NP-AERD compared than the measured in NM-C fibroblasts. As negative regulator of IL-1 signal transduction, IL-1Ra could be increased in cultured fibroblasts from NM-C in order to avoid an over-response to IL-1β. Considering the downregulation of IL-1R pathway in cultured fibroblasts from NP-AERD, high levels of IL-1Ra could block the already decreased capacity of these cells to respond to IL-1.

Akahoshi and co-workers (Akahoshi *et al.*, 1988) investigated the mechanism by which IL-1β upregulates the expression of its own receptor. In a human dermal fibroblast cell line, these authors concluded that this process was mediated by PGE_2 because the inhibition of COX enzymes by indomethacin was able to abolish the enhancement of IL-1RI expression. Using a human lung fibroblast cell line, Takii and collaborators (Takii *et al.*, 1992; Takii *et al.*, 1994) also demonstrated that IL-1β upregulates transcription, and the number of cell-surface IL-1RIs, through the induction of PGE_2 synthesis. In light of these findings, we analyzed whether the addition of exogenous PGE_2 in our model also stimulates the expression of IL-1RI. We found that PGE_2 dose-dependently induced IL-1RI expression in NM-C fibroblasts. In contrast, PGE_2 had hardly any effect on the induction of IL-1RI expression in NP-AERD fibroblasts, achieving a significant response only at high doses; nevertheless, the expression of IL-1RI remained lower in NP-AERD fibroblasts than in NM-C under the same conditions. Our results demonstrate that PGE_2 differentially induces IL-1RI expression in NM-C and NP-AERD fibroblasts. To confirm the effect of exogenous PGE_2 on IL-1RI expression, we incubated NM-C fibroblasts with IL-1β in the presence or absence of ASA, a non-selective COX inhibitor that stops the formation of PGE_2. In accordance with the aforementioned studies, we showed that ASA was able to block the induction of IL-1RI by IL-1β in control fibroblasts. These findings are consistent with those reported by Spriggs and co-workers (Spriggs *et al.*, 1990), who found that PGE_2 greatly increases the number of cell-surface IL-1RIs. These authors also reported that IL-1β depends on PGE_2 synthesis to stimulate IL-1RI expression because it is not able to directly induce IL-1RI expression by a PGE_2-independent mechanism.

Some effects of IL-1β can be mimicked by cAMP-elevating agents, which activate protein kinase A (PKA) (Chung *et al.*, 1999). The action of PGE_2 is mediated by four receptors (EP_1–EP_4), however only the activation of EP_2 and EP_4 receptors has the capacity to increase intracellular cAMP levels (Narumiya et al., 1999). Considering that, we first assessed the effect of IL-1β on intracellular cAMP production. Our results are in accordance with a previous study performed with human vascular smooth muscle cells in which the authors demonstrated that IL-1 induce the production of intracellular cAMP (Beasley 1999). Effectively, we observed increased intracellular cAMP levels in cultured fibroblasts from both NM-C and NP-AERD after cell treatment with IL-1β; however, in these conditions, intracellular cAMP production was significantly lower in cultured fibroblasts from NP-AERD when compared to NM-C, confirming thus the low responsiveness of cultured fibroblasts from NP-AERD to IL-1β as result of the low expression of its functional receptor.

We next evaluated the effect of EP_2 and EP_4 agonists on IL-1RI expression in cultured NM-C and NP-AERD fibroblasts. As expected, the activation of the EP_2 receptor through its agonist butaprost was able to dose-dependently induce IL-1RI expression in NM-C fibroblasts. In NP-AERD fibroblasts, as observed upon stimulation with PGE_2, a significant increase in IL-1RI expression was only detectable with a high concentration of butaprost, in contrast to NM-C fibroblasts. However, the increases in IL-1RI levels upon EP_4 receptor stimulation was identical for cultured NM-C and NP-AERD fibroblasts. Thus, despite the increased expression of EP_4 receptor in NP-AERD fibroblasts, its activation does not produce a greater increase in expression of IL-1RI in these cells compared to NM-C. In other words, it seems that this receptor subtype does not appear to contribute to the differences in PGE_2-induced IL-1RI expression between NM-C and NP-AERD fibroblasts. These results support our previous studies (study 1) in which we described that the high expression of EP_4 receptor in NP-AERD is not able to compensate for the low expression of EP_2 receptor also observed in these cells.

Forskolin is a well-known inducer of the second messenger cAMP (Shirakawa *et al.*, 1988) and is frequently used in *in vitro* protocols. In our experiments, we used forskolin to induce intracellular cAMP release in both NM-C and NP-AERD fibroblasts. We found that forskolin had the same capacity to induce IL-1RI expression in both NM-C and NP-AERD fibroblasts. Using a fibroblast cell line from mouse whole embryo cultures, Bonin and co-workers (Bonin *et al.*, 1990) showed that stimulation of the cAMP-dependent pathway leads to the induction of the IL-1RI, consistent with our results.

As previously described, EP_3 receptor has been considered to couple to G_i protein leading to reduction in intracellular cAMP production. However, activation of at least one EP_3 slice variant leads to an increase in cAMP levels, suggesting functional coupling to G_s protein (Israel *et al.*, 2009; White *et al.*, 2008). Despite our previous findings that showed no differences in the expression of EP_3 receptor between NM-C and NP-AERD, and a significant reduction of forskolin-induced intracellular cAMP production in the presence of an EP_3 agonist, we aimed to exclude the potential involvement of this receptor in the differences observed in IL-1RI expression between NM-C and NP-AERD when stimulated with PGE_2. We evaluated the effect of sulprostone on forskolin-induced IL-1RI levels and we found that the activation of EP_3 receptor through sulprostone inhibits forskolin-induced IL-1RI expression in cultured fibroblasts and this effect was similar between NM-C and NP-AERD. These results are in accordance with those reported by Bonin and collaborators (Bonin *et al.*, 1990) which demonstrated that the expression of IL-1RI is induced by a mechanism that involves the increase of intracellular cAMP.

Taken together, our findings suggest that defective PGE_2-related induction of IL-1RI occurs due to the low expression of EP_2 receptor subtype in NP-AERD fibroblasts, which results in reduced cAMP production.

To gain insight into the relationship between the expression of EP_2 and the induction of IL-1RI, and consequently of COX-2 and mPGES-1, cultured NP-AERD fibroblasts were transiently transfected with an expression vector of the human EP_2 receptor protein. We verified that the levels of IL-1RI did not differ between NP-AERD and NM-C fibroblasts transfected with EP_2 receptor and stimulated with PGE_2. This finding supports the notion that the reduced expression of EP_2 receptor in NP-AERD fibroblasts is responsible for the deficient induction of IL-1RI by PGE_2. These observations are consistent with the aforementioned studies that have demonstrated the crucial role played by the PGE_2/cAMP/PKA axis in the induction of IL-1RI expression. In this set of experiments, we also stimulated EP_2-transfected NP-AERD fibroblasts with IL-1β to demonstrate that the normalization of EP_2 receptor expression in NP-AERD fibroblasts not only favours the induction of IL-1RI expression as also, consequently and significantly, increases the expression of both COX-2 and mPGES-1 in these cells.

In summary, our findings show that the reduced expression of COX-2 and mPGES-1 reported in patients with AERD is closely related to the low expression of EP_2 receptor also found in these patients. This observation suggests, for the first time, that the abnormal regulation of the autocrine loop regulating the COX pathway, which includes IL-1RI, COX-2, mPGES-1, EP_2 receptor and PGE_2, is involved in the reduced production of PGE_2 detected in AERD (**Figure 61**). Moreover, the low expression of EP_2 receptor appears to play a central role in the deregulated autocrine loop as deduced by the finding that the entire mechanism normalizes upon restoration of the normal function of the EP_2 receptor.

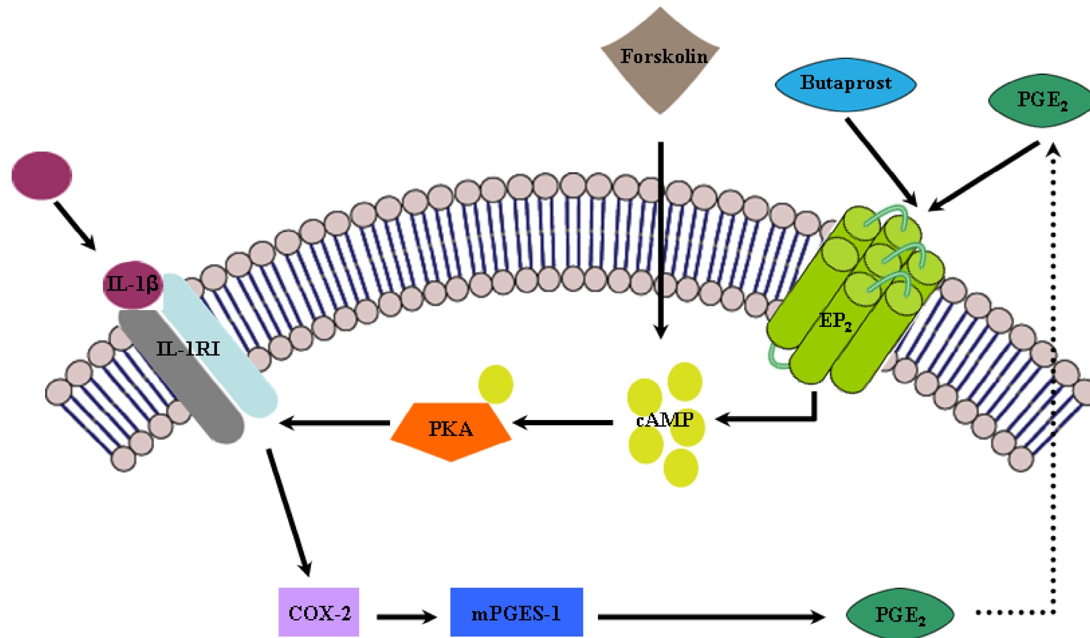


Figure 61. Schematic representation of the proposed mechanism underlying the regulation of COX pathway. IL-1β binds with high affinity to IL-1RI and stimulates the expression of both COX-2 and mPGES-1, increasing the production and secretion of PGE_2. PGE_2 (or selective EP_2 receptor agonists) activates EP_2 receptors and induces the increase in the secondary messenger cAMP, which is also stimulated by forskolin. The increase in cAMP production activates PKA, which stimulates the expression of IL-1RI, thereby indicating a potential positive feedback mechanism for this pathway. **cAMP:** cyclic adenosine monophosphate; **COX:** cyclooxygenase; **EP_2:** E-prostanoid 2; **IL-1β:** interleukin-1β; **IL-1RI:** interleukin-1 receptor type I; **mPGES-1:** microsomal prostaglandin E synthase-1; **PGE_2:** prostaglandin E_2; **PKA:** protein kinase A.

Interleukin-1β-stimulated cyclooxygenase pathway is inhibited by the inflammatory cytokines interleukin-4 and interferon-γ in aspirin exacerbated respiratory disease

AERD is a disease that is distinguished from aspirin tolerant asthma/chronic sinusitis by its most recognized characteristic – sensitivity to aspirin and other non-selective inhibitors of COX-1 (Steinke *et al.*, 2013). Recent studies have reported that other features, namely the pattern of cytokine expression could differentiate the two phenotypes. In contrast to asthmatic or eosinophilic sinusitis patients who tolerate aspirin, AERD seems to be more a mixture of Th2- and Th1-like milieu. Effectively, several observations have shown overexpressed IFN-γ and IL-4 in sinus tissue obtained from AERD patients when compared with control subjects and even with aspirin-tolerant patients (Steinke *et al.*, 2015). In this study, we were able to demonstrate a significant reduction in IL-1β-induced EP_2 receptor, IL-1RI, COX-2 and mPGES-1 protein expression after cell stimulation with a combination of IL-4 and IFN-γ in cultured fibroblasts from control subjects.

Taking into account the observations presented in study 2, in which we pointed out the central role of EP_2 receptor in the entire regulation of the autocrine loop that regulates the COX pathway in AERD, we first evaluated the effect of IL-4 and/or IFN-γ in the presence or absence of IL-1β on the expression of EP_2 receptor in cultured fibroblasts isolated from NM-C. To perform the experiments we only used cultured fibroblasts from NM-C because these cells show normal expression and regulation of the COX pathway components. We observed that alone or combined, neither IL-4 nor IFN-γ were able to modify the expression of EP_2 receptor, when compared to media-treated cells. On the other hand, the stimulation of cultured fibroblasts from NM-C with IL-1β significantly increased the expression of EP_2 receptor after 48 h. This result is in accordance with those reported by Roca-Ferrer (Roca-Ferrer *et al.*, 2011) in which the authors showed a time-dependent increase in EP_2 receptor expression after incubation of cultured fibroblasts from NM-C with the pro-inflammatory cytokine IL-1β. We then examined the effect of IL-4 and/or IFN-γ in IL-1β-induced EP_2 receptor expression and, under these conditions, significant differences were observed in IL-1β-stimulated fibroblasts treated with both IL-4 and IFN-γ when compared with IL-1β alone. We reported reduced protein levels of the EP_2 receptor, which were similar to those found in media-treated cells. In a recent review of the literature about the factors driving AERD phenotype, Steinke and collaborators (Steinke *et al.*, 2015) suggested that the molecular

mechanism underlying the impaired PGE_2 synthesis in AERD relies on the inhibition of the EP_2 receptor resulting from the over-expression of IL-4 present in the airways of those patients, setting aside IFN-γ. However, our results do not agree with this hypothesis, since we observed no differences in EP_2 receptor expression when cultured fibroblasts were stimulated with IL-4 alone or with the cytokine in the presence of IL-1β.

In this set of experiments we also assessed the effect of IL-4 and IFN-γ in the regulation of the expression of EP_3 and EP_4 receptors. We found no differences in the expression of these receptors, wherein its expression remained similar under all conditions compared to media-treated or IL-1β-stimulated cells. These observations reinforce the central role attributed to EP_2 receptor in the positive regulation of the COX pathway and demonstrate that the other PGE_2 receptors seem to have less relevance in the mechanism that regulates the COX pathway.

As suggested by us, the altered expression of EP_2 receptor in AERD could contribute to the malfunction of the feedback mechanism which regulates COX-2 and mPGES-1 induction via IL-1RI. Thus, in light of the aforementioned results, we sought to investigate whether this same cytokine combination is able to downregulate the other COX components, imperative to the correct function of the pathway.

As shown for EP_2 receptor protein expression, we found that the combination of IL-4 plus IFN-γ significantly diminished the expression of IL-1RI induced by IL-1β in cultured fibroblasts from NM-C. These observations give further support to our previous results describing that the low expression of EP_2 receptor in AERD, combined with its diminished capacity to increment intracellular cAMP levels upon activation, are crucial factors to impede the induction of IL-1RI in the presence of its agonist, IL-1β.

Following these findings, we also assessed the expression of inducible COX-2 and the expression of mPGES-1 in cultured fibroblasts from NM-C treated under the same conditions. It is well-known that both COX-2 and mPGES-1 are markedly induced in response to a wide variety of stimuli. In a study performed with human fibroblasts, Hayashi and co-workers (Hayashi *et al.*, 2000) studied the effect of IFN-γ and IL-4 on IL-1β-induced COX expression and terminal PGE_2 generation, and found that the pro-inflammatory cytokine IL-1β time- and dose-dependently induces the expression of COX-2, PGES-1 and the production of PGE_2 in human cultured fibroblasts. Effectively, the effect of IL-1β on COX-2 expression and PGE_2 production was also demonstrated in our *in vitro* model and was first described by Roca-Ferrer and co-workers (Roca-Ferrer *et al.*, 2011) in cultured fibroblasts isolated from NM-C and NP with or without

AERD. Hayashi and co-workers (Hayashi *et al.*, 2000) showed that, 48 h after cell treatment with IFN-γ or IL-4 in the presence or absence of IL-1β, neither IFN-γ nor IL-4 has an effect on COX-2 expression or PGE_2 production. However, the simultaneous treatment of cells with IL-1β and IFN-γ or IL-4 caused a significant decrease in both IL-1β-induced COX-2 expression and PGE_2 release. In another study performed with human alveolar macrophages and monocytes, Endo and collaborators (Endo *et al.*, 1998), by using northern and western blot analyses, reported that LPS-induced increases in COX-2 mRNA and protein were strongly attenuated by IL-4. The experiments also demonstrated that IFN-γ has a slight effect on LPS-induced COX-2 protein expression; however at this level no significant differences were found.

In conclusion, our results showed that neither IFN-γ nor IL-4 alone were able to inhibit IL-1β-induced increases in EP_2, IL-1RI, COX-2 or mPGES-1 expression in cultured fibroblasts from NM-C. Moreover, we also confirm that there is a finely-tuned autocrine mechanism that regulates the COX pathway of AA metabolism wherein any alteration in one of the components of the loop could disturb the normal function of the whole system. Moreover, it seems that the cytokine milieu characteristic of AERD has an important role in the entire process and could at least be part of the explanation of the observed deregulation of some of the components of the loop. These alterations could contribute to the severity of inflammation observed in AERD as well as accentuate the sensitivity of these individuals to the inhibition of PGE_2 synthesis associated with aspirin and other NSAIDs.

CONCLUSIONS & SUMMARY

CONCLUSIONS

The conclusions of the present thesis are the following:

Study 1

1. At baseline, there is decreased E-prostanoid 2 and increased E-prostanoid 4 receptor expression in both tissue samples and cultured fibroblasts obtained from nasal polyps of patients with aspirin exacerbated respiratory disease.
2. Cultured fibroblasts from nasal polyps of patients with aspirin exacerbated respiratory disease produced less intracellular cyclic adenosine monophosphate than fibroblasts from nasal mucosa of control subjects when stimulated with prostaglandin E_2.
3. The high levels of E-prostanoid 4 receptor in cultured fibroblasts from nasal polyps of patients with aspirin exacerbated respiratory disease are not sufficient to compensate for the deficient expression of E-prostanoid 2 receptor in these cells.
4. The deficient expression of E-prostanoid 2 receptor in cultured fibroblasts from nasal polyps of patients with aspirin exacerbated respiratory disease reduces the capacity of prostaglandin E_2 to suppress proliferation.
5. The deficient expression of E-prostanoid 2 receptor in cultured fibroblasts from nasal polyps of patients with aspirin exacerbated respiratory disease reduced the capacity of prostaglandin E_2 to inhibit the release of granulocyte-macrophage colony-stimulating factor.

Study 2

1. The reduced expression of cyclooxygenase-2 and microsomal prostaglandin E synthase-1 reported in aspirin exacerbated respiratory disease is closely related to the low expression of the E-prostanoid 2 receptor also found in these patients.

2. The abnormal regulation of the autocrine loop regulating the cyclooxygenase pathway is involved in the reduced production and secretion of prostaglandin E_2 detected in aspirin exacerbated respiratory disease.

3. The low expression of E-prostanoid 2 receptor appears to play a central role in the deregulated autocrine loop as deduced from the finding that by restoring the normal expression/function of this receptor the whole mechanism normalizes.

Study 3

1. The characteristics of the inflammatory airway milieu in aspirin exacerbated respiratory disease, consisting in the strong presence of interleukin-4 and interferon-γ, are responsible for the decrease in interleukin-1β-induced E-prostanoid 2 receptor expression.

2. The low expression of E-prostanoid 2 receptor in the presence of interleukin-4 and interferon-γ contributes to the malfunction of the feedback mechanism that regulates the induction of both cyclooxygenase-2 and microsomal prostaglandin E synthase-1 and consequently the production of prostaglandin E_2 via interleukin-1β/interleukin-1 receptor type I.

SUMMARY

This study provides evidence supporting the hypothesis that the inflammatory environment in the airways of patients with aspirin exacerbated respiratory disease induces alterations in the expression of the prostaglandin E_2 receptor E-prostanoid 2. This anomaly alters the induction of interleukin-1 receptor type I, the main factor responsible for the activation of cyclooxygenase-2 by interleukin-1β, which, in turn, results in a low production of prostaglandin E_2 which, associated with the low expression of the E-prostanoid 2 receptor, alters the autocrine feedback loop that regulates the cyclooxygenase pathway.

The E-prostanoid 2 receptor plays a central role in the alteration of prostaglandin E_2 synthesis, since its reestablishment can recover the normal function of the autocrine loop and thereby normalizing the expression and function of all the components that

constitute it (interleukin-1 receptor type I, cyclooxygenase-2, microsomal prostaglandin E synthase-1 and prostaglandin E_2).

The decrease in the synthesis of prostaglandin E_2 may contribute to the perpetuation of the eosinophilic inflammation and remodeling processes in the airways of patients with aspirin exacerbated respiratory disease.

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APPENDIX I. RESUMEN DE LA TESIS DOCTORAL

ESTUDIO DE LA EXPRESIÓN Y DE LA REGULACIÓN DE LOS COMPONENTES DEL BUCLE DE RETROALIMENTACIÓN AUTOCRINA DE LA VÍA DE LA CICLOOXIGENASA Y SU IMPLICACIÓN EN LA ENFERMEDAD RESPIRATORIA EXACERBADA POR ANTIINFLAMATORIOS NO ESTEROIDES

1. Introducción

La enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (EREA) se caracteriza por la presencia de asma bronquial, rinosinusitis crónica con pólipos nasales e hipersensibilidad a la aspirina y otros antiinflamatorios no esteroides. La inflamación de las vías aéreas en los pacientes con EREA se ha relacionada con diversas alteraciones en el metabolismo del ácido araquidónico (AA) las cuales podrían contribuir al incremento y perpetuación de los procesos inflamatorios. Además, estudios recientes también han demostrado que el perfil de citocinas (sobreexpresión de interferón (IFN)-γ e interleucina (IL)-4) en las vías aéreas, podría tener un papel relevante en la regulación del metabolismo del AA en la EREA.

La prostaglandina (PG) E_2, que es un producto del metabolismo del AA, que parece tener un papel importante en la regulación de los procesos inflamatorios. Su síntesis resulta de la actividad coordinada de las enzimas ciclooxigenasas (COX) y de las enzimas PGE sintasas (PGES). Ambas enzimas existen en forma constitutiva (COX-1 y PGES citosólica (cPGES)) e inducible (COX-2 y PGES microsomal-1 (mPGES-1)). En condiciones inflamatorias, la expresión de la COX-2 y de la mPGES-1 aumenta de manera concomitante para asegurar un incremento proporcional en la producción de PGE_2. En pacientes asmáticos con EREA se han descrito niveles reducidos de PGE_2. Esta baja producción de PGE_2 se ha atribuido a alteraciones en la regulación de las enzimas COX-2 y mPGES-1, aunque algunos estudios también han encontrado anomalías en la regulación de la COX-1. Los mecanismos responsables de estas alteraciones aún son desconocidos y una buena parte de los resultados descritos en la bibliografía han sido obtenidos usando un modelo con el que se prentende reproducir una situación inflamatoria *in vitro* mediante el uso de la IL-1β como estímulo proinflamatorio.

La IL-1β se une al receptor tipo I de la IL-1 (IL-1RI) que es el responsable de la transducción de señales de la IL-1β. Una segunda proteína de la superficie celular, la proteína accesoria del IL-1R, conocida como IL-1RAcP, se tiene que asociar al IL-1RI para que tenga lugar la transducción de señales. Este proceso puede ser anulado por dos mecanismos: uno mediante la unión de la molécula conocida como antagonista del receptor de la IL-1 (IL-1ra), que bloquea la transmisión de señales al unirse al IL-1RI y otro, a través de un segundo receptor, el IL-1RII, que inhibe las respuestas mediadas por la IL-1 secuestrando al IL-1RAcP y agotando la reserva disponible para asociarse con el IL-1RI. Estudios anteriores demostraron que en los procesos inflamatorios, la IL-1β es la principal citocina involucrada en la inducción de la enzima COX-2. Además, la IL-1β también es capaz de inducir la expresión de la mPGES-1. Así, las posibles alteraciones en la familia del IL-1R podrían ser responsables, al menos en parte, de las alteraciones descritas en las enzimas de la COX-2/mPGES-1 y consecuentemente en la producción de la PGE_2.

La PGE_2 puede actuar sobre 4 receptores denominados EP_1, EP_2, EP_3 y EP_4. La activación de estos receptores se considera responsable de los distintos efectos biológicos de la PGE_2. En concreto, en las vías respiratorias, la activación de los receptores EP_2 y EP_4 tiene efectos antiinflamatorios, mientras que la activación del los receptores EP_1 y EP_3 podría dar como resultado la activación de respuestas celulares inflamatorias. En pacientes con EREA se han observado alteraciones en la expresión de los receptores EP, en concreto se ha descrito una regulación a la baja del receptor EP_2. Como la activación de este receptor parece disminuir las respuestas inflamatorias, su expresión disminuida en la EREA podría reducir la capacidad protectora de la PGE_2 y contribuir a la perpetuación e intensidad del proceso inflamatorio presente en estos pacientes. Ahora bien, esta hipótesis no ha sido comprobada por ningún estudio realizado con células humanas.

En síntesis, el estudio de los mecanismos responsables de las alteraciones en la producción de la PGE_2 y el estudio de la regulación y expresión de sus receptores es de suma importancia, ya que dichas alteraciones podrían contribuir a explicar la presencia de una respuesta inflamatoria crónica en las vías respiratorias superiores e inferiores de los pacientes con EREA y a generar nuevos tratamientos farmacológicos con superior eficacia a la de los actualmente existentes.

2. Hipótesis General

La hipótesis general del estudio establece que las características del entorno inflamatorio de las vías aéreas (perfil de citocinas) en la enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (EREA) es responsable de la expresión deficiente del receptor E-prostanoide (EP) 2, modificando su capacidad de responder a la prostaglandina (PG) E_2, lo que provoca una alteración en la regulación del bucle de retroalimentación positivo autocrina de la vía de la ciclooxigenasa (COX), lo cual contribuye a la inflamación descontrolada y a los procesos de remodelado de las vías aéreas normalmente presentes en la EREA.

3. Objetivos Específicos y Resultados

Para validar esta hipótesis se llevaron a cabo los siguientes estudios:

3.1. Estudio 1

Evaluar la expresión de los receptores de la PGE_2 y su capacidad para mediar los efectos antiproliferativos y antiinflamatorios en la AERD.

- **Determinar la expresión de los receptores E-prostanoides (EP) en biopsias y fibroblastos de mucosa nasal de sujetos control (MN-C) y en pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

La expresión basal de las proteínas y del mRNA de los receptores EP_1, EP_2, EP_3 y EP_4 en biopsias y fibroblastos de MN-C y de PN-EREA se analizó mediante las técnicas de western blot y PCR cuantitativa en tiempo real, respectivamente. En las biopsias y los fibroblastos obtenidos de MN-C y de PN-EREA, detectamos la expresión de las proteínas y del mRNA de los receptores EP_2, EP_3 y EP_4, pero no del receptor EP_1. Verificamos que, a nivel basal, la expresión proteica y génica del receptor EP_2 estaba significativamente disminuida en las biopsias y los fibroblastos de PN-EREA comparada con la expresión en MN-C. Los niveles basales de las proteínas y del

ARNm del receptor EP_3 en las biopsias y los fibroblastos fueron similares entre MN-C y PN-EREA. Finalmente, la expresión proteica y génica del receptor EP_4 en las biopsias y en los fibroblastos de PN-EREA fueron significativamente superiores comparada a los observados en MN-C.

- **Determinar el efecto de la prostaglandina (PG) E_2 y de agonistas selectivos de los receptores E-prostanoides (EP) en la producción de adenosín monofosfato cíclico (AMPc) intracelular en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Los EP son receptores acoplados a proteína G cuya estimulación desencadena la producción o inhibición del segundo mensajero AMPc. Para determinar si el diferente patrón de expresión de los receptores EP tiene correlación con los niveles de cAMP liberado tras su activación, estudiamos el efecto de la PGE_2 y de agonistas selectivos de cada receptor sobre la producción de AMPc en fibroblastos de MN-C y de PN-EREA. La producción de AMPc se cuantificó mediante la técnica de ELISA con un kit comercial.

La incubación con PGE_2 indujo un incremento dosis-dependiente de los niveles de AMPc en los fibroblastos de MN-C y de PN-EREA en comparación con la situación control. Sin embargo, los niveles de AMPc generados en los fibroblastos de PN-EREA fueron significativamente inferiores a los de MN-C. La estimulación con un agonista selectivo del receptor EP_2 también incrementó significativamente la producción de AMPc en los fibroblastos de MN-C y de PN-EREA. En este caso, y de forma similar a la PGE_2, la producción de AMPc fue significativamente inferior en fibroblastos de PN-EREA en comparación con los de MN-C. Este resultado sugiere que la deficiente expresión del receptor EP_2 en fibroblastos de PN-EREA participa en la producción reducida de AMPc en estas células. Por otro lado, la estimulación con un agonista selectivo EP_4 incrementó de forma dosis-dependiente la producción de AMPc tanto en fibroblastos de MN-C como de PN-EREA. Sin embargo, este incremento fue significativamente superior en los fibroblastos de NP-AERD comparado con el observado en fibroblastos de NM-C. Estos resultados sugieren la existencia de una correlación entre la expresión de los receptores EP y la producción de cAMP.

Para determinar si la expresión aumentada del receptor EP_4 en PN-EREA es capaz de compensar la deficiencia en la expresión del EP_2 en este grupo, estudiamos el efecto

de la suma de los agonistas de los receptores EP_2 y EP_4 en la producción de AMPc. Verificamos que a pesar de que la producción de cAMP es mayor en fibroblastos de NP-AERD cuando son estimulados con el agonista EP_4, la suma de los dos agonistas EP_2 y EP_4 no fue capaz de igualar los niveles de AMPc generados en fibroblastos de NM-C. Con estos resultados podemos concluir que el incremento de la expresión de los receptores EP_4 en fibroblastos de PN-EREA no es capaz de corregir la expresión deficiente del receptor EP_2 en estas células.

Para confirmar que las diferencias en la producción de AMPc en estas células solo se deben a las diferencias en la expresión de los receptores, estudiamos el efecto del aumento del AMPc por mecanismos independientes de los receptores EP. La síntesis del AMPc fue inducida por forskolin, que es un inductor directo de la enzima adenilato ciclasa (AC) responsable de la producción de AMPc. Observamos que el forskolin indujo la producción de AMPc en fibroblastos de MN-C y de PN-EREA sin diferencias entre los dos grupos. Estos resultados indican que las diferencias en la producción de AMPc se deben únicamente al desequilibrio en la expresión de los receptores EP entre los fibroblastos de MN-C y los de PN-EREA.

También se evaluó el efecto de la activación específica del receptor EP_3 en la producción de AMPc. Dado que la activación del receptor EP_3 inhibe la producción de AMPc, estimulamos previamente los fibroblastos con el forskolin para inducir la producción de AMPc y a continuación añadimos el agonista selectivo EP_3. Observamos que el agonista EP_3 fue capaz de reducir significativamente la producción de AMPc de manera dosis-dependiente tanto en fibroblastos de MN-C como de NP-EREA, sin diferencias entre los dos grupos. Este resultado indica que el receptor EP_3 no contribuye a las diferencias observadas en la producción de AMPc entre fibroblastos de MN-C y de PN-EREA.

- **Evaluar la capacidad de la prostaglandina (PG) E_2 y de un agonista selectivo del receptor E-prostanoide (EP) 2 para mediar los efectos antiproliferativos en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

La literatura describe que la PGE_2 es capaz de inhibir la proliferación celular y este efecto es mediado por el receptor EP_2 que, cuando es estimulado, activa la enzima AC y aumenta la producción de AMPc. Considerando que en nuestro modelo describimos

una expresión reducida del receptor EP_2 en PN-EREA y que el aumento de AMPc generado por la activación del receptor EP_4 no es suficiente para compensar la deficiencia de EP_2, evaluamos la capacidad de la PGE_2 y de un agonista selectivo del EP_2 para mediar los efectos antiproliferativos en fibroblastos obtenidos de NM-C y de PN-EREA. La proliferación celular fue determinada midiendo la incorporación del análogo de la timidina (EdU) mediante citometría de flujo con un kit comercial, en fibroblastos de MN-C y de PN-EREA.

Observamos que la estimulación con PGE_2 inhibe de manera dosis-dependiente la proliferación celular inducida por el suero bovino fetal (FBS) al 2.5% en fibroblastos de MN-C y de PN-EREA. Sin embargo, esta inhibición por parte de la PGE_2 fue significativamente inferior en fibroblastos de PN-EREA comparada con la observada en fibroblastos de MN-C. Cuando los fibroblastos fueron estimulados con el agonista selectivo del receptor EP_2, también observamos una inhibición de la proliferación en fibroblastos de MN-C y de PN-EREA. De forma similar a la PGE_2, la capacidad del agonista EP_2 para inhibir la proliferación celular fue significativamente inferior en fibroblastos de NP-AERD. Estos resultados indican que la baja expresión del receptor EP_2 en fibroblastos de PN-EREA puede contribuir al proceso de remodelado de las vías aéreas presente en los pacientes con EREA.

- **Estudiar la capacidad de la prostaglandina (PG) E_2 y de un agonista selectivo del receptor E-prostanoide (EP) 2 para mediar los efectos antiinflamatorios en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Los pacientes con la EREA frecuentemente presentan una inflamación eosinofílica masiva resultante de un incremento en la migración, activación y supervivencia de los eosinófilos en las vías respiratorias. Estos procesos son modulados por diversas citocinas tales como el GM-CSF. Estudios anteriores demostraron que la PGE_2 por la vía unión con su receptor EP_2 es capaz de reducir la liberación de GM-CSF en células musculares lisas de los bronquios humanos. En nuestro modelo de fibroblastos obtenidos de MN-C y de PN-EREA, evaluamos la capacidad de la PGE_2 y de un agonista selectivo del EP_2 para inhibir la secreción de GM-CSF. La liberación de GM-CSF fue medida en el sobrenadante de los fibroblastos de MN-C y de PN-EREA mediante ELISA con un kit comercial.

Nuestros resultados demostraron que la PGE_2 inhibió la secreción de GM-CSF inducida por FBS al 5% tanto en fibroblastos de MN-C como de PN-EREA. Sin embargo, el efecto de la PGE_2 fue significativamente inferior en los fibroblastos de PN-EREA comparado con los fibroblastos de MN-C. También evaluamos el efecto de un agonista selectivo del receptor EP_2 en la secreción de GM-CSF y observamos que el agonista EP_2 ocasionaba una inhibición en la secreción de GM-CSF en fibroblastos de MN-C y de PN-EREA. De forma similar a la PGE_2, el efecto inhibitorio del agonista EP_2 fue significativamente inferior en los fibroblastos de PN-EREA comparado con el observado en fibroblastos de MN-C. Estos datos indican que la deficiencia en la expresión del receptor EP_2 en fibroblastos de PN-EREA puede contribuir a perpetuar la inflamación eosinofílica característica de estos pacientes.

3.2. Estudio 2

Determinar los mecanismos involucrados en la regulación alterada de la vía de la ciclooxigenasa (COX) en la enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (EREA).

- **Evaluar la expresión basal e inducida por un estímulo proinflamatorio (IL-1β) de la familia del receptor de la interleucina-1 (IL-1RI, IL-1RII, IL-1RAcP, IL-1Ra) en los fibroblastos de mucosa nasal de sujetos control (NM-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Varios estudios previos han descrito una estrecha interdependencia en la regulación de la IL-1β, del IL-1RI, la COX-2, la mPGES-1 y la PGE_2. Empezamos por evaluar la expresión de los miembros de la familia IL-1R en fibroblastos obtenidos de MN-C y de PN-EREA. El receptor IL-1RI es el receptor funcional que permite la transducción de señales de la vía de la IL-1β. Observamos que a nivel basal la expresión del IL-1RI era significativamente inferior en fibroblastos de PN-EREA comparado con la de los fibroblastos de MN-C, tanto por western blot como por PCR cuantitativa en tiempo real. La incubación con la IL-1β (10 ng/mL) durante 24 h aumentó significativamente la expresión del IL-1RI en fibroblastos de MN-C y de PN-EREA. Sin embargo, en los fibroblastos de PN-EREA los niveles del IL-1RI eran significativamente inferiores en comparación con los de los fibroblastos de MN-C. Estos resultados obtenidos por

western blot y PCR cuantitativa en tiempo real también se confirmaron por inmunofluorescencia. Concluimos que existe una alteración en la expresión del IL-1RI en los fibroblastos de PN-EREA.

A continuación, evaluamos también posibles alteraciones en la expresión del IL-1RII, de la IL-1RAcP y, finalmente, del IL-1Ra en fibroblastos obtenidos de MN-C y de PN-EREA. El IL-1RII actúa como un inhibidor de la respuesta a la IL-1, una vez que no permite la transducción de señales debido a la ausencia del dominio citoplasmático TIR. Por otro lado, la disponibilidad de la IL-1RAcP es un mecanismo de control del efecto de la IL-1, ya que su presencia en la membrana es fundamental para que se lleve a cabo la transducción de señales. Por otro lado, el IL-1Ra, como antagonista natural al receptor de la IL-1, es capaz de inhibir la actividad de la IL-1.

A nivel basal, no se encontraron diferencias en la expresión proteica y en la expresión de mRNA del IL-1RII y de la IL-1RAcP entre fibroblastos de MN-C y de PN-EREA. El tratamiento con la IL-1β (10 ng/mL, 24 h) fue capaz de incrementar significativamente la expresión del mRNA del IL-1RII y de la IL-1RAcP tanto en fibroblastos de MN-C como de PN-EREA, pero no se observó incremento en la expresión proteica.

En este conjunto de experimentos evaluamos también la expresión del IL-1Ra en fibroblastos de MN-C y de PN-EREA en presencia o ausencia de la IL-1β. Observamos que en ausencia de la IL-1β, la expresión del IL-1Ra era significativamente inferior en fibroblastos de PN-EREA comparada con los de MN-C. Además, la incubación con la IL-1β fue capaz de incrementar significativamente la expresión del IL-1Ra en fibroblastos de MN-C pero no en los de PN-EREA.

- **Confirmar la expresión de la ciclooxigenasa (COX)-2 en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

A continuación, evaluamos la expresión de la COX-2 mediante western blot y PCR cuantitativa en tiempo real para confirmar los resultados ya descritos en la bibliografía. Confirmamos que la expresión proteica de la COX-2 es significativamente inferior en respuesta a la IL-1β a 10 ng/mL en fibroblastos de PN-EREA comparada con los de MN-C. Además, el análisis del mRNA de la COX-2 inducida por la IL-1β también reveló que los niveles de la COX-2 eran significativamente inferiores en los fibroblastos de PN-EREA comparados con los medidos en los de MN-C.

- **Evaluar la expresión de la prostaglandina E sintasa-1 microsomal (mPGES-1) en los fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Teniendo en cuenta que el incremento en la producción de PGE_2, en situaciones inflamatorias, depende directamente de la inducción de las enzimas COX-2 y mPGES-1, evaluamos también la expresión de la mPGES-1 en fibroblastos de MN-C y de PN-EREA mediante western blot. Observamos que la incubación con IL-1β (10 ng/mL) fue capaz de aumentar significativamente la expresión de la mPGES-1 en los fibroblastos de MN-C y de PN-EREA. Sin embargo, la inducción de la mPGES-1 por IL-1β fue inferior en los fibroblastos de PN-EREA en comparación a la observada en los fibroblastoas de MN-C.

- **Establecer una correlación entre la expresión del receptor tipo 1 de la interleucina-1 (IL-1RI) y entre la expresión de la ciclooxigenasa (COX)-2 y de la prostaglandina E sintasa-1 microsomal (mPGES-1) en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Observamos que existe una correlación significativa entre la expresión del IL-1RI y de la COX-2 (coeficiente de correlación de Pearson: 0.8181; p=0.0002, dos colas) y entre la expresión del IL-1RI y de la mPGES-1 (coeficiente de correlación de Pearson: 0.9313; p<0.0001, dos colas). Para confirmar estos resultados silenciamos la expresión del IL-1RI en fibroblastos de MN-C usando la técnica de silenciamiento génico y medimos los niveles de la COX-2 y de la mPGES-1 tras estimulación con la IL-1β. En condiciones experimentales optimizadas, la expresión del IL-1RI fue totalmente inhibida por el silenciador génico específico para el IL-1RI. También usamos un silenciador no específico para comprobar la especificidad y efectividad de la técnica. Observamos que en las células tratadas con el silenciador génico específico para el IL-1RI, la incubación con IL-1β no fue capaz de incrementar ni los niveles de la COX-2 ni los niveles de la mPGES-1, lo que sugiere que la expresión inducida por la IL-1β de las proteínas de la COX-2 y de la mPGES-1 es directamente dependiente de la expresión del IL-1RI.

- **Estudiar la expresión del receptor tipo 1 de la interleucina-1 (IL-1RI) y de la ciclooxigenasa (COX)-2 en biopsias de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Evaluamos si las alteraciones observadas en la expresión del IL-1RI y de la COX-2 en fibroblastos de PN-EREA también están presentes en el tejido de MN-C y de PN-EREA. Demostramos que la expresión tanto del IL-1RI como de la COX-2 es significativamente inferior en muestras de tejido de PN-EREA comparada con la observada en tejido de MN-C, lo que sugiere que estas alteraciones están presentes no solamente en fibroblastos, sino que también lo están en otros tipos celulares.

- **Estudiar el efecto de la prostaglandina (PG) E_2 en la regulación de la expresión del receptor tipo 1 de la interleucina-1 (IL-1RI) en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Para evaluar el mecanismo por el cual la IL-1β incrementa su propio receptor, incubamos los fibroblastos obtenidos de MN-C y de PN-EREA con o sin PGE_2 a diferentes concentraciones y medimos los niveles de expresión proteica del IL-1RI. La estimulación con la PGE_2 causó un incremento dosis-dependiente en la expresión del IL-1RI en los fibroblastos de MN-C. Sin embargo, en los fibroblastos de PN-EREA, la incubación con la PGE_2 apenas indujo un incremento en la expresión del IL-1RI a la máxima concentración de la PGE_2 usada. Además, la expresión del IL-1RI fue significativamente inferior en los fibroblastos de PN-EREA comparada con los de MN-C.

Para confirmar el efecto de la PGE_2 en la regulación de la expresión del IL-1RI, los fibroblastos de MN-C fueron incubados con la IL-1β (10 ng/mL) en presencia o ausencia de diferentes concentraciones de ácido acetilsalicílico (ASA), un fármaco usado para bloquear la producción de la PGE_2. Observamos que la incubación con el ASA (10^{-5} M y 10^{-3} M) fue capaz de inhibir el incremento en la expresión del IL-1RI producido por la IL-1β. Estos resultados indican que la IL-1β es capaz de incrementar y modular la expresión de su propio receptor a través de un mecanismo dependiente de la PGE_2.

- **Evaluar el efecto de la interleucina-1 (IL-1)β en la producción de adenosín monofosfato cíclico (AMPc) en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (PN-EREA).**

Para evaluar el efecto de la IL-1β en la producción de AMPc, los fibroblastos obtenidos de MN-C y de PN-EREA fueron tratados en presencia o ausencia de la IL-1β (10 ng/mL). Observamos que la estimulación celular con la IL-1β indujo un incremento significativo en los niveles de AMPc en fibroblastos de MN-C y de PN-EREA. Sin embargo, los niveles de AMPc generados en estas condiciones fueron significativamente inferiores en fibroblastos de PN-EREA comparados con los de MN-C.

- **Estudiar el receptor involucrado en la regulación del receptor tipo 1 de la interleucina-1 (IL-1RI) por la prostaglandina (PG)E_2 en fibroblastos de mucosa nasal de sujetos control (MN-C) y de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (NP-EREA).**

Para evaluar el receptor EP responsable de las diferencias observadas en la regulación de la expresión del IL-1RI por la $PGE_{2,}$ evaluamos el efecto de agonistas selectivos de los receptores EP en la expresión del IL-1RI. La incubación de los fibroblastos con un agonista selectivo del EP_2 incrementó significativamente y de manera dosis-dependiente la expresión del IL-1RI en fibroblastos de MN-C. De forma similar al efecto de la PGE_2, el agonista selectivo del EP_2 fue capaz de incrementar la expresión del IL-1RI en fibroblastos de PN-EREA, pero tan solo a la máxima concentración del agonista. Además, se observaron diferencias significativas entre los dos grupos a todas las concentraciones usadas del agonista, lo que sugiere que la deficiencia en la expresión del receptor EP_2 en los fibroblastos de PN-EREA es probablemente la causa de la inducción disminuida de la expresión del IL-1RI mediada por la PGE_2 en estas células.

La incubación con un agonista selectivo del EP_4 también causó un incremento significativo en la expresión del IL-1RI en fibroblastos de MN-C y de PN-EREA. Sin embargo, no se encontraron diferencias entre los dos grupos.

En estos experimentos usamos también el forskolin para confirmar el papel del AMPc en la regulación de la expresión del IL-1RI. Observamos que la incubación de las células con el forskolin fue capaz de inducir la expresión del IL-1RI en los fibroblastos de MN-C y de PN-EREA sin diferencias entre los dos grupos, confirmando que la falta de inducción de los niveles del IL-1RI en fibroblastos de PN-EREA, tras estimular las células con la PGE_2, se debe a la expresión disminuida del receptor EP_2 en estas células.

Evaluamos también el efecto de la activación del receptor EP_3 en los niveles de expresión del IL-1RI. Ya que la inducción del IL-1RI es AMPc dependiente y que el receptor EP_3 inhibe la producción de este mediador intracelular, incrementamos previamente los niveles de AMPc usando el forskolin y a continuación estimulamos las células con un agonista selectivo del EP_3 y medimos la expresión proteica del IL-1RI. Observamos que el agonista selectivo del EP_3 fue capaz de reducir significativamente la expresión del IL-1RI en fibroblastos de MN-C y de PN-EREA, sin diferencias significativas entre los dos grupos.

- **Estudiar la expresión del receptor tipo 1 de la interleucina-1 (IL-1RI) en fibroblastos de pólipos nasales de pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (NP-EREA) transfectados con el receptor E-prostanoide (EP) 2.**

Con los estudios de los agonistas demostramos que las diferencias en la inducción de la expresión del IL-1RI entre los fibroblastos de MN-C y de PN-EREA, tras la estimulación con la PGE_2, pueden deberse a la deficiencia en la expresión del receptor del EP_2 en los fibroblastos de PN-EREA. Para confirmarlo, transfectamos los fibroblastos obtenidos de PN-EREA con el receptor EP_2 para incrementar su expresión en estas células. A continuación, incubamos las células transfectadas con la PGE_2 o con la IL-1β y medimos la expresión del IL-1RI y de la COX-2, respectivamente. Observamos que, en los fibroblastos transfectados con el receptor EP_2, el tratamiento con la PGE_2 era capaz de inducir significativamente la expresión del IL-1RI de manera dosis-dependiente. Además, no se observaron diferencias en la inducción del IL-1RI entre los fibroblastos de PN-EREA transfectados y los fibroblastos de MN-C sometidos al mismo tratamiento con la PGE_2 exógena.

Usando las mismas condiciones de transfección, evaluamos si la expresión inducida por la IL-1β de las enzimas de la COX-2 y de la mPGES-1 también se modificaba.

Encontramos que los fibroblastos de PN-EREA transfectados con el receptor EP_2 expresaban más COX-2 y más mPGES-1 en presencia de la IL-1β comparados con los fibroblastos no transfectados. Además, la expresión inducida por la IL-1β de la COX-2 y de la mPGES-1 en fibroblastos de PN-EREA transfectados con el receptor EP_2 fue similar a la que se observó en los fibroblastos de MN-C. Estos resultados indican que la normalización del receptor EP_2 en los fibroblastos de PN-EREA es capaz de recuperar la inducción de la expresión del IL-1RI por la PGE_2 y la expresión de las enzimas de la COX-2 y de la mPGES-1 inducidas por la IL-1β.

2.3. Estudio 3

Estudiar el efecto del entorno inflamatorio característico de la enfermedad respiratoria exacerbada por antiinflamatorios no esteroides (EREA) en la regulación de la vía de la ciclooxigenasa (COX) en los fibroblastos de mucosa nasal de sujetos control (MN-C).

- **Determinar el efecto de la interleucina (IL)-4 y del interferon (IFN)-γ en la expresión del receptor tipo 1 de la IL-1 (IL-1RI), de la ciclooxigenasa (COX)-2 y de los receptores E-prostanoides (EP) en los fibroblastos de mucosa nasal de sujetos control (MN-C).**

Estudios recientes han demostrado que el entorno inflamatorio (perfil de citocinas) en pacientes con EREA es distinto del observado en pacientes asmáticos o con rinosinusitis crónica eosinofílica tolerantes a la aspirina. En la EREA se observa una sobreexpresión de las citocinas IFN-γ e IL-4.

La hipótesis de este estudio estableció que el entorno inflamatorio presente en las vías aéreas de los pacientes con EREA es el responsable del desarrollo de las alteraciones del metabolismo del AA observado en estos pacientes.

Con el objetivo de estudiar el efecto de estas citocinas sobre la regulación de la vía de la COX, estimulamos los fibroblastos obtenidos de MN-C con IL-4 (10 ng/mL) y/o IFN-γ (10 ng/mL) en presencia o ausencia de la IL-1β (10 ng/mL) durante 48 h y medimos la expresión proteica de los receptores EP_2, EP_3, EP_4, del IL-1RI y de la COX-2.

Observamos que la incubación de los fibroblastos de MN-C con la IL-4, el IFN-γ o con la combinación de las dos citocinas no fue capaz de modificar la expresión de ninguno de los receptores EP. Sin embargo, el tratamiento de los fibroblastos de MN-C con la

IL-1β incrementó significativamente la expresión del receptor EP_2, pero no se observaron diferencias en los receptores EP_3 y EP_4. Evaluamos, a continuación, el efecto de las citocinas IL-4 e IFN-γ en presencia de la IL-1β. En estas condiciones, verificamos que la incubación de los fibroblastos de NM-C con la IL-4 o el IFN-γ no era capaz de modificar la expresión del receptor EP_2 inducida por la IL-1β. Sin embargo, la co-estimulación con la IL-4 y el IFN-γ fue responsable de una disminución significativa de la expresión inducida por la IL-1β del receptor EP_2. La expresión de los receptores EP_3 y EP_4 no sufrió alteraciones en estas condiciones.

A continuación, y teniendo en cuenta que la expresión del receptor EP_2 afecta a diferentes componentes de la vía de la COX en la EREA, también evaluamos si en estas condiciones, existen modificaciones en la expresión proteica del IL-1RI y de la COX-2. La incubación de los fibroblastos de MN-C con la IL-4 y con el IFN-γ o con la combinación de las dos citocinas no fue capaz de modificar la expresión del IL-RI. Además, en estas condiciones, no detectamos expresión de la proteína de la COX-2. Como fue descrito en el estudio anterior, la estimulación de los fibroblastos con la IL-1β incrementó significativamente la expresión proteica del IL-1RI y de la COX-2. Observamos que la incubación de los fibroblastos de MN-C con la IL-4 o el IFN-γ en presencia de la IL-1β no fue capaz de modificar la expresión del IL-1RI o de la COX-2. Sin embargo, verificamos que el co-tratamiento con la IL-4 y el IFN-γ en presencia de la IL-1β fue responsable de una disminución significativa de los niveles de expresión del IL-1RI y de la COX-2. Estos resultados demuestran que el entorno inflamatorio presente en la AERD podría contribuir de manera significativa a la reducción de la expresión del receptor EP_2, contribuyendo con ello a la desregulación de la vía de la COX.

4. Discusión de los resultados

En el estudio 1 se ha investigado la expresión y funcionalidad de los receptores EP así como el papel que desempeñan en los efectos mediados por la PGE_2 en las biopsias y en los fibroblastos de MN-C y de PN-EREA. Demostramos que el patrón de expresión de los receptores EP es diferente entre MN-C y PN-EREA, tanto en las muestras de tejido como en los cultivos de fibroblastos. Observamos que en los PN-EREA la expresión del receptor EP_2 era significativamente inferior comparada con la observada en las MN-C, mientras que la expresión del receptor EP_4 era superior en el grupo PN-EREA en comparación al de MN-C. Estos resultados concuerdan con los

provenientes de otros estudios que describen una expresión deficiente del receptor EP_2 en pacientes con EREA.

La activación de los receptores EP_2 y EP_4 provoca un aumento en la producción de AMPc. En las vías aéreas, este mediador intracelular es responsable de la mayoría de los efectos “protectores” de la PGE_2. En este estudio demostramos que a pesar de la presencia de niveles elevados del receptor EP_4 en los PN-EREA, su activación no fue capaz de generar suficiente AMPc para compensar el que resulta de la deficiente expresión del receptor EP_2. Estas observaciones concuerdan con estudios anteriores en los cuales se comparó la producción de AMPc tras la activación de los receptores EP_2 y EP_4. Los autores de estos estudios verificaron que, comparado con el receptor EP_2, el receptor EP_4 tiene un acoplamiento funcional menos eficiente al AMPc. Nuestros resultados indican que el mecanismo celular potencial para compensar la falta del receptor EP_2 en los PN-EREA, a través de la sobreexpresión del receptor EP_4, no es suficiente para normalizar los niveles de AMPc producidos en estas células.

En este estudio también se describe el impacto que tiene la expresión reducida del receptor EP_2 en los PN-EREA sobre los efectos antiproliferativos y antiinflamatorios mediados por la PGE_2. La bibliografía describe que la PGE_2 vía el EP_2 y consecuentemente a través del incremento en la producción de AMPc es capaz de inhibir la proliferación celular. Efectivamente, en nuestro modelo, observamos que el tratamiento con la PGE_2 y con un agonista selectivo del receptor EP_2 son capaces de inhibir significativamente la proliferación de los fibroblastos inducida por suero tanto en MN-C como en PN-EREA. Sin embargo, el efecto observado en la inhibición de la proliferación fue significativamente inferior en los PN-EREA comparado con el medido en MN-C y estas diferencias se deben probablemente a la expresión deficiente del receptor EP_2 en los PN-EREA. De esta manera demostramos que los fibroblastos de PN-EREA tienen resistencia a los efectos antiproliferativos mediados por la PGE_2 debido a la expresión reducida del EP_2.

Estudios anteriores describieron un papel importante de la PGE_2 y de su receptor EP_2 en la inhibición de la secreción de citocinas proinflamatorias, como por ejemplo el GM-CSF. Efectivamente, los resultados de nuestro estudio demostraron que la PGE_2 por medio del EP_2 es capaz de inhibir la secreción de GM-CSF en fibroblastos de MN-C y de PN-EREA, siendo este efecto significativamente inferior en los fibroblastos de PN-EREA. Este hecho podría contribuir a explicar la inflamación eosinofílica masiva frecuentemente presente en pacientes con EREA.

En el estudio 2 describimos, por primera vez, un mecanismo potencialmente capaz de explicar las alteraciones presentes en la vía de la COX en los pacientes con EREA. Teniendo en cuenta que la IL-1β, actuando sobre su receptor IL-1RI, es capaz de inducir la expresión de las enzimas COX-2 y mPGES-1 y consecuentemente la producción de PGE_2, estudiamos la expresión y regulación del receptor IL-1RI en la EREA. Observamos que en biopsias y fibroblastos de PN-EREA, la expresión del receptor IL-1RI estaba significativamente disminuida comparada con la expresión medida en MN-C, lo que podría impedir el incremento de la expresión de COX-2 y mPGES-1 tras la estimulación con la IL-1β. Estudios anteriores demostraron que la IL-1β es capaz de inducir la expresión de su propio receptor. Nuestros resultados concuerdan con estos estudios ya que observamos que en los fibroblastos tanto de MN-C como de PN-EREA la estimulación con la IL-1β fue capaz de aumentar significativamente la expresión del receptor IL-1RI. Sin embargo, en los fibroblastos de PN-EREA el aumento de la expresión fue estadísticamente inferior comparada con la observada en los fibroblastos de MN-C.

Diversos estudios han demostrado la capacidad de la IL-1β para regular la expresión de la COX-2 y de la mPGES-1. En nuestro modelo observamos que la inducción de estas dos enzimas es un mecanismo directamente dependiente de la expresión del receptor IL-1RI existiendo una correlación positiva entre la expresión del IL-1RI y la expresión de la COX-2 y de la mPGES-1. Además, en ausencia del receptor IL-1RI observamos que la IL-1β no era capaz de inducir la expresión de estas dos enzimas. Estos resultados indican que la expresión disminuida del receptor IL-1RI en PN-EREA podría ser la responsable de la falta de inducción de la COX-2 y de la mPGES-1 en estos pacientes, lo que daría como resultado final una producción disminuida de la PGE_2.

En el estudio 2 también investigamos el mecanismo por el cual la IL-1β induce a su propio receptor. De acuerdo con la literatura, observamos que la PGE_2, por medio del EP_2, es capaz de inducir la expresión del IL-1RI en MN-C y en PN-EREA, siendo este efecto inferior PN-EREA debido a la expresión reducida del receptor EP_2. Así, estos resultados muestran que la inducción deficiente del receptor IL-1RI por la PGE_2 en PN-EREA se debe a una expresión y funcionalidad reducida del receptor EP_2.

Para arrojar un poco más de luz en relación al papel del receptor EP_2 en la regulación de la vía de la COX, estudiamos los efectos de la recuperación de la expresión del receptor EP_2 en la regulación de la vía de la COX en PN-EREA. Observamos que la normalización de la expresión del receptor fue capaz de aumentar tanto la expresión

del receptor IL-1RI como la expresión de las enzimas COX-2 y mPGES-1, que alcazaron niveles similares a las de MN-C. Con estos resultados hemos aportado evidencias que muestran los mecanismos involucrados en la autorregulación de la vía de la COX, y las alteraciones observadas en los pacientes con EREA.

Finalmente, en el estudio 3 hemos demostrado que el entorno inflamatorio característico de la EREA, que consiste en la sobreexpresión de las citocinas IL-4 e IFN-γ, puede ser responsable de la alteraciones en la vía de la COX descritas en los estudio 1 y 2. Efectivamente, observamos que en los fibroblastos de MN-C incubados con IL-4 e IFN-γ, la IL-1β no es capaz de aumentar la expresión del EP_2, del IL-1RI, de la COX-2 y de la mPGES-1, lo que consecuentemente impediría el aumento de la producción de la PGE_2 en condiciones inflamatorias. Así, parece que el entorno inflamatorio de la EREA tiene un papel importante en el proceso de regulación de los componentes de la vía de la COX, en la cual la alteración de un solo componente, el receptor EP_2, es capaz por si solo de perturbar el correcto funcionamiento de todo el sistema.

5. Resumen de los resultados

1. A nivel basal, la expresión del receptor EP_2 está disminuida en las biopsias y en los fibroblastos de PN-EREA, mientras que la expresión del receptor EP_4 está aumentada.
2. En comparación con los fibroblastos de MN-C, los fibroblastos de PN-EREA producen menos cAMP intracelular tras la estimulación con la PGE_2.
3. Los niveles aumentados del receptor EP_4 en los fibroblastos de PN-EREA no son suficientes para compensar la expresión deficiente del receptor EP_2 en estas células.
4. La expresión disminuida del receptor EP_2 en los fibroblastos de PN-EREA reduce la capacidad antiproliferativa de la PGE_2.
5. La expresión disminuida del receptor EP_2 es los fibroblastos de PN-EREA reduce la capacidad de la PGE_2 para inhibir la liberación de GM-CSF.

6. La inducción deficiente de la COX-2 y de la mPGES-1 en los fibroblastos de PN-EREA está estrechamente relacionada con la expresión disminuida del receptor EP_2 en estas células.

7. La regulación anormal del bucle que regula la vía de la COX está involucrada en la producción reducida de PGE_2 observada en la EREA.

8. La expresión reducida del receptor EP_2 tiene un papel central en la regulación anormal de la vía de la COX observada en la EREA ya que la recuperación de la expresión y consecuente funcionalidad del receptor EP_2 es capaz de normalizar todo el mecanismo.

9. Las características del entorno inflamatorio en la EREA que consiste en la fuerte presencia de las citocinas IL-4 y IFN-γ, bloquean la inducción del receptor EP_2 por la IL-1β observado en los fibroblastos de MN-C.

10. La expresión reducida del receptor EP_2 en la presencia de IL-4 y IFN-γ podría contribuir al funcionamiento anormal del bucle de regulación de la vía de la COX.

6. Conclusión

Este estudio aporta evidencias que soportan la hipótesis de que el entorno inflamatorio en las vías aéreas de los pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides induce la alteración en la expresión del receptor E-prostanoide 2 de la prostaglandina E_2. Esta anomalía altera la inducción del receptor tipo I de la interleucina-1, principal responsable de la activación de la ciclooxigenasa-2 por la citocina interleucina-1β, lo que, a su vez, da como resultado una baja producción de prostaglandina E_2 que asociada a la baja expresión de su receptor E-prostanoide 2 altera el bucle de retroalimentación autocrina que regula la vía de la ciclooxigenasa.

El receptor E-prostanoide 2 juega un papel central en la alteración de la síntesis de la prostaglandina E_2 ya que su reparación permite recuperar el funcionamiento normal del bucle autocrino y con ello la normalización en la expresión y funcionamiento de todos los componentes del mismo (receptor tipo I de la interleucina-1, ciclooxigenasa-2, prostaglandina E sintasa microsomal-1 y prostaglandina E_2).

La disminución en la síntesis de prostaglandina E_2 contribuye a perpetuar la inflamación eosinofílica y el proceso de remodelado de las vías aéreas de los pacientes con enfermedad respiratoria exacerbada por antiinflamatorios no esteroides.

APPENDIX II. Publications

Machado-Carvalho *et al. Respiratory Research* 2014, **15**:100
http://respiratory-research.com/content/15/1/100

RESPIRATORY RESEARCH

REVIEW Open Access

Prostaglandin E2 receptors in asthma and in chronic rhinosinusitis/nasal polyps with and without aspirin hypersensitivity

Liliana Machado-Carvalho[1,2,3*], Jordi Roca-Ferrer[1,2,3] and César Picado[1,2,3]

Abstract

Chronic rhinosinusitis with nasal polyps (CRSwNP) and asthma frequently coexist and are always present in patients with aspirin exacerbated respiratory disease (AERD). Although the pathogenic mechanisms of this condition are still unknown, AERD may be due, at least in part, to an imbalance in eicosanoid metabolism (increased production of cysteinyl leukotrienes (CysLTs) and reduced biosynthesis of prostaglandin (PG) E_2), possibly increasing and perpetuating the process of inflammation. PGE_2 results from the metabolism of arachidonic acid (AA) by cyclooxygenase (COX) enzymes, and seems to play a central role in homeostasis maintenance and inflammatory response modulation in airways. Therefore, the abnormal regulation of PGE_2 could contribute to the exacerbated processes observed in AERD. PGE_2 exerts its actions through four G-protein-coupled receptors designated E-prostanoid (EP) receptors EP1, EP2, EP3, and EP4. Altered PGE_2 production as well as differential EP receptor expression has been reported in both upper and lower airways of patients with AERD. Since the heterogeneity of these receptors is the key for the multiple biological effects of PGE_2 this review focuses on the studies available to elucidate the importance of these receptors in inflammatory airway diseases.

Keywords: Aspirin exacerbated respiratory disease, Asthma, Chronic rhinosinusitis, Nasal polyps, Prostaglandin E_2, Prostaglandin E_2 receptors

Introduction

The purpose of this review is to offer a global overview of the significant literature available about prostaglandin (PG) E_2 receptors in asthma, chronic rhinosinusitis (CRS) and nasal polyposis, with and without aspirin hypersensitivity.

Asthma

Pathophysiologically, asthma is a multifactorial and complex chronic inflammatory disorder of the lung and is characterized by epithelial disruption, airway smooth muscle hypertrophy and hyperplasia, increased mucus secretion, basement thickening, increased cytokine production and chronic infiltration of inflammatory cells [1,2]. Depending on the severity of the disorder, it manifests clinically with repeated, variable, and episodic attacks of cough, wheezing and breathlessness [3,4]. The most effective drugs used in asthma control are inhaled corticosteroids. Although recommended and clinically effective in most asthma patients, airway remodelling changes can be resistant to the conventional pharmacological approach [5]. Various factors can trigger and/or develop asthma attacks: allergens, exercise, cold exposure, chemical sensitizers, air pollutants, and respiratory viral infections [3]. Conventionally, classification into atopic and nonatopic asthma is based on the presence or absence of clinical symptoms precipitated by one or more allergens. The presence of allergen-specific antibodies can be identified by skin prick testing or by measuring the level of specific immunoglobulin (Ig) E in serum [6,7].

Airway inflammation in asthma is associated with a massive influx of inflammatory and immune cells within the airways, including eosinophils, T helper (Th) 2 lymphocytes, mast cells, neutrophils and macrophages. Local overproduction of Th2 cytokines such as interleukin (IL)-

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4, IL-5, IL-9 and IL-13 by Th2 cells plays an important role in its pathophysiology [6]. IL-4 promotes Th2 cell differentiation, induces IgE production and increases IgE receptors; IL-5 is responsible for promoting eosinophil development, differentiation, recruitment, activation and survival; finally, IL-13 mediates allergen-induced airway hyperresponsiveness [8].

Chronic rhinosinusitis and nasal polyposis

According to the *European Position Paper on Rhinosinusitis* (EPOS) [9] rhinosinusitis is defined as an inflammatory process of the nose and the paranasal sinuses characterized by two or more symptoms: nasal blockage/obstruction/congestion or nasal discharge, as well as facial pain/pressure or reduction/loss of smell [9]. This disorder may be classified into two forms, according to the duration of the symptoms, as acute or chronic [9–11]. In fact, the chronic form that persists beyond 12 weeks without complete resolution is associated with a lower quality of life and constitutes one of the most common health care problems [12]. CRS is subdivided itself into CRS with or without nasal polyps (NPs). Chronic rhinosinusitis with nasal polyps (CRSwNP) is a clinical phenotype found in up to 4% of the population [13]. The condition consists of loose connective tissue, oedema, inflammatory cells, and some glands and capillaries leading to nasal obstruction, secretion, loss of smell and headache [14]. Although the eosinophils are the most common cells in NPs, other cell types are also present, such as neutrophils, mast cells, plasma cells, lymphocytes, monocytes and fibroblasts [15,16].

Aspirin exacerbated respiratory disease

Aspirin exacerbated respiratory disease (AERD) is a clinical syndrome characterized by hypersensitivity to aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs), bronchial asthma and CRS with recurrent NPs [17,18]. AERD affects 10-20% of the asthmatic patient population and 8-26% of those diagnosed with CRSwNP [19]. The ingestion of aspirin or other NSAIDs in these patients provokes bronchoconstriction and exacerbates bronchospasms with attacks of asthma and rhinitis [20]. Effectively the characteristic symptoms of this disorder include moderate to severe asthma, massive eosinophilic infiltration and high prevalence of CRS associated with nasal polyposis [21].

The pathogenic mechanism underlying this disorder is believed to involve, at least in part, alterations in the eicosanoid metabolism and altered eicosanoid receptor expression [22–24]. However, and despite all the efforts, the pathogenicity of AERD is still not fully understood.

Arachidonic acid metabolism

Arachidonic acid (AA) is a 20-carbon polyunsaturated fatty acid and is the main precursor of eicosanoids, mediating important functions in homeostasis, inflammation and immunoregulation [25]. Under normal conditions AA is not freely available and its concentration within the cell is very low. The availability of free AA is essential for the biosynthesis of eicosanoids. Upon activation of the cell and by the action of various phospholipases (preferentially cytosolic phospholipase A_2) AA is released from the membrane phospholipids [26]. Therefore, once released, AA is rapidly metabolized through three enzymatic pathways namely cyclooxygenase (COX), lipoxygenase (LO), and cytochrome oxidases (hydrolase, epoxygenase) and one non-enzymatic pathway [27].

Lipoxygenase pathway

AA, which is esterified on plasma membrane phospholipids, is released and converted into leukotriene (LT) A_4 through 5-LO activity (Figure 1). LTA_4 is subsequently converted by LTA_4 hydrolase into LTB_4 and by LTC_4 synthase, which conjugates reduced glutathione into LTC_4. This is metabolized into LTD_4, which is then metabolized into LTE_4. LTC_4, LTD_4 and LTE_4 are designated as cysteinyl leukotrienes (CysLTs). LTs are synthesized upon cellular activation and the intracellular expression and distribution of 5-LO varies considering the cell type. In the airways, 5-LO is present in several types of leukocytes and becomes activated during allergic inflammation [8]. CysLTs activate three receptors with differential selectivity ($CysLT_1$, $CysLT_2$ and GPR17) and the stimulation of these receptors, principally $CysLT_1$, account for most of its effects [21]. This receptor is expressed in a large variety of cells which include monocytes, macrophages, eosinophils, basophils, mast cells, neutrophils, T cells, B lymphocytes, pluripotent hematopoietic cells, interstitial cells of the nasal mucosa (NM), airway smooth muscle cells, bronchial fibroblasts and vascular endothelial cells [28] and its activation contributes to most of the effects of CysLTs that are relevant to the pathophysiological changes observed in patients with asthma [8].

Cyclooxygenase pathway

AA can be metabolized through the COX pathway by the action of the COX enzymes (Figure 1). COX is a bifunctional enzyme with two active sites, one with COX activity that catalyzes the reduction of AA to form PGG_2 and the other with peroxidase activity involved in the reduction of peroxidase group in PGG_2 to hydroxyl group forming PGH_2. These enzymes catalyze the reactions responsible for the production of several bioactive prostanoids, such as PGs, prostacyclins and thromboxane [29]. Studies show the presence of two isoforms of COX enzymes, namely COX-1 and COX-2. COX-1, the dominant source of prostanoids that serves a number of physiologic "housekeeping" functions, presents a uniform expression in almost all tissue and is generally considered constitutive

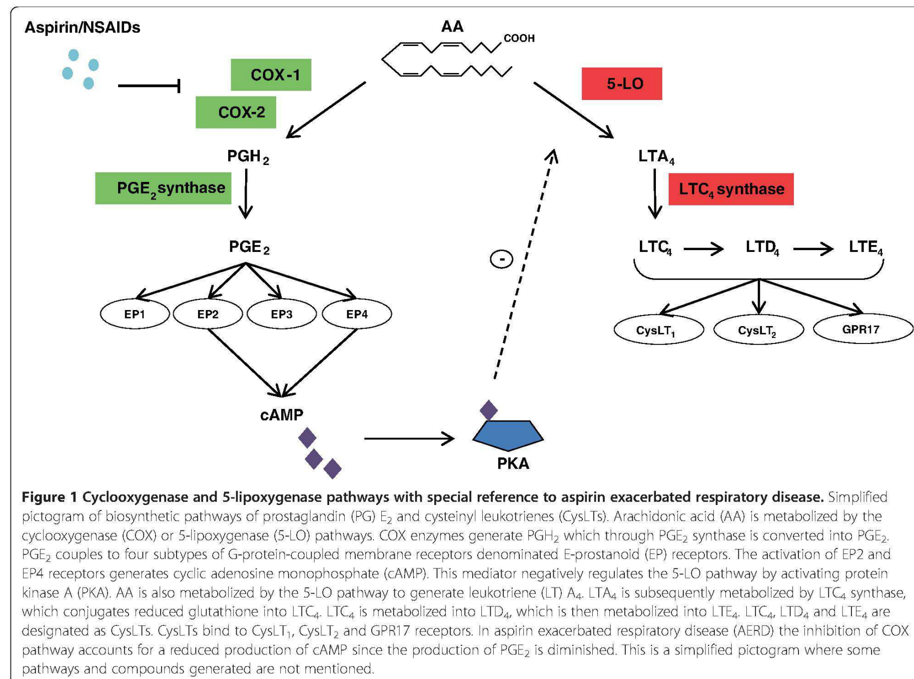

Figure 1 Cyclooxygenase and 5-lipoxygenase pathways with special reference to aspirin exacerbated respiratory disease. Simplified pictogram of biosynthetic pathways of prostaglandin (PG) E_2 and cysteinyl leukotrienes (CysLTs). Arachidonic acid (AA) is metabolized by the cyclooxygenase (COX) or 5-lipoxygenase (5-LO) pathways. COX enzymes generate PGH_2 which through PGE_2 synthase is converted into PGE_2. PGE_2 couples to four subtypes of G-protein-coupled membrane receptors denominated E-prostanoid (EP) receptors. The activation of EP2 and EP4 receptors generates cyclic adenosine monophosphate (cAMP). This mediator negatively regulates the 5-LO pathway by activating protein kinase A (PKA). AA is also metabolized by the 5-LO pathway to generate leukotriene (LT) A_4. LTA_4 is subsequently metabolized by LTC_4 synthase, which conjugates reduced glutathione into LTC_4. LTC_4 is metabolized into LTD_4, which is then metabolized into LTE_4. LTC_4, LTD_4 and LTE_4 are designated as CysLTs. CysLTs bind to $CysLT_1$, $CysLT_2$ and GPR17 receptors. In aspirin exacerbated respiratory disease (AERD) the inhibition of COX pathway accounts for a reduced production of cAMP since the production of PGE_2 is diminished. This is a simplified pictogram where some pathways and compounds generated are not mentioned.

[30]. COX-2 is described, in diverse studies, as highly induced and only expressed in response to certain inflammatory stimuli [31–35]. Furthermore, COX-2 is the more important source of prostanoid formation in inflammatory processes and proliferative diseases [36]. Both isoforms are located in the endoplasmatic reticulum and nuclear envelope, COX-2 is more highly concentrated in the nuclear envelope [37]. PGE_2 is one of the most abundant prostanoids produced in the body [36]. Montuschi and co-workers [38] evaluated the effects of COX inhibition on exhaled eicosanoids in patients with chronic obstructive pulmonary disease. The authors found that in exhaled breath condensate, PGE_2 is primarily derived from COX-1 activity. PGE_2 exhibits pleiotropic and contrasting effects in different cell types and organs. According to Vancheri *et al.* [39] the lung is considered a privileged site for the action of PGE_2. Although in this organ PGE_2 can exert anti-inflammatory, anti-fibrotic and immune restrictive actions, it can also mediate pro-inflammatory responses [39,40].

Prostaglandin E_2 receptors

The activity of PGE_2 is mediated by a group of rhodopsin-type G-protein-coupled membrane receptors (GPCRs) denominated E-prostanoid (EP) receptors [41]. There are four GPCR subtypes: EP1, EP2, EP3 and EP4. The physiological and contrasting effects of PGE_2 depend on the expression or the co-expression of more than one receptor or isoform [42]. Additionally, each receptor may be differentially expressed in tissues. EP receptors differ in terms of intracellular signalling [43,44]. These receptors could be classified according to their intracellular signalling and second messenger [45]. The EP1 receptor signals via Ca^{2+} mobilization with slight phosphatidylinositol activity [46,47]. Distribution of this receptor in human tissues and cells is restricted and has been demonstrated in the myometrium, pulmonary veins, mast cells, colonic longitudinal muscle and keratinocytes. EP1 exerts mostly constrictive functions, however and compared with other prostanoid receptors, it seems to be less studied [47]. EP2 and EP4 receptors increase intracellular cyclic adenosine monophosphate (cAMP) through activation of adenyl cyclase [39,48]. Functional studies have demonstrated that the EP2 receptor is widely distributed [49] and it seems to be involved in processes of relaxation such as bronchodilation [50] and anti-inflammation [51]. On the other hand, EP4 is also widely distributed

[44]. In a direct comparison with EP2 receptor subtype signalling, the EP4 receptor demonstrated a less efficient functional coupling to cAMP [52,53]. Effectively, studies have reported several cAMP-independent signalling pathways for EP4 receptor activation [54–59]. EP4 mediates vasorelaxation of pulmonary arterial veins and also promotes anti-inflammatory effects [60]. Consistent with its bronchoprotective action, PGE_2 inhibited proliferation and migration of bronchial smooth muscle cells through the action of the EP4 receptor [61]. Studies have revealed that the EP3 receptor shows a wide distribution in almost all tissues and consists of multiple isoforms generated by numerous alternative splicing of the C-terminal [62–64]. Signalling through this receptor reduces the levels of cAMP and increases intracellular Ca^{2+} [44]. EP3 exerts mainly contractile functions such as the constriction of human pulmonary artery in the lung [65]. Moreover, this receptor has been implicated in inflammation, pain and cough [47,66].

In summary, EP2 and EP4 receptor signalling promotes the accumulation of cAMP which is normally related to inhibition of cell functions. On the other hand, EP1 and EP3 receptors that increase intracellular calcium could be associated with cellular activation.

Arachidonic acid metabolism in aspirin exacerbated respiratory disease

The pathogenesis of AERD is not fully understood but several studies have reported that the pathogenic mechanisms of this condition may be due, at least in part, to marked imbalance in eicosanoid metabolism possibly increasing and perpetuating the process of inflammation [20,22,67]. Aspirin and other NSAIDs block the COX pathway by acetylation of the COX enzyme and consequently inhibit conversion of AA to PG. The dependency on COX products to modulate and maintain homeostasis over 5-LO activity is a unique feature of AERD [68]. Effectively in this disorder the inhibition of COX-1 results in the overproduction of CysLTs. CysLTs are potent bronchoconstrictors that contribute to the pathophysiological changes observed in patients with asthma. CysLTs, by increasing pulmonary microvascular permeability and mucus hypersecretion can contribute to bronchial obstruction in asthmatic patients [69–72]. Diamant *et al.* [73] reported that the inhalation of CysLTs increases eosinophilia in sputum of asthmatic patients and induces the recruitment of eosinophils into the airway mucosa. Indeed, CysLTs might participate in the process of airway remodeling, including eosinophilic airway inflammation, airway smooth muscle cell hyperplasia, mucus gland hyperplasia, mucus hypersecretion, and fibrous collagen depositions [69,70]. Several studies have described the biological effects and the contribution of these lipid mediators in AERD. After challenging AERD patients with oral, intravenous and intranasal aspirin treatments, the levels of CysLTs are significantly increased [74,75]. Moreover, patients with AERD excrete higher levels of LTE_4 in their urine when compared with asthmatic patients without aspirin intolerance [76,77]. Pérez-Novo *et al.* [78] reported that the nasal tissue of patients with CRSwNP presents elevated levels of CysLTs when compared with NM from aspirin-tolerant (AT) asthmatic patients and this increased production is associated with the elevated expression of LTC_4 synthase, the terminal enzyme in the production of CysLTs. In summary, the overproduction of CysLTs reported in AERD seems to play an important role in the pathogenesis of the disease, since the levels of this mediator are comparatively reduced in asthmatic patients without aspirin intolerance.

PGE_2 formed from COX-dependent conversion of AA have demonstrated inhibitory effects on CysLTs production. PGE_2 administration blocks bronchoconstriction and inhibits the increase in urinary LTE_4 that occur with aspirin challenge in subjects with AERD [79,80]. Pharmacological studies suggest that most urinary PGE_2 metabolites in AT asthmatic patients and healthy subjects derive from COX-2 [81]. Several studies have demonstrated that NP tissue from subjects with or without aspirin sensitivity shows impaired expression of COX-2 [82,83] and hypermethylation of the PGE_2 synthase *(PTGES)* gene in patients with AERD when compared with polyps from AT patients [84]. Moreover, experiments in *vitro* have shown a reduced production of PGE_2 in epithelial cells from NPs [85], bronchial fibroblasts [86], and peripheral blood leukocytes from patients with AERD [87]. The combination of a reduced expression of COX-2 in inflammatory conditions in subjects with AERD with hypermethylation of the *PTGES* gene reported could be responsible for the low production of PGE_2 observed in these subjects. Thereby, in patients with AERD, when COX-1 is inhibited by aspirin or other NSAIDs, the diminished availability of PGE_2 will contribute to the exacerbations of the characteristic symptoms of this pathology.

Prostaglandin E_2 receptors in asthma and chronic rhinosinusitis with nasal polyps

Few studies have been performed to elucidate the importance of this receptor family in upper and lower respiratory airway diseases. The literature mainly describes the expression and cellular distribution of these receptors at different levels of the airways, using for that purpose diverse type of samples, such as biopsies, cultured cells or peripheral blood and different techniques.

Asthma

Additional file 1: Table S1 shows the main publications on EP receptors in asthma [see Additional file 1: Table

S1]. Ying and co-workers [88] used immunocytochemistry to compare the expression and cellular distribution of the EP receptors in induced sputum cells from asthmatic patients and control subjects. They reported that sputum cells showed immunoreactivity for all receptors in both patients with asthma and control subjects. However, in patients with asthma, they found a high immunoreactivity for EP2 and EP4, but not EP1 and EP3 receptors on macrophages when compared with control subjects. The investigators concluded that the pattern of EP receptor expression is particularly increased in airway macrophages of patients with asthma.

In a study performed with bronchial biopsies [89] from both AERD and AT asthmatic patients and control subjects, the authors reported that, compared with AT, patients with AERD have increased bronchial mucosal neutrophil and eosinophil numbers but reduced percentages of T cells, macrophages, mast cells and neutrophils expressing EP2. In contrast, quantitative analysis of EP receptor mRNA expression in peripheral blood mononuclear cells isolated from these patients showed no significant differences between the two groups.

Chronic rhinosinusitis and nasal polyps

In Additional file 2: Table S2 we highlight the studies that have been performed to elucidate the importance of the EP receptors on the upper airways [see Additional file 2: Table S2]. Pérez-Novo *et al.* [90] studied the possible link between the expression of prostanoid receptors and the eosinophilic inflammation characteristic of paranasal sinus diseases by means of real-time PCR. The results of this study showed a high mRNA expression of EP2 and EP4 receptors in nasal tissue from both CRS without NP (CRSsNP) and CRSwNP patients when compared with control subjects. On the other hand, EP1 and EP3 receptors seem to be downregulated in nasal tissue from CRSwNP when compared with CRSsNP patients and control subjects.

Ying and co-workers [91], in a study based on immunohistochemistry, analysed the expression pattern of EP receptors in nasal biopsies from CRSwNP patients with AERD and AT and control subjects. The extensive analysis showed that, globally, mucosal expression of EP1 and EP2, but not EP3 and EP4 was significantly elevated in nasal biopsies from both patients with AERD and AT patients when compared with nasal biopsies extracted from control subjects. The researchers attribute the results principally to the high percentage of epithelial cells and goblet cells expressing these receptors. In inflammatory cells, the findings reported were different. They showed that the percentages of neutrophils, mast cells, eosinophils and T cells expressing EP2, but not EP1, EP3, or EP4, were significantly reduced in AERD patients when compared with AT patients.

Effectively, EP2 receptor downregulation seems to be common in both upper and lower airways of patients with AERD. Adamusiak and collaborators [92] described that, in NPs from AERD patients, the density of cells expressing the EP2 receptor was significantly lower when compared with NP from AT patients.

Using the Western blot technique, Roca-Ferrer *et al.* [83], using fibroblast cell cultures isolated from NP of both AERD and AT patients, described that there were low levels of EP2 protein receptor expression under inflammatory conditions when compared with fibroblasts isolated from NM of control subjects.

Apart from these expression studies, various polymorphisms in EP2 gene (*PTGER2*) were described [93,94] and they could possibly be related to AERD. In an extensive candidate gene analysis study to identify susceptibilities to AERD in the Japanese population, Jinnai and co-workers [93] showed the association of AERD with a functional single-nucleotide polymorphism in *PTGER2* that decreases the transcription level of the receptor *in vitro*.

As previously described, the ability of PGE_2 to induce or suppress various mechanisms involved in inflammatory processes indicates the complex activities of its receptor. The activation of EP2 and EP4 (Figure 1) initiates the production of cAMP, a secondary messenger that acts by activating protein kinase A (PKA). Once activated, PKA has the capacity to regulate 5-LO [95,96] by phosphorylation of serine-523 in 5-LO, suppressing its function. Effectively, Luo and co-workers [96] described that the mutation of serine-523 on human 5-LO prevents phosphorylation by PKA and promotes the abnormal synthesis of LTs. The dysfunctional signalling through cAMP and PKA contributes to a variety of diseases, including those characterized by chronic inflammation. In 1983, Ham and colleagues [97] showed for the first time that PGE_2 inhibited LT biosynthesis in activated neutrophils, and the inhibition was mediated by an increment of cAMP levels. Indeed, the mechanisms by which the enhancement of cAMP (by PGE_2 or other cAMP-elevating agents) are able to down-regulate LT biosynthesis involve the inhibition of the translocation of 5-LO to the nuclear envelope in human polymorphonuclear leukocytes [80]. The mechanisms and receptors by which PGE_2 modulates the activation of human mast cells have also been assessed [95]. The investigators described that PGE_2 can attenuate through EP2 receptors the generation of CysLTs in activated mast cells. The effect of low levels of EP2 on the downstream signalling pathway as well as the polymorphic variants of its gene are still unclear and further studies are needed to determine the functional repercussion of these alterations. Nevertheless, considering the regulatory effect of PGE_2 on 5-LO through EP2 receptors these alterations could

contribute, at least in part, to the exacerbation of the inflammatory processes demonstrated in patients with AERD.

Targeting prostaglandin E_2 receptors

All the available information about the role of EP receptor subtypes in inflammatory airway diseases or in a totally different disorder comes partly from genetic ablation of prostanoid receptors or from studies performed with selective EP receptor agonists or antagonists. The latter strategy includes the development of small-molecule ligands that target a specific EP receptor and whose purpose is receptor inhibition or activation. All EP receptors are activated by their natural agonist PGE_2 or by a number of PGE_2 analogues named agonists and inhibited by antagonists. Over the years several studies have been performed to develop these compounds, which are used to find a possible therapeutic approach to treat numerous diseases [47].

As described previously, the EP2 receptor exerts many inhibitory functions. PGE_2 has been considered to be a bronchodilator and anti-inflammatory natural substance with potential for treating asthma and other respiratory diseases. The use of developed selective agonists improved this viewpoint. Effectively, the bronchodilator effect of PGE_2 is mediated by the EP2 receptor, which promotes airway relaxation and inhibits IgE-dependent mast cell activation [98]. Recent studies have also shown that the EP4 receptor mediates bronchodilation supporting the idea that targeting this receptor may be a novel therapeutic approach for obstructive airway diseases [99]. Patients with AERD characteristically showed a critical deficiency in PGE_2/EP2 signalling. Considering the protective and beneficial effects of the PGE_2/EP2 axis in airways, the use of specific commercially developed agonists could correct this deficit and ameliorate the inflammation scenario in these patients. The potential use of inhibitors of the EP3 receptor in the treatment of chronic cough has also been recently proposed [66].

Conclusions

Differential regulation and expression patterns of PGE_2 receptors were observed in each of the chronic inflammatory airway diseases presented in this review. Although these alterations may worsen the already diminished levels of PGE_2, additional studies are necessary to reveal further information about the role of these receptors in asthma and CRS with or without NPs or aspirin hypersensitivity. Moreover, EP receptors represent potential targets for therapeutic approaches. The use of PGE_2 analogues and synthetic drugs, which can selectively and specifically agonize or antagonize signalling from EP receptor subtypes, has proved very useful for a deeper understanding of the pathologic mechanisms where PGE_2 and its receptors are involved.

Additional files

Additional file 1: Table S1. Prostaglandin E_2 receptor expression in lower airways.

Additional file 2: Table S2. Prostaglandin E_2 receptor expression in upper airways.

Abbreviations

AA: Arachidonic acid; AERD: Aspirin exacerbated respiratory disease; AT: Aspirin-tolerant; cAMP: Cyclic adenosine monophosphate; COX: Cyclooxygenase; CRS: Chronic rhinosinusitis; CRSwNP: Chronic rhinosinusitis with nasal polyp; CRSsNP: Chronic rhinosinusitis without nasal polyp; CysLTs: Cysteinyl leukotrienes; EP: E-prostanoid; GPCR: G-protein-coupled membrane receptor; Ig: Immunoglobulin; IL: Interleukin; LO: Lipoxygenase; LT: Leukotriene; NM: Nasal mucosa; NP: Nasal polyp; NSAID: Non-steroidal anti-inflammatory drug; PG: Prostaglandin; PKA: Protein kinase A; Th: T helper.

Competing interests

The authors declared that there are no competing interests in relation to this manuscript.

Authors' contributions

All authors wrote and revised the manuscript, and approved the final version.

Acknowledgements

LMC was supported by a grant from the Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias from Instituto de Salud Carlos III, Madrid, Spain.

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Received: 23 April 2014 Accepted: 13 August 2014
Published: 26 August 2014

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doi:10.1186/s12931-014-0100-7
Cite this article as: Machado-Carvalho *et al.*: **Prostaglandin E2 receptors in asthma and in chronic rhinosinusitis/nasal polyps with and without aspirin hypersensitivity.** *Respiratory Research* 2014 **15**:100.

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