Regulació del metabolisme de l'àcid araquidònic i senyalització cel·lular en un model d'asma i intolerància als AINEs

Francesc J. García García

Regulació del metabolisme de l'àcid araquidònic i senyalització cel·lular en un model d'asma i intolerància als AINEs.

Memòria presentada per

Francesc J García García

Per optar al grau de

Doctor per la Universitat de Barcelona
Programa de Biomedicina
Departament de Medicina
Bienni 2007-2009

Tesi dirigida pel **Dr. Jordi Roca Ferrer** i el **Dr. Cèsar Picado Vallés** realitzada al laboratori d'Immunoal·lèrgia Respiratòria Clínica i Experimental, Fundació Clínic per la Recerca Biomèdica, Hospital Clínic, IDIBAPS, Barcelona.

Dr Jordi Roca Ferrer

Dr Cèsar Picado Vallés

Francesc J. García García
2013

A mi madre,
porque hoy serías muy feliz

“Daría todo lo que sé por
la mitad de lo que ignoro.”

René Descartes

“Quien no haya experimentado
la irresistible atracción de la
ciencia, no podrá comprender
su tiranía.”

Frankenstein o el moderno
Prometeo.

Mary Shelley

Agraïments

Arribats al punt en que una tesi es troba pràcticament finaliztada cal girar la vista enrera i fer una justa menció a tots aquells que heu fet possible, de moltes maneres diferents, que aquest projecte hagi arribat a bon port. Vull donar les gràcies a tots i cadascú de vosaltres que heu compartit (sovint patit) el llarg camí que hem recorregut plegats durant aquesta tesi doctoral.

En primer lloc, et vull agraïr l'oportunitat que em vas donar ara ja fa alguns anys de formar-me tan acadèmica com professionalment. Després, us vull donar les gràcies a vosaltres dos que heu tingut infinita paciència i sàbies paraules en molts dels moments de desesperació que m'han invaït. Gracias por todo lo que me has enseñado durante estos años, pero no solo compartir conmigo tus conocimientos sinó también tu espacios, en la poyata, en cultivos y en los congeladores, has hecho todo mucho más fácil. Gràcies a tu, per donar l'empenta necessària per finalitzar aquest projecte, dedicant bona part del poc temps que tens a corregir, rellegir i reorientar de forma intel·ligent els resultats obtinguts treient un rendiment més que òptim. I sobretot gràcies per posar seny i la teva experiència no només professional, sinó també personal, davant totes les dificultats sorgides. Si m'hagués deixat portar pels meus impulsos molt probablement no podríes estar llegint això, molt probablement aquest projecte no hagués finalitzat.

Gràcies a tu que has buscat la millor resposta davant tots els dubtes que m'han sorgit durant aquest procés deixant de banda sempre tota la feina que tenies entre mans. Gràcies també a tu per la fe que em vas manifestar i enviar per la publicació de l'article. Tambien quiero agradecerte a ti todo lo que compartimos, todo lo que me regalaste, durante el tiempo de tu tesis en Barcelona. Y darte las gracias a tí por los buenos momentos compartidos durante el máster, los días en el laboratorio y sobretodo durante los congresos. Muchos ánimos ahora que eres tú el que encara la recta final de esta aventura.

I would also show all my gratitude to the people that make my time in UK easier. It was a pleasure to stay, work and learn a lot from you in the Lab in Nottingham. Many thanks to my office partners for tolerate my continuous

compplaints with lot of humour. Specially to you for the surrealistic smoking moments between the garbage. And of course I also want to thank you all your support, attention, availability, good advices and tutoring during my Nottingham time. También daros las gracias a vosotros que os convertisteis en compañeros de la inclemencia metereológica británica, de alguna que otra pinta, la mejor dieta del mundo y muchos buenos momentos fuera del laboratorio. Como bien sabeis en Barcelona teneis una casa.

També vull donar les gràcies a tots i totes les companyes de la Facultat de Medicina, Hospital Clínic, IDIBAPS, CEK i recentment, CELLEX, amb els que hem compartit passadissos, penúries i alegries. No hi ha prou paraules per expressar el meu agraïment a tots els que m'heu acollit amb els braços oberts en els últims temps i m'heu fet un raconet allà on pràcticament no hi havia ni oxigen. Vull donar-vos les gràcies a tots i cadascun de vosaltres per fer que em sentís com a casa, per escoltar, aconsellar, guiar, (fumar!?!?!), ajudar, suportar, estimar, esperar, comprendre, compartir, patir... Si hi ha una cosa que tinc molt clara (i que també em sap molt de greu) és que trobaré a faltar l'increïble "rutina" que m'heu regalat en aquest tram final de la meva tesi, heu aconseguit que tot sigui molt més fàcil. Em sento molt afortunat de poder dir que m'heu deixat formar part d'un grup amb una qualitat humana excepcional, tan de bo els nostres camins professionals es tornin a creuar. Jo faré tot el possible per a que els nostres camins personals no se separin. Us trobaré molt a faltar.

Gràcies també a vosaltres pel vostre recolçament fora del laboratori, hem compartit molt, moltíssim durant tots aquests anys des que vam començar la nostra formació el segle passat. Els anys passen i vosaltres seguiu allà, al peu del canó, encara que jo us tingui una mica abandonats. Gràcies per estar i deixar-me estar als moments importants, gràcies per tot el que m'heu donat, gràcies per perdonar tot el que últimament no us he donat. Gràcies també a tu per involucrar-me, comptar i confiar en mi per nous projectes relacionats un camp tan desconegut per mi com la divulgació de la ciència. Gracias por ayudarme a hacer esta tesis como quería. També et vull donar les gràcies a tu amb qui puc comptar sempre estiguis al lloc del món que estiguis, gràcies a la tecnologia et sento propera. Grazie pure a vuoi che siete lontani, ma vi

sento viccino al cuore. Tutto ha fatto un bel giro quando vi ho trobato, con vuoi ho deciso da fare una delle cose piú belle, cosa che ha scambiatto mia vita, grazie. Mi mancate.

Gracias a vosotras que os convertisteis en la *famiglia* y siempre habeis estado muy cerca pese a la distancia que nos separa con algunas. Gracias a todas por abrir las puertas de vuestras casas allá donde esteis, gracias por tener siempre abiertas las puertas de vuestro corazón. Gracias por todos estos años creciendo juntos, por la ilusión y el amor que me habeis transmitido siempre.

Por supuesto, también muchas gracias a vosotros, por el apoyo incondicional y constante a mis decisiones, por vuestra paciencia y por vuestra comprensión. También por perdonar mis desapariciones y por no exigirme nada a cambio de tanto, sobre todo en estos últimos tiempos. També a la resta, per fer pinya quan més us necessitava, per cuidar-me i estimar-me (encara que no em deixi gaire), per cuidar-nos i estimar-nos, per ser al nostre costat.

Gracias a todos los que te rodean y que han demostrado con creces su apoyo, su estima y su cariño. Finalmente, gracias a ti por estar a mi lado durante todo este tiempo. Gracias por toda la paciencia, la comprensión y el apoyo que me has regalado. Gracias por conseguir que lo difícil se convierta en sencillo y lo imposible en posible. Gracias por estar siempre. Gracias por todo. Gracias.

Finalment, espero no oblidar-me a ningú, però si ho he fet demano disculpes i li envio des d'aquestes últimes línies els meus més profunds agraïments.

Gràcies a tothom.

Backward

Aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used drugs in the world. They are usually well tolerated by most subjects but there is an exception in a particular subset of asthmatic patients. These patients suffer from so-called aspirin-induced asthma (AIA), a clinical syndrome characterized by asthma attacks triggered by the ingestion of NSAIDs. Furthermore, these patients frequently suffer from chronic rhinosinusitis with nasal polyposis (CRSwNP). In fact, the prevalence of CRSwNP in AIA patients is about 60-70%.

The pathogenesis of AIA remains poorly understood but it is accepted that alterations in the arachidonic acid metabolism may be involved. An overactive 5-lipoxygenase pathway has been reported in these patients, resulting in increased cysteinyl leukotriene production. In contrast, studies focusing on the cyclooxygenase pathway in aspirin-induced asthma subjects have reported contradictory results, ranging from reduced to increased activity of the cyclooxygenase pathway.

This doctoral thesis examines the arachidonic acid metabolism in the upper airways of patients suffering from AIA. This research has particularly focused on the study of the cyclooxygenase pathway and its regulation in fibroblasts cultured from nasal mucosa and nasal polyps of patients with or without AIA.

Table of contents

Abbreviations

AA	Arachidonic acid
AIA	Aspirin-induced asthma
AR	Allergic rhinitis
ATA	Aspirin-tolerant asthma
ATP	Adenosine 5'-triphosphate
CF	Cystic fibrosis
CFTR	CF transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxigenase
CRE	cAMP-response element
CREB	cAMP-response element binding protein
CRS	Chronic rhinosinusitis
CS	Corticosteroids
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESS	Endoscopic sinus surgery
FBS	Foetal bovine serum
FCS	Foetal calf serum
GCs	Glucocorticoids
GRE	Glucocorticoid response elements
HETEs	Hydroxyeicosatetraeinoic acids
Ig	Immunoglobulin
IL	Interleukin
IκKβ/γ	IκB kinase
JAK	Janus kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
NSAID	Nonsteroidal anti-inflammatory drug
NF-kB	Nuclear factor κB

NM	Nasal mucosa
NP	Nasal polyp
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PL	phospholipase
PLA_2	Phospholipase A2
LTs	Leukotrienes
PGs	Prostaglandins
PGI_2	Prostacyclin
TxA_2	Thromboxane A2
COX	Cyclooxigenase
LOX	Lipoxygenase
ROS	Reactive oxygen species
TAK	TGF activated kinase
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TR	Tandem repeats
VNTR	Variable number of tandem repeats
SFM	Serum-free media

1. Introduction

Chapter 1. Chronic rhinosinusitis with nasal polyposis.

1. The airways.

The main purpose of the respiratory system is to supply the body with oxygen and remove carbon dioxide, in order to promote gas exchange between our body and the external environment. The airways comprise a large, continuous structure that extends from the nasal vestibule to there alveoli. The airways are divided by an imaginary line into two subdivisions: upper and lower airways. The upper airways, also known as the upper respiratory system, include the nose, nasal cavity, paranasal sinuses, nasopharynx and oropharynx, while the lower respiratory system (lower airways) consists of the larynx, trachea, bronchi and lungs (Scadding et al. 2009), as can be observed in **Figure 1**.

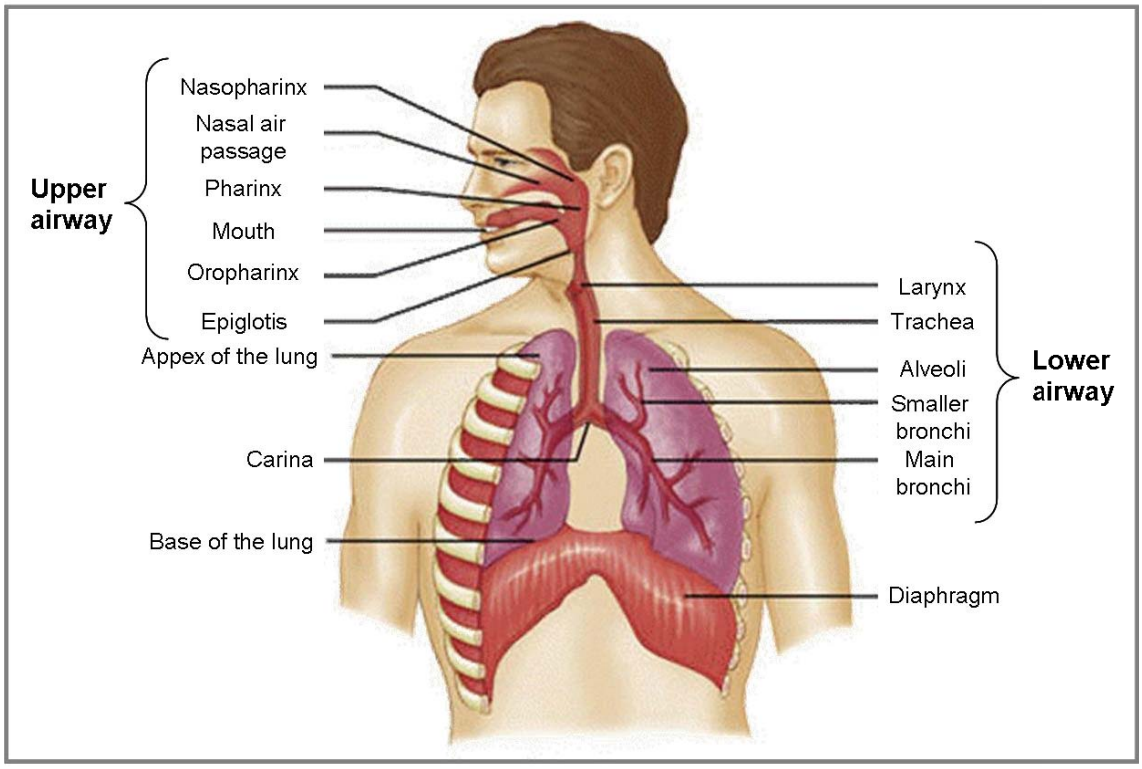

Figure 1. Schematic representation of the respiratory system. The figure represents the two sub-divisions of the respiratory system into upper and lower airways (adapted from http://www.emergencymedicaled.com/Illustrations/TheRespiratorySystem.htm).

2. The nose.

The nose is a double organ composed of two nasal cavities divided by a septum. These cavities contain three prominent structures, called upper, middle and lower turbinates. Both the nasal septum and the turbinates are covered by respiratory mucosa (Braunstahl 2011). This organization is essential to the provision of the air resistance required for the correct functioning of the lung and the preparation of inhaled air, by filtering, warming and moistening it before it reaches the lungs. In fact, the nose and their associated structures are the first site of allergen, microbial and particle deposition, and therefore play an important role in the immunological response. Accordingly, pathological conditions in the nasal mucosa (NM) interfere with the nose's functions, which can lead to increased exposure of the lower airways to allergens and subsequent airway inflammation (Mygind 1990).

2. The nasal mucosa.

3.1. Histology. The nasal mucosa is composed of a respiratory epithelium, a basement membrane and a submucosa **(Figure 2)**.

- Respiratory epithelium. This is a pseudostratified columnar epithelium that contains globet, basal, ciliated and non-ciliated cells. Moreover, inflammatory, immune and phagocytic cells migrate to, remain within or pass through it on their way to the lumen.

- Basement membrane. This is produced by epithelial cells and subepithelial fibroblasts and comprises various types of collagen, laminin and fibronectin (Paulsson 1992).

- Submucosa. Situated under the epithelium, separated by the basement membrane, the submucosa contains:
    a. An external area rich in fenestrated blood capillaries;

b. Submucosal glands, composed of serous and mucous cells and glandular ducts that drain secretions to the nasal lumen;
c. Venous sinusoids that form the erectile tissue. In the connective tissue around submucosal glands, a blood vessel net is responsible for nasal congestion and decongestion. Finally, there is also a bony structure to which the nasal mucosa is attached.

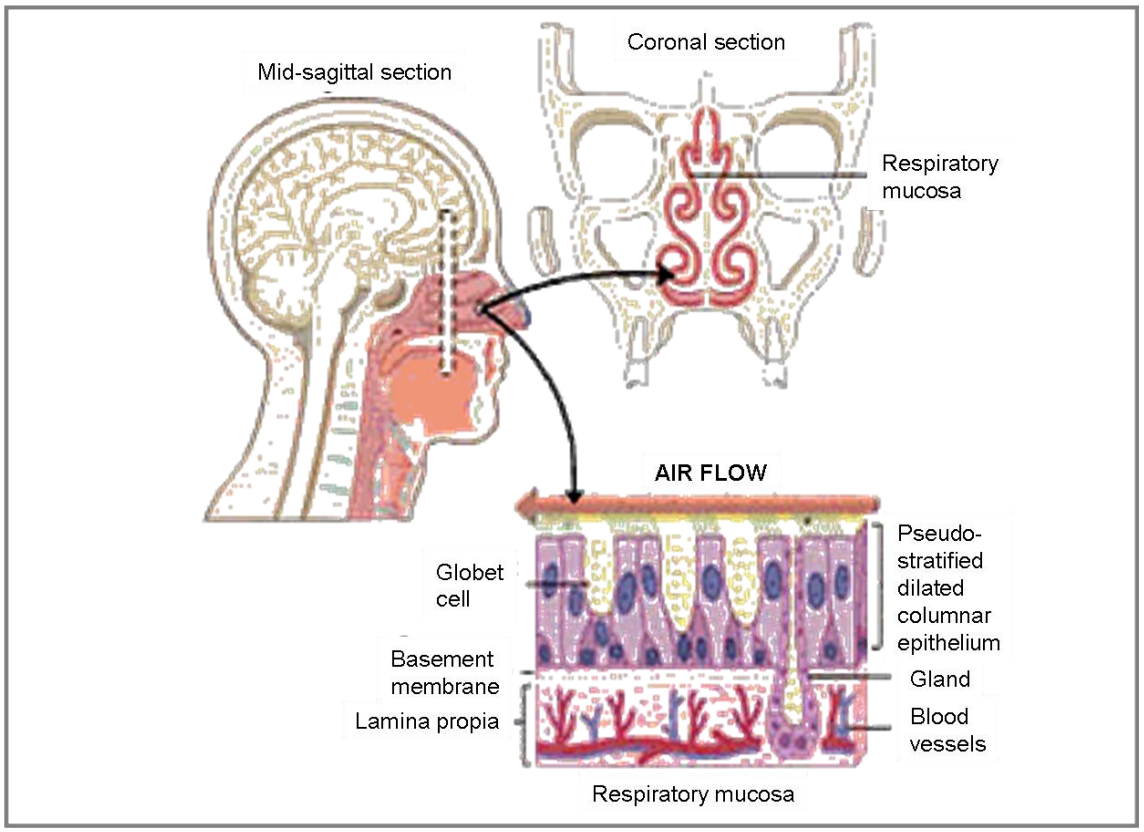

Figure 2. Schematic representation of the nasal respiratory mucosa. The figure represents the mid-sagittal section, the coronal section and the nasal mucosa tissue structure (adapted from http://emptynosesyndrome.org/turbinates_tutorial3.php).

3.2. Physiology. In homeostatic situations, the respiratory epithelium is covered by a mucus layer. The main role of this mucus is to cover and protect the respiratory tract by trapping pathogens and irritants and facilitate their removal via mucociliary clearance ((Cauna et al. 1969); (Cauna 1970)).

Moreover, the nasal mucosa, by means of its epithelium, has other important functions, such as the formation of a physical barrier, transport, secretion and inflammatory modulation (**Table 1**).

Table 1. Functions of the nasal respiratory mucosa.

Functions	Result
1. Physical protection	• Selective absorption. • Humidification and warming of inhaled air. • Entrapping of noxious agents.
2. Transport	• Transport of mucus from lung to the throat.
3. Secretion	• Mucins, cytokines, adhesion molecules, growth factors.
4. Target of proinflammatory and antiinflammatory agents	• Response to cytokines, glucocorticoids, chromones, antihistamines.

To sum up, the nasal mucosa is the organ involved in the preparation of inhaled air by filtering, warming and humidifying it before reaching the lungs. This allows it to protect the airways from external irritants and pathogens.

4. Chronic rhinosinusitis.

Rhinosinusitis is defined as an inflammatory process involving the mucosa and one or more sinuses (Scadding et al. 2008). Sinusitis involves the inflammation of sinus linings and rarely occurs without simultaneous rhinitis. Moreover, rhinosinusitis is a significant and increasing health problem that affects about 15% of the population in Western countries and it has direct medical costs, as well as a severe impact on lower airway diseases and general health outcomes (Meltzer et al. 2004). The International Classification of Diseases divides rhinosinusitis into two forms: acute and chronic, according to the duration of symptoms. The acute form lasts up to 12 weeks, with a complete resolution of symptoms, whereas the chronic form persists beyond 12 weeks and is associated with significant morbidity and a lower quality of life (Fokkens 2005).

CRS is characterized by chronic inflammation of the nasal and paranasal sinus mucosa, cytokine release and tissue remodelling, including changes in the extracellular matrix (ECM), protein deposition and tissue structure.

CRS is a multifactorial disease and is subdivided into two classes: CRS without nasal polyposis and CRS with nasal polyposis (Ferguson 2004). NP and CRS are often consideredthe samee disease, however, because differentiating between them is very complicated and, in fact, nearly impossible. Nasal polyposis was generally considered the endpoint of the evolution of CRS without nasal polyps, but nowadays there is growing evidence that, despite clinical similarities, these entities are completely distinct and presentdifferent inflammatory pathways, cytokine profiles and tissue remodelling (Eloy et al. 2011).

Clinically, the diagnosis of CRS requires two or more of the following symptoms: nasal blockage, anterior or postnasal drip, facial pain or pressure and reduced or absent sense of smell. An endoscopic intervention is also available, recording the presence or absence of inflammation (Pawankar et al. 2007).

5. Chronic Rhinosinusitis with nasal polyps.

Chronic rhinosinusitis with nasal polyps (CRSwNP) is defined as an inflammation of the nose and paranasal sinuses characterized by two or more long-term symptoms, one of which should be either nasal blockage/obstruction/congestion or nasal discharge, with/without facial pain/pressure or reduction (hyposmia) or loss of smell (anosmia) (Fokkens et al. 2007).

Nasal polyps can be divided into different subgroups based on clinical aspects, aetiology, histopathology (Stammberger 1999) and mediator content (Bachert et al. 1998). Nasal inflammatory polyps can be classified as follows (Bachert et al. 2003):

a) Idiopathic unilateral or bilateral. Mostly eosinophilic polyps with no involvement of the lower airways.

b) Bilateral eosinophilic polyps with concomitant asthma and/or aspirin sensitivity.

c) Polyposis associated with other diseases such as cystic fibrosis, Churg-Strauss syndrome or Kartagener syndrome.

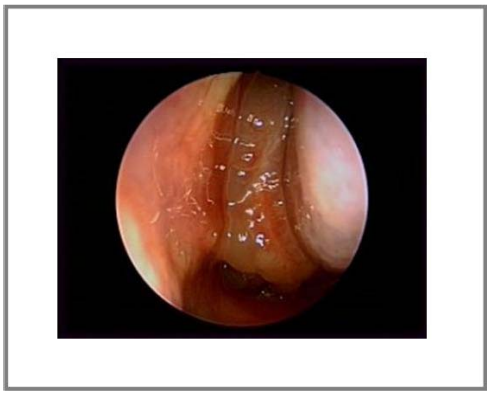

Figure 3. Endoscopic image of a nasal polyp. From www.ohiosinus.com/patient-info/nasal-polyps.

6. Histopathology of inflammatory nasal polyps.

Histologically, polyps are covered by a respiratory pseudostratified columnar epithelium with some areas of squamous metaplasia. There is frequent epithelial damage (epithelium shedding) and varying stages of thickened basement membrane, as we can observe in **Figure 4**.

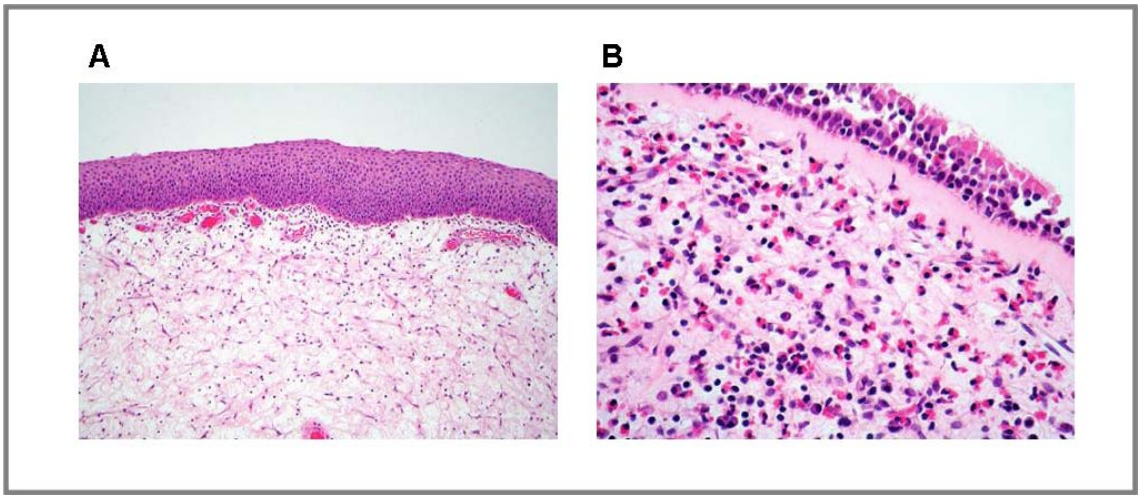

Figure 4. Histopathology of nasal polyps. A. Immature squamous metaplasia on the surface of an inflammatory polyp. **B.** Inflammatory nasal polyp covered by respiratory epithelium with basement membrane thickening and an eosinophil-rich inflammatory infiltrate (Helliwell 2010).

Pseudocyst formation and oedema are two major characteristics of nasal polyps. The pseudocysts contain albumin and other plasma proteins (Watelet et al.

2006). The number of vessels and glands is reduced, and there is virtually no neuronal structure. Fibroblasts and infiltrating inflammatory cells are localized around pseudocyst formations. Activated eosinophils are usually located around vessels, and glands are predominant in about 80% of patients with nasal polyps. There is a large number of degranulating epithelial mast cells (Ponikau et al. 2003).

7. Epidemiology and co-morbodities.

The exact prevalence of nasal polyposis in the general population is not known, because there are few epidemiological studies and their results depend on the study population selected and the diagnostic methods used. In fact, any estimate of the prevalence of CRSwNP remains speculative. Data published by the American General Health Survey show that patients seeking medical advice owing to chronic rinosinusitis-related symptoms represent 14.7% of the American population, but nasal polyposis affects 2 to 5% of the general population (Moloney et al. 1977; Moloney 1977). Other relevant publications mention a nasal polyposis prevalence of 4.3% in the general population in Western countries (Bauchau et al. 2004). The incidence is higher in men than in women and significantly increases above the age of 40 years. CRS with nasal polyposis is very difficult to treat, while its aetiology and pathophysiology are still unclear. It can frequently reappear, even after surgery and medical treatment. Patients with NP often present other related diseases, such as cystic fibrosis, allergic rhinitis, asthma or aspirin intolerance (Hadfield et al. 2000; Pawankar et al. 2007).

7.1. 7.1. Cystic fibrosis. Cystic fibrosis (CF) is the most common deadly recessive genetic disease in Caucasian population, with an incidence rate varying from 1 per 2,000 to 1 per 6,500 newborn babies. It is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7, which leads to the production of a defective chloride channel (Yamaya et al. 1991). Defective expression of the CFTR in CF epithelial cells is associated with mucus hypersecretion, inflammation and infection that begin in early life and lead to a marked cyclical airway obstruction and infection responsible for the morbidity and mortality of patients with CF (Loebinger et al. 2009; Kovell et al. 2011)

Patients suffering from CF present a higher prevalence of nasal polyposis than the general population, ranging from 6 to 48%. Furthermore, 92 to 100% of CF patients present radiological signs of sinonasal diseases. In children, bilateral NP is often a clinical sign of CF (Marshak et al. 2011). Fifty percent of the children between 4 and 16 years of age who suffer from nasal polyposis also present CF (Kovell et al. 2011).

7.2. Allergic rhinitis. Allergic rhinitis (AR) is an allergic inflammation of the nasal airways. AR is a heterogeneous disorder characterized by the presence of one or more of the following nasal symptoms: sneezing, itching, rhinorrhea and nasal congestion. Allergic rhinitis and CRS share the same trend of increasing prevalence and are frequently associated (Jones et al. 1998). The incidence of AR is 18-29% in the general European population, and between 0.5 and 4.5% of subjects with this pathology have NP (Fokkens 2011), while the prevalence of allergy in patients with NP has been reported as varying from 10% to 64% (Krause 2003; Bauchau et al. 2005).

7.3. Asthma. Asthma is a complex multifactorial disease of the airways and is currently a worldwide problem, with an estimated 300 million people affected. Asthma is defined by the Global Initiative for Asthma as: “a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment” (Munoz-Lopez 2010).

The disease may develop early in childhood or later in life, and there are many factors that can precipitate asthma attacks. These factors include allergy, viral infections, exercise and airbone irritants such as smoke cigarette or dusts. Asthma can be intermittent, or persistently mild, moderate or severe. The severity of the pathology varies from one subject to another, and it can also change in one individual over time (O'Byrne 2008).

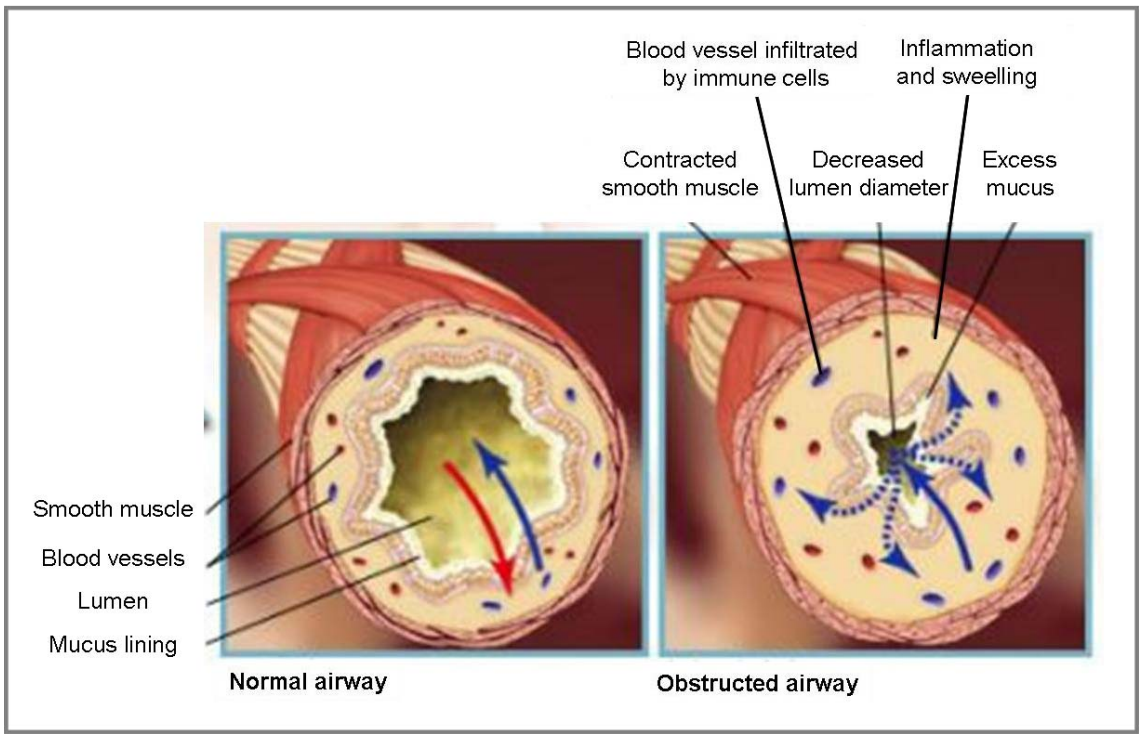

Figure 5. Pathophysiology of an asthma attack. The image shows a normal airway on the left and obstructed airway on the right (adapted from http://tatjana-mihaela.hubpages.com/hub/asthma-children).

Pathophysiologically, asthma is characterized by epithelial disruption, airway smooth muscle hypertrophy and hyperplasia, increased mucus secretion, basement membrane thickening, increased cytokine production and chronic infiltration of inflammatory cells **(Figure 5)**. These changes that occur on a structural level are often referred to as remodelling, which defines complex morphological changes involving all structures within the bronchial wall (Fireman 2003; Blake 2006; Hamid et al. 2007).

CRSwNP and asthma are frequently associated (Bousquet et al. 2001): seven percent of asthma patients have NP compared to lower percentages in the non-asthma population (Settipane et al. 1977). The association with asthma was stronger in those reporting both CRS and allergic rhinitis (Jarvis et al. 2012). Asthma prevalence in the Spanish and Catalan general population is 7% and rises to 30% in a population of patients with nasal polyposis (Klossek et al. 2005). Furthermore, up to 60 % of patients with NP have lower airway involvement, assessed by history, pulmonary function and histamine provocation tests (Ragab et al. 2004).

7.4. Aspirin-induced asthma. Aspirin-induced asthma (AIA) is a distinct clinical syndrome characterized by the association of chronic rhinosinusitis with nasal polyposis (CRSwNP), asthma and bronchoconstriction episodes triggered by the intake of non-steroidal-anti-inflammatory drugs (NSAIDs) (Stevenson et al. 2006). The first case of AIA was reported in 1922 by Widal and col, when an episode of bronchospasm was observed following the ingestion of acetyl salicylic acid (aspirin) in a subject suffering from asthma. In the late 60s Samter and Beers described this peculiar syndrome with its clinical triad of asthma, nasal polyposis and aspirin intolerance, in greater detail (Samter et al. 1968).

Aspirin and other NSAIDs are among the most widely used medication in the world. They are usually prescribed to treat and prevent heart diseases, as well as being used for antipyretic, anti-inflammatory and analgesic therapy. These drugs are normally well tolerated by most people but a subset of asthmatic patients is an exception. In these patients aspirin and other NSAIDs are able to precipitate asthma attacks (Szczeklik et al. 2006). Accordingly, AIA is recognized as an aggressive phenotype of airway disease that often runs a protracted course. When compared with aspirin-tolerant (AT) subjects, patients with AIA are more susceptible to irreversible airway flow obstruction and frequent exacerbations, as well as being more likely to be diagnosed with severe asthma (Mascia et al. 2005; Koga et al. 2006).

Exposure to aspirin or other NSAIDs does not trigger or perpetuate the underlying respiratory inflammatory disease. Since the disease is ongoing, however, these drugs do induce the release or synthesis of critical mediators. After ingestion of aspirin or NSAIDs, patients with adverse reactions develop symptoms within 2-3 hours. These generally consist of bronchospasm, profuse rhinorrea, conjunctival injection, periorbital oedema and generalized flushing (Szczeklik et al. 2003; Morwood et al. 2005).

Aspirin sensitivity is not considered an immunological reaction, because the pathology did not involve an immunoglobulin (Ig) E-mediated reaction. The pathological mechanism responsible for the development of AIA has not been completely elucidated (Pfaar et al. 2006), but several findings suggest that

abnormalities in the regulation of arachidonic acid metabolism may be involved in the adverse response to NSAIDs in patients with this syndrome (Stevenson et al. 2006; Yoshimura et al. 2008).

This peculiar syndrome has an estimated prevalence of one percent in the general population and it is present in approximately 3 to 20% of the adult asthmatic population andmore frequently in women than in men (Szczeklik et al. 2003). Furthermore, in women the symptoms normally appear earlier, and the disease seems to be more aggressive (Szczeklik et al. 2000), while the children of patients with aspirin sensitivity had CRSwNP more often than the children of a control population (May et al. 2000). Patients with aspirin/NSAID-induced respiratory reactions often have an underlying history of asthma, nasal polyposis, and/or rhinosinusitis, although some patients may not have any predisposing diseases (Gollapudi et al. 2004). Subjects who develop the aspirin triad typically develop persistent rhinitis in their third or fourth decade of life and, approximately 2-5 years later, there is a progression to asthma, aspirin sensitivity and nasal polyposis (Hamad et al. 2004). The prevalence of CRSwNP in aspirin sensitivity patients has been reported as varying from 36 to 96% (Caplin et al. 1971; Spector et al. 1979; Ogino et al. 1986) and up to 96% have radiographic changes affecting their paranasal sinuses (Szczeklik et al. 1999). Sensitivity to aspirin has been reported in 35-52% of patients with NPs and in as many as 65% of patients who suffer from both bronchial asthma and nasal polyposis (Samter's triad) (Schiavino et al. 2000).

8. Clinical aspects and diagnosis of nasal polyposis.

The symptoms in acute and chronic rhinosinusitis, as well as in CRSwNP, are similar, although their pattern and intensity may vary. The general symptoms are: nasal blockage, congestion or stuffiness, nasal discharge or postnasal drip, facial pain or pressure and reduction/loss of sense of smell (Fokkens et al. 2007).

Nasal polyps may cause nasal congestion, which can give rise to a feeling of pressure and fullness in the nose and paranasal cavities. This is typical of ethmoidal polyposis, which in severe cases can cause widening of the nasal and paranasal

cavities, as demonstrated radiologically. Disorders of smell are more prevalent in patients with nasal polyps than in other CRS patients (Vento et al. 2000).

Clinically, nasal polyp diagnosis is based on clinical symptoms and on endoscopy and CT-scan of the paranasal sinuses showing the presence of endoscopically visible bilateral polyps growing from the middle meatus into the nasal cavities, affecting the ethmoidal and maxillary sinuses (Benninger et al. 2003; Fokkens et al. 2007). During the last decade more attention has been paid not only to symptoms but also to their effect on patients' quality of life (QoL) (Benninger et al. 1997; Metson et al. 2000).

8.1. AIA diagnosis. The diagnosis of ASA hypersensitivity is based on a history of adverse reaction precipitated byASA or other NSAIDs. In asthmatic patients with a negative history, and/ or those who have never been exposed to NSAIDs but have additional risk factors (rhinosinusitis, nasal polyposis, history of near fatal reactions), the risk of adverse reaction is further increased and provocation test may be required (Nizankowska-Mogilnicka et al. 2007). Oral challenge is the reference standard for the diagnosis of hypersensitivity to aspirin and other NSAIDs, and several protocols for oral aspirin provocation have been developed and described (McDonald et al. 1972; Stevenson 2000). Inhalation challenge with lysine-aspirin (a soluble form of acetylsalicylic acid) was introduced by Blanco et al in 1977 and this is often used in Europe to confirm/exclude aspirin sensitivity in patients with bronchial asthma. An inhalation test is faster and safer to perform than an oral challenge (the reaction can be easily reversed by nebulised β_2-agonists) and both tests have a similar sensitivity and specificity (Dahlen et al. 1990; Nizankowska et al. 2000). A nasal provocation test with lysine aspirin is also a possible tool for diagnosing hypersensitivity to aspirin, providing the clinical symptoms are combined with the standardized objective technique of airflow measurement for assessment of the result (Milewski et al. 1998). The test is rapid and safe and can be performed in an outpatient setting; even in asthmatic patients with a reduced pulmonary function ineligible for bronchial provocation it results approach those of a bronchial challenge (Milewski et al. 1998; Casadevall et al. 2000).

9. Management of nasal polyposis.

The management of nasal polyps is extremely individual and must be discussed case by case, as the expectations of one patient are not necessarily the same of those of another. The treatment can be either pharmaceutical or surgical (Bachert et al. 2005).

The goals of treatment are to re-establish the nasal airway and nasal breathing, minimize symptoms, improve the sense of smell, treat co-existing diseases such as asthma, improve quality of life and prevent complications. Recurrence is the norm, however, but compliance with the treatment can postpone any relapse of the disease for as long as possible. There are various treatment recommendations, depending on the severity of the symptoms, but topical and oral corticosteroids are the basis for an optimal treatment of nasal polyposis (Fokkens et al. 2012). Management of asthma and rhinosinusitis in AERD patients should follow general guidelines, but several specific measures for AERD should be considered. Patient education and careful avoidance of ASA and other NSAIDs in sensitive patients seem to be of the utmost importance as aspirin can cause severe asthmatic attacks (Dahlen et al. 1990).

9.1. Glucocorticoids. Glucocorticoids (GCs) are the most effective drugs in the prevention and suppression of inflammation originated by mechanical, chemical, infectious and immunological stimuli. GCs inhibit various inflammatory aspects by inducing or reducing gene transcription and expression mediators, receptors, adhesion molecules and cytokines (Pujols et al. 2004; Pujols et al. 2007).

There is good evidence to show that topical and systemic GCs are effective in the management of CRSwNP. Considering the chronicity of CRSwNP, however, many treatments will need to be continuous, as in the case of local GC therapy in asthma. Local therapy appears to be effective but the ability to effectively deliver intranasal GCs to the paranasal sinuses may largely determine the response to treatment. The use of topical GCs taken on a daily basis for several months to years is considered the first-line therapy in mild-to-moderate nasal polyps, as it reduces symptoms and avoids

the need for surgery. Furthermore, other drugs, such as long-term antibiotics, nasal vasoconstrictors, antihistamines and antileukotriens, can occasionally be used in combination with GCs (Fokkens et al. 2012).

GCs have a proven therapeutic effect on the symptoms of nasal polyposis and they can reduce the underlying cause of nasal polyposis, i.e., mucosal inflammation. Symptoms such as nasal obstruction, rhinorrhea and, occasionally, hyposmia are reduced during the treatment period, especially in obstructive polyposis (Lund et al. 1998; Tos et al. 1998; Benitez et al. 2006). Furthermore, GCs delay any recurrence after surgery (Mygind 1999), but surgery needs to be considered in cases of treatment failure, side effects or unwillingness on the part of patients to adhere to the drug treatment (Bachert et al. 2003).

9.2. Endoscopic polypectomy and sinus surgery. Endoscopic sinus surgery (ESS) has as its main objectives the restoration of nasal ventilation and the unblocking of the natural drain of the paranasal sinuses, in order to re-establish the physiological purging of its secretions. The restoration of ventilation and the recovery of mucocilliary functions are priorities for curing the disease and maintaining a healthy sinonasal mucosa (Dalziel et al. 2003).

Extensive postoperative care and follow-up is required to preserve the postoperative results and prevent the relapse of polyps. The long-term efficacy of surgery is mostly influenced by the treatment regime prescribed postoperatively and subsequent compliance with it (Fokkens et al. 2012). Nevertheless, nasal polyposis is a chronic disease with a high rate of recurrence (40% after 5 years), even after careful medical and surgical treatment (Vento et al. 2000). AIA patients tend to suffer from more extensive sinus disease. They benefit from sinus surgery, but to a lesser extent than patients without AIA. They are more prone to disease recurrence and undergo revision surgery more frequently than aspirin-tolerant CRS patients (Mendelsohn et al. 2011).

In summary, nasal polyposis is a common upper-airway inflammatory disease that affects the nasal mucosa and is frequently associated with asthma and aspirin

sensitivity. Nasal polyposis is also characterized by high inflammatory cell infiltration, and consequently an elevated number of inflammatory mediators are found among NP. Several findings in the literature suggest that alterations in the regulation of the arachidonic acid metabolism would be responsible for the adverse response to NSAIDs in patients with AIA. Arachidonic acid metabolism and cyclooxygenase regulation will be examined in Chapter 2.

Chapter 2. The arachidonic acid metabolism.

1. Inflammation.

Inflammation is a protective response triggered after injury from physical damage or infection by microorganisms (Ricciotti et al. 2011). While both systemic and local responses can be activated, inflammation is an essential biological process seeking to eliminate aberrant factors, promote tissue repair/wound healing and establish memory, which enables the host to mount a faster and more specific response in the event of any future encounter (Stables et al. 2011).

An acute inflammatory response is a complex and coordinated sequence of events involving a large number of molecular, cellular and physiological changes. If these events occur in the correct sequence the acute inflammation will resolve itself, giving rise to complete restoration of the inflamed tissue's physiological functioning and reinstating homeostasis. If, however, defects arise during any part of this sequence, inflammation will persist and become chronic, lasting for longer periods (days, months or years) and leading to excess tissue damage. This is characteristic of the pathogenesis of various prevalent diseases in modern Western civilisation, such as rheumatoid arthritis and periodontal disease (Van Dyke et al. 2003), cardiovascular diseases such as myocardial infarction (Anselmi et al. 2004) and atherosclerosis (Libby 2002).

Diseases characterized by airway inflammation, excessive airway secretion and airway obstruction affect a great proportion of worldwide population. Excessive airway production of chemokines, cytokines and growth factors in response to irritants, infectious agents and inflammatory mediators may play an important role in the modulation of acute and chronic airway inflammation. The four main signs of airway inflammation are oedema (swelling), vasodilatation (redness), cellular infiltration and pain (increased airway responsiveness) (Levine 1995).

Although it is widely accepted that pro-inflammatory mediators generated in the inflamed tissue drive acute inflammation, there is also a systemic and local

production of local mediators that counter-balance these pro-inflammatory events; these have evolved to avoid the development of pathologies such as those mentioned before. Lipid mediators derived from polyunsaturated fatty acids such as arachidonic acid (AA) are synthesized during normal cell homeostasis or, more often, after cell activation and under conditions of stress. The lipid mediators activate counter-regulatory, anti-inflammatory and pro-resolution mechanisms. These immune-modulator effects are also found in a family of lipids derived from AA, including, which are known to be involved in driving some of the cardinal signs of inflammation, such as heat, redness, swelling, pain and loss of function (Stables et al. 2011).

2. Arachidonic acid metabolism.

Arachidonic acid (AA), a 20-carbon unsaturated fatty acid, is the predominant precursor of the eicosanoids, a family of lipid mediators (Smyth et al. 2009). Most AA is stored in the membranes of the cells as a part of the phospholipids. Eicosanoid biosynthesis begins with the release of AA, esterified in the sn-2 domain of membrane phospholipids, through the action of phospholipase enzymes in response to various stimuli (physical, chemical, hormonal, cytokines, etc). The main enzyme involved in the release of AA is phospholipase A_2 (PLA_2). AA, in its turn, is rapidly metabolized in several enzymatic and non-enzymatic pathways and released from the source cell, acting in an autocrine/paracrine manner on target cells.

AA can be metabolized via three main enzymatic pathways:

- Lipoxygenase (LOX) pathway; this produces leukotrienes (LTs) and lipoxins. LTs are involved in various procedures, such as asthma, allergic inflammation and innate immunity, and they play an important role in the inflammatory response. The functions of LTs include inducing chemotaxis, exerting a potent bronchoconstriction effect and increasing vascular permeability (Hallstrand et al. 2010).

- Cyclooxygenase (COX) pathway; this produces prostanoids. Prostanoids include prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane A2 (TxA_2). Prostanoids participate in several physiological functions, such as gastric

epithelial cytoprotection, maintenance of renal function and hemostasis. However, they also have an important role in pathological conditions, modulating fever, pain or inflammation. Their production is enhanced by chronic inflammatory diseases such as arthritis, cystic fibrosis and chronic obstructive pulmonary disease (Simmons et al. 2004).

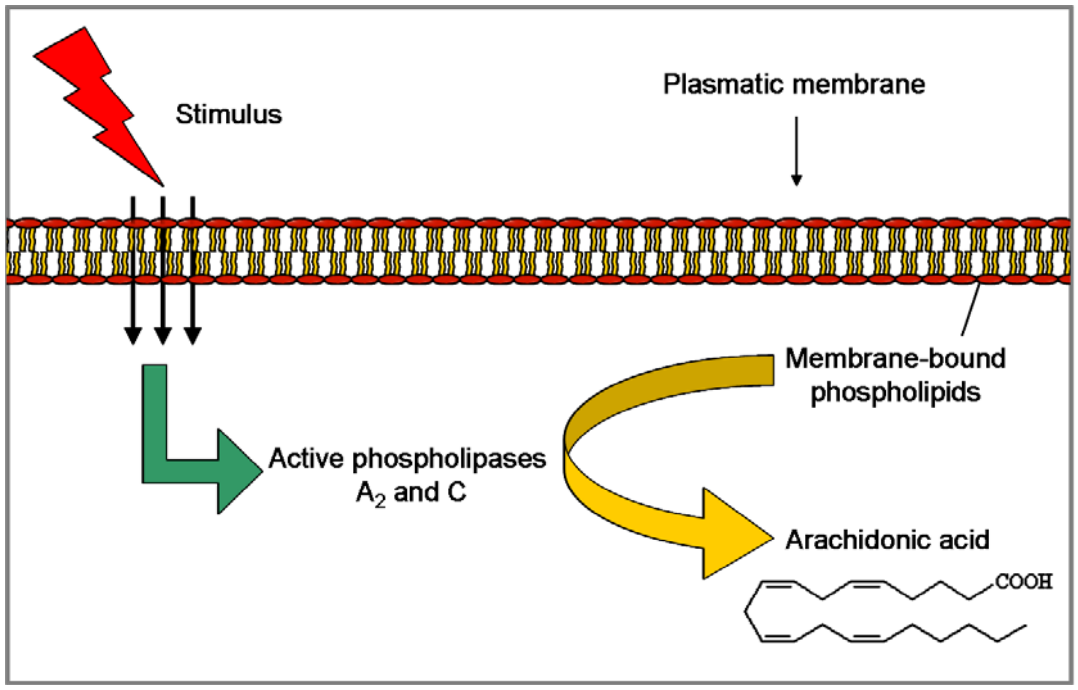

Figure 6. Schematic representation of AA release process. Membrane-bound phospholipids are converted to AA by the action of phospholipase enzymes, which are activated in response to external stimuli (adapted from Stratton et al. 2002).

3. Lipoxygenase (LOX) pathway.

The 5-Lipoxygenase pathway also plays an important role in inflammation as it is the responsible for the synthesis of leukotrienes (LTs). The 5-LOX, with the help of 5-LOX activating protein (FLAP), is responsible for the production of anaphylactic substances such as LTC_4, LTD_4 and LTE_4, the Cys-LTS, which are potent mediators of allergic response, and LTB_4, which is a powerful polymorphonuclear leukocyte chemoattractant. Upon activation, 5-LOX interacts with FLAP, allowing the oxygenation of AA and resulting in LTA_4 synthesis. LTA_4 can then be transformed in some cells into LTB_4 or into LTC_4, which is then transported to the exterior of the cells, where it is metabolized into LTD_4, which can be further modified into LTE_4. 5-LOX, in contrast with

the COX-1 enzyme, is inactive in quiescent cells but becomes enzymatically functional when the cells are activated by an increase in intracellular calcium (Stables et al. 011).

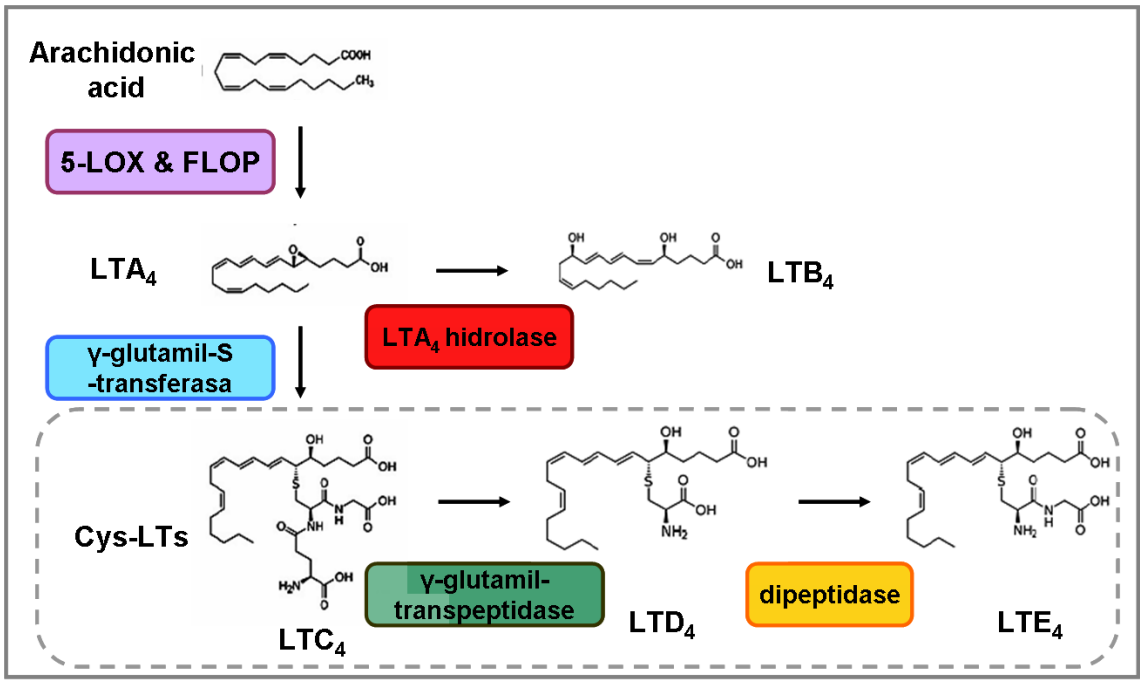

Figure 7. Schematic representation of the LT metabolism. AA is converted into LTA_4 by the action of 5-LOX and FLAP. The LTA_4 can be transformed into LTB_4 or LTC_4. LTC_4, in its turn, is metabolized into LTD_4, which is further transformed into LTE_4 (adapted from Stables et al. 2011).

The Cys-LTs act through specific receptors of the rhodopsin class, located on the outer leaflet of the plasma membrane (Cys-LTs receptor 1 and 2). Once they are bound to a specific receptor, a signal is sent via G-protein in the cytoplasm to increase intracellular calcium and block the formation of cAMP, which alters several cellular activities. The Cys-LTs play a role in the development of bronchoconstriction, mucus secretion, and oedema in airways during an asthmatic crisis (Picado 2006).

4. Cyclooxigenase (COX) pathway.

Cyclooxygenases. In the COX pathway AA is converted into PGH_2, the common precursor of prostanoids, via the action of COX enzymes. COXs are glycoproteins with a haemo group that catalyzes two reactions: cyclooxygenase reaction, generating the intermediate metabolite PGG_2, which in its turn is converted into PGH_2 via a peroxidase reaction (Hata et al. 2004).

COX enzymes are also known as prostaglandin-endoperoxide synthases (PGHSs) and there are at least 2 isoforms, COX-1 and COX-2. The gene for COX-1 is located in chromosome 9 while the COX-2 gene is located in chromosome 1. Both these enzymes, COX-1 and COX-2, are closely related in their amino acid sequence and crystal structure. There is a 65% homology between the two isoforms in the same species and they have a similar catalytic activity. They do differ, however, in their regulation of activity, and the COX-1 and COX-2 proteins have a different location.

Cyclooxygenase (COX)-1. Generally speaking, the COX-1 isoform is located in the endoplasmatic reticular membrane. It is considered a housekeeping enzyme that is expressed in most of the cell envelope (Chandrasekharan et al. 2004; Stables et al. 2011). The prostaglandin production of COX-1 is essential to the maintenance of physiological functions such as vascular homeostasis and the gastric and renal functions. There is a splice variant of COX-1 that retains intron one and has a frameshift mutation; this enzyme is usually called COX-1b or COX-1 variant (COX-1v), although it can also be found in the literature as COX-3. The expression of this COX-1v is basically confined to the cerebral cortices, kidneys and neuronal tissues (Snipes et al. 2005).

COX-1 gene contains 11 exons and generates a 2.8 Kb mRNA that is translated, in its turn, into a 70 KDa protein. The COX-1 promoter does not have a TATA box, although it does contain some Sp1 sites at 5'. The promoter structure of the COX-1 gene is typical of housekeeping genes. Although COX-1 expression is constitutive in most tissues; induction of gene expression has been described in some cell types under certain conditions (Chandrasekharan et al. 2004).

4.3. Cyclooxygenase (COX)-2. Generally speaking, COX-2 is situated in the perinuclear envelope. It is usually considered an inducible gene, despite being expressed constitutively in some specific areas of the brain and kidneys (Chandrasekharan et al. 2004; Stables et al. 2011). The expression of COX-2 is typically increased in pathological conditions such as inflammation, tumour development and chronic inflammatory diseases such as arthritis rheumatoid, atherosclerosis and cystic fibrosis (Baigent et al. 2003; Roca-Ferrer et al. 2006). In fact, several studies have demonstrated that COX-2 transcription can be increased via the action of different molecules, as summarized in **Table 2**.

Table 2. Molecules increasing COX-2 transcription.

Molecule	Cell type	Methodology	Reference
IL-1β	CNS cells	RT-PCR/ IHC	Samad et al. 2001
IL-1β	SMCs, ECs	WB	Briones et al. 2005
TGF-β	Artery SMCs	WB	El-Haroun et al. 2004
TGF-β	Epithelial Cell	WB	Tian et al. 2010
TNF-α	HCS-2/8	RT-PCR	Sakai et al. 2001
TNF-α	Carcinoma cells	WB/ RT-PCR	Grau et al. 2004
INF-γ	RAW 264.7	WB	Vila-del Sol et al. 2005
INF-γ	RAW 264.7	WB/RT-PCR	Wu et al. 2005
IL-1β, TNF-α, INF-γ	NPs explants	WB/RT-PCR	Mullol et al. 2002
IGF-1	Osteoblasts	WB/RT-PCR	Cao el at. 2007
LPS	Macrophages	WB/ RT-PCR	Eliopoulos et al. 2002
Angiontensin II	Vascular SMCs	WB/ RT-PCR	Alvarez et al. 2007
Angiontensin II	Aortic fibroblasts	WB/ RT-PCR	Beltran et al. 2009
Endothelin	A549	WB	Peng et al. 2008

IL-1β, interleukin-1β; CNS, Central Nervous System; RT-PCR, Real Time- Polymerase Chain Reaction; IHC, Immunohistochemistry; SMCs, Smooth Muscle Cells; ECs, Endothelial Cells; WB, Western Blot; TGF-β, Transforming Growth Factor-β; TNF-α, Tumour Necrosis Factor-α; HCS-2/8, Human Chondrocytes-like Cells; IFN-γ, Interferon- γ; RAW 264.7, Macrophages like-Cells; NPs, Nasal Polyps; IGF-1, Insulin Growth Factor-1; LPS, Lipopolysaccharide.

Consequently, the increase in prostanoid production observed in these inflammatory and tumoral diseases could be attributed to COX-2 induction (Warner et al. 2004).

5. Transcriptional regulation of COX-2.

The COX-2 gene comprises 10 exons, which are converted in their turn into 4.6 Kb transcript and translated into a protein with a similar molecular weight to COX-1 (70 KDa). COX-2 expression is regulated at the transcriptional, post-transcriptional and also post-traductional levels (Chandrasekharan et al. 2004). The COX-2 promoter has different binding sites for transcription factors, strongly conserved in humans, mice and rats (Klein et al. 2007). COX-2 transcriptional regulation involves complex mechanisms such as MAPK signalling, and occasionally the end result is the recruitment of different transcription factors, such as nuclear-factor kB (NF-kB) and CCAAT-enhancer binding protein (C/EBP), which induce the complete activation of the gene. The COX-2 promoter has various regulatory elements, including a TATA box, a binding site for NF-IL6, a binding site for C/EBP, two binding sites for (NF-kB), a CRE motif (response element to cAMP) and an E-box site (Klein et al. 2007).

5.1 MAPKs. MAPKs phosphorylate specific serine and threonine residues of target protein substrates and regulate cellular activities such as gene expression, mitosis, movement, metabolism and programmed death. The large number of important cellular functions controlled by MAPKs has led to extensive research into their role in maintaining homeostasis and their involvement in human disease. MAPK-catalyzed phosphorylation functions as a switch that turns the activity of their target proteins on and off (Dong et al. 2002). Substrates of MAPKs include other protein kinases, phospholipases, transcription factors and cytoskeletal proteins. Protein phosphatases remove the phosphates transferred to the protein substrate by MAPKs. The actions of MAPKs and phosphatases therefore alter, reciprocally and rapidly, the behaviour of cells as they respond to changes in their environment. In multicellular organisms there are three well-characterized subfamilies of MAPKs. These include ERKs (ERK1 and ERK2), JNKs (JNK1, JNK2 and JNK3), and the four p38 enzymes (p38α, p38β, p38γ and p38δ) (Raman et al. 2007).

There is substantial evidence that MAPK family members play a role in COX-2 gene expression. In fact, most of the previously mentioned molecules, such as IL-1β, LPS, TNF-α and INF-γ, induce COX-2 expression trough a signal cascade modulated by MAPK. It has been reported that the ERK 1/2 and p38 MAPK'sspecific inhibitors attenuated LPS-induced COX-2 mRNA expression as well as PGE_2 production in a concentration-dependent manner in RAW 264.7 cells (Lo 2003). In human keratinocytes, the specific inhibitor for p38, SB202190, attenuated UVB-induced COX-2 mRNA expression, but the specific inhibitor for ERK 1/2, PD98059, failed to significantly alter COX-2 levels (Chen et al. 2001). Other inhibitors, such as UO126, the specific blocker of the MEK1/2, blocked PGE_2 production and COX-2 mRNA expression induced by LPS in monocytes (Scherle et al. 2000). In HeLa cells stimulated with bacterial LPS and IL-1, inhibition of p38 destabilized COX-2 mRNA (Ridley et al. 1998). In fibroblast cultures (Beltran et al. 2009) and epithelial intestinal cells (Slice et al. 2005), the induction of COX-2 through angiontensin-II is only reduced in the presence of p38 MAPK inhibitor. In CMLV p38, however, MAPKs and ERK contribute to the expression of COX-2 induced by angiotensin-II (Ohnaka et al. 2000). Furthermore, a contribution from JNK to the regulation of COX-2 expression has been observed in human chondrocytes induced by IL-1β(Nieminen et al. 2005).

Finally, several works have demonstrated the post-transcriptional regulation of COX-2 through MAPKs. These studies have shown that p38 MAPK also contributes to the rise in COX-2 expression by increasing the stability of the mRNA, and subsequently their expression (Duque et al. 2006; Di Mari et al. 2007).

All in all, these different results suggest that the effect of MAPKs on the transcriptional regulation of COX-2 expression is cell-type- and stimulus-dependent.

5.2. NF-kB. The transcription factor NF-kB is an inducible and ubiquitously expressed transcription factor responsible for regulating the expression of genes involved in cell survival, inflammation and differentiation (Shishodia et al. 2002; Chen et al. 2007). In fact, NF-kB is one of the principal factors regulating COX-2 expression. Active NF-kB complexes are dimmers of various combinations of the Rel family of polypeptides, consisting of p50, p52, c-Rel, v-Rel, Rel A (p65) and Rel B. In most resting

cells, NF-kB is retained in the cytoplasm by binding to one of the inhibitory IkB proteins (IkBα, IkBβ, IkBγ, p105 and p100), which blocks the nuclear localization sequences of NF-kB (Baldwin 2001). NF-kB is activated in response to a wide variety of stimuli promoting the dissociation of IkBα through phosphorylation. Thus, unmasking the nuclear localization sequence of NF-kB allows NF-kB to enter the nucleus and bind to kB-regulatory elements. The phosphorylation of IkBα, as a critical event in NF-kB activation, is catalyzed by an IKK complex (Pahl 1999).

There is considerable evidence that NF-kB plays a central role in general inflammatory, as well as immune, responses. The promoter region of COX-2 contains ~~to~~ kB binding sites. Thus, NF-kB has been shown to be a positive regulator of COX-2 expression in several cell types, such as murine macrophages (Eliopoulos et al. 2002) and human colon adenocarcinoma lines exposed to LPS (Kojima et al. 2000). Accordingly, other studies using specific NF-kB inhibitors demonstrated a reduction in PGE_2 secretion and COX-2 expression (Yan et al. 2002).

All in all, these different results suggest that NF-kB activation plays a role in COX-2 transcriptional regulation.

5.3. C/EBP. These transcription factors are also involved in regulating the activity of the COX-2 promoter. There is a C/EBP binding site on the human COX-2 promoter. The three main members of the C/EBP family include C/EBPα, C/EBPβ and C/EBPγ, which all recognize the same DNA sequence. They have a common structure, with an N-terminal domain bearing the transactivation sequence, a basic DNA-binding domain and a C-terminal domain containing a leucine zipper that allows for the homo- or heterodimerization of these factors (Williams et al. 1997).

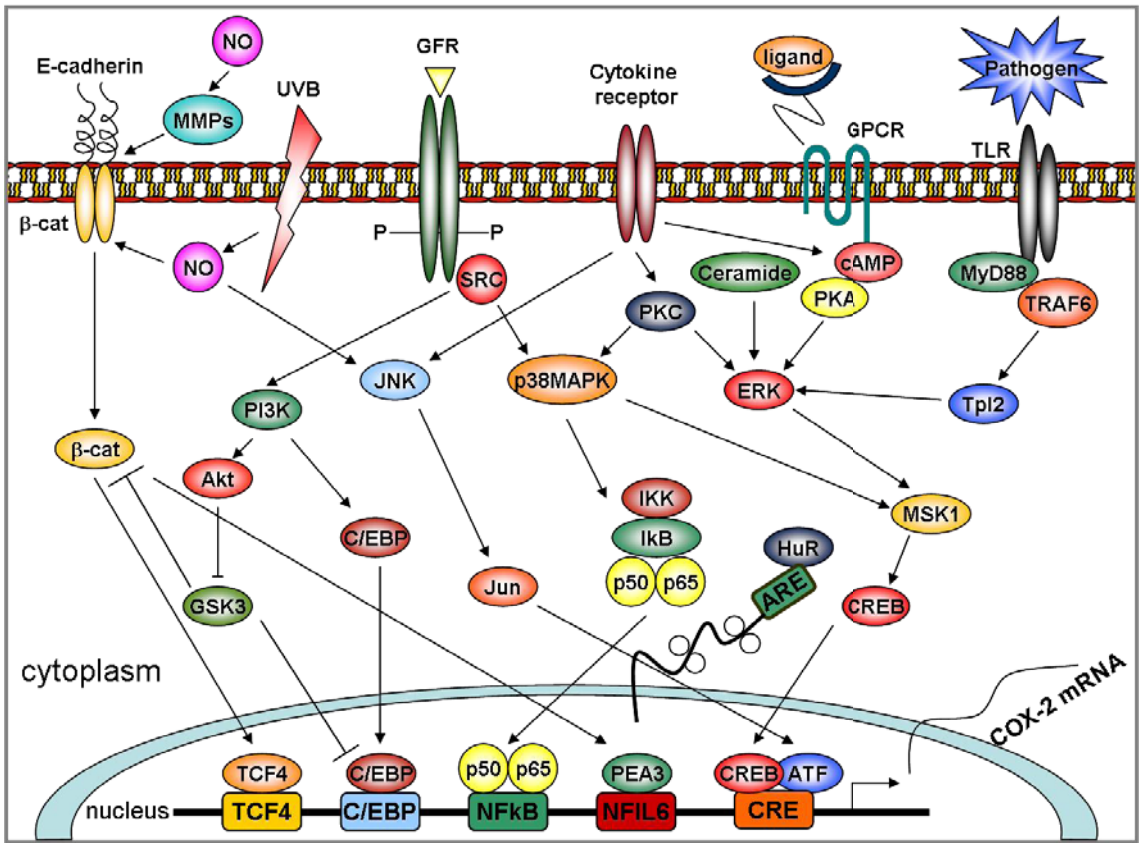

Figure 8. Schematic represantion of signalling pathways involved in COX-2 expression. Adapted from Tsatsanis et al. 2006.

C/EBPα, C/EBPβ and C/EBPγ primarily act to regulate genes involved in mediating inflammation and cell proliferation, and they tend to be upregulated during the acute phase response. It is difficult to generalize, however, about the functions of C/EBP family members in COX-2 expression since, depending on the cell type, the same C/EBP isoform displays opposite effects (Chandrasekaran et al. 1993). Many studies have shown either the transition of protein binding from C/EBPα to C/EBPβ and γ or a change in their relative level of expression between C/EBPα mRNA and C/EBPβ and γ messages when genes are activated. Gorgoni and cols demonstrated that COX-2 mRNA induction and promoter activity were profoundly impaired in C/EBPβ (-/-) macrophages but could be rescued by the expression of C/EBPβ (Gorgoni et al. 2002). Other studies have also demonstrated the involvement of C/EBPβ in the transcriptional activation of COX-2 in murine and human cells through proinflammatory mediators (Saunders et al. 2001; Wu et al. 2005).

All in all, these different results suggest that the role of C/EBP in the COX-2 transcriptional regulation should be deeply investigated.

6. Prostaglandin synthases and prostanoids.

6.1. Prostaglandin synthases. The formation of biologically active prostanoids from PGH_2 occurs through the prostaglandin synthases with an isomerization reaction. These synthases include prostaglandin D synthase (PGDS), prostaglandin E synthase (PGES), prostaglandin F synthase (PGFS), prostaglandin I synthase (PGIS) and thromboxane A synthase (TXAS), which form PGE_2, $PGF_2\alpha$, PGI_2 (also known as prostacyclin) and TxA_2, respectively, as represented in **Figure 9**. The differential expression of these enzymes in each tissue and cell determines the profile of prostanoid production (Smyth et al. 2009; Stables et al. 2011).

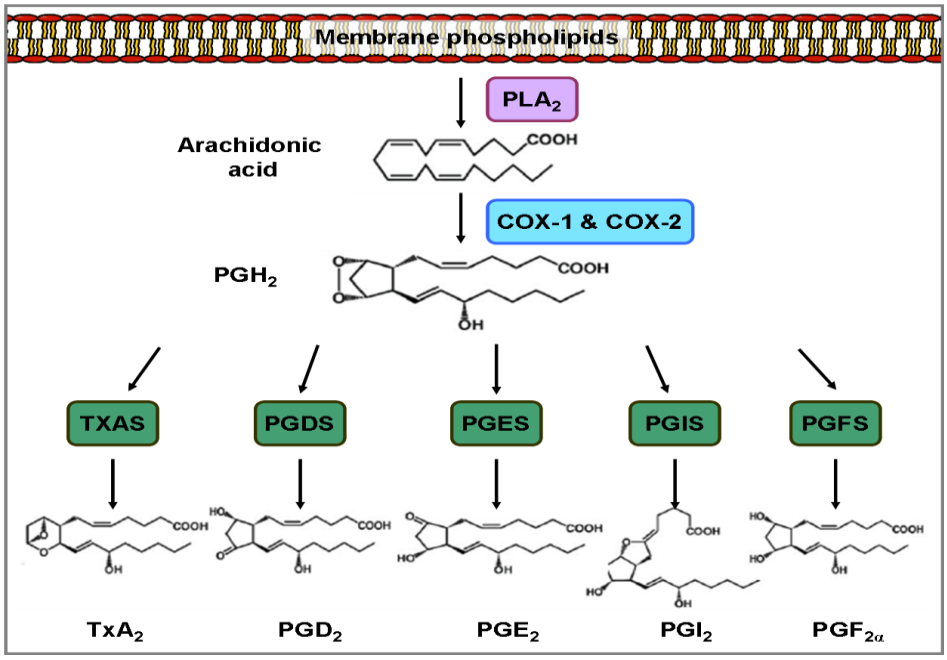

Figure 9. Biosynthethic pathway of prostanoids.

Isoform-specific preference for downstream enzymes has been reported in heterologous expression systems, although their biological relevance is unknown. COX-1 couples preferentially, but not exclusively, with TXAS, PGFS and the cytosolic PGES isozymes. COX-2 prefers PGIS and the microsomal (m) PGES isozymes, both of which are induced by cytokines and tumour promoters. The production of prostanoids is determined by the different PG synthases existing in every cell type, by the availability

of AA and by cyclooxygenase activity, among other factors (Smyth et al. 2009; Stables et al. 2011).

6.2 Prostanoids. The prostanoids (PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and TxA_2) are metabolites that exert their biological effects in the proximity of the sites of their synthesis, in autocrine or paracrine manner. These mediators play an important role in the inflammatory process. In inflamed tissues, their biosynthesis is significantly increased and they contribute to the development of the main signs of acute inflammation. Moreover during an inflammatory response, the level and profile of prostanoids production change significantly (Ricciotti et al. 2011).

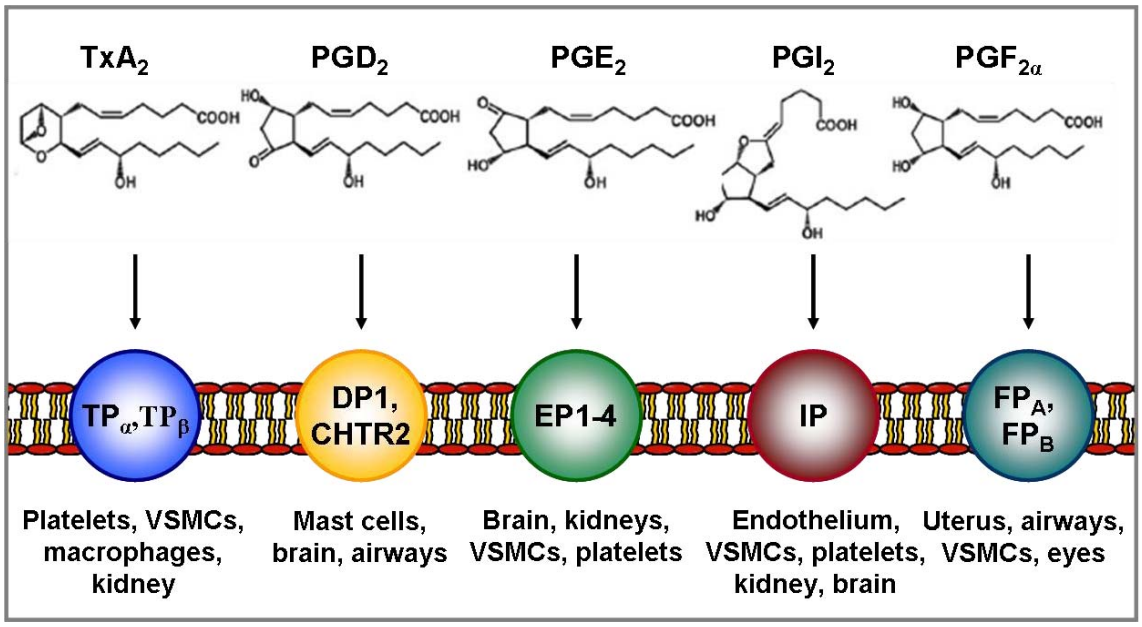

Figure 10. Prostanoids receptors subtypes and tissue expression.

The prostanoids activate G-protein-coupled receptors or nuclear receptors in target cells. The prostanoid receptor subfamily comprises eight members (DP, EP1-4, FP, IP and TP), classified according to the prostanoid ligand with whichthey bind with the greatest affinity, as is represented in **Figure 10** (Hata et al. 2004).

7. Prostaglandin E2 (PGE_2).

PGE_2 is one of most abundant prostaglandins produced in the body and exhibits a multiplicity of biological activities. The physiological effects of PGE_2 include control of

gastric acid secretion and regulation of renal blood flow, as well as several aspects of the female reproductive function, such as ovulation and fertilization (Rocca 2006).

Moreover, PGE_2 has been shown to play an important role in regulating inflammatory processes. Commonly considered a potent proinflammatory mediator, PGE_2 is actively involved in the pathogenesis of several diseases, ranging from inflammatory states such as periodontitis, UVB-mediated cutaneous inflammation and rheumatoid arthritis to cancer growth (Vancheri et al. 2004).

7.1. Prostaglandin E2 receptors (EP). As previously mentioned, the activity of PGE_2 is mediated by four receptors, termed prostaglandin E_2 receptors (EP) (EP1-EP4), which are encoded by distinct genes and have divergent aminoacid sequences. Thus, the multiple receptor subtypes allow PGE_2 to trigger several intracellular signal transduction pathways and have diverse end resultts, which sometimes seem to be in opposition, functionally, within the same cell or organ.

The complexity of PGE_2 responses is further complicated by evidence that multiple EP receptors are often co-expressed or induced in the same cell or organ. The regulation of this co-expression is still unknown, but the response to PGE_2 appears to be modulated on the basis of the activation of different pathways by various EP-receptor subtypes (Rocca 2006).

In the carboxy-terminal region the EP receptors are coupled to G proteins (GPCRs) that transmit various intracellular signals capable of activating different signal transduction pathways. The homology of the carboxy-terminal region is low between EP receptors, so there is a differential recruitment of different G proteins, which would explain why EP receptors can transmit different types of intracellular signalling (Sugimoto et al. 2007).

The EP1 receptor activates phosphotidylinositol metabolism, leading to the formation of inositol triphosphate (IP_3), along with the mobilization of intracellular free calcium (Ca^{2+}) (Vancheri et al. 2004). The EP1 receptor increases intracellular Ca^{2+} levels through $G_{\alpha q}$ protein association, and it is involved in pain perception and blood pressure regulation (Stock et al. 2001).

The EP2 and EP4 receptors stimulate adenylate cyclase (AC), leading to the production of cyclic adenosine 3', 5'-monophosphate (cAMP), which activates the cAMP-dependent protein kinase A (PKA). Stimulation of EP4 also activates phosphoinositide-3'-kinase (PI3K) (Vancheri et al. 2004). The EP2 and EP4 receptors are coupled to $G_{\alpha s}$ proteins, which increase intracellular cAMP levels. EP2 regulates vasodilatation, tumor proliferation and angiogenesis, while EP4 acts by regulating kidney function and osteoclastogenesis (Breyer et al. 2000; Regan 2003). According to the inflammatory response, EP2 and EP4 are the receptors responsible for PGE_2 activity during immune response (Fujino et al. 2003).

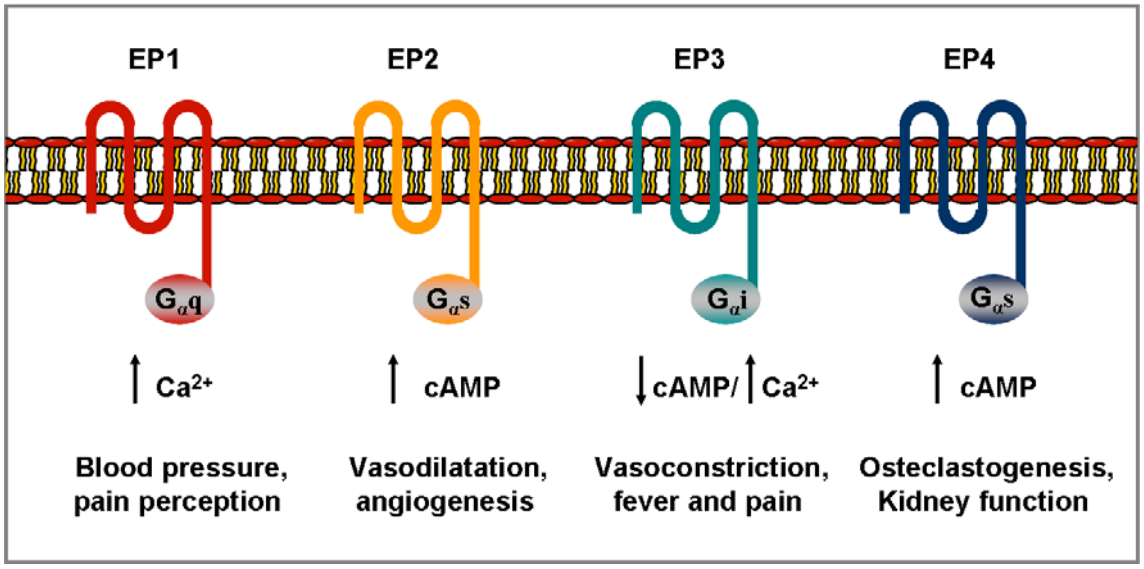

Figure 11. Schematic representation of EP receptor signalling and their functions.

EP3 is the only receptor that possesses multiple splice variants and the different isoforms couple to multiple G proteins, producing either inhibition of AC or stimulation of AC activity (Vancheri et al. 2004). Finally, the signal transduction mediated by EP3 is the most complex of all as there are 8 different isoforms in humans that, according to the length of the carboxy-terminal region, could couple to $G_{\alpha s}$, $G_{\alpha i}$ o $G_{\alpha q}$ (Bilson et al. 2004). EP3 participates in the generation of fever and pain, and in vasoconstriction (Kobayashi et al. 2002).

8. Protective role of PGE_2 in the airways.

Commonly considered a potent pro-inflammatory mediator, PGE_2 has been reported to have several protective effects in the airways of asthmatic patients, in contrast to the rest of the body, (Vancheri et al. 2004; Jaffar et al. 2007).

The anti-asthmatic effect of PGE_2 has been reported in patients since the 1970s (Smith 1975; Szczeklik et al. 1977). Clinical studies have demonstrated that PGE_2 administered as an aerosol in asthmatic patients prevents inflammation and hyperreactivity in the airways, in both allergic asthma and non-allergic asthma (Sestini et al. 1996; Gauvreau et al. 1999). Pavord and cols have shown that inhaled PGE_2 protects against bronchial hyperreactivity (Pavord et al. 1991). Many subsequent studies have observed the bronchodilator effect of PGE_2 in normal subjects and in patients with asthma and chronic bronchitis, showing that PGE_2 attenuates bronchoconstriction (Sastre et al. 2012). Moreover, it has been reported that PGE_2 modulates airway remodelling in chronic asthma and lung fibrosis (Pierzchalska et al. 2003). It has been also demonstrated that PGE_2 inhibits collagen synthesis, fibroblasts-myofibroblast transition and regulation in the reparation of the airway epithelium (Burgess et al. 2004; Petkova et al. 2004; Vancheri et al. 2004).

The protective effects of PGE_2 are mainly mediated by EP2 and EP4 receptors. It has been reported that PGE_2 mediates bronchodilatation via the EP2 receptor (Kay et al. 2006), as well as anti-inflammatory effects via the EP2 and/or EP4 receptor, by inhibiting the production of some chemokines (Takayama et al. 2002). When PGE_2 interacts with EP2 in smooth muscle cells it induces relaxation and inhibits the proliferation of those cells involved in AHR, broncochonstriction and remodelling (Mori et al. 2011).

9. Arachidonic acid metabolism alterations in CRSwNP and AIA.

Several studies have described abnormalities in the arachidonic acid metabolism in patients with aspirin-induced asthma patients, where the interplay between the COX and the LOX pathways is evident and results in several alterations in

the biosynthesis of eicosanoids. Several studies have also reported changes in the 15-LOX and lipoxin pathways in these patients (Vancheri et al. 2004; Picado 2006).

9.1. Alterations in the 5-LOX pathway in CRSwNP and AIA. Various studies have clearly demonstrated that the LOX pathway is more active in asthmatic patients than in control subjects. Moreover, this pathway seems to be more highly activated in patients with AIA than in aspirin-tolerant patients (Picado 2006). It has been suggested that, in patients suffering from AIA, the inhibition of the COX pathway by aspirin or some other NSAID may cause shunting of AA into the 5-LOX pathway. This condition could trigger the synthesis of Cys-LTs (LTC_4, LTD_4 and LTE_4) by the 5-LOX/LTC_4 synthase pathway, generating an increased amount of these substances with bronchoconstrictor and vasoactives properties (Vancheri et al. 2004; Adamjee et al. 2006). It has been demonstrated that the enzyme LTC_4 synthase is much more highly expressed in the bronchial biopsies and nasal polyps of patients with AIA than in aspirin-tolerant (AT) asthmatic patients and control subjects (Cowburn et al. 1998; Adamjee et al. 2006; Farooque et al. 2009). Moreover, it has been also demonstrated that the Cys-LTs receptor 1 is overexpressed in nasosinusal mucosa from AIA patients (Perez-Novo et al. 2005).

Thus, the abnormal activity of the 5-LOX pathway leads to the baseline over-production of Cys-LTs in patients with CRSwNP, especially those with AIA, which is further increased when patients are exposed to aspirin or other NSAIDs (Picado et al. 1999; Kowalski et al. 2000).

9.2. Alterations in the COX pathway in CRSwNP and AIA. Alterations in the COX pathway have been also described in patients with CRSwNP and AIA (Picado 2006; Stevenson et al. 2006). However, the precise role of the COX pathway in the pathogenesis of CRS and AIA remains to be elucidated. While aspirin and other NSAIDS have precipitated asthma attacks in aspirin-sensitive patients, some clinical studies have demonstrated that selective COX-2 inhibitors such as rofecoxib do not precipitate bronchoconstriction in AIA patients (Dahlen et al. 2001; Stevenson et al. 2001), although COX-2 expression and activity are reduced in AIA patients (Szczeklik et al. 2006). So, the reduced PGE_2 concentrations in airways from AIA patients seem to be

related to the combination of both low COX-2 expression and general COX inhibition by aspirin or other NSAIDs.

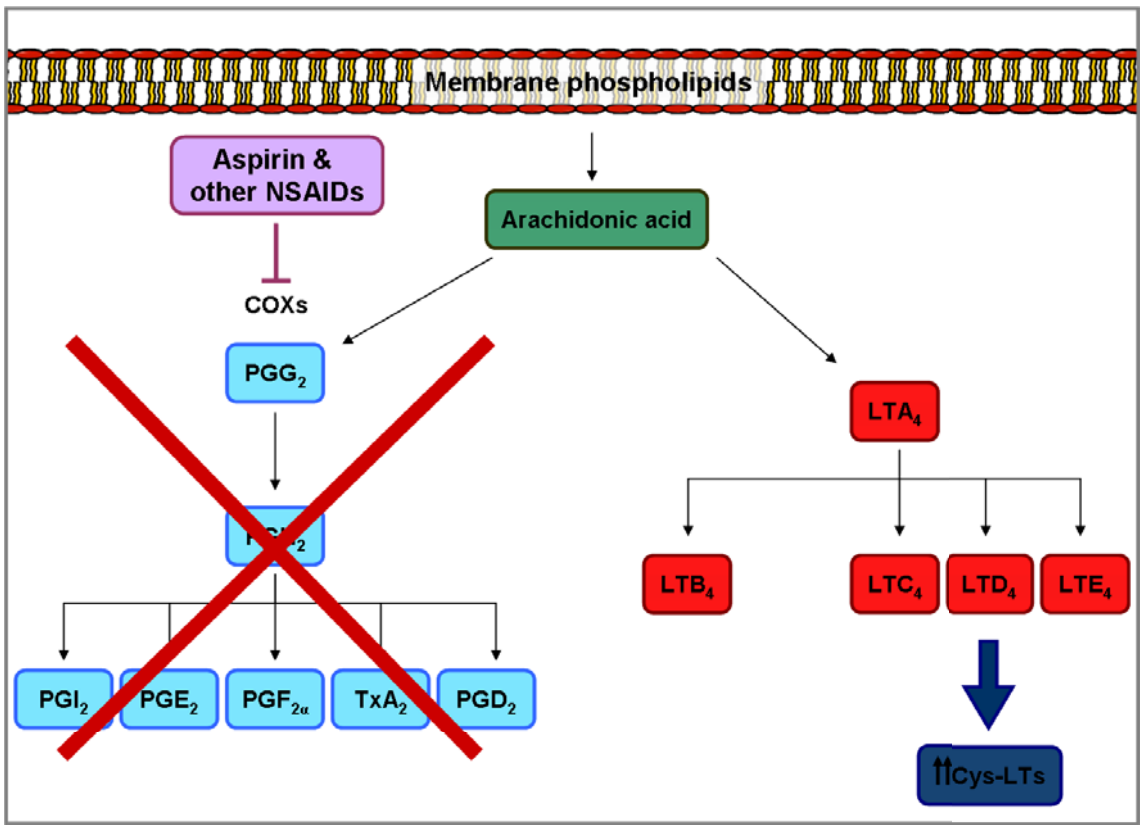

Figure 12. **Schematic representation of the effects of aspirin/NSAIDs on AIA patients.** The image shows the inhibition of the COX pathway by NSAIDs and the increase in Cys-LT production described in AIA patients along the 5-LOX pathway.

However, and in contrast with the published findings on the lipoxygenase pathway, some, but not all, studies, have reported anomalies in the regulation of the COX pathway. Various studies have reported a reduced production of PGE_2, associated with a downregulation in the expression of COX enzymes in NP tissue, as well as in epithelial cells derived from the NPs of patients with aspirin-induced asthma (Picado et al. 1999; Pujols et al. 2004; Perez-Novo et al. 2005; Yoshimura et al. 2008). All in all, these studies suggest that dysregulation of COX could play a role in both AIA and CRSwNP, since these alterations have been found in the upper and lower airways. I contrast, studies that used other models and methodologies, such as bronchial tissue or bronchial epithelial cells, have not found any differences in the expression of either COX-1 or COX-2 enzymes between patients with AIA or ATA and control subjects (Cowburn et al. 1998; Pierzchalska et al. 2003; Pierzchalska et al. 2007). In summary,

whether anomalies in the regulation of the COX pathway play a role in the pathogenesis of AIA is still a matter of debate.

2. Hypothesis and objectives

Hypothesis

As mentioned above, several anomalies in the lypoxygenase pathway have been extensively reported in aspirin-induced asthma patients. In contrast to the reported findings in the lypoxigenase pathway, the altered regulation of cyclooxygenase enzymes has been reported in some but not all the published studies, and consequently their role in the pathogenesis of aspirin-induced asthma is a matter of debate. There are no clear explanations for the observations reported in the regulation of COX enzymes in patients with asthma, nasal polyps and aspirin intolerance.

We hypothesized that the reported discrepancies are due to the use of different techniques and methods, as well as the lack of studies focusing on the simultaneous assessment of the multiple factors involved in the COX pathway (prostaglandins, enzymes and prostaglandin receptors).

Our hypothesis also establishes a definitive clarification of the controversy requires a comprehensive study using different techniques to assess the regulation of the COX pathway in tissues obtained from healthy airways and from inflamed airways of asthma patients with and without aspirin intolerance.

General objectives

1) To study the regulation of the cyclooxygenase pathway, at baseline and under inflammatory conditions in asthma patients with and without aspirin intolerance.

2) To study the anomalies in the signal transduction pathways that can account for the the abnormal regulation of COX-2 expression in asthma and aspirin-induced asthma

Specific objectives

Study 1. To study the cyclooxygenase pathway in fibroblasts from nasal polyps of patients with and without aspirin-induced asthma.

- To measure cyclooxygenase (COX-1 and -2) expression and PGE_2 secretion in fibroblasts from human nasal mucosa (control group) and nasal polyps of patients with and without aspirin-induced asthma at baseline level and under inflammatory conditions (IL-1β).

- To measure the expression of PGE_2 receptors in fibroblasts from the nasal polyps of patients with and without aspirin-induced asthma at baseline level and under inflammatory conditions (IL-1β).

Study 2. To study the cyclooxygenase pathway in fibroblasts from the human nasal mucosa of patients with aspirin-induced asthma.

- To measure cyclooxygenase (COX-1 and -2) expression and PGE_2 secretion in fibroblasts from the human nasal mucosa of subjects with and without aspirin-induced asthma at baseline level and under inflammatory conditions (IL-1β).

Study 3. To investigate the role of signal transduction pathways (MAPKs, NF-κB, and C/EBP) in the abnormal regulation of COX-2 in fibroblasts from asthma patients with and without aspirin intolerance.

3. Research work

List of Publications

Study 1. Roca-Ferrer, J., F. J. Garcia-Garcia, J. Pereda, M. Perez-Gonzalez, L. Pujols, I. Alobid, J. Mullol and C. Picado (2011). "Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma." *J Allergy Clin Immunol* 128(1): 66-72 e61. IF: 11.003

Study 2. Roca-Ferrer, J., M. Perez-Gonzalez, F. J. Garcia-Garcia, J. Pereda, L. Pujols, I. Alobid, J. Mullol and C. Picado (2013). **"**Low Prostaglandin E2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics." *Respirology* (accepted 10/12/2012). IF: 2.416

Study 3. Garcia-Garcia FJ, Mullol J, Perez-Gonzalez M, Pujols L, Alobid I, Mullol J, Picado C (2012). Signal Transduction Pathways (MAPKs, NF-κB, and C/EBP) Regulating COX-2 Expression in Nasal Fibroblasts from Asthma Patients with Aspirin Intolerance. PLoS ONE 7(12): e51281. doi:10.1371/journal.pone.0051281 IF: 4.092

The total impact factor accomplished is: 17.511

"Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma."

Roca-Ferrer, J., F. J. Garcia-Garcia, J. Pereda, M. Perez-Gonzalez, L. Pujols, I. Alobid, J. Mullol and C. Picado.

J Allergy Clin Immunol 2011; 128(1): 66-72 e61. IF: 11.003

Estudi 1. Reducció en l'expressió de COXs i producció de prostaglandina E(2) en pacients amb poliposi nasal amb o sense asma induïda per aspirina.

Antecedents i objectiu: existeix debat en com la regulació de les ciclooxigenases (COX-1 i COX-2), que medien la producció de les prostaglandines (PGs), afecta en la patogènesi dels pòlips nasals (NPs) i l'asma induïda per aspirina (AIA). L'objectiu d'aquest estudi va ser investigar el paper de la PGE_2, la COX-1 i la COX-2, així com els receptors de PGE_2 en el desenvolupament de NPs i AIA mitjançant la mesura de la seva expressió en fibroblasts derivats de mucosa nasal (NM) i NPs per tal de contribuir a clarificar el debat.

Mètodes: van aïllar-se els fibroblasts de NM d'individus sense asma que patien desviació septal, hipertròfia dels cornets nasals, o totes dues afectacions (individus control=7), i de NPs procedents tant de pacients no asmàtics tolerants a l'aspirina (n=7) com de pacients amb asma i intolerància a l'aspirina (n=7). Les mostres de pòlips nasals van recollir-se durant cirurgia endoscòpica. Els cultius de fibroblasts obtinguts van estimular-se amb IL-1β (10 ng/mL) durant 72 hores. La secreció de PGE_2, l'expressió de COX-1 i de COX-2 i l'expressió dels receptors de PGE_2 (EP1-4), van mesurar-se mitjançant ELISA, Western blot i immunofluorescència.

Resultats: després del tractament amb IL-1β, la secreció de PGE_2 va ser significativament menor als fibroblasts procedents de pacients amb NPs i tolerància a l'aspirina, i encara més reduïda en aquells fibroblasts procedents de pacients amb NPs i AIA, comparada amb la secreció dels fibroblastes d'individus control (NM). De forma similar, l'exposició a IL-1β va induir de forma significativa l'expressió de COX-1 i COX-2 als fibroblasts obtinguts de NM de subjectes controls, aquesta inducció va ser moderada als fibroblasts de NPs de pacients no asmàtics tolerats a l'aspirina, i pràcticament no va tenir efecte en els fibroblasts procedents de NPs de pacients amb AIA. La incubació amb IL-1β també va induir l'expressió del receptor EP2 als fibroblasts controls procedents de NM, però no en aquells fibroblasts procedents de NPs de pacients no asmàtics tolerants a l'aspirina ni d'aquells amb AIA.

Conclusions: Aquest estudi confirma les alteracions descrites en la via de la COX (com ara, reducció en la producció de PGE_2 i increment disminuït de l'expressió de COX-1 i COX-2, així com del receptor EP2 en condicions inflamatòries) en el pòlips nasals en pacients amb o sense AIA, les quals podem contribuir a la inflamació persistent de les vies aèries i al desenvolupament del fenomen de la intolerància a l'aspirina.

Reduced expression of COXs and production of prostaglandin E_2 in patients with nasal polyps with or without aspirin-intolerant asthma

Jordi Roca-Ferrer, PhD,[a,b] Francesc J. Garcia-Garcia, MS,[a,b] Javier Pereda, PhD,[a,b] Maria Perez-Gonzalez, MS,[a,b] Laura Pujols, PhD,[a,b] Isam Alobid, MD, PhD,[a,b,c] Joaquim Mullol, MD, PhD,[a,b,c]* and Cesar Picado, MD, PhD[a,b,d]*

Barcelona, Spain

Background: Researchers have debated whether regulation of the COX enzymes (COX-1 and COX-2), which mediate production of prostaglandins (PGs), affects the pathogenesis of nasal polyps (NPs) and aspirin-intolerant asthma (AIA).

Objective: We investigated the roles of PGE_2, COX-1 and COX-2, and PGE_2 receptors in the development of NPs and AIA by measuring their expression in fibroblasts derived from nasal mucosa (NM) and NPs.

Methods: Fibroblasts were isolated from the NM of subjects without asthma who had septal deviation, turbinate hypertrophy, or both (control subjects, n = 7); NPs of aspirin-tolerant nonasthmatic patients (n = 7); and NPs of patients with asthma who were intolerant of aspirin (n = 7). Polyp samples were collected during endoscopic surgery. Cultures were stimulated with IL-1β (10 ng/mL) for 72 hours. We used ELISA, immunoblotting, and immunofluorescence analyses to measure secretion of PGE_2, expression of COX-1 and COX-2, and expression of the PGE_2 receptors EP1 to EP4.

Results: Compared with NM from control subjects, PGE_2 concentrations were significantly lower in IL-1β–stimulated fibroblasts from patients with NPs who were tolerant to aspirin and even lower in polyps from patients with AIA. Similarly, IL-1β exposure induced the expression of COX-1 and COX-2 in fibroblasts from NM of control subjects, had only moderate effects on fibroblasts from NPs of aspirin-tolerant nonasthmatic patients, and almost no effect on fibroblasts from NPs of patients with AIA. IL-1β also induced expression of EP2 in fibroblasts from control NM but not in fibroblasts from NPs of aspirin-tolerant nonasthmatic patients or those with AIA.

Conclusion: Alterations in the COX pathway (ie, reduced production of PGE_2 and lack of upregulation of COX-1, COX-2, and EP2 under conditions of inflammation) are associated with NPs in patients with or without AIA. (J Allergy Clin Immunol 2011;128:66-72.)

Key words: *Aspirin intolerance, asthma, chronic rhinosinusitis, cyclooxygenase, fibroblast, nasal mucosa, nasal polyp, prostaglandin E_2, α-smooth muscle actin*

In mammalian cells activation of phospholipase A_2 results in release of arachidonic acid (AA) from membrane phospholipids. AA is in turn transformed by COX and lipoxygenase pathways into eicosanoids. There are at least 2 isoforms of COX enzymes. COX-1 is generally constitutively expressed and considered to be a housekeeping enzyme,[1] whereas COX-2 is induced under chronic inflammatory conditions, such as arthritis,[2] periodontitis,[3] cystic fibrosis,[4] and *chronic obstructive pulmonary disease.*[5,6] COX-2 activation leads to synthesis of several prostaglandins (PGs). Of these, PGE_2 seems to have an important role in inflammatory processes. COX-1 and COX-2 are the targets of classical nonsteroidal anti-inflammatory drugs (NSAIDs), and there are selective inhibitors of COX-2.[1]

Bronchial asthma is a chronic inflammatory disorder that, unlike other inflammatory diseases, does not respond to the anti-inflammatory effects of COX inhibitors, such as NSAIDs. In fact, ingestion of NSAIDs even induces bronchoconstriction in some patients,[7] which is called aspirin-intolerant asthma (AIA). The pathogenic mechanism of AIA is believed to involve altered metabolism of AA, although the putative biochemical defects have not been completely elucidated.[7] Interestingly, selective inhibitors of COX-2 appear to be well tolerated by patients with AIA.[8,9] These observations indicate that prostanoids are regulated differently in patients with AIA than in those with other inflammatory diseases.[7,9] Moreover, these abnormalities in AA metabolism might not occur in only the lower airways because these patients typically have chronic rhinosinusitis with nasal polyps (NPs).[7,9]

Many studies have reported increased production of cysteinyl leukotrienes in bronchial mucosa and NPs of patients with AIA.[7,9] Increased release of cysteinyl leukotrienes correlates with upregulated expression of enzymes in the lipoxyenase pathway, particularly of leukotriene C_4 synthase.[7] Altered expression of COX has also been described in the airways of patients with AIA. However, and in contrast to the reported findings in the lipoxygenase pathway, altered regulation of COX enzymes has

From [a]Immunoallèrgia Respiratòria Clínica i Experimental, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS); [b]Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES); [c]Unitat de Rinologia i Clínica de l'Olfacte, Servei d'Otorrinolaringologia, Hospital Clínic; and [d]Servei de Pneumologia i Allèrgia Respiratoria, Hospital Clínic, Universitat de Barcelona.

*These authors contributed equally to this work.

Supported by grants from the Spanish Ministry of Health (FIS PI030033, FIS PI080249), Fundació Catalana de Pneumologia (FUCAP), and Fundación Respira (Spanish Respiratory Society).

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication October 10, 2010; revised January 14, 2011; accepted for publication January 18, 2011.

Available online March 12, 2011.

Reprint requests: Cesar Picado, MD, PhD, Servei de Pneumologia, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. E-mail: cpicado@ub.edu.

0091-6749/$36.00

doi:10.1016/j.jaci.2011.01.065

Abbreviations used
AA: Arachidonic acid
AI: Aspirin intolerant
AIA: Aspirin-intolerant asthma
AT: Aspirin tolerant
ATA: Aspirin-tolerant asthma
EP: Prostaglandin E receptor
NM: Nasal mucosa
NP: Nasal polyp
NSAID: Nonsteroidal anti-inflammatory drug
PG: Prostaglandin
SFM: Serum-free media

been reported in some but not all studies; their role in the pathogenesis of AIA is a matter of debate.[7,9] Several studies that assessed PGE_2 production in patients with AIA produced different results, ranging from reduced production[10-15] to normal production[16-18] or overproduction.[19] Because PGE_2 production requires expression of COX-2 and the PGE_2 synthases, the changes in levels of PGE_2 detected in patients with AIA might result from altered expression of these enzymes. Similar to the disparate results reported in levels of PGE_2 in patients with AIA, COX-2 expression has been reported to be downregulated in the upper airways of these patients[20-22] and upregulated in airway mast cells[23]; other studies did not detect differences in COX-2 expression among patients with AIA, patients with aspirin-tolerant asthma (ATA), patients with chronic rhinosinusitis, or control subjects.[11,24-26] Furthermore, decreased levels of PGE_2 synthase 1 were detected in nasal tissues of patients with chronic rhinosinusitis.[27]

Although COX-1 is not considered to be involved in inflammatory responses,[1] some studies found altered expression in airways of patients with aspirin sensitivity,[11,21,28] and other studies did not report differences.[20] PGE_2 signals through 4 G protein–coupled receptors (prostaglandin E receptors 1-4 [EP1-EP4]).[29] Expression of EP2 has been reported to be reduced in nasal mucosal inflammatory cells of patients with AIA.[30]

There are no clear explanations for the reported discrepancies in regulation of COX enzymes in patients with asthma and NPs; they might be caused, at least in part, by differences in study methods.[21] Regulation of multiple factors in the COX pathway (ie, PGs, enzymes, and PG receptors) has not been assessed in patients with NPs and AIA, and therefore the relationships among different components of the metabolic pathways have not been determined for this disorder. We investigated the levels of the COX enzymes, PGE_2, and EP1 through EP4 in nasal fibroblasts from patients with polyps and from patients with NPs and AIA. Previous studies have shown that alterations in the production of PGE_2 in NPs can occur in the absence of associated asthma.[21,22] However, isolated nasal polyposis in patients with aspirin intolerance is rarely seen because it is almost always associated with asthma.[7]

METHODS

Nasal mucosa (NM) specimens were obtained from 7 subjects without asthma who had septal deviations, turbinate hypertrophy, or both and underwent corrective surgery (control-NM). All the control subjects had taken normal doses of aspirin or NSAIDs without adverse reactions (asthma, rhinitis, urticaria, angioedema, or anaphylaxis). NP specimens were collected from 7 patients with asthma, chronic rhinosinusitis, and aspirin intolerance (NP-AI) who were treated with endoscopic surgery. NP samples were also collected, by using the same procedure, from 7 patients without asthma who tolerated aspirin (NP-AT). The diagnosis of aspirin intolerance was confirmed by means of lysine-aspirin nasal challenge, as previously described.[31] None of the subjects received oral or intranasal corticosteroids for at least 1 week before the operation or had upper airway infections in the 2 weeks before the operation. All patients provided informed consent to participate in the study, which was approved by the scientific and ethics committee of our institution. Demographic and clinical characteristics of subjects are shown in Table I.

TABLE I. Epidemiologic characteristics of control subjects and patients with NPs

Characteristics	Control-NM fibroblasts	NP-AT fibroblasts	NP-AI fibroblasts
Fibroblast cultures (no.)	7	7	7
Age (y)	33.9 ± 2.9	44.1 ± 16.6	48.1 ± 2.7
Female sex, no. (%)	1 (14.3)	2 (28.6)	4 (57.1)
Asthma (%)	0	0	100
Aspirin intolerance (%)	0	0	100
Atopy, no. (%)	3 (42.8)	2 (28.6)	0 (0)
Blood eosinophilia (%)	2.0 ± 1.2	4.7 ± 3.6	10.0 ± 1.8
Intranasal corticosteroid, no. (%)	0 (0)	6 (85.7)	7 (100)

For more information on tissue handling and cell culture; experimental protocols; PGE_2 and COX-2 ELISA, immunoblotting, and immunofluorescence; and statistical analysis, see the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Culture characterization

Cultures of control-NM, NP-AT, and NP-AI fibroblasts incubated with serum-free media (SFM) for 24 hours did not contain cytokeratin-positive cells, whereas all cells were positive for vimentin (Fig 1). No significant differences were observed in the percentages of myofibroblasts in these cultures (control-NM, 1.5% [25th-75th interquartile, 1.3% to 3.0%]; NP, 4.7% [25th-75th interquartile, 2.4% to 8.7%]; and NP-AIA, 4.5% [25th-75th interquartile, 2.2% to 5.7%]; n = 5).

PGE_2 production

There were no significant differences in the production of PGE_2 at baseline in cultured fibroblasts isolated from control-NM or NP samples. Quiescent cells were incubated with SFM in the presence or absence of 10 ng/mL IL-1β for 24 hours, and PGE_2 secretion was analyzed in culture supernatants collected after 4 and 24 hours. Compared with cells in only SFM, control-NM cells incubated with IL-1β had an increased ratio of PGE_2/total protein after 4 and 24 hours. In contrast, the ratio of PGE_2/total protein did not increase in NP-AT or NP-AI cells after 4 hours incubation with IL-1β, and there was a slight increase after 24 hours. The lowest ratio of PGE_2/total protein was observed in cells from NP-AI tissues; values were significantly different from those of control-NM cells at 4 and 24 hours and from those of NP-AT cells at 4 hours (Fig 2).

COX expression

PGE_2 secretion requires COX-1 and COX-2 activity, and therefore we measured the expression of both enzymes in quiescent

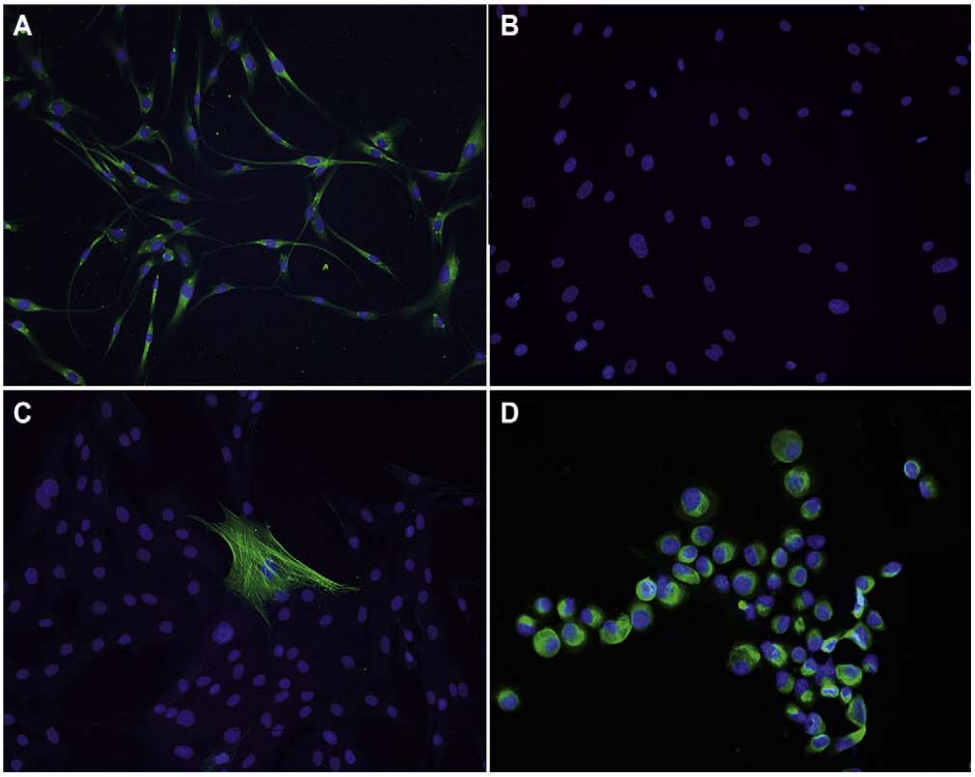

FIG 1. Characterization of cultured cells. Representative images from immunofluorescence analysis of quiescent control-NM fibroblasts. **A**, Presence of vimentin fibers (green) confirms the fibroblast phenotype of cells in culture. **B**, Absence of staining for cytokeratins demonstrates the lack of epithelial cells. **C**, Detection of α-smooth muscle actin fibers confirms the presence of myofibroblasts. **D**, Positive control for cytokeratin in NM epithelial cells (original magnification ×200).

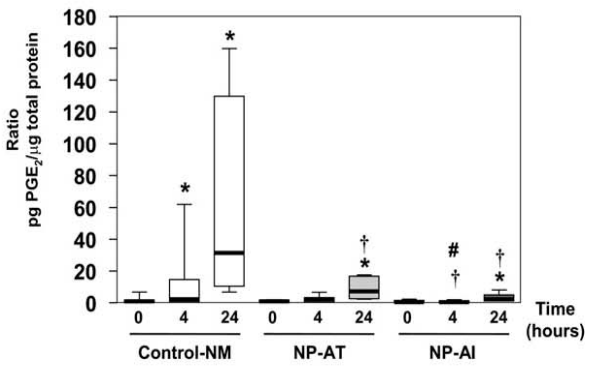

FIG 2. Time course of IL-1β on secretion of PGE_2 protein. Fibroblasts were incubated with 10 ng/mL IL-1β. The concentration of PGE_2 (n − 7) was measured by means of ELISA. *$P < .05$ compared with control media. †$P < .05$ compared with control-NM fibroblasts incubated with IL-1β. #$P < .05$ compared with NP-AT fibroblasts incubated with IL-1β.

fibroblasts incubated in SFM in the presence or absence of 10 ng/mL IL-1β. Previous studies used various methods to measure COX-2 expression in NP samples and had varying results, and therefore we used several different techniques to quantify expression of COX-2: ELISA, immunoblotting, and immunofluorescence analyses. Measured by means of ELISA, exposure of control-NM fibroblasts to IL-1β increased expression of COX-2 after 4 and 24 hours. In contrast, the cytokine had a slight nonsignificant effect on the induction of COX-2 in NP-AT and NP-AI fibroblasts. Compared with control-NM samples, the mean increase in the expression of COX-2 protein was significantly lower in NP-AT fibroblasts after 24 hours and in NP-AI fibroblasts after 4 and 24 hours. The lowest expression level of COX-2 was observed in fibroblasts from patients with NP-AI, which was significantly lower than that from NP-AT tissue at 24 hours (Fig 3, *A*).

In immunoblot analyses, compared with fibroblasts cultured in SFM, IL-1β increased the ratio of COX-2/β-actin in only control-NM fibroblasts. COX-2 expression was significantly higher in IL-1β–stimulated control-NM fibroblasts than in NP-AT or NP-AI fibroblasts at 24 hours (Fig 3, *B*). Immunofluorescence analysis demonstrated a significant increase in the number of COX-2–positive fibroblasts from all tissues after incubation with IL-1β compared with cells incubated in SFM. However, the percentage of COX-2 control-NM fibroblasts was significantly greater than that of NP-AT or NP-AI fibroblasts after 24 hours incubation with IL-1β (Fig 3, *C* and *D*).

Although COX-1 is considered a noninducible enzyme, its expression can increase moderately in proinflammatory conditions. In contrast with COX-2, COX-1 protein is very stable and remains in cultured cells for several hours after its synthesis. We measured the effect of 10 ng/mL IL-1β on expression of COX-1 protein at 24, 48, and 72 hours. Compared with control-NM cells cultured in SFM, control-NM cells incubated with IL-1β expressed significantly higher levels of COX-1 protein after 72 hours. However, fibroblasts from NP-AT or NP-AI tissues did not alter expression of COX-1 during incubation with IL-1β (Fig 4).

EP expression

There were no differences in baseline expression levels of EP1 through EP4 among fibroblasts from control-NM, NP-AT, or

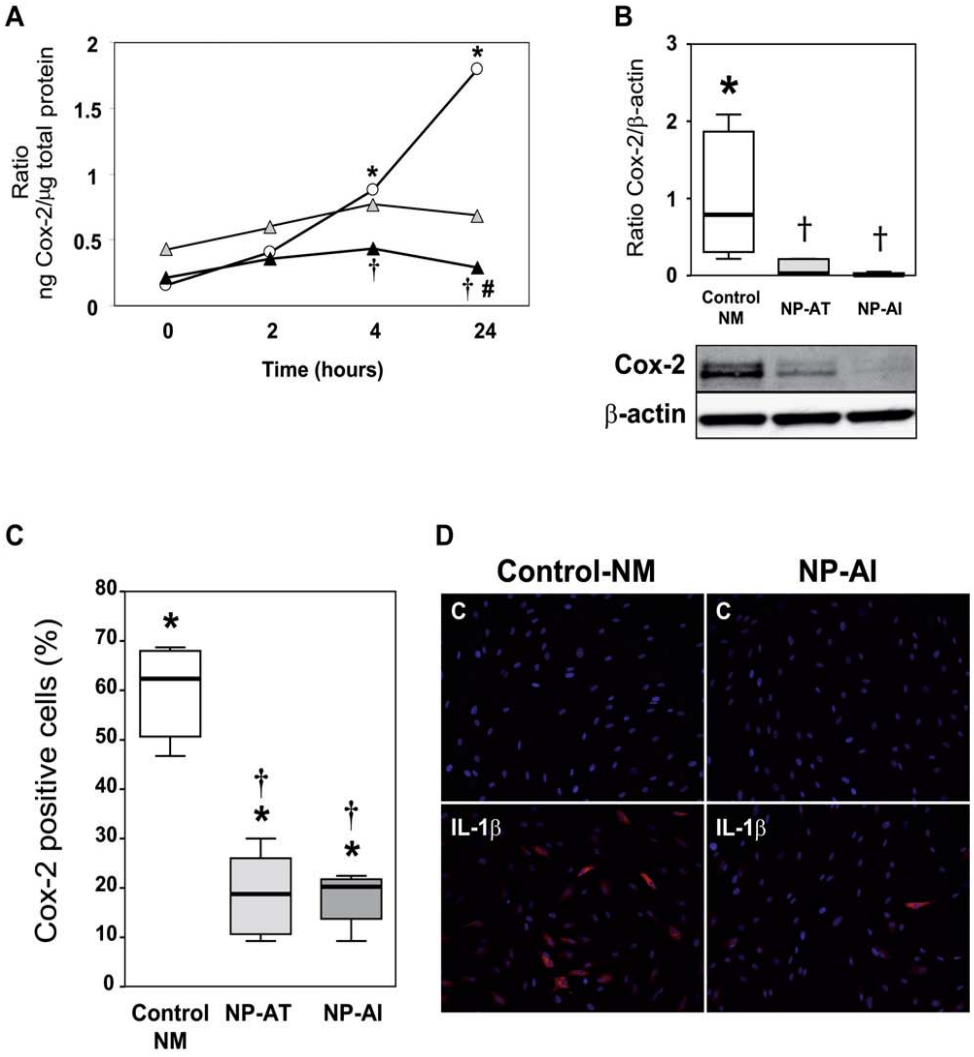

FIG 3. Effect of IL-1β on expression of COX-2 protein. Fibroblasts were incubated with 10 ng/mL IL-1β (n = 7). **A**, ELISA analysis of COX-2 protein expression in control-NM *(white circles)*, NP-AT *(gray triangles)*, and NP-AI *(black triangles)* fibroblasts. **B-D**, Immunoblot (Fig 3, *B*) and immunofluorescence (Fig 3, *C* and *D*) analyses of COX-2 protein (red) after fibroblasts were incubated for 24 hours with IL-1β. *$P < .05$ compared with control media. †$P < .05$ compared with control-NM fibroblasts incubated with IL-1β. #$P < .05$ compared with NP-AT fibroblasts incubated with IL-1β.

NP-AI tissues cultured in SFM (Table II). However, the ratio of EP2/β-actin increased during incubation of control-NM fibroblasts with 10 ng/mL IL-1β for 24, 48, and 72 hours. However, this ratio did not increase in fibroblasts from NP-AT or NP-AI tissues compared with that seen in cells cultured in SFM (Fig 5). Expression of EP1, EP3, and EP4 protein did not change in any fibroblast culture during incubation with IL-1β (data not shown).

DISCUSSION

We investigated secretion of PGE_2 and expression of COX enzymes and EP1 through EP4 in cultured fibroblasts isolated from control NM and NP samples from patients without asthma who are tolerant to aspirin and patients with asthma who are intolerant to aspirin.

We observed that IL-1β stimulates production of PGE_2 in fibroblasts from control-NM tissues but has a lower stimulatory effect on fibroblasts from NP-AT tissues and hardly any effect in fibroblasts from NP-AI tissues. Expression of COX-2 protein increased after incubation of fibroblasts from control-NM tissues with IL-1β but only slightly in fibroblasts from NP-AT tissues and hardly at all in fibroblasts from NP-AI tissues. IL-1β stimulation increased COX-1 protein expression in fibroblasts from control-NM tissue and to a smaller extent in fibroblasts from NP-AT tissue but did not alter COX-1 expression in fibroblasts from NP-AI tissues. IL-1β–upregulated EP2 in fibroblasts from control-NM tissues did not induce significant changes in levels of EP2 in fibroblasts from NPs. These results indicate that the COX pathway of AA metabolism is altered in fibroblasts from NPs of patients without asthma and those of patients with AIA.

Altered production of PGE_2 is a characteristic of nasal polyposis and AIA; *in vitro* studies have reported reduced production of PGE_2 in epithelial cells from NPs of patients with AIA,[12] bronchial fibroblasts from patients with AIA,[11] and peripheral blood leukocytes from patients with AIA,[13] as well as in NP samples from patients tolerant to aspirin or from patients with AIA.[14,22] *In vivo* studies have, however, reported varying results, with higher,[8] similar,[16,17] or lower,[10] levels of PGE_2 in nasal or bronchoalveolar fluids from patients with AIA compared with patients with ATA or healthy control subjects. These discrepancies

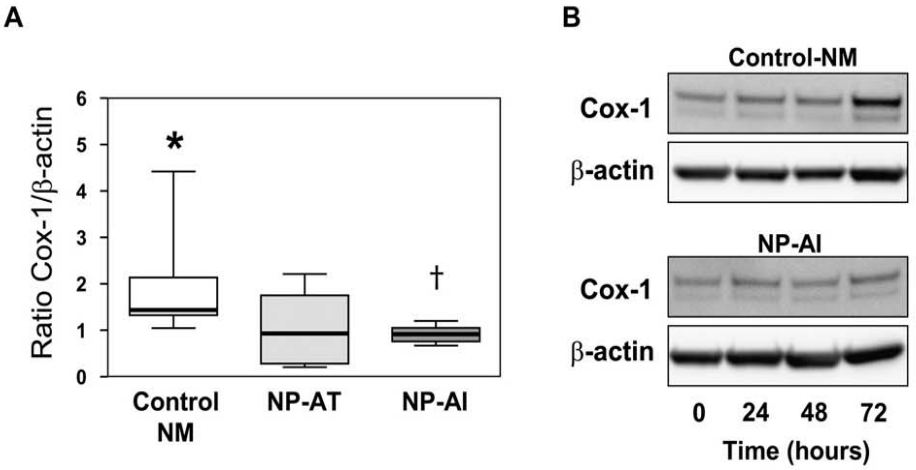

FIG 4. Effect of IL-1β on COX-1 protein expression. Fibroblasts were incubated with 10 ng/mL IL-1β for 72 hours (n – 7). **A**, COX-1 protein expression analyzed by means of immunoblotting. **B**, Representative immunoblot. *$P < .05$ compared with control media (COX-1/β-actin ratio, 1). †$P < .05$ compared with control-NM fibroblasts incubated with IL-1β.

TABLE II. Baseline levels of EP1 to EP4 in cultured fibroblasts

PG receptor subtypes	Control-NM fibroblasts (n = 7)	NP-AT fibroblasts (n = 7)	NP-AI fibroblasts (n = 7)
EP1	0.86 (0.66-1.22)	1.44 (0.91-1.91)	1.22 (0.86-1.82)
EP2	0.83 (0.55-1.02)	1.84 (0.62-2.19)	1.11 (0.29-1.39)
EP3	0.88 (0.63-0.96)	1.16 (0.73-1.44)	1.10 (0.82-1.50)
EP4	1.01 (0.91-1.45)	0.95 (0.80-1.54)	1.20 (1.00-1.24)

Data are represented as medians (25th-75th interquartiles) of the EP receptor/β-actin ratio. The Mann-Whitney *U* test was used for between-group comparisons. No significant differences were found.

probably result from limitations in bronchoalveolar and nasal lavage methodologies and the varying effects of dilution of lavage fluids. The presence of airway obstruction and inflammation in patients compared with control subjects could also affect collection of fluid samples. Studies that quantified levels of PGE_2 in urine had discrepant results: 1 study reported significantly lower concentrations of PGE_2 in urine from patients with AIA compared with those with ATA,[15] whereas another study reported no differences.[18] Studies that measured prostanoid production by using concentrated, exhaled air methodologies reported that patients with ATA and AIA had levels of PGE_2 that were similar to healthy control subjects.[32]

Findings of similar levels of PGE_2 among patients with AIA or ATA and healthy subjects have been used to argue against dysregulated PGE_2 synthesis in asthma.[32,33] However, production of PGE_2 should increase under conditions of inflammation; the similar production of PGE_2 in patients with asthma and healthy control subjects is an anomaly rather than a normal finding. PGE_2 release is increased, however, in other airway inflammatory diseases, such as cystic fibrosis[34] and chronic obstructive pulmonary disease.[6] The prostanoid pathway is therefore abnormally regulated in patients with NPs, especially among those with AIA.

We found that expression of COX-2 increased significantly in NM fibroblasts stimulated with a proinflammatory cytokine (IL-1β), but this response was reduced in fibroblasts from NP-AT tissue and almost undetectable in fibroblasts from NP-AI tissue. This observation concurs with the findings of Pérez-Novo et al[22] and Pujols et al,[21] who also observed reduced levels of COX-2 mRNA in NPs, especially from those of patients with AIA. Other studies, however, did not report abnormal regulation of COX-2 in the airways of patients with ATA or AIA. How can these discrepancies be explained? Pierzchalska et al[11] reported very low production of PGE_2 in bronchial fibroblasts from patients with AIA but did not detect differences in COX-2 expression in fibroblasts isolated from the same patients, control subjects, or patients with ATA. However, they measured COX-2 levels at only 1 time point (4 hours). Our study and other studies showed the importance of collecting samples at a number of time points to detect changes in levels of COX-2 in patients with AIA; the fact that Pierzchalska et al[11] did not use this approach could contribute to their findings.

Studies that used immunohistochemistry to quantify COX-2 expression have yielded contradictory results. In one study COX-2 was reportedly upregulated in mast cells from airways of patients with AIA,[23] whereas other studies reported no differences in expression of COX-2 in cells from upper[25,26] or lower airways[24] of patients with AIA, patients with ATA, or patients with chronic rhinosinusitis compared with healthy control subjects. The static characteristics of immunohistochemical analyses used in these studies[23-26] might account for their discrepancies with findings from studies that measured COX-2 kinetics levels by means of immunoblotting or RT-PCR assays.[21,35] Discrepancies might also result from the use of polyclonal versus mAbs to quantify levels of COX-2.[36] The accuracy of immunohistochemical analyses can also be limited by histological differences among tissues; COX-2 is located in the nuclear membrane, and antibodies used to detect it might penetrate the cells of the NM differently cells from NPs. To overcome these technical limitations, we studied COX-2 expression in isolated and stimulated fibroblasts using ELISA and immunoblot analyses and used immunostaining to confirm observed alterations in COX-2 expression in NP-AT and NP-AI tissues.

COX-1 is considered a constitutively expressed enzyme that responds to the physiological needs of cells but is not involved in inflammatory responses.[1] Some studies, however, reported small increases in its expression in inflamed tissues.[37] COX-1 was reported to be substantially upregulated in NPs from patients with chronic rhinosinusitis associated with cystic fibrosis, indicating

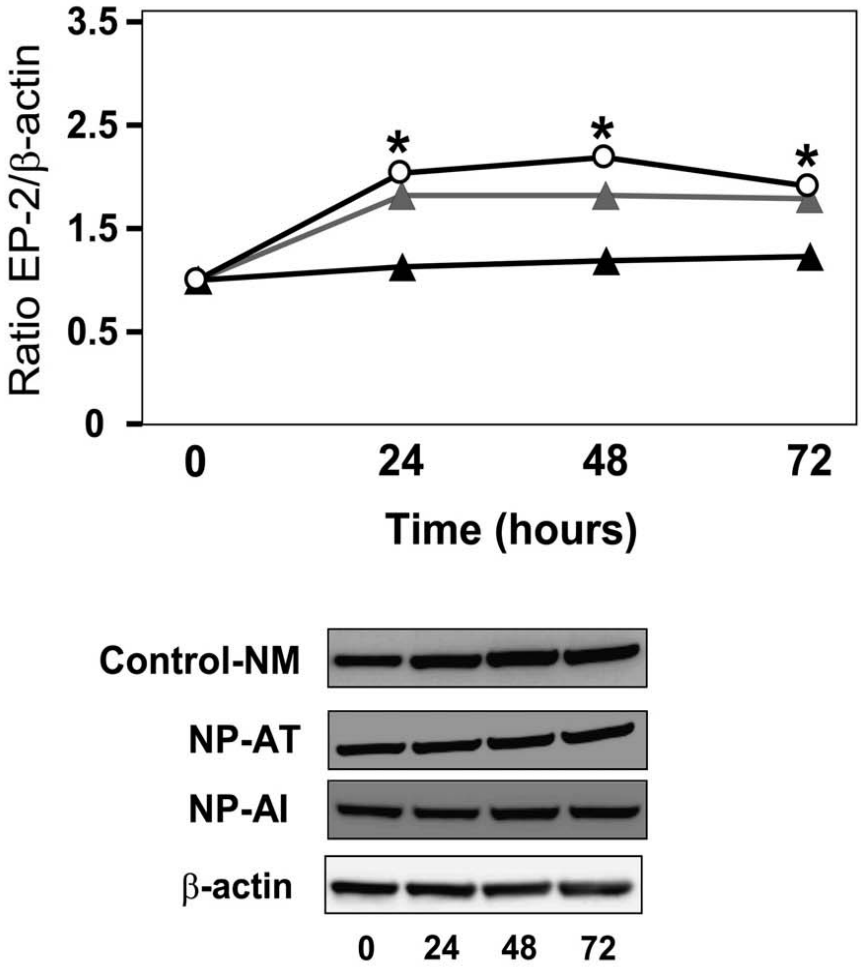

FIG 5. Time course of IL-1β on EP2 protein expression. Fibroblasts were incubated with 10 ng/mL IL-1β for 72 hours (n = 7). EP2 protein expression in control-NM *(white circles)*, NP-AT *(gray triangles)*, and NP-AI *(black triangles)* fibroblasts was analyzed by means of immunoblotting. Representative immunoblots are shown. $*P < .05$ compared with control media.

that airway COX-1 is sensitive to inflammatory stimuli,[4] but studies of the regulation of COX-1 in NPs of patients with and without AI have yielded contradictory results. Some studies reported no differences in COX-1 expression between NM and NPs from patients with ATA or AIA,[20,26] whereas others found higher levels of COX-1 in NPs from patients with AIA compared with healthy NM.[21] Pierzchalska et al[11,28] found significantly lower levels of COX-1 mRNA in bronchial epithelial cells and fibroblast from asthmatic patients compared with healthy subjects, and this difference was greater in patients with AIA. Kinetic studies have shown that COX-1 is upregulated, either spontaneously or after stimulation with cytokines, in samples of NM but not NPs from AT subjects.[35]

We used stimulated fibroblasts to investigate the regulation of COX-1 in NPs from patients with AIA. Previous studies found a delay between transcription of mRNA and increased levels of COX-1 protein.[38,39] Interestingly, and in contrast to COX-2 protein, COX-1 protein has a very long half-life.[38,39] On the basis of these observations, we measured COX-1 protein levels up to 72 hours after fibroblast stimulation with IL-1β and found increased levels of COX-1 protein in control NM tissue but not in NP-AI tissue. Altered regulation of COX-1, along with the lack of upregulation of COX-2, might contribute to the low levels of PGE_2 detected in NPs from patients with AIA in this and other studies.

PGE_2 has functions in the lung that limit inflammatory responses and control tissue repair.[40] The ability of PGE_2 to induce or suppress various mechanisms involved in inflammatory responses indicates the complex activities of its receptors.[39] EP1 through EP4 each signal through distinct pathways[29]; some require cell activation,[29] and therefore we studied their levels in fibroblasts stimulated with IL-1β. IL-1β increased the expression of EP2, but not EP3, EP4, or EP5, in control-NM fibroblasts. In contrast, IL-1β had no significant effect on EP2 expression in NP-AI fibroblasts, which is in agreement with the results of a previous study reporting a reduced number of eosinophils that express EP2 in NP-AI samples.[30] Most of the anti-inflammatory effects of PGE_2 are reportedly mediated by stimulation of EP2.[29,40] We speculate that reduced release of PGE_2 (which has anti-inflammatory properties in airways) and lower expression of its EP2 receptor increases the inflammatory process in the airways of patients with AIA. Studies are needed to determine whether regulation of EP2 expression contributes to AIA.

In summary, we have demonstrated alterations in the COX pathway of fibroblasts isolated from NPs, especially in those derived from patients with AIA. Observed alterations include very low production of PGE_2, lack of the expected upregulation of COX-2 on stimulation with a proinflammatory cytokine, and differences in the regulation of COX-1 and EP2 in IL-1β–stimulated

fibroblast from patients with AIA. Alterations in the COX pathway might be involved in the pathogenesis of NPs, asthma, and AI.

Key messages

- **Reduced production of PGE_2, which can have anti-inflammatory and tissue-repairing effects, might contribute to the development of NPs.**
- **Deficient regulation of COX-1 and COX-2 might be involved, at least in part, in the predisposition of some asthmatic patients to exacerbations induced by NSAIDs.**

REFERENCES

1. Simmons DL, Botting RM, Hla T. Cyclooxygnease isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 2004;56:387-437.
2. Siegle I, Klein T, Backman JT, Saal JG, Nüsing RM, Fritz P. Expression of cyclooxygenase 2 in human synovial tissue. Differential elevation of cyclooxygenase 2 in inflammatory joint diseases. Arthritis Rheum 1998;41:122-9.
3. Lohinai Z, Stachlewitz R, Székely AD, Fehér E, Dézsi L, Szabó C. Evidence for the expression of cyclooxygenase-2 enzyme in periodontitis. Life Sci 2001;70: 279-90.
4. Roca-Ferrer J, Pujols L, Gartner S, Moreno A, Pumarola F, Mullol J, et al. Upregulation of COX-1 and COX-2 in nasal polyps in cystic fibrosis. Thorax 2006;61: 592-6.
5. Xaubet A, Roca-Ferrer J, Pujols L, Ramírez J, Mullol J, Marin-Arguedas A, et al. Cyclooxygenase-2 is up-regulated in lung parenchyma of chronic obstructive pulmonary disease and down-regulated in idiopathic pulmonary fibrosis. Sarcoidosis Vasc Diffuse Lung Dis 2004;21:35-42.
6. Togo S, Holz O, Liu X, Sugiura H, Kamio K, Wang X, et al. Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. Am J Respir Crit Care Med 2008;178:248-60.
7. Stevenson DD, Szczeklik A. Clinical and pathologic perspectives on aspirin sensitivity and asthma. J Allergy Clin Immunol 2006;118:773-86.
8. Gyllfors P, Bochenek G, Overholt J, Drupka D, Kumlin M, Sheller J, et al. Biochemical and clinical evidence that aspirin-intolerant asthmatic subjects tolerate the cyclooxygenase 2-selective analgesic drug celecoxib. J Allergy Clin Immunol 2003;111:1116-21.
9. Picado C. Mechanisms of aspirin sensitivity. Curr Allergy Asthma Rep 2006;6: 198-202.
10. Langmack EL, Wenzel SE. Mast cell and eosinophil response after indomethacin in asthmatics tolerant and intolerant to aspirin. In: Szczeklik A, Gryglewski RJ, Vane J, editors. Eicosanoids, aspirin and asthma. New York: Marcel Dekker, Inc; 1998. p. 337-50.
11. Pierzchalska M, Szabó Z, Sanak M, Soja J, Szczeklik A. Deficient prostaglandin E_2 production by bronchial fibroblasts of asthmatic patients, with special reference to aspirin-induced asthma. J Allergy Clin Immunol 2003;111:1041-8.
12. Kowalski ML, Pawliczak R, Wozniak J, Siuda K, Poniatowska M, Iwaszkiewicz J, et al. Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients. Am J Respir Crit Care Med 2000;161:391-8.
13. Jedrzejczak-Czechowicz M, Lewandowska-Polak B, Bienkiewicz B, Kowalski ML. Involvement of 15-lipoxygenase and prostaglandin EP receptors in aspirin-triggered 15-hydroxyeicosatetraenoid acid generation in aspirin-sensitive asthmatics. Clin Exp Allergy 2008;38:1108-16.
14. Yoshimura T, Yoshikawa M, Otori N, Haruna S, Moriyama H. Correlation between the prostaglandin D2/E2 ratio in nasal polyps and the recalcitrant pathophysiology of chronic rhinosinusitis associated with bronchial asthma. Allergol Int 2008;57: 429-36.
15. Higashi N, Mita H, Ono E, Fukutomi Y, Yagamuchi H, Kajiwara K, et al. Profile of eicosanoids generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers. J Allergy Clin Immunol 2010;125:1084-91.
16. Picado C, Ramis I, Rosello J, Prat J, Bulbena O, Plaza V, et al. Release of peptide leukotriene into nasal secretions after local instillation of aspirin in aspirin-sensitive asthmatic patients. Am Rev Respir Dis 1992;145:1028-9.
17. Szczeklik A, Sladek K, Dworski R, Nizankowska E, Soja J, Sheller J, et al. Bronchial aspirin challenges causes specific eicosanoid response in aspirin-sensitive asthmatics. Am J Respir Crit Care Med 1996;154:1608-14.
18. Mastalerz L, Sanak M, Gawlewicz-Mrocka A, Gielicz A, Cmiel A, Szczeklik A. Prostaglandin E_2 production in patients with asthma and without aspirin hypersensitivity. Thorax 2008;63:27-34.
19. Sladek K, Dworski R, Soja, Sheller JR, Nizankowska E, Oates JA, et al. Eicosanoids in bronchoalveoalar lavage fluid of aspirin-intolerant patients with asthma after aspirin challenge. Am J Respir Crit Care med 1994;149:940-6.
20. Picado C, Fernandez-Morata JC, Juan M, Roca-Ferrer J, Fuentes M, Xaubet A, et al. Cyclooxygenase-2 mRNA is downexpressed in nasal polyps from aspirin-sensitive asthmatics. Am J Respir Crit Care Med 1999;160:291-6.
21. Pujols L, Mullol J, Alobid I, Roca-Ferrer J, Xaubet A, Picado C. Dynamics of COX-2 in nasal mucosa and nasal polyps from aspirin-tolerant and aspirin-intolerant patients with asthma. J Allergy Clin Immunol 2004;114:814-9.
22. Pérez-Novo CA, Watelet JB, Claeys C, Van Cauwenberge P, Bachert C. Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis. J Allergy Clin Immunol 2005;115:1189-96.
23. Sousa A, Pfister R, Christie PE, Lane SJ, Nasser SM, Schmitz-Schumann M, et al. Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma. Thorax 1997;52: 940-5.
24. Cowburn AS, Saldek K, Soja J, Adamek L, Nizankowska E, Lam BK, et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. J Clin Invest 1998;15:834-46.
25. Demoly P, Crampette L, Lebel B, Campbell AM, Mondain M, Bousquet J. Expression of cyclo-oxigenases 1 and 2 proteins in upper respiratory mucosa. Clin Exp Allergy 1998;28:278-83.
26. Adamjee J, Suh YJ, Park HS, Choi JH, Penrose JF, Lam BK, et al. Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma. J Pathol 2006;209:392-9.
27. Okano M, Fujiwara T, Yamamoto M, Sugata Y, Matsumoto R, Fukushima K, et al. Role of prostaglandin D2 and E2 terminal synthases in chronic rhinosinusitis. Clin Exp Allergy 2006;36:1028-38.
28. Pierzchalska M, Soja J, Woś M, Szabó Z, Nizankowska-Mogielnicka E, Sanak M, et al. Deficiency of cyclooxygenases transcripts in cultured primary bronchial epithelial cells of aspirin-sensitive asthmatics. J Physiol Pharmacol 2007;58:207-18.
29. Sugimoto Y, Narumiya S. Prostaglandin E receptors. J Biol Chem 2007;282: 11613-7.
30. Ying S, Meng Q, Scadding G, Parikh A, Corrigan CJ, Lee TH. J Aspirin-sensitive rhinosinusitis is associated with reduced E-prostanoid 2 receptor expression on nasal mucosal inflammatory cells. J Allergy Clin Immunol 2006;117:312-8.
31. Casadevall J, Ventura PJ, Mullol J, Picado C. Intranasal challenge with aspirin in the diagnosis of aspirin intolerant asthma: evaluation of nasal response by acoustic rhinometry. Thorax 2000;55:921-4.
32. Antczak A, Montuschi P, Khaaratinov S, Gorski P, Barnes PJ. Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. Am J Respir Crit Care Med 2002;166:301-6.
33. Farooque SP, Lee TH. Aspirin-sensitive respiratory disease. Annu Rev Physiol 2009;71:465-87.
34. Zakrzewski JT, Barnes NC, Piper PJ, Costello JF. Detection of sputum eicosanoids in cystic fibrosis and in normal saliva by bioassay and radioimmunoassay. Br J Clin Pharmacol 1987;23:19-27.
35. Mullol J, Fernàndez-Morata JC, Roca-Ferrer J, Pujols L, Xaubet A, Benitez P, et al. Cyclooxygenase 1 and cyclooxygenase 2 expression is abnormally regulated in human nasal polyps. J Allergy Clin Immunol 2002;109:824-30.
36. Sampson AP. Aspirin-intolerant asthma. New insights from bronchial mucosal biopsies. In: Szczeklik A, Gryglewski RJ, Vane J, editors. Eicosanoids, aspirin and asthmaNew York: Marcel Dekker, Inc; 1998. p. 371-89.
37. McAdam BF, Mardinin IA, Habid A, Burke A, Lawson A, Kapoor A, et al. Effect of regulated expression of human cyclooxygenase isoforms on eicosanoid production in inflammation. J Clin Invest 2000;105:1473-82.
38. Bunimov N, Smith JE, Gosselin D, Laneuville O. Translational regulation of PGHS-1 mRNA: 5′ unstraslated region and first two exons conferring negative regulation. Biochim Biophys Acta 2007;1769:92-105.
39. Kang YJ, Mbonye UR, DeLong CJ, Wada M, Smith WL. Regulation of intracelular cyclooxygenase levels by gene transcription and protein degradation. Prog Lipid Res 2007;46:108-25.
40. Vancheri C, Mastruzzo C, Sortino MA, Crimi N. The lung as a privileged site for the beneficial actions of PGE_2. Trends Immunol 2004;25:40-6.

METHODS

Tissue handling and cell culture

NM and NP samples were cut into 3 × 3–mm fragments and placed in 6-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco modified Eagle medium supplemented with 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, Calif), and 2 μg/mL amphotericin B (Sigma, St Louis, Mo). Cultures were kept in a 5% CO_2 humidified incubator at 37°C. When fibroblasts began to proliferate and covered 50% of the well surface, tissue fragments were removed, and the first passage was performed by adding 0.05% trypsin/0.02% EDTA (Invitrogen) for 5 minutes. The reaction was stopped with 10% FBS-supplemented media, and cells were centrifuged (400*g* for 5 minutes), seeded in 75-cm^2 flasks (NUNC), and grown to 80% confluence. At passages 3 to 7, fibroblasts were cultured to subconfluence in CultureSlides and 150-cm^2 flasks (NUNC, Rochester, NY) to perform culture characterization and experimental protocols. Culture characterization was performed by means of immunofluorescence for vimentin (fibroblasts), cytokeratins (epithelial cells), and α-smooth muscle actin (myofibroblasts) in CultureSlides incubated with SFM for 24 hours. The same batch of FBS was used for the whole experimental period, and mycoplasma contamination was tested by means of PCR in all cultures; none were found to be positive.

Experimental protocols

It was reported that COX expression, in response to exogenous stimuli, is more robust in quiescent cells than in proliferating cells,[E1] and therefore experimental protocols were started when cultures were subconfluent and after incubation with SFM for 24 hours. Then the culture media were changed, and the cells were incubated with SFM in the presence or absence of 10 ng/mL IL-1β (R&D Systems, Minneapolis, Minn) for different times. Cell lysates were used to analyze COX-1, EP1, EP2, EP3, and EP4 receptor protein expression by means of immunoblot analysis; COX-2 protein expression was measured by using ELISA and immunoblot analyses. In addition, COX-2 protein was studied with immunofluorescence by using CultureSlides.

Culture supernatants and cell lysates

Culture supernatants were collected, centrifuged at 400*g* for 10 minutes at 4°C, sterilized through 0.22-μm filters, and stored at −80°C until PGE_2 concentrations were measured. Total protein was obtained by collecting the cells after 2 washes with ice-cold PBS. The cells were centrifuged (1500*g* for 5 minutes at 4°C) and resuspended in 0.4 mL of ice-cold lysis buffer (Complete protease inhibitor cocktail tablet in 50 mL of 0.05 mol/L Hepes buffer solution, 0.05% vol/vol Triton X-100, and 625 μmol/L phenylmethylsulfonyl fluoride). Cells were sonicated twice for 15 seconds in a sonifier (Branson, Danbury, Conn) and centrifuged (12,000*g* for 10 minutes at 4°C).

ELISAs for PGD_2, PGE_2, and COX-2

Concentrations of PGE_2 in supernatants were measured with enzyme immunoassay kits (Cayman Chemical, Ann Arbor, Mich). The assay range was 7.81 to 1000 pg/mL. COX-2 protein concentrations were measured with an enzyme-linked immunosorbent sandwich assay (Zymed Laboratories, San Francisco, Calif). The assay range was 2.15 to 275 ng/mL. Concentrations of PGE_2 and COX-2 were normalized to the total protein content in the cell lysate from corresponding samples.

Immunoblot analysis

Thirty micrograms of protein extract in loading buffer (NuPAGE LDS sample buffer) was denaturalized in a thermocycler (70°C for 10 minutes), loaded in 7% TRIS-acetate gels, and run at 125 V for 90 minutes in a Novex XCell II Mini-Cell (Invitrogen) to quantify expression of COX-1, COX-2, and PG receptors (EP1-EP4). The proteins were transferred (20 V for 2 hours) to a 0.45-μm pore-size nitrocellulose membrane, and nonspecific binding sites were blocked with blocking buffer (5% nonfat dry milk and 0.1% Tween 20 in 10 nmol/L PBS) for 1 hour at room temperature in an orbital shaker. Membranes were incubated with the primary antibody against COX-1 (SC-1752, Santa Cruz), COX-2 (SC-1745, Santa Cruz), EP1 (101740, Cayman), EP2 (101750, Cayman), EP3 (101760, Cayman), or EP4 (45863; Abcam, Inc, Cambridge, Mass) in blocking buffer (1:1000); washed 4 times in 0.5% Tween 20 in 10 nmol/L PBS; and incubated with peroxidase-conjugated secondary antibody in blocking buffer (1:3000). After 4 washes, immunoreactive bands were visualized by using a chemiluminiscent method (Supersignal West Pico Chemiluminescent Substrate, Rockford, Ill), and light emissions were detected with the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software and normalized to intensities of β-actin and assessed from the same samples.

Immunofluorescence analysis

CultureSlides were washed with PBS, fixed with paraformaldehyde 4% for 15 minutes, and permeabilizated with 0.2% Triton for 10 minutes. Nonspecific binding sites were blocked with 1% BSA-PBS for 1 hour. Antibodies against α-smooth muscle actin (M0851; DAKO, Glostrup, Denmark) diluted 1:500, vimentin diluted 1:100 (V5255; Sigma, Saint Louis, Mo), pancytokeratin diluted 1:200 (C2562, recognizing human cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19; Sigma), or COX-2 (SC-1745, Santa Cruz Biotechnology) were added for 1 hour at 37°C. After 3 washes, a fluorescent secondary antibody was added for 1 hour. Nuclei were stained with 4′-6-diaidino-2-phenylindole dihydrochloride (1/10,000 dilution). Finally, slides were mounted with Prolong gold antifade reagent (Invitrogen). Percentages of positive cells were quantified by using fluorescence microscopy.

Statistical analyses

Immunofluorescence data are expressed as medians (25th-75th interquartiles) of positive-cell percentage among total cells. ELISA results are shown as medians (25th-75th interquartiles) of picograms of PG or nanograms of COX-2 per microgram of total proteins. Finally, immunoblot results are depicted as medians (25th-75th percentiles) of band intensities normalized for β-actin. The nonparametric statistical Mann-Whitney *U* test was used for between-group comparisons, and the Wilcoxon test was used for paired comparisons after confirming differences with the Friedman test. Statistical significance was set at a *P* value of less than .05.

REFERENCE

E1. Wu KK. Differential cyclooxygenase-2 transcriptional control in proliferating versus quiescent fibroblasts. Prostaglandins Other Lipid Mediat 2007;83:175-81.

“Low Prostaglandin E2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics.”

Roca-Ferrer, J., M. Perez-Gonzalez, F. J. Garcia-Garcia, J. Pereda, L. Pujols, I. Alobid, J. Mullol and C. Picado

. *Respirology* (2013) (accepted 10/12/2012). IF: 2.416

Estudi 2. Baixa producció de Prostaglandina E2 i expressió de Ciclooxigenases en fibroblasts aïllats de mucosa nasal de pacients amb asma induïda per aspirina.

Antecedents i objectius: La bibliografia descriu alteracions en la regulació de les ciclooxigenases (COX-1 i -2) en pòlips nasals de pacients amb asma induïda per aspirina (AIA). Tot i això, encara és poc clar si aquestes anomalies són específiques dels pòlips nasals o afecten a tota la mucosa nasal (MN) a les vies respiratòries superiors. L'objectiu d'aquest estudi va ser demostrar que les alteracions descrites a la via de la COX en el pòlips es troben també present en la mucosa nasal (MN) de pacients amb AIA.

Mètodes: van aïllar-se fibroblasts de NM de 5 pacients amb AIA (AIA-NM) i 5 individus control (control-NM). Les cèl·lules obtingudes van incubar-se durant 72 hores amb IL-1β a 10 ng/ml. Va mesurar-se la producció de la prostaglandina E_2 (PGE_2) mitjançant ELISA, l'expressió de la COX-1 a través de Western blot, i l'expressió de la COX-2 mitjançant les tècniques d'ELISA, Western blot i immunofluorescència.

Resultats: la incubació amb IL-1β va incrementar significativament la producció de PGE_2, així com l'expressió de COX-1 al grup de fibroblasts control-NM, però no van observar-se canvis en els fibroblasts procedents d'AIA-NM. La incubació amb IL-1β va causar un increment significatiu temps depenent en l'expressió de la proteïna COX-2 als fibroblasts control-NM, però va tenir un efecte molt més moderat en l'expressió de la COX-2 als fibroblasts del grup AIA-NM.

Conclusions: les dades obtingudes demostren que les anomalies observades a la via de la COX no són un fenòmen exclusiu dels pòlips nasals sinó que també són presents a tota la mucosa nasal dels pacients AIA. Aquestes anomalies podrien estar implicades en la patogènesi inflamatòria de les vies respiratòries i la intolerància als AINEs en pacients asmàtics amb rinosinusitis crònica i poliposi nasal.

Low Prostaglandin E_2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics

Jordi Roca-Ferrer, PhD[1,2]

Maria Pérez-Gonzalez, MS[1,2]

Francesc J Garcia-Garcia, MS[1,2]

Javier Pereda. PhD[1,2]

Laura Pujols, PhD [1,2]

Isam Alobid, MD, PhD[1,2,3]

Joaquim Mullol, MD, PhD[1,2,3,*]

Cesar Picado, MD, PhD[1,2,4,*]

(*) Both authors contributed equally to this work with senior responsibilities.

1. Immunoal·lèrgia Respiratòria Clínica i Experimental, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). Casanova 134, 08036-Barcelona. Spain.

2. Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES). Casanova 134, 08036-Barcelona. Spain.

3. Unitat de Rinologia i Clínica de l'Olfacte, Servei d'Otorrinolaringologia. Hospital Clínic. Villarroel 170, 08036-Barcelona, Spain.

4. Servei de Pneumologia i Al·lèrgia Respiratoria. Hospital Clínic. Universitat de Barcelona, Villarroel 170, 08036-Barcelona, Spain

Corresponding author: J. Roca-Ferrer, PhD.

Immunoal·lèrgia Respiratòria Clínica i Experimental, IDIBAPS. Casanova 134, 08036 Barcelona. Spain.

e-mail: idibaps402@clinic.ub.es

Telephone number: 34 93 227 54 00 (2906)

Fax number: 34 93 227 98 13

Summary at a glance

Our study demonstrates that the cyclooxygenase pathway of arachidonic acid metabolism is altered in nasal mucosa of aspirin-intolerant asthmatic patients, suggesting that abnormalities in the cyclooxygenase metabolisms are present in all the nasal mucosa of these patients. Our results also provide further support to the consensus term of rhinosinusitis.

Abstract

Background and objective: Anomalies in regulation of cyclooxygenases (COX)-1 and-2 have been described in nasal polyps of aspirin-induced asthma (AIA). Whether these anomalies are specific to nasal polyps or affect all the nasal mucosa (NM) of upper airways is still unclear. The objective of this study was to compare the COX pathway in NM of AIA patients with the NM of control subjects.

Methods: Fibroblasts were isolated from NM of 5 AIA patients (AIA-NM) and 5 control subjects (control-NM). Cells were treated with 10 ng/ml IL-1β for up to 72 hours. Prostaglandin E_2 (PGE_2) production was measured by ELISA, expression of COX-1 protein by Western blot, and COX-2 protein by ELISA, Western blot and immunofluorescence techniques.

Results: IL-1β increased PGE_2 production and COX-1 protein expression in control-NM fibroblasts, but no changes were found in AIA-NM. IL-1β provoked a significant time-dependent increase in COX-2 protein expression in control-NM fibroblasts but had a very mild effect on COX-2 protein expression in AIA-NM.

Conclusions**:** Our data suggest that abnormalities in the COX pathway are not a phenomenon exclusive to nasal polyp mucosa as they are also present in all the nasal mucosa of AIA patients. These anomalies may be involved in the pathogenesis of airway inflammation and NSAID intolerance in asthma patients with chronic rhinosinusitis and nasal polyposis.

Key words

Aspirin intolerance, cyclooxygenase, fibroblasts, nasal mucosa, prostaglandin E_2

Short Title

COX Pathway in ASA-sensitive Asthma

Introduction

Aspirin-induced asthma (AIA) is a syndrome characterized by the association of asthma, chronic rhinosinusitis with nasal polyps (NP), and episodes of bronchospasm precipitated by non-steroidal anti-inflammatory drugs (NSAIDs).[1] Although the mechanism responsible for AIA has not been completely elucidated, anomalies in the regulation of arachidonic acid (AA) metabolism seem to be involved in the adverse response to NSAIDs in these patients.[1,2]

Upon stimulation, activated phospholipase A_2 releases AA from membrane phospholipids. AA is then converted by enzymatic and non-enzymatic pathways into a large number of eicosanoids. The major enzymatic routes include the lipoxygenase (LO) pathway, which is responsible for the formation of leukotrienes, 15-HETE, and lipoxins; the cyclooxygenase (COX) pathway, involved in the formation of prostaglandins (PG), thromboxanes and prostacyclin; and the cytochrome P450 pathway, responsible for the formation of 20-HETE and other metabolites.[3,4]

In the human lung, the 5-LO pathway is responsible for the synthesis of cysteinyl-leukotrienes (CysLTs). Various studies have reported that this pathway is up-regulated in the upper and lower airways of AIA patients. The expression of the enzyme LTC_4 synthase, which is involved in the synthesis of LTC_4, is much higher in the airways of AIA patients than in those of aspirin-tolerant asthma patients and healthy controls.[5,6] The increased activity of the 5-LO pathway results in overproduction of CysLTs in AIA, which is further increased when patients are exposed to NSAIDs.[7,8]

This over-expression of CysLT is a finding reported by almost all the authors who have studied the mechanism of AIA. However, the presence of anomalies in the regulation of the COX metabolism has been reported in only some of these studies, and their role in the pathogenesis of AIA remains to be clarified.[1,2] Accordingly, this study has focused solely on the COX pathway in AIA.

The COX pathway converts AA into PGs, including PGE_2, PGD_2 and $PGF_{2\alpha}$. Two functional COX isoforms have been identified. COX-1 is constitutively expressed in most tissues and generates PGs that are primarily involved in the regulation of homeostatic functions. COX-2 is inducible by several cytokines and growth factors, including interleukin (IL)-1β and tumor necrosis factor α .[3,4]

Various anomalies have been reported in the COX pathway of AIA.[1,2] Low production of PGE_2 associated with down-regulated expression of COX enzymes has been reported in NP tissue and in both fibroblasts and epithelial cells from the NP of AIA patients,[9-15] suggesting that abnormalities in AA metabolism could play a role in AIA etiology.

However, there are no reports to date of such alterations in the NM of AIA patients. In contrast to the results obtained in NP, studies using bronchial biopsies and cultured bronchial fibroblasts from AIA and aspirin-tolerant asthma have not been able to find any differences in the expression of COX-1 and COX-2 when compared to healthy controls.[5,16] There are no clear explanations for the reported discrepancies between the results obtained in the upper and lower airways. These discrepancies might be due, at least in part, to the fact that the regulation of COX enzymes has been extensively studied by comparing NP with NM from control subjects[10-14] but has yet to be studied by comparing NM from AIA patients with NM from control subjects. In other words, since inflammatory polyps are structures found in the upper airways but never seen in the lower airways, it is reasonable to ask whether the anomalies reported in the regulation of COX-1 and COX-2 expression in the upper airways are specific to NP and therefore not representative of either the NM surrounding the NP structure or the bronchial mucosa.

The objective of this study was to compare the COX pathway in the NM of AIA patients with the NM of control subjects. With this aim in mind, we studied and compared PGE_2 production and COX-1 and 2 expression in fibroblasts derived from the NM of both AIA and control subjects.

Methods

Study population

We obtained NM specimens from 5 non-asthmatic subjects with either septal deviation or turbinate hypertrophy who had undergone nasal corrective surgery (control-NM). All the control subjects had taken aspirin or NSAIDs at clinical dosage without any untoward reactions (asthma and/or rhinitis, urticaria, angioedema or anaphylaxis). NM specimens were also collected from 5 asthmatic patients with chronic rhinosinusitis and aspirin intolerance (AIA-NM) who had undergone endoscopic sinus surgery. The clinical and demographic characteristics of the subjects are shown in Table 1. The diagnosis of aspirin intolerance was confirmed by lysine-aspirin nasal challenge, as previously described.[17] None of the control subjects had had any oral or intranasal corticosteroid treatment for at least one month before surgery. None of the patients had any upper airway infection in the 2 weeks before surgery. All patients gave informed consent to participate in the study, which was approved by the Scientific and Ethics Committee of our Institution.

Tissue handling and cell culture

NM tissue was cut into 3 x 3 mm fragments and placed in six-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen, Carlsbad, California, USA) and 2 µg/ml amphotericin B (Sigma, St Louis, MO, USA). Cultures were placed in a 5% CO_2 humidified incubator at 37ºC. Once the fibroblasts had grown, tissue fragments were removed and the first

passage was performed by adding 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, California, USA) for 5 min. The reaction was stopped with 10% FBS-supplemented DMEM. Cells were then centrifuged (400g, 5 min) and seeded in 150 cm^2 flasks (NUNC). At passages 3 to 7, fibroblasts were cultured in CultureSlides® and flasks to perform culture characterization and experimental protocols. The same batch of FBS was used for the whole experimental period. Mycoplasma contamination was tested by PCR in the cultures and all of them were negative.

Culture characterization

Culture characterization was performed by immunofluorescence for vimentin (fibroblasts), cytokeratins (epithelial cells) and alpha smooth muscle actin (myofibroblasts) in CultureSlides® incubated with serum-free media (SFM) for 24 hours.

Experimental protocols

When cultures reached 80% confluence, FBS-supplemented media was switched to serum-free media (SFM) for 24 hours. Cells were then incubated with SFM in the presence or absence of 10 ng/ml IL-1β (R&D Systems Minneapolis, MN, USA) for different times. CultureSlides® were used to study COX-2 protein expression by immunofluorescence. Flask culture supernatants were centrifuged (400g, 10 min at 4ºC), sterilized through 0.22 µm filters, and stored at -80ºC until the PGE_2 concentrations were measured. Total proteins were obtained by scraping the flasks after two washes with cold PBS. The cells were centrifuged (1,500g, 5 min at 4ºC) and resuspended in 0.4 ml cold lysis buffer (Complete™ protease inhibitor tablet in 50 ml of 0.05 M Hepes buffer solution, 0.05% v/v Triton X-100, and 625 µM PMSF). Cells

were sonicated twice for 15 seconds in a sonifier (Branson, Danbury, CT, USA) and centrifuged (12,000g, 10 min at 4ºC). Cell lysates were used to analyze protein expression by Western blot and ELISA.

Immunofluorescence

This technique was performed as previously reported [14]. The primary antibodies were against alpha-smooth muscle actin (M0851, DAKO, Glostrup, Denmark) at dilution 1:500, vimentin at 1:100 (V5255, Sigma, Saint Louis, Missouri, USA), pan-cytokeratin at 1:200 (C2562, recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19, Sigma) or COX-2 (SC-1745, Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). The percentage of positive cells was quantified using fluorescence microscopy.

PGE_2 and COX-2 ELISA

Concentrations of PGE_2 in supernatants were measured using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI, USA). The assay range was 7.81-1000 pg/ml. COX-2 protein concentrations were measured using an enzyme-linked immunosorbent sandwich assay (Zymed Laboratories, San Francisco, CA, USA). The assay range was 2.15-275 ng/ml. The concentrations of PGE_2 and COX-2 were normalized to the total protein content in the cell lysate from corresponding samples.

Western Blot

To analyze COX-1 and COX-2 protein expression, we performed the technique we have previously reported.[14] The primary antibodies used were against COX-1 (SC-1752,

Santa Cruz) and COX-2 (SC-1745, Santa Cruz) at dilution 1:1000. Immunoreactive bands were visualized using a chemoluminiscent method (Supersignal West Pico Chemiluminescent Substrate, Rockford, IL, USA). Light emissions were detected by the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software and normalized by β-actin band intensities assessed in the same samples.

Statistical analysis

Data are reported as follows: immunofluorescence data are expressed as median and 25-75th percentile of positive cell percentage among total cells. ELISA results are shown as median and 25-75th interquartile of pg of PG or ng of COX-2/μg total proteins. Finally, Western blot results are depicted as median and 25-75th percentile of band intensities normalized by β-actin. The nonparametric statistical Mann-Whitney U-test was used for between-group comparisons and the Wilcoxon test was used for paired comparisons, after confirming differences with the Friedman test. Statistical significance was set at $P<0.05$.

Results

Culture characterization

All the cells in culture had fibroblast phenotype since 100% of cells were positive to vimentin. There was a total absence of epithelial cells in the fibroblast cultures since no cytokeratin-positive cells were found. Smears of nasal mucosa epithelial cells were used as a positive control (Figure 1). No significant differences were observed in the myofibroblast percentage in fibroblast cultures (control-NM=1.6%, 1.3-3.3; AIA-NM=3.5%, 2.0-3.6, N=5).

Prostaglandin E_2 production

There were no significant differences in the production of PGE_2 at baseline in cultured fibroblasts derived from either control-NM or AIA-NM. Compared to SFM-treated cells, IL-1β significantly increased PGE_2 production at 24 hours in control-NM, but had no effect on PGE_2 secretion in fibroblasts derived from AIA-NM (Figure 2).

COX expression

COX-1 basal expression was not different between control-NM and AIA-NM. Compared to SFM-treated cultures, however, IL-1β induced a significant time-dependent increase in COX-1 protein expression in control-NM fibroblasts from 24 to 72 hours. In contrast, a tendency to decreased COX-1 expression was found in AIA-NM fibroblasts (Figure 3).

COX-2 expression was measured by ELISA, Western blot and immunofluorescence techniques. COX-2 protein expression was not detected in SFM-treated cultures,

because the level of the protein was under the level of detection of the ELISA. No cells showed fluorescence and no detectable signals were present in Western blot. When cells were incubated with IL-1β, however, there was a significant time-dependent increase in COX-2 expression from 2 to 24 hours (measured by ELISA) in control-NM fibroblasts. In contrast, IL-1β had a very mild and non-significant effect on the induction of COX-2 in AIA-NM fibroblasts (Figure 4A). Similarly, when analyzed by Western blot and compared to SFM treated cultures, IL-1β increased the ratio of COX-2/β-actin protein expression only in control-NM (Figure 4B). Finally, when studied by immunofluorescence, there was a significant increase in COX-2 positive cells in response to IL-1β in both control-NM and AIA-NM cultures. The percentage of COX-2 positive cells in IL-1β-treated cultures was significantly higher, however, in control-NM than in AIA-NM fibroblasts (Figure 4C).

Discussion

The main findings of our study were: (1) IL-1β markedly stimulated the production of PGE_2 in control-NM fibroblasts but had a non-significant effect on fibroblasts obtained from AIA-NM; (2) IL-1β stimulation increased COX-1 protein expression in fibroblasts isolated from control-NM, but not in fibroblasts obtained from AIA-NM; and (3) there was a marked increase in COX-2 protein expression following IL-1β exposure in control-NM fibroblasts, but almost no effect on fibroblasts derived from AIA-NM.

This is the first study to demonstrate anomalies in the COX pathway of AA metabolism in nasal mucosa from AIA patients. Previous studies have reported decreased production of PGE_2 in nasal polyps – an anomaly that is even more marked in the nasal

polyps of patients with AIA.[10,11,16] In line with this finding, various studies have reported a lack of up-regulation of COX-2 in nasal polyps from both aspirin-tolerant asthma and AIA.[11-15,18] For reasons that remain unclear, other studies have been unable to find any differences in the expression of COX-2 between nasal polyps and control nasal mucosa.[6,19] As most of the studies that did not find any differences in the expression of COX-2 in AIA were performed with immunohistochemistry techniques, we used three different methods of analysis (ELISA, Western blot and immunofluorescence) to assess the level of COX-2 expression.

In our study, the three methods presented similar outcomes and confirmed that COX-2 protein is not detected in non-stimulated fibroblasts and almost fails to be up-regulated by inflammatory stimuli in the nasal mucosa of AIA. These results are in keeping with other studies showing undetectable COX-2 protein in unstimulated nasal mucosa and polyp fibroblasts.[14] In contrast, increased expression of COX-2 has been reported in nasal polyps from cystic fibrosis patients, as expected in inflammatory diseases.[20,21] This observation adds further support to the notion that, for reasons that are still unknown, COX-2 is abnormally regulated in the nasal mucosa of patients with AIA.

Previous studies suggest that an alteration in the regulation of COX-1 may also coexist with anomalies in the regulation of COX-2 in the nasal polyps of patients with and without associated AIA.[14,18] In this study we report that these anomalies are also present in fibroblasts derived from the nasal mucosa of AIA patients. Although it is generally accepted that COX-1 plays a limited role in inflammatory responses, mild

modifications can occur in the expression of the enzyme in cells and tissues when they are stimulated by pro-inflammatory cytokines or injury.[22] The induction of COX-1 in inflammatory conditions might help to mitigate, at least in part, the deficient up-regulation of COX-2 in the mucosa of AIA patients. However, this compensatory mechanism could not occur in NM fibroblasts obtained from AIA patients as COX-1 is not induced by IL-1β.

PGE_2 exerts various anti-inflammatory and anti-fibrotic effects, including suppression of eosinophil infiltration, reduction of CysLT release, inhibition of fibroblast proliferation, myofibroblast transformation and collagen synthesis.[23]. The limited production of PGE_2 found in the NM of AIA patients may contribute to the intensification of the inflammatory process in their airways and may account for the reported association of aspirin intolerance with increased asthma and nasal-polyp severity.[10,24,25]

Moreover, when the COX-2 and PGE_2 concentrations obtained by ELISA in cultures treated with IL-1β were compared, the values obtained in the NM of AIA were similar to those previously reported in NP of AIA patients[14], indicating that these alterations are present to a similar degree in all the nasal mucosa of AIA patients.

In contrast, our study reported down-regulation of arachidonic acid metabolism in fibroblasts of nasal mucosa from AIA patients. In keeping with our findings, several studies have already reported that the COX pathways are also abnormally regulated in nasal[9] and bronchial[26] epithelial cells from aspirin-sensitive asthmatics.

The mechanisms responsible for the reported alterations in the regulation of COX-2 found in our study remain to be clarified. It is well known that inflammatory stimuli induce cellular responses through the activation of mitogen-activated protein kinases (MAPKs), and that COX-2 gene expression is also regulated by the action of transcription factors such as NF-κB and C/EBP, among others. The role of these proteins has recently been studied in AIA-NP fibroblasts and showed no differences in MAPK phosphorylation and transcription factor nuclear translocation, compared to control nasal mucosa cells.[27] These findings suggest that the mechanisms involved in COX down-regulation should be related to other mechanisms, such as transcription factor bindability to gene promoter, the activity of histone acetyltransferases and deacetylases, and mRNA stability.

Furthermore, the absence of any differences in the myofibroblast percentage in cultures suggests that changes in cell phenotype do not play a role in the COX pathway abnormalities found in our study.

Finally, our findings, along with data from previous studies showing that IL-5 and ECP levels are increased in both sinusal (ethmoidal) and nasal (lower turbinate) samples from patients with chronic rhinosinusitis and NP,[28] give further support to the consensus term of rhinosinusitis recommended by the European Position Paper on Rhinosinusitis and Nasal Polyps (EP^3OS).[29]

In conclusion, we found significant differences in the regulation of PGE_2, COX-1 and COX-2 in the nasal mucosa fibroblasts of patients with AIA. Our data suggest that

abnormalities in the COX pathway are present in all the nasal mucosa of AIA patients and are not exclusively confined to the nasal polyp mucosa. This study also demonstrates that the nasal mucosa of AIA patients cannot be used as surrogate of healthy nasal mucosa.

Acknowledgements

Supported by grants from the Spanish Ministry of Health (FIS PI030033, FIS PI080249), Fundació Catalana de Pneumologia, and Fundación Respira (Spanish Respiratory Society)

References

1. Stevenson DD, Sczceklik A. Clinical and pathologic perspectives on aspirin sensitivity and asthma. *J Allergy Clin Immunol* 2006; **118**: 773-86.

2. Picado C. Mechanisms of aspirin sensitivity. *Curr Allergy Asthma Rep* 2006; **6**: 198-202.

3. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. *Progress Lipid Res* 2011; **50**: 35-51.

4. Simmons DL, Botting RM, Hla T. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol Rev* 2004; **56**: 387-437.

5. Cowburn AS, Saldek K, Soja J, *et al*. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998; **101**: 834-46.

6. Adamjee J, Suh YJ, Park HS, *et al*. Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma. *J Pathol* 2006; **209**: 392-99.

7. Kumlin M, Dalhén B, Björck T, *et al*. Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B2 in response to bronchial provocation with allergen,

aspirin, leukotriene D4, and histamine in asthmatics. *Am Rev Respir Dis* 1992; **146**: 96-103.

8. Picado C, Ramis I, Rosello J, *et al*. Release of peptide leukotriene into nasal secretions after local instillation of aspirin in aspirin-sensitive asthmatic patients. *Am Rev Respir Dis* 1992; **145**; 65-9.

9. Kowalski ML, Pawliczak R, Wozniak J, *et al*. Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients. *Am J Respir Crit Care Med* 2000; **161**: 391-8.

10. Yoshimura T, Yoshikawa M, Otori N, *et al*. Correlation between the prostaglandin D2/E2 ratio in nasal polyps and the recalcitrant pathophysiology of chronic rhinosinusitis associated with bronchial asthma. *Allergol International* 2008: **57**: 429-36.

11. Pérez-Novo CA, Watelet JB, Claeys C, *et al*. Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis. *J Allergy Clin Immunol* 2005; **115**: 1189-96.

12. Picado C, Fernandez-Morata JC, Juan M, *et al*. Cyclooxygenase-2 mRNA is downexpressed in nasal polyps from aspirin-sensitive asthmatics. *Am J Respir Crit Care Med* 1999; **160**: 291-6.

13. Pujols L, Mullol J, Alobid I, *et al*. Dynamics of COX-2 in nasal mucosa and nasal polyps from aspirin-tolerant and aspirin-intolerant patients with asthma. *J Allergy Clin Immunol* 2004; **114**: 814-9.

14. Roca-Ferrer J, Garcia-Garcia FJ, Pereda J, *et al*. Reduced expression of COXs and production of prostaglandin E2 in patients with nasal polyps with or without aspirin-intolerant asthma. *J Allergy Clin Immunol* 2011; **128**: 66-72.

15. Adamusiak AM, Stasikowska-Kanicka O, Lewandowska-Polak A, *et al*. Expression of Arachidonate Metabolism Enzymes and Receptors in Nasal Polyps of Aspirin-Hypersensitive Asthmatics. *Int Arch Allergy Immunol* 2012; **157**: 354-62.

16. Pierzchalska M, Szabó Z, Sanak M, *et al*. Deficient prostaglandin E_2 production by bronchial fibroblasts of asthmatic patients, with special reference to aspirin-induced asthma. *J Allergy Clin Immunol* 2003; **111**: 1041-8.

17. Casadevall J, Ventura PJ, Mullol J, *et al*. Intranasal challenge with aspirin in the diagnosis of aspirin intolerant asthma: evaluation of nasal response by acoustic rhinometry. *Thorax* 2000; **55**: 921-4.

18. Mullol J, Fernàndez-Morata JC, Roca-Ferrer J *et al*. Cyclooxygenase 1 and cyclooxygenase 2 expression is abnormally regulated in human nasal polyps. *J Allergy Clin Immunol* 2002; **109**: 824-30.

19. Demoly P, Crampette L, Lebel B, *et al*. Expression of cyclo-oxigenases 1 and 2 proteins in upper respiratory mucosa. *Clin Exp Allergy* 1998; **28**: 278-83.

20. Owens JM, Shroyer KR, Kingdom TT. Expression of cyclooxygenase and lypoxygenase enzymes in sinonasal mucosa of patients with cystic fibrosis. *Arch Otolarygol head Neck Surg* 2008; **134**: 825-31.

21. Roca-Ferrer J, Pujols L, Gartner S, *et al*. Upregulation of COX-1 and COX-2 in nasal polyps in cystic fibrosis. *Thorax* 2006; **61**: 592-6.

22. Kang YJ, Mbonye UR, DeLong CJ, *et al*. Regulation of intracellular cyclooxygenase levels by gene transcription and protein degradation. *Progress Lipid Res* 2007; **46**: 108-25.

23. Vancheri C, Mastruzzo C, Sortino MA, *et al*. The lung as a privileged site for the beneficial actions of PGE_2. *Trends Immunol* 2004; **25**: 40-6.

24. Lee H, Haselkorn T, Borish L, *et al*. Risk factors associated with persistent airflow limitation in severe or difficult-to-treat asthma: insights from the TENOR study. *Chest* 2007: **132**: 1882-9.

25. Mascia K, Haselkorn T, Deniz YM, *et al*. Aspirin sensitivity and severity of asthma: evidence for irreversible airway obstruction in patients with severe or difficult-to-treat asthma. *J Allergy Clin Immunol* 2005; **116**: 970-5.

26. Pierzchalska M, Soja J, Woś M, *et al*. Deficiency of cyclooxygenases transcripts in cultured primary bronchial epithelial cells of aspirin-sensitive asthmatics. *J Physiol Pharmacol* 2007; **58**: 207-18.

27. FJ Garcia-Garcia, J Mullol, M Perez-Gonzalez, et al. Signal transduction pathways (MAPKs, NF-KB, and C/EBP) regulating COX-2 expression in nasal fibroblasts from asthma patients with aspirin intolerante. *PLoS One* 2012; DOI: 10.1371/journal.pone.0051281.

28. Van Crombruggen K, Van Bruaene N, Holtappels G, *et al*. Chronic sinusitis and rhinitis: clinical terminology "Chronic Rhinosinusitis" further supported. *Rhinology* 2010; **48**: 54-8

29. Fokkens W, Lund V, Mullol J. European position paper on rhinosinusitis and nasal polyps 2007. *Rhinology* 2007; **20**: 1-136.

Table 1. Epidemiological characteristics of control subjects and AIA patients.

Characteristics	Control-NM	NM-AIA
Fibroblast cultures, N	5	5
Age, years (mean±sem)	32.2±4.2	45.2±4.0
Female, N (%)	1 (20)	3 (60)
Asthma, N (%)	0 (0)	5 (100)
Aspirin intolerance, N (%)	0 (0)	5 (100)
STP positive, N (%)	1 (20)	0 (0)
Blood eosinophilia, % (mean±sem)	1.7±0.5	9.8±1.7
CRS with nasal polyps, N (%)	0 (0)	5 (100)
Intranasal corticosteroid, N (%)	0 (0)	3 (60)

AIA, aspirin-intolerant asthmatic.
CRS, chronic rhinosinusitis.
NM, nasal mucosa.
SPT, skin prick test.

Figures and legends

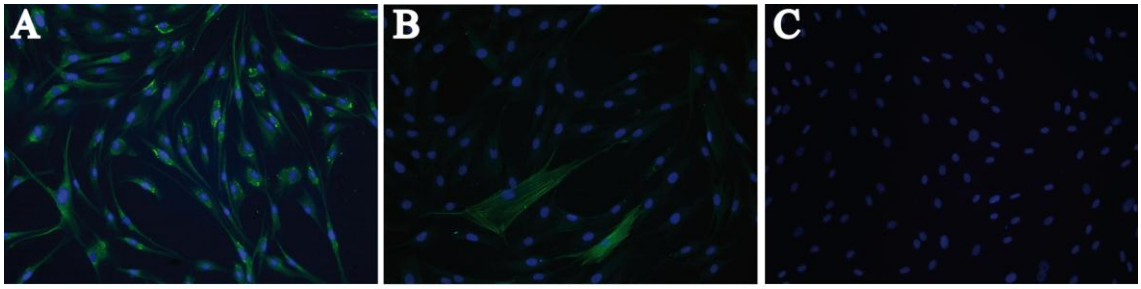

Figure 1. Culture characterization.

Representative immunofluorescent staining in control nasal mucosa fibroblasts. **A)** Fibroblast phenotype of cells in culture was confirmed by the presence of vimentin fibers. **B)** Myofibroblast phenotype of cells in culture was confirmed by the detection of α-smooth muscle actin fibers. **C)** Absence of epithelial cells demonstrated by the absence of cytokeratin staining in cultures. Original magnification x 200.

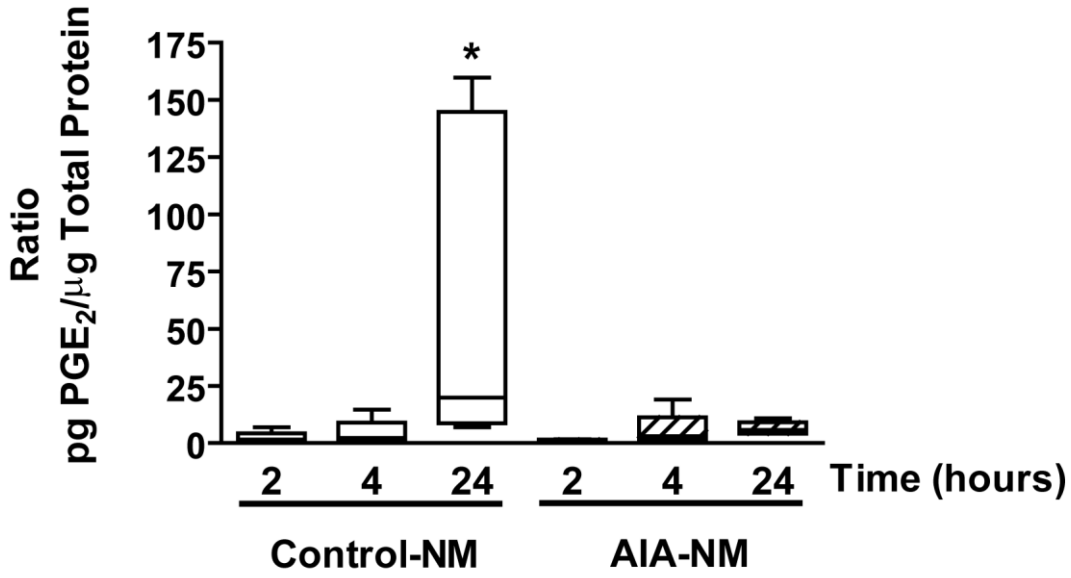

Figure 2. Time course of IL-1β effects on PGE_2 protein production

Fibroblasts were incubated with serum-free media with IL-1β at 10 ng/ml for 4 and 24 hours. PGE_2 (N=5) concentration was measured by ELISA. Wilcoxon signed-rank test was used for analysis. * $P<0.05$ compared to non-stimulated cells. AIA, aspirin-induced asthma; NM, nasal mucosa.

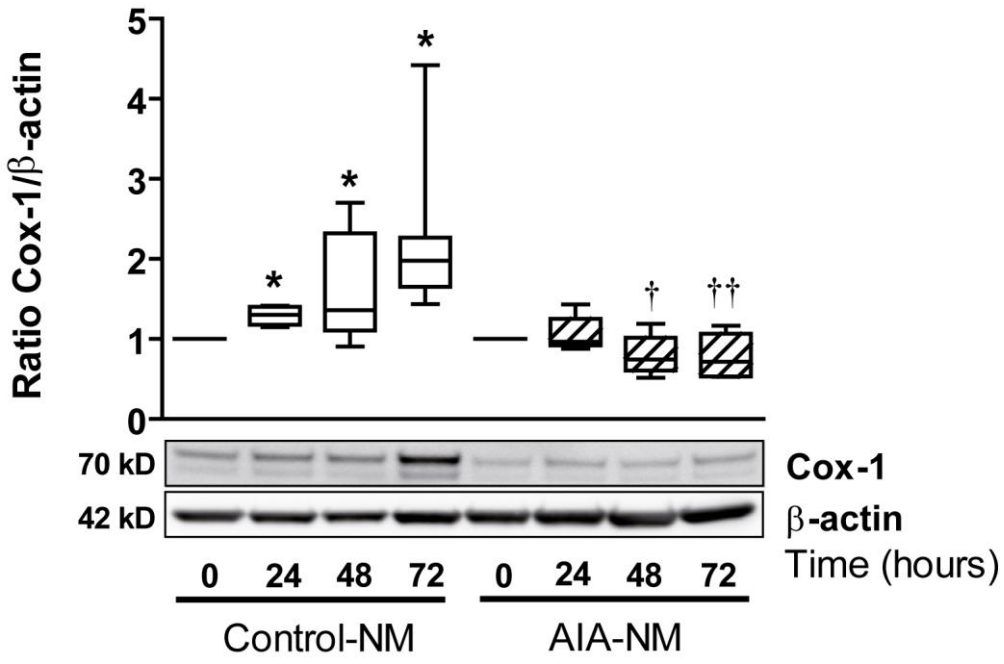

Figure 3. Effects of IL-1β on COX-1 protein expression.

Fibroblasts were incubated with serum-free media with IL-1β at 10 ng/ml for up to 72 hours (N=5). COX-1 protein expression was analyzed by Western blot. Mann-Whitney U-test was used for between-group comparisons and Wilcoxon test for paired comparisons. *, P<0.05 compared to non-stimulated cells (ratio COX-1/β-actin=1); †, P<0.05 and ††, P<0.01 compared to IL-1β treated control-NM. AIA, aspirin-induced asthma; NM, nasal mucosa.

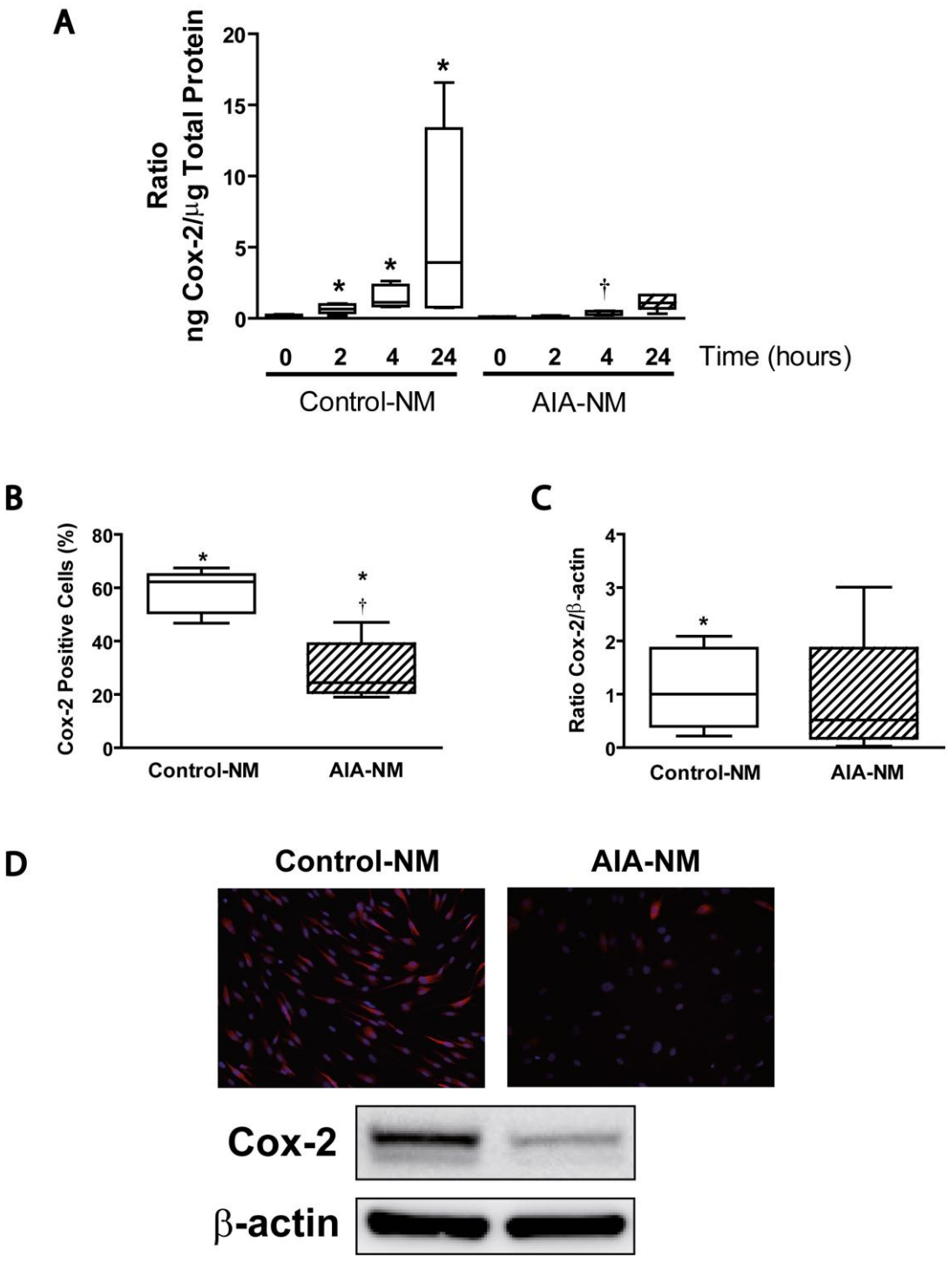
A
Ratio
ng Cox-2/µg Total Protein
20
15
10
5
0
*
*
*
†
0 2 4 24 0 2 4 24
Time (hours)
Control-NM
AIA-NM
B
Cox-2 Positive Cells (%)
80
60
40
20
0
*
*
†
Control-NM
AIA-NM
C
Ratio Cox-2/β-actin
4
3
2
1
0
*
Control-NM
AIA-NM
D
Control-NM
AIA-NM
Cox-2
β-actin

Figure 4. Effects of IL-1β on COX-2 protein expression.

Fibroblasts were incubated with serum-free media with IL-1β at 10 ng/ml (N=5). **A)** Time course of IL-1β on COX-2 protein expression analyzed by ELISA. Immunofluorescence **(B)** and Western blot **(C)** analysis of COX-2 protein expression in cultures incubated for 24 hours with IL-1β. **(D)** Representative COX-2 detection by immunofluorescence and Western blot in fibroblast cultures. Mann-Whitney U-test was used for between-group comparisons and Wilcoxon test for paired comparisons. *, P<0.05 compared to non-stimulated cells; † P<0.05 compared to IL-1β-treated control-NM. AIA, aspirin-induced asthma; NM, nasal mucosa.

Signal Transduction Pathways (MAPKs, NF-κB, and C/EBP) Regulating COX-2 Expression in Nasal Fibroblasts from Asthma Patients with Aspirin Intolerance.

Garcia-Garcia FJ, Mullol J, Perez-Gonzalez M, Pujols L, Alobid I, Mullol J, Picado C

PLoS ONE 2012; 7(12): e51281. doi:10.1371/journal.pone.0051281

IF: 4.092

Estudi 3. Estudi de les vies de transducció de senyal (MAPKs, NF-κB i C/EBP) reguladores de l'expressió de la COX-2 en fibroblasts nasals de pacients asmàtics amb intolerància a l'aspirina.

Antecedents i objectiu: estudis recents han evidenciat que l'expressió de la ciclooxigenasa-2 (COX-2) es troba regulada a la baixa en pacients amb asma induïda per aspirina (AIA). Les vies de senyalització (MAPKs, NF-kB and C/EBP) implicades en la regulació de la COX-2 són diverses. L'objectiu d'aquest estudi va ser investigar la regulació de la COX-2 a través de l'activació de la senyalització per vies MAPK i la translocació nuclear de factors de transcripció en l'asma induïda per aspirina.

Mètodes: van aïllar-se fibroblasts de mucosa nasal (NM, N=5) i de pòlips nasals d'invividus amb AIA (NP-AIA, N=5). Després d'incubar les cèl·lules amb IL-1β (1 ng/ml), van mesurar-se les formes fosforilades de les MAPKs ERK, JNK i p38, així com l'expressió de COX-2 mitjançant Western blot. El paper de les MAPKs en l'inducció de l'expressió de COX-2 va analitzar-se mitjançant el tractament dels fibroblasts amb inhibidors específics d'ERK (PD98059), JNK (SP600125) i p38 (SB203580), previ a la incubació amb IL-1β. La translocació al nucli de les subunitats de NF-kB i C/EBP va mesurar-se mitjançant Western blot i TransAm® després d'incubar els fibroblasts amb IL-1β (10 ng/ml).

Resultats: no van observar-se diferències entre els fibroblasts procedents de NM i NP-AIA, pel que fa a les corbes temps resposta de fosforilació de les MAPKs estudiades. L'inhibidor de la MAPK p38 a 10 mM va ser l´únic que va reduir significativament l'expressió de la COX-2 (85%) induïda per IL-1β en fibroblasts de NM. Als fibroblasts procedents de NP-AIA la inhibició de la COX-2 (65%) no va ser estadísticament significativa després del tractament amb l'inhibidor específic de p38 MAPK a 1 i 10 mM comparat amb les cèl·lules no tractades. Els inhibidors específics de les MAPKs JNK i ERK no van tenir efectes significatius sobre l'expressió de la COX-2 en els fibroblasts de NM ni tampoc en els de NP-AIA. Els efectes de la IL-1β sobre la translocació de les subunitats de NF-kB i C/EBP va ser similar als fibroblasts de NM i als de NP-AIA.

Conclusions: aquests resultats suggereixen que la MAPK p38 és l'única que es troba involucrada en l'expressió de la COX-2 a través de la inducció amb IL-1β. Els fibroblasts aïllats de NM i de NP-AIA presenten dinàmiques semblants pel que fa a la fosforilació de MAPKs i la translocació de factors de transcripció (NF-kB i C/EBP). La disminució de l'expressió de COX-2 descrita als pacients amb AIA no sembla causada per diferències en les dinàmiques d'activació de MAPKs ni la translocació de factors de transcripció reguladors de la COX-2.

PLOS ONE

Signal Transduction Pathways (MAPKs, NF-κB, and C/EBP) Regulating COX-2 Expression in Nasal Fibroblasts from Asthma Patients with Aspirin Intolerance

Francesc Josep Garcia-Garcia[1,2], Joaquim Mullol[1,2,3], Maria Perez-Gonzalez[1,2], Laura Pujols[1,2], Isam Alobid[1,2], Jordi Roca-Ferrer[1,2☯], Cesar Picado[1,2,4*☯]

1 Immunoal·lèrgia Respiratòria Clínica i Experimental (IRCE), Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Catalonia, Spain, **2** Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES), Barcelona, Catalonia, Spain, **3** Unitat de Rinologia i Clínica de l'Olfacte, Servei d'Otorinolaringologia, Hospital Clínic, Barcelona, Catalonia, Spain, **4** Servei de Pneumologia i Al·lèrgia Respiratòria, Hospital Clinic, Universitat de Barcelona, Barcelona, Catalonia, Spain

Abstract

Background: Recent studies have revealed that cyclooxygenase-2 (COX-2) expression is down-regulated in aspirin-induced asthma (AIA). Various signal pathways (MAPKs, NF-κB and C/EBP) are involved in COX-2 regulation.

Objective: To investigate the regulation of COX-2 expression through MAP-kinase pathway activation and nuclear factor translocation in aspirin-induced asthma (AIA).

Methods: Fibroblasts were isolated from specimens of nasal mucosa (NM, N = 5) and nasal polyps (NP, N = 5). After IL-1β (1 ng/ml) incubation, COX-2 and phosphorylated forms of ERK, JNK and p38 MAPK were measured by Western blot. MAPK's role in IL-1β-induced COX-2 expression was assessed by treating cells with ERK (PD98059), JNK (SP600125) and p38 MAPK (SB203580) inhibitors (0.1–10 μM) prior to IL-1β exposure. NF-κB and C/EBP nuclear translocation was measured by Western blot and TransAM® after IL-1β (10 ng/ml) exposure.

Results: No differences were observed in the MAPK phosphorylation time-course between NM and NP-AIA fibroblasts. The p38 MAPK inhibitor at 10 μM significantly reduced IL-1β-induced COX-2 expression in NM fibroblasts (85%). In NP-AIA fibroblasts the COX-2 inhibition (65%) at 1 and 10 μM was not statistically significant compared to non-treated cells. ERK and JNK inhibitors had no significant effect in either the NM or NP-AIA cultures. The effect of IL-1β on NF-κB and C/EBP subunits' nuclear translocation was similar between NM and NP-AIA fibroblasts.

Conclusions: These results suggest that p38 MAPK is the only MAPK involved in IL-1β-induced COX-2 expression. NM and NP-AIA fibroblasts have similar MAPK phosphorylation dynamics and nuclear factor translocation (NF-κB and C/EBP). COX-2 downregulation observed in AIA patients appears not to be caused by differences in MAPK dynamics or transcription factor translocation.

Citation: Garcia-Garcia FJ, Mullol J, Perez-Gonzalez M, Pujols L, Alobid I, et al. (2012) Signal Transduction Pathways (MAPKs, NF-κB, and C/EBP) Regulating COX-2 Expression in Nasal Fibroblasts from Asthma Patients with Aspirin Intolerance. PLoS ONE 7(12): e51281. doi:10.1371/journal.pone.0051281

Editor: Thomas H. Thatcher, University of Rochester Medical Center, United States of America

Received August 3, 2012; **Accepted** October 31, 2012; **Published** December 11, 2012

Funding: This work was supported by grants from the Spanish Ministry of Health (FIS PI030033, FIS PI080249), Fundació Catalana de Pneumologia (FUCAP), and Fundación Respira (Spanish Respiratory Society). The funders had no role in the study design, data collection or analysis, or the decision to publish, or the preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: idib402@clinic.ub.es

☯ These authors contributed equally to this work.

Introduction

Aspirin-induced asthma (AIA) is a syndrome clinically characterized by chronic rhinosinusitis with nasal polyposis (CRSwNP), asthma and bronchoconstriction episodes triggered by the intake of non-steroidal-anti-inflammatory drugs (NSAIDs) [1]. A close relationship has been demonstrated between CRSwNP and AIA, since the prevalence of CRSwNP in AIA may be as high as 60 70%, while in the population of aspirin-tolerant asthmatics it is less than 10% [2].

The pathogenesis of AIA remains poorly understood but accumulated evidence suggests that abnormalities in arachidonic acid metabolism may play a role [2,3]. Both an overactive 5-lipoxygenase pathway (5-LO) and reduced COX expression have been demonstrated, resulting in increased cysteinyl leukotriene production and reduced PGE_2 release in AIA [1,4 7]. There are two well-characterized COX enzymes: COX-1, considered a constitutive form involved in cell homeostasis [8], and COX-2, an inducible form activated by pro-inflammatory mediators, growth factors and cytokines. These alterations in AIA patients seem to be present in both the lower [6] and upper airways [4]. In fact,

previous studies have reported COX-2 down-regulation in airway fibroblasts obtained from AIA patients [7]. In contrast with asthma, increased COX-2 expression has been reported in other airway inflammatory diseases such as cystic fibrosis [9] and chronic obstructive pulmonary disease [10].

The mechanisms responsible for the reported alterations in the regulation of COX-2 in inflamed NP tissue remain to be clarified. It is well known that inflammatory stimuli elicit cellular responses through the activation of mitogen-activated protein kinases (MAPKs) by phosphorylation. MAPKs regulate various cellular activities, including gene expression, mitosis and programmed death. MAPK-catalyzed phosphorylation functions as a switch for turning the activity of their target proteins on/off [11,12].

In pluricellular organisms, there are three well-characterized subfamilies of MAPKs: extracellular-signal-regulated kinases, p42/44 (ERK1/2), c-jun amino terminal kinases (JNKs) and p38 MAPKs [11,12]. So far, it has been demonstrated that MAPK family members play a role in COX-2 gene expression in various cell types, such as HUVECs [13], airway smooth muscle cells [14] and chondrocytes [15]. However, the role of the various MAPKs regulating COX-2 in AIA has never been studied.

COX-2 gene expression is also regulated by the action of several transcription factors, such as NF-κB [16 18] and C/EBP [19]. It has been widely demonstrated that NF-κB regulates cell survival and inflammatory responses by acting, at least in part, on the two active κB binding sites described in the COX-2 promoter gene [16,20 22]. Active NF-κB complexes are dimers of combinations of Rel family polypeptides (p50, p52 and p65) that respond to a wide variety of stimuli. The composition of NF-κB dimmers partially determines their biological effects by conditioning nuclear translocation and binding to the κB-regulatory elements [16,20].

There is also a C/EBP binding site on the human COX-2 promoter, which is involved in COX-2 induction. The three main members of the C/EBP family are C/EBPα, C/EBPβ and C/EBPγ. Their nuclear translocation is induced by pro-inflammatory stimuli, but although all C/EBPs subunits recognize the same DNA sequence, the balance between them and the cell type will determine the activation or repression of the gene expression [23 25]. However, the regulation of NF-κB and C/EBP transcription factors in fibroblasts from AIA patients has not been studied.

We hypothesized that the COX-2 down-regulation observed in AIA patients is caused by alterations in the mechanisms regulating the activation of MAPKs and nuclear factor translocation (NF-κB, and C/EBPs). As we have demonstrated in previous studies that the anomalies in the regulation of COX-2 are present in cultured fibroblasts obtained from the NP of AIA patients, the objective of our study was to examine the activation of MAPKs and nuclear factor translocation (NF-κB, and C/EBPs) in fibroblasts derived from nasal mucosa, and from the NP of AIA patients.

Materials and Methods

Study Population

NM specimens were obtained from the lower turbinate of 5 non-asthmatic subjects with either septal deviation, turbinate hypertrophy or both who were undergoing nasal corrective surgery. All control subjects had taken aspirin or NSAIDs at clinical dosage without any untoward reactions (asthma and/or rhinitis, urticaria, angioedema or anaphylaxis). NP specimens were collected from patients with aspirin intolerance (NP-AIA) who had undergone endoscopic sinus surgery. The clinical and demographic characteristics of the subjects are shown in **Table 1**. The diagnosis of aspirin intolerance was confirmed by lysine-aspirin nasal challenge, as previously described [26]. None of the control subjects had received oral or intranasal corticosteroid treatment for at least one month before surgery. None of the control subjects or patients had any upper airway infection in the 2 weeks before surgery.

Table 1. Epidemiological characteristics of control subjects and patients with NP and AIA.

Characteristics	NM fibroblasts	NP-AIA fibroblasts
Fibroblast cultures, N	5	5
Age, years (mean ± SD)	55.6⁺14.5	59.6⁺11.9
Females, N (%)	3 (60)	4 (80)
Asthma, N (%)	0 (0)	5 (100)
Aspirin intolerance, N (%)	0 (0)	5 (100)
Atopy, N (%)	0 (0)	2 (40)
Intranasal corticosteroid, N (%)	0 (0)	3 (60)

SD, standard deviation.
doi:10.1371/journal.pone.0051281.t001

Ethical Declaration

All patients gave their written informed consent to participate in the study, which was approved by the Scientific and Ethics Committee (Comité Étic d'Investigació Clínica) of our Institution (Hospital Clínic de Barcelona).

Tissue Handling and Cell Culture

NM and NP tissues were cut into 3×3 mm fragments and placed in six-well plates (NUNC, Wiesbaden, Germany) containing Dulbecco's modified Eagle's media (DMEM) supplemented by 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, California, USA) and 2 μg/ml amphotericin B (Sigma, St Louis, MO, USA). Cultures were placed in a 5% CO_2 humidified incubator at 37°C and the culture media were changed every 2 days. Once the fibroblasts had grown, tissue fragments were removed and the first passage was performed by adding 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, California, USA) for 5 min. The reaction was stopped with 10% FBS-supplemented DMEM. Cells were then centrifuged (400 g, 5 min) and seeded in 150 cm^2 flasks (NUNC). At passages 5 to 6, fibroblasts were cultured in CultureSlides® and flasks to perform culture characterization and experimental protocols. The same batch of FBS was used for the whole experimental period. Mycoplasma contamination was tested by PCR in the cultures and all of them were negative.

Cell Characterization

Characterization of cultured cells was performed by immunofluorescence for fibroblasts (vimentin) and epithelial cells (cytokeratins) on CultureSlides® incubated with serum-free media (SFM) for 24 h. Immunofluorescence assessment was performed as previously reported [11]. The primary antibodies were against vimentin at dilution 1:100 (V5255, Sigma, Saint Louis, Missouri, USA) and pan-cytokeratin at 1:200 (C2562, recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19, Sigma). The percentage of positive cells was quantified using fluorescence microscopy.

Study Design

When cultures placed in 150 cm^2 flasks reached 80% confluence, FBS-supplemented media were switched to serum-free

media (SFM) for 24 h. To analyze COX expression, the dynamics of MAPK phosphorylated forms and transcription factor nuclear translocation, cells were incubated with SFM in the presence or absence of IL-1β (R&D Systems Minneapolis, MN, USA) at different concentrations and for different times, depending on the protein analyzed. Meanwhile, in experiments on MAPK inhibition fibroblasts were pre-treated with ERK (PD98059), JNK (SP600125) and p38 MAPK (SB203580) inhibitors from Calbiochem (La Jolla, CL, USA) at different concentrations (0.1 10 μM) for 1 h, prior to the addition of 1 ng/ml IL-1β for 24 h. Total proteins were obtained by scraping the flasks after two washes with cold PBS. The cells were centrifuged (400 g, 5 min at 4°C) and then resuspended in different buffers, depending on the protein quantified and the analytical method.

Analysis of COX Expression by Western Blot

Fibroblast cultures were incubated with IL-1β (1 ng/ml) from 0 to 24 h in time-course experiments, and for 24 h with IL-1β (0 10 ng/ml) in dose-response experiments. Cell pellet was resuspended in 0.4 ml ice-cold lysis buffer (Complete™ protease inhibitor cocktail tablet in 50 ml of 0.05 M Hepes buffer solution, 0.05% v/v Triton X-100, and 625 μM PMSF). Cells were sonicated twice for 15 sec in a sonifier (Branson, Danbury, CT, USA) and centrifuged (12,000 g, 10 min at 4°C). Supernatant containing total proteins was quantified by Lowry's method and used to analyze COX-1 and COX-2 protein expression by Western blot, as described previously (11). The primary antibodies used were against COX-1 (SC-1752, Santa Cruz) or COX-2 (SC-1745, Santa Cruz) at dilution 1:1000. Immunoreactive bands were visualized using a chemoluminiscent method (Supersignal West Pico Chemiluminescent Substrate, Rockford, IL, USA). Light emissions were detected by the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software, and normalized by β-actin band intensities assessed in the same samples.

Analysis of the Dynamics of MAPK Phosphorylated Forms by Western Blot

Fibroblast cultures were incubated with 1 ng/ml IL-1β from 0 to 60 min. Cell pellet was resuspended with an insulin syringe in ice-cold Rippa buffer (TrisHcl 50 mM+Nacl 150 mM, ph 7.4, apropine 1:1000, leupeptine 1:1000, ortovanadate 1:1000, NaF 1 mM, DTT 1 mM, pefabloc 100 mg/ml, igepal 1%, SDS 0.1% and Na deoxicolat 0.5%). The samples were in ice for 1 h and then centrifuged (12,000 g, 10 min at 4°C). Supernatant containing total proteins was quantified by Lowry's method and used to analyze phosphorylated and non-phosphorylated forms of MAPKs by Western blot. Briefly, 20 μg of proteins in loading buffer were denaturalized in a thermocycler (70°C, 10 min), loaded in 12% TRIS-glycine gels and ran (125 V, 90 min) in a Novex XCell II Mini-Cell. Proteins were transferred (20 V, 2 h) to a 0.45 μm pore size nitrocellulose membrane and non-specific binding sites were blocked using blocking buffer (5% BSA, 0.1% Tween 20, in 10 nM TBS) for 1 h at room temperature in an orbital shaker. The membranes were then washed three times in 0.1% Tween 20, in 10 nM TBS and incubated overnight with the primary antibody buffer (5% BSA and 0.1% Tween 20 in 10 nM TBS). The primary antibodies used were p-p38 MAPK at 1:1000 (9215, Cell Signalling Technology, Inc, Beberly, Mass, USA), p38 MAPK at 1:1000 (9212, Cell Signalling), p-JNK at 1:1000 (4668, Cell Signalling), JNK at 1:1000 (9258, Cell Signalling), p-ERK at 1:2000 (4370, Cell Signalling) and ERK at 1:2000 (4695, Cell Signalling). The membranes were then washed four times in washing buffer (0.1% Tween 20 in 10 nM TBS) and incubated with peroxidase-conjugated secondary antibody (1:3000) diluted in blocking buffer. After four washes, immunoreactive bands were visualized using a chemoluminiscent method (Supersignal West Dura Chemiluminescent Substrate, Rockford, IL, USA). Light emissions were detected by the CCD Camera System LAS 3000 (Fujifilm, Tokyo, Japan). Band intensities were quantified with Fujifilm Image Gauge 4.0 Software and normalized by the non-phosphorylated form of the respective MAPKs studied. The β-actin protein was assessed as a loading control.

MAPK Inhibition Analysis by Western Blot

Cell pellet was resuspended in 0.4 ml ice-cold lysis buffer, sonicated twice for 15 sec and centrifuged (12,000 g, 10 min at 4°C). Supernatant containing total proteins was quantified by Lowry's method and used to analyze COX-2 protein expression by Western blot, as described above.

Isolation of Nuclear Proteins

Fibroblasts cultures were incubated with 10 ng/ml IL-1β from 0 to 60 min. Since activated transcription factors translocate to the nucleus, the Active Motif Nuclear Extract Protocol (Active Motif, Carlsbad, CA, USA) was used to isolate nuclear proteins. The purity of the nuclear fractions was assayed using LDH (AB 1222, Millipore, Chemicon International, Temecula, CA, USA) as a cytosolic marker.

Analysis of Transcription Factors Translocation by ELISA-based Kits

The presence in nuclear extracts of p50, p52, p65, c/EBPα and C/EBPβ was measured with the ELISA-based kit TransAM® (Active Motif, Carlsbad, CA, USA), according to the manufacturer instructions. The colorimetric reading at 450 nm was determined in a microplate reader MultiScan Ascent (Thermo, Rockford, IL, USA). The positive-control Jurkat nuclear extract provided with the kit was used to quantify the samples and assess assay specificity.

Analysis of Transcription Factor Translocation by Western Blot

Since Western blot was performed to confirm the TransAM® findings, only the transcription factors that showed an increase in translocation with this ELISA-based method were analyzed. Nuclear extracts (30 μg) quantified by the Lowry method were denaturalized in thermocycler, loaded in 7% TRIS-acetate gel and run (125 V for 90 min) in a Novex XCell II Mini-Cell. The protein was then transferred (20 V 2 h) to an 0.45 μm pore-size nitrocellulose membrane and non-specific sites were blocked with blocking buffer for 1 h at RT in an orbital shaker. The membranes were incubated with the primary antibody against p65 (SC-372-G, Santa Cruz), p50 (SC-1190, Santa Cruz) in blocking buffer (1:1,000). The membranes were then washed 4 times in 0.5 Tween 20 in 10 nmol/L PBS and incubated with the peroxidase-conjugated secondary antibody (1:1000) diluted in blocking buffer. The bands were visualized as described in the COX Western blot section.

Statistic Analysis

The data obtained from MAPK phosphorylation are expressed as median of the ratio phosphorylated:non-phosphorylated form. Results obtained from MPAK inhibition are expressed as medians and 25^{th} 75^{th} interquartiles of the COX-2 expression, compared to IL-1β treated cells. Data obtained from ELISA-based kits are expressed as fold change increase median of the ratio transcription factor versus positive control. The non-parametric statistical

Mann-Whitney U-test was used for between-group comparisons and the Wilcoxon test was used for paired comparisons. Statistical significance was set at $p<0.05$.

Results

COX Expression

Basal COX-1 expression was not different between NM and NP-AIA fibroblasts. After IL-1β incubation in time-course and dose-response experiments, COX-1 expression was not modified in NM (N = 3) and NP-AIA (N = 3) cultured fibroblasts (**Figure 1**). COX-2, basal protein expression was not detected in either the NM or NP-AIA fibroblasts. However, fibroblasts from NM showed an increase in COX-2 expression, in a time-course and dose-response manner, after IL-1β incubation, with the highest COX-2 expression coming after 24 h of treatment with IL-1β at 1 and 10 ng/ml. In contrast, incubation with IL-1β at 24 h did not change COX-2 protein expression in cultured fibroblasts from the NP of AIA, compared to the baseline (**Figure 1**).

MAPK Activation

A trend towards increased ratios of phosphorylated versus non-phosphorylated forms of the studied MAPKs (p38 MAPK, JNK and ERK) were observed after 5 min of IL-1β incubation in both NM and NP-AIA fibroblast cultures. In NM, the phosphorylation kinetics of p38 MAPK, JNK and ERK reached their maximum at 15 min. However, in NP-AIA the maximum phosphorylation level was observed at 5 min for both p38 MAPK and ERK, and at 15 min for JNK. The phosphorylation level of p38 MAPK, JNK and ERK reverted to close to the basal level at 60 min in both the NM and NP-AIA fibroblast cultures. Nevertheless the observed trends did not achieve statistical significance compared to baseline (**Figure 2**). A comparison of the ratios of phosphorylated versus non-phosphorylated MAPK forms showed no significant differences between NM and NP-AIA fibroblasts.

MAPK Inhibition

In NM, incubation with p38 MAPK inhibitor SB203580 at 10 μM significantly decreased IL-1β-induced COX-2 protein expression (85%). In NP-AIA, p38 MAPK specific inhibitor decreased IL-1β-induced COX-2 protein expression at 1 μM (65%) and 10 μM (65%), although statistical significance was not achieved ($p=0.06$), probably due to the low COX-2 induction levels in these cells. As regards JNK and ERK inhibition, both failed to modify COX-2 protein expression in NM and NP-AIA fibroblasts (**Figure 3**). Finally, when comparing the effects of the different specific inhibitors on COX-2 expression, no significant differences were found between NM and NP-AIA fibroblasts.

Transcription Factor Nuclear Translocation

The analysis by TransAM® of p50 and p65 subunits showed the presence of these subunits at basal level in both NM and NP-AIA fibroblasts. Treatment with IL-1β induced the nuclear translocation of subunits p50 and p65, with a trend towards maximum translocation at 30 min and a decrease at 60 min in NM fibroblasts. In NP-AIA the trend towards maximum translocation was reached at 15 min and a plateau effect was maintained until 60 min. However, the observed trends did not achieve statistical significance compared to baseline. Western blot was performed with the same samples and the obtained images suggested similar kinetics (**Figure 4**). TransAM® measures revealed that the nuclear presence of C/EBPα, C/EBPβ (**Figure S1**) and p52 was not modified after IL-1β incubation of NM and NP-AIA fibroblasts. No statistically significant differences were found compared at any time in p50, p65, p52, C/EBPα and C/EBPβ protein expression between NM and NP-AIA fibroblasts.

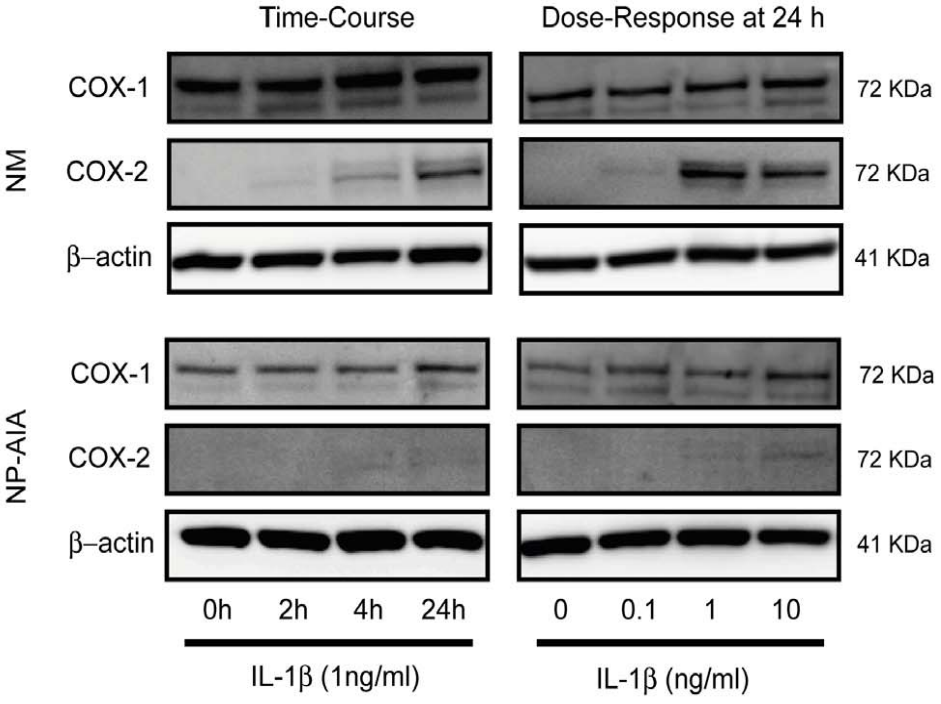

Figure 1. Time-course and dose-response of IL-1β in COX-1 and COX-2 protein expression. Fibroblasts from nasal mucosa (NM) and nasal polyps from AIA patients (NP-AIA) were incubated at different times and concentrations with IL-1β. COX-1 expression was not altered by IL-1β treatment in either NM or NP-AIA fibroblasts. In NM fibroblasts, COX-2 expression was induced in a time-dependent and dose-response manner. In NP-AIA, COX-2 protein expression was not significantly increased, compared to baseline level. The image that is shown is representative of NM and NP-AIA Western blot.
doi:10.1371/journal.pone.0051281.g001

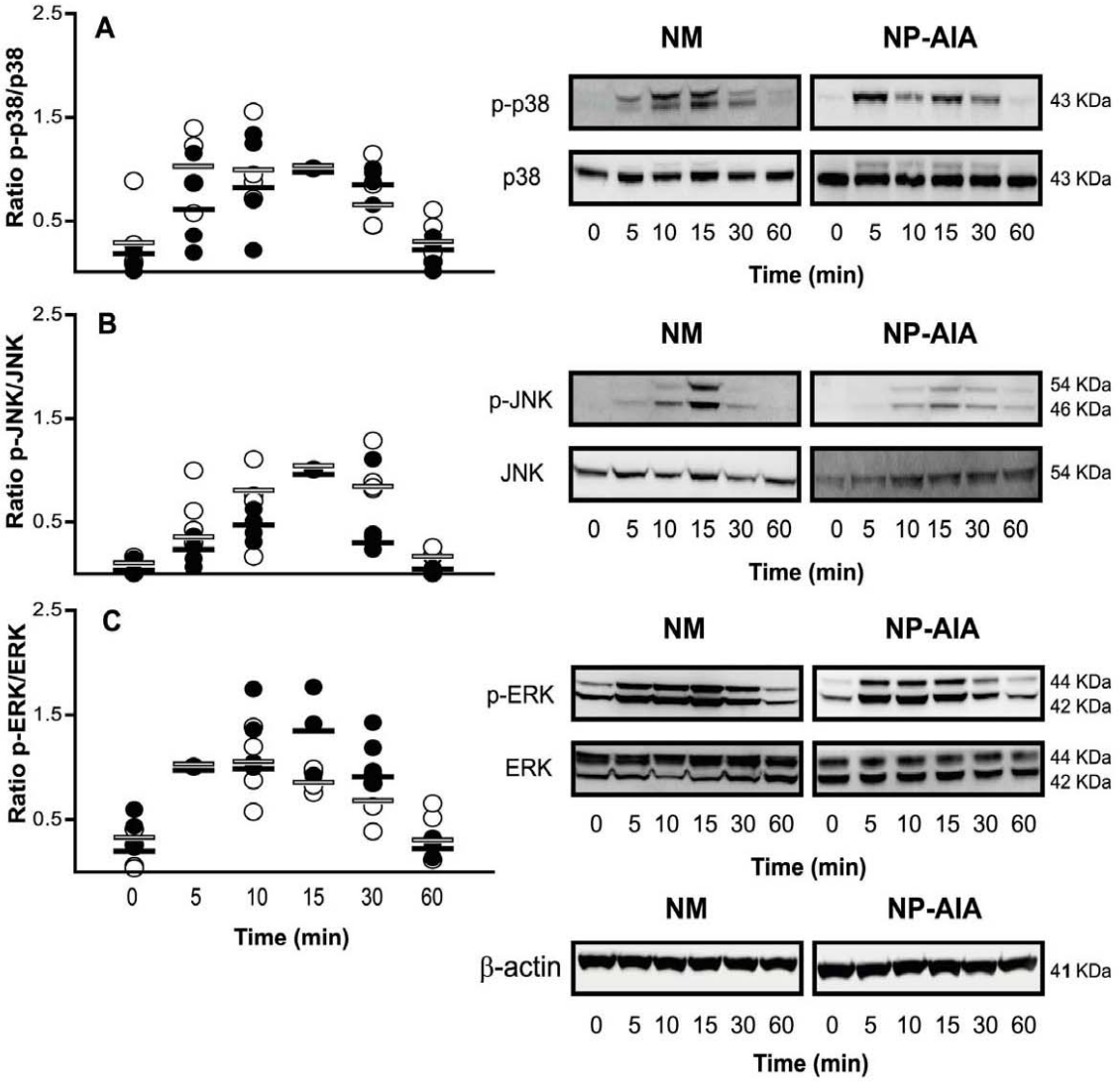

Figure 2. Time-course of MAPK activation by IL-1β in nasal fibroblasts cultures. Fibroblasts from nasal mucosa (NM, N = 4, black spots) and nasal polyps from AIA patients (NP-AIA, N = 4, white spots) were incubated with IL-1β (1 ng/ml) for 5 to 60 min. Phosphorylated and non-phosphorylated forms of p38 MAPK (A), JNK (B), and ERK (C) were measured by Western blot. Results are expressed as the ratio of phosphorylated *versus* non-phosphorylated MAPK forms. Graph shows individual experimental results and lines indicate the medians values. MAPK dynamic activation was not different (NS, Mann-Whitney U-test) between NM and NP-AIA fibroblasts. Insets show representative Western blot images of p38 MAPK, JNK, and ERK phosphorylation dynamics and β-actin as loading control.
doi:10.1371/journal.pone.0051281.g002

Discussion

Reduced COX-2 expression, and consequently decreased PGE_2 concentration, has previously been reported in peripheral blood leukocytes (PBLs) [27], urine [28], nasal polyps [29] and nasal polyp fibroblasts [7] in a variety of cells and samples from AIA patients, although the mechanism involved in this alteration remain unclear. To investigate the potential mechanisms underlying differences in the COX pathway of arachidonic acid in AIA, we used a previously published *in vitro* model [7] in which IL-1β induced lower COX-2 expression in NP-AIA fibroblasts compared with NM fibroblasts.

We hypothesized that the observed anomalies of COX-2 expression under inflammatory conditions were caused by differences in the signalling pathways, MAPK activation or nuclear transcription factor translocation between fibroblasts derived from NM and those obtained from NP of AIA patients.

The transient increase in phosphorylated levels in NP-AIA, peaking at 5 min (p38 MAPK and ERK) and 15 min (JNK) post-stimulation, were similar to those previously reported in stromal cells [30] and keratocyst fibroblasts [31], demonstrating the ability of IL-1β to activate MAPK signalling. To examine the involvement of the three main MAPK pathways in COX-2 gene expression regulation, NM and NP-AIA fibroblasts were incubated with selective MAPK inhibitors. ERK and JNK specific inhibitors had no significant effect on COX-2 induced expression. In contrast, a selective p38 MAPK inhibitor significantly reduced the IL-1β-induced COX-2 expression in NM fibroblasts, while only a tendency towards the inhibition of COX-2 expression was observed in NP. The latter finding can be explained by the difficulties in demonstrating differences in the inhibition of the expression of a gene (COX-2 gene) that changes very little after stimulation. All in all, these findings suggest that the p38 MAPK pathway is the transcription factor most involved in the regulation of COX-2 expression in nasal fibroblasts.

Our finding concurs with Chen *et al.* [32], who demonstrated the importance of p38 MAPK in COX-2 regulation in human pulmonary epithelial cells and the lack of involvement of ERK.

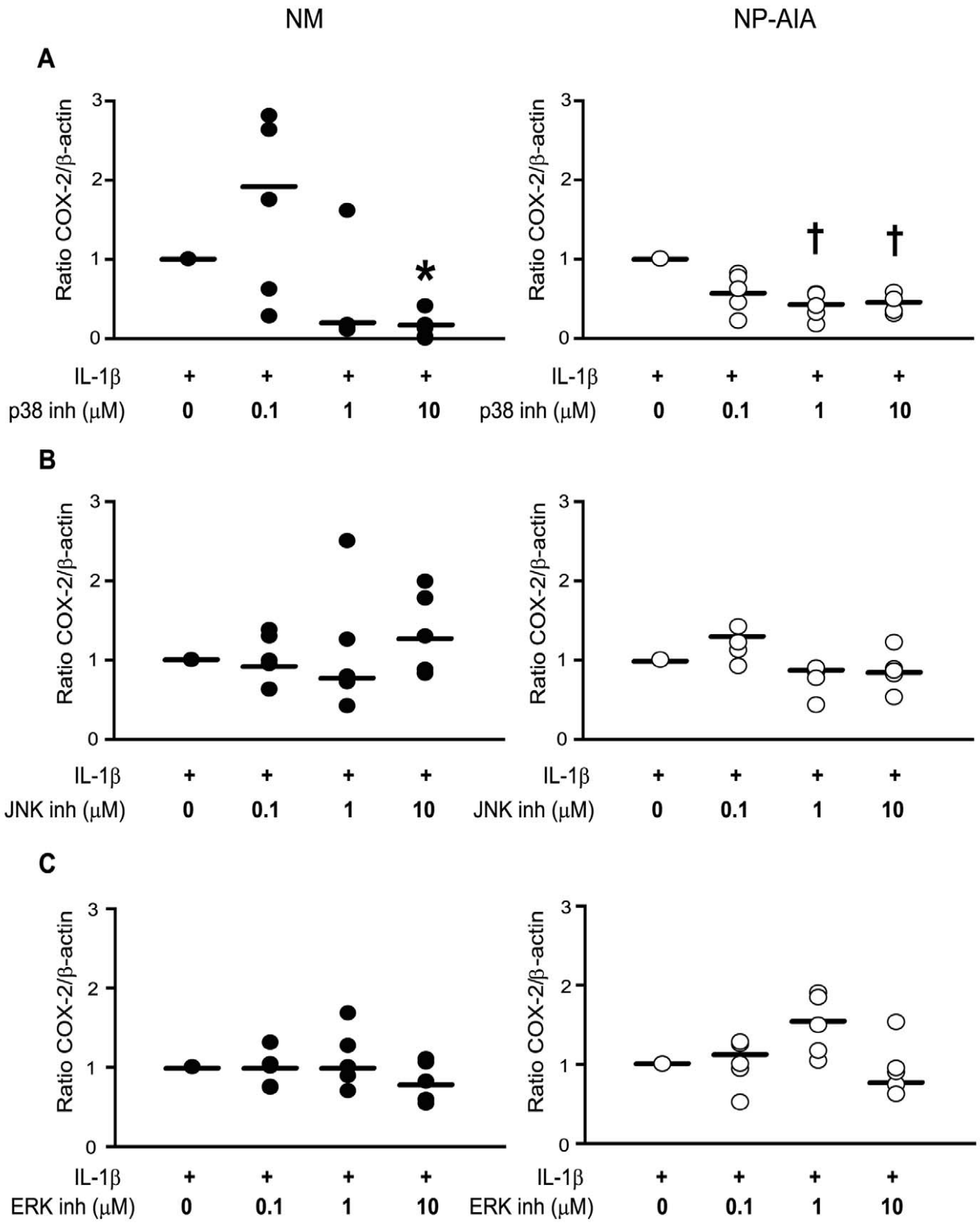

Figure 3. Effect of MAPK specific inhibitors on COX-2 protein expression. Fibroblasts from nasal mucosa (NM, N = 5, black spots) and nasal polyps from AIA patients (NP-AIA, N = 5, white spots) were pre-treated with p38 (A) MAPK (SB203580), (B) JNK (SP600125), and (C) ERK (PD98059) specific inhibitors at different concentrations (0.1–10 μM) for 1 h before addition of IL-1β (1 ng/ml) for 24 h. COX-2 and β-actin protein expression was analyzed by Western blot. Only the p38 MAPK inhibitor significantly blocked IL-1β-induced COX-2 expression in NM fibroblasts. Results are presented as COX-2/β-actin ratio. Graph shows individual experimental results and lines indicate the medians values. * $p<0.05$, † $p = 0.06$ compared to IL-1β treatment by Wilcoxon test.
doi:10.1371/journal.pone.0051281.g003

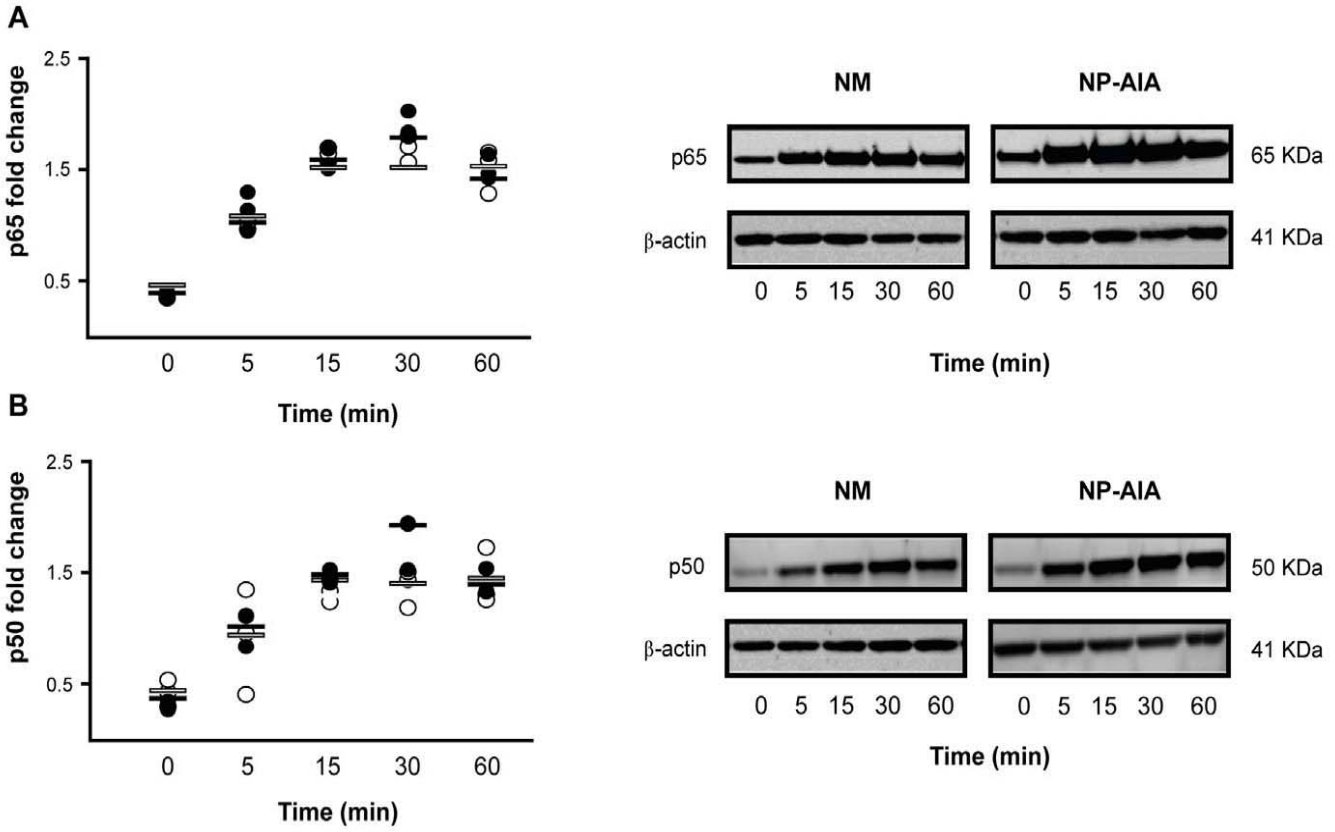

Figure 4. Time-course of p65 and p50 nuclear translocation induced by IL-1β in nasal fibroblast cultures. Fibroblasts from nasal mucosa (NM, N = 3, black spots) and nasal polyps from AIA patients (NP-AIA, N = 3, white spots) were incubated with IL-1β (10 ng/ml) for 5 to 60 min. p65 (A) and p50 (B) nuclear translocation were measured by TransAM® and Western blot. Graphs show the fold change increase from individual experimental results and lines indicate the medians values. Insets show representative Western blot images of p65 (A) and p50 (B) subunits from both NM and NP-AIA fibroblasts and the loading control β-actin. No significant differences (NS by Mann-Whitney U-test) were found at any time between NM and NP-AIA fibroblasts.
doi:10.1371/journal.pone.0051281.g004

Similarly, Ulivi *et al.* [21] also showed that p38 MAPK plays a critical role in COX-2 regulation in chondrocytes under inflammatory conditions.

IL-1β treatment induced rapid nuclear translocation of p50 and p65 NF-κB subunits in both NM and NP-AIA fibroblasts. In keeping with this result, previous reports have shown similar findings in several cell types, such as ASM cells [33], HT-29 [34] and lung fibroblasts [35], demonstrating p50 and p65 NF-κB involvement in gene expression regulation under inflammatory conditions, such as COX-2 induction. There were no statistical differences in translocation dynamics and nuclear concentrations of p65 and p50 proteins between NM and NP-AIA fibroblasts, suggesting that the nuclear translocation of NF-κB transcription factors is not altered in NP-AIA fibroblasts. In contrast, Picado *et al.* [36] reported a reduced NF-κB activity in NP-AIA samples when measuring p65 and p50 subunits. These discrepancies could be explained by the samples used (whole NP tissue explants), the experimental design followed (unstimulated samples) and the heterogeneity of NP-AIA tissues (some of the patients were receiving nasal corticosteroids before surgery).

In our study, the results obtained for the NF-κB subunit p52 did not show any variation after IL-1β induction, suggesting that p52 is not activated under the experimental conditions used in the study. Similarly, C/EBP isoform assessment revealed no changes after IL-1β incubation, suggesting that C/EBPs are not activated through the IL-1β pathway in either NM or NP-AIA fibroblasts. C/EBP involvement in COX-2 regulation has been demonstrated in several cell models, such as macrophages [37] and human foreskin fibroblasts [38]. However, the stimuli used in these studies were endotoxin and PMA, respectively, and this difference may account for the C/EBP activation found in these experimental models.

We could not find any differences in the nuclear translocation of p65, p50 and C/EBP isoforms in NP-AIA fibroblasts compared to NM fibroblasts after IL-1β induction. This observation concurs with the findings of Coward *et al.* [35], who demonstrated that the expression of p65 and C/EBPβ was unaltered in fibroblasts from idiopathic pulmonary fibrosis patients compared to control fibroblasts, a disease that is also characterized by down-regulated COX-2 expression in fibroblasts.

In summary, our study demonstrates that only p38 MAPK plays a role in COX-2 induction by IL-1β. Moreover, there were similar nuclear translocation dynamics in the NF-κB subunits, and no differences in C/EBP regulation were found in fibroblasts from either NP-AIA or NM. In conclusion, in the present study we did not find any alterations responsible for the COX-2 down-regulation described in NP-AIA fibroblasts.

Finally, future studies should assess other mechanisms, such as transcription factors binding ability to gene promoter, the activity of histone acetyltransferases and deacetylases and mRNA stability, which could be crucial to understanding the observed down-regulation of COX-2 in aspirin-induced asthma.

Supporting Information

Figure S1 Time-course of c/EBPα and c/EBPβ nuclear translocation induced by IL-1β in nasal fibroblast cultures. Fibroblasts from nasal mucosa (NM, N = 3, black spots) and nasal polyps from AIA patients (NP-AIA, N = 3, white spots) were incubated with IL-1β (10 ng/ml) for 5 to 60 min. c/EBPα (A) and c/EBPβ (B) nuclear translocation were measured by TransAM®. Graphs show the fold change increase from individual experimental measures and the medians. No significant differences (NS by Mann-Whitney U-test) were found at any time between NM and NP-AIA fibroblasts.
(TIF)

Author Contributions

Conceived and designed the experiments: FJG JRF LP JM CP. Performed the experiments: FJG MPG JRF LP. Analyzed the data: FJG JRF MPG LP. Wrote the paper: FJG JRF LP JM CP. Patient characterization and sample collection: IA JM CP. Reviewed and critically revised text and interpretation of a late version of the manuscript: JM CP JRF.

References

1. Stevenson DD, Szczeklik A (2006) Clinical and pathologic perspectives on aspirin sensitivity and asthma. J Allergy Clin Immunol 118: 773 86.
2. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, et al. (2012) EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. Rhinology 50: 1 12.
3. Picado C (2006) Mechanisms of aspirin sensitivity. Curr Allegy Asthma Rep 6: 198 202.
4. Kowalski ML, Pawliczak R, Wozniak J, Siuda K, Poniatowska M, et al. (2000) Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients. Am J Respir Crit Care Med 16: 391 8.
5. Schäfer D, Schmid M, Göde UC, Baenkler HW (1999) Dynamics of eicosanoids in peripheral blood cells during bronchial provocation in aspirin-intolerant asthmatics. Eur Respir J 13: 638 46.
6. Pierzchalska M, Szabó Z, Sanak M, Soja J, Szczeklik A (2003) Deficient prostaglandin E2 production by bronchial fibroblasts of asthmatic patients, with special reference to aspirin-induced asthma. J Allergy Clin Immunol 111: 1041 8.
7. Roca-Ferrer J, Garcia-Garcia FJ, Pereda J, Perez-Gonzalez M, Pujols L, et al. (2011) Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma. J Allergy Clin Immunol 128: 66 72.
8. Simmons DL, Botting RM, Hla T (2004) Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev 56: 387 437.
9. Roca-Ferrer J, Pujols L, Gartner S, Moreno A, Pumarola F, et al. (2006) Upregulation of COX-1 and COX-2 in nasal polyps in cystic fibrosis. Thorax 61: 592 6.
10. Xaubet A, Roca-Ferrer J, Pujols L, Ramírez J, Mullol J, et al. (2004) Cyclooxygenase-2 is up-regulated in lung parenchyma of chronic obstructive pulmonary disease and down-regulated in idiopathic pulmonary fibrosis. Sarcoidosis Vasc Diffuse Lung Dis 21: 35 42.
11. Dong C, Davis RJ, Flavell RA (2002) Map kinases in the immune response. Ann Review Immunol 20: 55 72.
12. Raman M, Chen W, Cobb MH (2000) Differential regulation and properties of MAPKs. Oncogene 26: 3100 12.
13. Nieminen R, Leinonen S, Lahti A, Vuolteenaho K, Jalonen U, et al. (2005) Inhibitors of mitogen-activated protein kinases downregulate COX-2 expression in human chondrocytes. Mediators Inflamm 5: 249 55.
14. Caughey GE, Cleland LG, Penglis PS, Gamble JR, James MJ (2001) Roles of Cyclooxygenase (COX)-1 and COX-2 in Prostanoid Production by Human Endothelial Cells: Selective Up-Regulation of Prostacyclin Synthesis by COX-2. Journal Immunol 167: 2831 8.
15. Laporte JD, Moore PE, Lahiri T, Schwartzman IN, Panettieri RA Jr, et al. (2000) p38 MAP kinase regulates IL-1 beta responses in cultured airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 279: L932 41.
16. Chun K-S, Surh Y-J (2004) Signal transduction pathways regulating cyclooxygenase-2 expression: potential molecular targets for chemoprevention. Biochem Pharmacol 68: 1089 100.
17. Ackerman WE 4th, Summerfield TLS, Vandre DD, Robinson JM, Kniss DA (2008) Nuclear factor-kappa B regulates inducible prostaglandin E synthase expression in human amnion mesenchymal cells. Biol Reprod 78: 68 76.
18. Griffin BD, Moynagh PN (2006) Persistent interleukin-1beta signaling causes long term activation of NFkappaB in a promoter-specific manner in human glial cells. J Biol Chem 281: 10316 26.
19. Branski RC, Zhou H, Sandulache VC, Chen J, Felsen D, et al. (2010) Cyclooxygenase-2 signaling in vocal fold fibroblasts. Laryngoscope 120: 1826 31.
20. Syeda F, Grosjean J, Houliston RA, Keogh RJ, Carter TD, et al. (2006) Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways. J Biol Chem 281: 11792 804.
21. Ulivi V, Giannoni P, Gentili C, Cancedda R, Descalzi F (2008) p38/NF-kB-dependent expression of COX-2 during differentiation and inflammatory response of chondrocytes. J Cell Biochem 104: 1393 406.
22. Birkenkamp KU, Tuyt LM, Lummen C, Wierenga AT, Kruijer W, et al. (2000) The p38 MAP kinase inhibitor SB203580 enhances nuclear factor-kappa B transcriptional activity by a non-specific effect upon the ERK pathway. Br J Pharmacol 131: 99 107.
23. Wu KK, Liou J-Y, Cieslik K (2005) Transcriptional Control of COX-2 via C/EBPbeta. Arterioscler Thromb Vasc Biol 25: 679 85.
24. Joo M, Park GY, Wright JG, Blackwell TS, Atchison ML, et al. (2004) Transcriptional regulation of the cyclooxygenase-2 gene in macrophages by PU.1. J Biol Chem 279: 6658 65.
25. Cho YH, Lee CH, Kim SG (2003) Potentiation of Lipopolysaccharide-Inducible Cyclooxygenase 2 Expression by C2-Ceramide via c-Jun N-Terminal Kinase-Mediated Activation of CCAAT/Enhancer Binding Protein β in Macrophages. Molecular Pharmacol 63: 512 23.
26. Casadevall J, Ventura PJ, Mullol J, Picado C (2000) Intranasal challenge with aspirin in the diagnosis of aspirin intolerant asthma: evaluation of nasal response by acoustic rhinometry. Thorax 55: 921 4.
27. Jedrzejczak-Czechowicz M, Lewandowska-Polak A, Bienkiewicz B, Kowalski ML (2008) Involvement of 15-lipoxygenase and prostaglandin EP receptors in aspirin-triggered 15-hydroxyeicosatetraenoic acid generation in aspirin-sensitive asthmatics. Clin Exp Allergy 38: 1108 16.
28. Higashi N, Mita H, Ono E, Fukutomi Y, Yamaguchi H, et al. (2010) Profile of eicosanoid generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers. J Allergy Clin Immunol 125: 1084 1091.e6.
29. Pujols L, Mullol J, Alobid I, Roca-Ferrer J, Xaubet A, et al. (2004) Dynamics of COX-2 in nasal mucosa and nasal polyps from aspirin-tolerant and aspirin-sensitive asthmatics. J Allergy Clin Immunol 114: 814 9.
30. Wu MH, Wang CA, Lin CC, Chen LC, Chang WC, et al. (2005) Distinct regulation of cyclooxygenase-2 by interleukin-1beta in normal and endometriotic stromal cells. J Clin Endocrinol Metab 90: 286 95.
31. Ogata S, Kubota Y, Yamashiro T, Takeuchi H, Ninomiya T, et al. (2007) Signaling pathways regulating IL-1alpha-induced COX-2 expression. J Dent Res 86: 186 91.
32. Chen P, Cai Y, Yang ZG, Zhou R, Zhang GS, et al. (2006) Involvement of PKC, p38 MAPK and AP-2 in IL-1β eta-induced expression of cyclooxygenase-2 in human pulmonary epithelial cells. Respirology 11: 18 23.
33. Nie M, Pang L, Inoue H, Knox AJ (2003) Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. Mol Cell Biol 23: 9233 44.
34. Liu W, Reinmuth N, Stoeltzing O, Parikh AA, Tellez C, et al. (2003) Cyclooxygenase-2 is up-regulated by interleukin-1 beta in human colorectal cancer cells via multiple signaling pathways. Cancer Res 63: 3632 6.
35. Coward WR, Watts K, Feghali-Bostwick CA, Knox A, Pang L (2009) Defective histone acetylation is responsible for the diminished expression of cyclooxygenase 2 in idiopathic pulmonary fibrosis. Mol Cell Biol 29: 4325 39.
36. Picado C, Bioque G, Roca-Ferrer J, Pujols L, Mullol J, et al. (2003) Nuclear factor-kappaB activity is down-regulated in nasal polyps from aspirin-sensitive asthmatics. Allergy 58: 122 6.
37. Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR (2000) Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 275: 6259 66.
38. Saunders MA, Sansores-Garcia L, Gilroy DW, Wu KK (2001) Selective suppression of CCAAT/enhancer-binding protein beta binding and cyclooxygenase-2 promoter activity by sodium salicylate in quiescent human fibroblasts. J Biol Chem 276: 18897 904.

Summary of the results

- The expression of the cyclooxygenase (COX-1 and -2) enzymes and the secretion of PGE_2 are reduced in response to an inflammatory situation (IL-1β) in fibroblasts from nasal polyps, especially those of aspirin-induced asthma patients.

- The expression of the PGE_2 receptor EP2 is reduced in response to an inflammatory situation (IL-1β) in fibroblasts from nasal polyps, especially those ofom aspirin-induced asthma patients.

- The expression of the cyclooxygenase (COX-1 and -2) enzymes and the secretion of PGE_2 are also reduced in response to an inflammatory situation (IL-1β) in nasal mucosa fibroblasts from aspirin-induced asthma patients.

- p38 MAPK is the main signal transduction pathway regulating COX-2 originating after IL-1β incubation in human nasal fibroblasts.

- The p38 MAPK signal transduction pathway originating after IL-1β incubation is not altered in nasal polyp fibroblasts from aspirin-induced asthma patients.

- Transcription factors C/EBPα and C/EBPβ are not activated after IL-1β incubation in human nasal fibroblasts.

- NF-kB subunits p65 and p50 are rapidly translocated to the nucleus after IL-1β incubation in human nasal fibroblasts, although there are no alterations in these fibroblasts in aspirin-induced asthma patients.

Resum de resultats

- L'expressió de les ciclooxigenases (COX-1 i -2) i la secreció de PGE_2 de es troben reduïdes en resposta a una situació inflamatòria (IL-1β) en fibroblasts procedents de pòlips nasals, especialment en aquells procedents de pacients amb asma induïda per aspirina.

- L'expressió del receptor EP2 de la PGE_2 es troba reduïda en resposta a una situació inflamatòria (IL-1β) als fibroblasts de pòlips nasals, especialment en aquells procedents de pacients amb asma induïda per aspirina.

- L'expressió de les ciclooxigenases (COX-1 i -2) i la secreció de PGE_2 es troben també reduïdes en resposta a una situació inflamatòria (IL-1β) en fibroblasts aïllats de mucosa nasal de pacients amb asma induïda per aspirina.

- La MAPK p38 és la principal via de transducció de senyal reguladora de COX-2 originada després de la incubació amb IL-1β en fibroblasts nasals humans.

- La via de transducció de senyal de la MAPK p38 originada després de la incubació amb IL-1β no es troba alterada en els fibroblasts de pacients amb asma induïda per aspirina.

- Els factors de transcripció C/EBPα and C/EBPβ no es troben activats després de la incubació amb IL-1β en fibroblasts nasals humans.

- Les subunitats p65 i p50 de NF-kB transloquen ràpidament al nucli després de la incubació amb IL-1β en fibroblasts nasals humans sense alteracions en aquells fibroblasts procedents de pacients amb asma induïda per aspirina.

4. Discussion

Aspirin-induced asthma is a clinical syndrome in which the interplay between two arachidonic acid enzymatic pathways (COX and LOX) is evident, being characterized by several abnormalities in the biosynthesis of their mediators and receptors. Several findings in the literature confirm that the LOX pathway is more highly activated in asthmatic patients, especially those with aspirin-induced asthma. However, alterations in the cyclooxygenase pathway have been reported in some studies (although not all of them), and consequently their role in the pathogenesis of aspirin-induced asthma is still controversial.

For a better understanding of the cyclooxygenase pathway in nasal polyposis and aspirin-induced asthma, in the present thesis:

a) Cyclooxygenase expression patterns and PGE_2 secretion have been studied and compared in fibroblasts isolated from the nasal mucosa of control subjects and nasal polyps of both non-asthmatic and aspirin-induced asthma patients.

b) Similarly, cyclooxygenase expression patterns and PGE_2 secretion have been studied and compared in fibroblasts cultured from the nasal mucosa of control subjects and aspirin-induced asthma patients.

c) Moreover, the prostaglandin receptor (EP) expression patterns and PGE_2 effects on cyclooxygenases under inflammatory conditions have been studied and compared in fibroblasts obtained from the nasal mucosa of control subjects and nasal polyps of both non-asthmatic and aspirin-induced asthma.

d) Finally, the signal transduction pathways regulating COX-2 expression have been studied and compared in fibroblasts isolated from the nasal mucosa of control subjects and the nasal polyps of asthma-induced aspirin patients.

These studies will contribute to a better understanding of the cyclooxygenase pathway role in the ethiopathogenesis of the nasal polyposis, asthma and aspirin-induced asthma.

1. Cyclooxygenase pathway in fibroblasts from nasal polyps with and without aspirin-induced asthma.

The cyclooxygenase pathway has been extensively studied in aspirin-induced asthma using both “in vivo” and “in vitro” models. Discordant and contradictory results have been obtained, however, probably due to the diverse methodologies and experimental protocols used in the various studies.

In Study 1 presented in the current thesis, we have demonstrated that the COX pathway is altered in nasal polyp fibroblasts, especially in those derived from aspirin-intolerant asthma patients. We have shown changes at different levels of the COX pathway, including: very low production of PGE_2; lack of the expected upregulation of COX-2 on inflammatory stimulation; and differences in the regulation of COX-1 (Roca-Ferrer et al. 2011).

As has been previously mentioned, PGE_2 is considered a potent mediator of inflammation. It is increased in some airway inflammatory diseases, such as CF (Zakrzewski et al. 1987) and COPD (Togo et al. 2008). However, PGE_2 does not seem to act as a pro-inflammatory mediator as a general rule, since low levels have been reported in cells and tissues from asthmatic patients. Furthermore, a protective effect has been also described for PGE_2 in the airways of patients suffering from AIA and idiopathic pulmonary fibrosis (IPF) (Vancheri et al. 2004).

Analysis of the role of PGE_2 in airway inflammatory diseases shows that *in vivo* studies measuring PGE_2 in urine (Mastalerz et al. 2008; Higashi et al. 2010) or bronchoalveolar lavages (BAL) (Langmack et al. 1998) have found lower PGE_2 concentrations in AIA patients compared with those with ATA or healthy control subjects. Similarly, *in vitro* studies using bronchial fibroblasts found reduced PGE_2 secretion in asthma patients, especially those with AIA (Pierzchalska et al. 2003). However, other *in vivo* studies analyzing measurements of concentrated exhaled air (Antczak et al. 2002), BAL (Szczeklik et al. 1996), induced sputum (Pavord et al. 1999) and urine (Mastalerz et al. 2008) did not found any differences in PGE_2 production when ATA and AIA patients were compared with healthy control subjects.

Furthermore, no differences were found between ATA and control subjects when comparing PGE_2 secretion from bronchial epithelial cells (Pierzchalska et al. 2007).

In summary, low PGE_2 production, or no difference at all, has been found in the lower airways of asthma and AIA patients. PGE_2 downregulation has been found, however, in almost all the studies investigating *in vivo* and *in vitro* secretion from upper airways. For instance, reduced PGE_2 secretion has been found in sinonasal tissue (Perez-Novo et al. 2005), NP explants (Kowalski et al. 2000) and NP epithelial cells (Kowalski et al. 2000; Yoshimura et al. 2008). In line with some of the findings reported in the upper airways, the downregulation of PGE_2 secretion is especially marked in AIA patients.

Table 1. PGE_2 secretion in nasal polyposis, asthma and aspirin induced asthma.

Airways	Study type	Sample	Methods	Result	Reference
Lower	In vivo	BAL	GC-MS	No ≠ ATA vs AIA	Sczezklik et al. 1996
Lower	In vivo	Induced sputum	GC-MS	No ≠ ATA vs Ctrl	Pavord et al. 1999
Lower	In vivo	Induced sputum	GC-MS	No ≠ ATA vs Ctrl	Brightling et al. 2000
Lower	In vivo	Expired breath condensated	ELISA	No ≠ ATA vs AIA	Antczak et al. 2002
Lower	In vivo	Urine	ELISA	No ≠ ATA/AIA vs Ctrl	Mastalerz et al. 2008
Lower	In vivo	Urine	ELISA	Lower in AIA	Higashi et al. 2010
Lower	In vitro	Bronchial fibroblasts	GC-MS	No ≠ ATA/AIA vs Ctrl	Pierzchalska et al. 2003
Lower	In vitro	Bronchial epithelial cells	GC-MS	No ≠ ATA vs Ctrl	Pierzchalska et al. 2007
Upper	In vivo	Sinonasal tissue	ELISA	Low in NP and AIA	Perez-Novo et al. 2005
Upper	In vitro	NP epithelial cells	ELISA	Low in AIA vs ATA	Kowalski et al. 2000
Upper	In vitro	NP explants	ELISA	Low in AIA	Yoshimura et al. 2008
Upper	In vitro	NP fibroblasts	ELISA	Low in NP and AIA	Roca-Ferrer et al. 2011

BAL, Bronchoalveolar Lavage; GC-MS, Gas Chromatography- Mass Spectrometry; ATA, Aspirin-Tolerant Asthamtic; AIA, Aspirin-induced Asthma; Ctrl, Control; ELISA, Enzyme-Linked ImmunoSorbent Assay; NP, Nasal polyp.

Our findings, using fibroblast cultures isolated from the NP of patients with and without AIA, concur with those outlined above, demonstrating that the prostanoid pathway is abnormally regulated in nasal polyposis, especially in those patients with AIA. Thus, according to the classification of PGE_2 as a pro-inflammatory mediator, the unchanged or reduced production of PGE_2 in CRSwNP, asthma and AIA should be considered an anomaly rather than an expected finding.

Since PGE_2 synthesis is regulated, at least in part, by COX activity, and since COX expression is expected to be increased under inflammatory conditions, the next question is: What is the expression of COX enzymes in patients with CRSwNP, particularly in those with AIA?

As previously mentioned, COX-1 is considered a constitutive enzyme expressed in most cells and it has functions that regulate homeostatic procedures (Snipes et al. 2005). However, COX-1 expression has proved to be increased in some inflamed tissues (Chandrasekharan et al. 2004). In fact, when analyzing samples from asthma and AIA patients, *in vitro* studies demonstrate a reduced COX-1 expression in bronchial fibroblasts (Pierzchalska et al. 2003) and bronchial epithelial cells (Pierzchalska et al. 2007), compared to ATA and control subjects. So, despite the absence of any differences, or reduced PGE_2 secretion in asthma and AIA, COX-1 seems to be downregulated.

In the upper airways, some studies using NP tissue did not manage to find any differences in COX-1 expression when comparing ATA and AIA with control subjects (Picado et al. 1999; Adamjee et al. 2006; Okano et al. 2006). However, other studies analyzing the NP tissue of patients with CRS and CF found COX-1 expression either increased or decreased, depending on the experimental conditions. Increased COX-1 expression has been reported in NP from ATA and AIA samples over short times (Pujols et al. 2004), suggesting that COX-1 is sensitive to inflammatory stimuli. Other studies, however, have found reduced COX-1 expression when comparing NP tissue to control subjects over longer time-courses (Mullol et al. 2002). Based on the long half-life of COX-1 protein, the measurements in Study 1 were performed after 72 hours of induction, demonstrating an increase in COX-1 expression in fibroblasts from healthy

subjects that was slightly observed in fibroblasts from NP and not observed at all in NP-AIA fibroblasts (Roca-Ferrer et al. 2011).

Table 2. COX-1 expression in nasal polyposis, asthma and aspirin induced asthma.

Airways	Study type	Sample	Methods	Result	Reference
Lower	In vitro	Bronchial fibroblasts	RT-PCR/ WB	Low in AIA	Pierzchalska et al. 2003
Lower	In vitro	Bronchial epithelial cells	RT-PCR	Low in AIA	Pierzchalska et al. 2007
Upper	In vivo	NP tissue	RT-PCR	No ≠ATA/AIA vs Ctrl	Picado et al. 1999
Upper	In vivo	NP tissue	IHC	No≠ ATA vs AIA	Adamjee et al. 2006
Upper	In vivo	Sinonasal tissue	RT-PCR	No ≠ATA/NP vs Ctrl	Okano et al. 2006
Upper	In vitro	NP explants	RT-PCR/ WB	Low in NP	Mullol et al. 2002
Upper	In vitro	NP explants	RT-PCR	High in ATA/AIA	Pujols et ak 2004
Upper	In vitro	NP fibroblasts	WB	Low in NP and AIA	Roca-Ferrer et al. 2011

RT-PCR, Real Time-Polimerase Chain Reaction; WB, Western Blot; AIA, Aspirin-Induced Asthma; NP, Nasal polyp; ATA, Aspirin-Tolerant Asthma; Ctrl, Control; IHC, Immunohistochemistry.

The expression of COX-2 is usually increased under inflammatory conditions. In fact, diseases characterized by high levels of PGE_2 are usually associated with increased expression of COX-2 (Simmons et al. 2004). However, the decreased production of PGE_2 in airways described in patients suffering from CRSwNP, asthma and AIA, would lead us to expect a reduced COX-2 expression.

The studies measuring COX-2 expression in the lower airways did not find any differences when comparing the bronchial tissue (Cowburn et al. 1998), bronchial epithelial cells (Pierzchalska et al. 2007) and bronchial fibroblasts (Pierzchalska et al. 2003) of patients suffering from asthma and AIA with control subjects. So, although no differences or reduced PGE_2 secretion have been found in these patients, a similar COX-2 expression was reported.

In contrast, several *in vivo* studies measuring COX-2 expression in upper airways, found reduced COX-2 expression in NPs, specially those of AIA patients (Picado et al. 1999; Okano et al. 2006). Similar results were obtained in some *in vitro* studies that demonstrated reduced COX-2 expression in NP explants compared to nasal mucosa. In line with the *in vivo* studies, a greater reduction was reported in NP from AIA patients (Mullol et al. 2002; Pujols et al. 2004). These results concur with the reduced COX-2 expression reported in Study 1, which used fibroblasts isolated from healthy nasal mucosa, nasal polyps and nasal polyps from AIA patients. Moreover, in Study 1 the same model was observed with three different methodologies - Western blot, ELISA and immunohistochemistry - and similar results were obtained (Roca-Ferrer et al. 2011). The literature also reveals some studies using immunohistochemistry that did not find any differences in COX-2 expression in cells from upper or lower airways when comparing AIA, ATA or CRS with control subjects (Sousa et al. 1997; Cowburn et al. 1998; Demoly et al. 1998; Adamjee et al. 2006). Discrepancies with these other studies might account for the static characteristics of the immunohistochemical analyses performed, compared to the kinetic ones observed in Western blot or RT-PCR assays (Mullol et al. 2002; Pujols et al. 2004).

Finally, altered regulation of COX-1, along with the lack of response of COX-2 upregulation, might contribute to the low levels of PGE_2 detected in NPs, especially in patients with AIA.

Table 3. COX-2 expression in nasal polyposis, asthma and aspirin induced asthma.

Airways	Study type	Sample	Methods	Result	Reference
Lower	In vivo	Bronchial tissue	IHC	No ≠ ATA/AIA vs Ctrl	Cowburn et al. 1998
Lower	In vitro	Bronchial fibroblasts	RT-PCR/ WB	No ≠ ATA/AIA vs Ctrl	Pierzchalska et al. 2003
Lower	In vitro	Bronchial epithelial cells	RT-PCR	No ≠ ATA/AIA vs Ctrl	Pierzchalska et al. 2007
Upper	In vivo	NP tissue	RT-PCR	Low in AIA vs ATA/Ctrl	Picado et al. 1999
Upper	In vivo	Sinonasal tissue	RT-PCR	Low in NP and AIA	Perez-Novo et al. 2005
Upper	In vivo	NP tissue	RT-PCR	Low in ATA	Okano et al. 2006
Upper	In vivo	NP tissue	IHC	No ≠ ATA vs AIA	Adamjee et al. 2006
Upper	In vitro	NP explants	RT-PCR/ WB	Low in NP	Mullol et al. 2002
Upper	In vitro	NP explants	RT-PCR	Low in ATA/AIA	Pujols et al. 2004
Upper	In vitro	NP fibroblasts	IHC/ ELISA/ WB	Low in NP and AIA	Roca-Ferrer et al. 2011

IHC, Immunohistochemistry; ATA, Aspirin-Tolerant Asthma; AIA, Aspirin-Induced Asthma; Ctrl, Control; RT-PCR, Real Time-Polimerase Chain Reaction; WB, Western Blot; NP, Nasal polyp;

2. Cyclooxygenase pathway in fibroblasts from aspirin-induced asthma nasal mucosa.

In Study 2 presented in the current thesis we reported alterations to the cyclooxygenase pathway in nasal mucosa fibroblasts from aspirin-intolerant asthmatic patients compared to control nasal mucosa.

This study helps clarify whether the reported anomalies described in Study 1 (Roca-Ferrer et al. 2011) were representative of the whole nasal mucosa or specific to nasal polyps. So, since our study 1, along with most of the studies undertaken with nasal polyp specimens, we planned and performed a series of experiments using the nasal mucosa surrounding nasal polyps.

We found a general reduced response to an inflammatory situation regulating PGE_2 secretion and COX-1 and COX-2 expression in fibroblasts from NM fibroblasts of control subjects compared to NM from AIA patients.

The measures of PGE_2 secretion in NM fibroblasts from AIA patients were significantly reduced compared to those from NM fibroblasts from control subjects. These findings concur with the observations made in Study 1 on fibroblasts isolated from the NP of AIA patients. The limited production of PGE_2 found in NM-AIA fibroblasts may contribute to the intensification of the inflammatory process in the airways and may account for the reported association between NSAID intolerance, asthma and nasal polyp severity.

As several studies have not found any difference in the levels of COX-2 expression in AIA, we measured COX-2, in keeping with Study 1 (Roca-Ferrer et al. 2011), with three different methodologies (Western blot, ELISA and immunohistochemistry). The three methods used in the present study showed similar outcomes and demonstrated that COX-2 protein is not upregulated by inflammatory stimuli in fibroblasts from the NM of AIA patients. These findings support those of Study 1 (Roca-Ferrer et al. 2011) as regards COX-2 expression in NP-AIA fibroblasts. These observations would endorse the notion that, for reasons as yet unknown, COX-2 is downregulated in AIA patients. Moreover, these results would contradict the generally accepted theory of the increase in the COX pathway in inflammatory diseases.

With respect to COX-1 regulation in AIA, Study 2 showed that alterations in COX-1 expression were also present in fibroblasts obtained from the NM of AIA patients. This observation concurs with the observations made in Study 1 (Roca-Ferrer et al. 2011) and will support the studies confirming a general deregulation of the COX pathway in AIA.

To sum up, we found a significant reduction in PGE_2 secretion and COX-1 and -2 expression in the NM from AIA patients compared to the NM of control subjects. The findings presented in Studies 1 (Roca-Ferrer et al. 2011) and 2 might help clarify the

involvement of alterations to the COX pathway in the pathogenesis of NPs, asthma and AIA. Furthermore, our data suggest that abnormalities in the COX pathway are present in the whole nasal mucosa of AIA patients, and they are not only confined to the NP mucosal tissue, demonstrating that the nasal mucosa of AIA patients cannot be used as a surrogate for healthy nasal mucosa. Finally, our results also support the consensus term of rhinosinusitis suggested by the European Position Paper on Rhinosinusitis and Nasal Polyps (Fokkens et al. 2012).

3. Expression of the EP receptors and PGE_2 effects over COX expression in fibroblasts from nasal polyps with or without aspirin-induced asthma.

In Study 1 (Roca-Ferrer et al. 2011), the expression of PGE_2 receptors (EP1-4) was measured and compared in fibroblasts isolated from NM and NP fibroblasts of patients with and without AIA at baseline and after 72 h of IL-1β induction.

Baseline measures of the EP receptor expression (1 to 4) by Western blot did not reveal any differences between the fibroblasts from the NM and NP of patients with or without AIA. Moreover, the expression of the EP1, EP3 or EP4 receptors in the fibroblast populations studied was not changed by induction with IL-1β for 72 h. On the contrary, the expression of the EP2 receptor was significantly increased in control NM fibroblasts, while the response was reduced and absent in fibroblasts from NP and NP-AIA, respectively. This downregulation of the EP-2 expression in NP and AIA could play a role in the inflammatory process present in the airways of AIA patients, since it has been reported that most of the anti-inflammatory effects of PGE_2 are mediated by stimulation of EP2 receptor (Kay et al. 2006). Moreover, a reduced number of eosinophils expressing EP2 as also been reported in samples of the NP of AIA patients (Ying et al. 2006). So, the decreased release of PGE_2 described in Studies 1 and 2 and the lower expression of its receptor EP2 might increase the inflammatory process in the airways of AIA patients.

In keeping with this observation, Study 3 of the present thesis was designed to describe and compare the effects of exogenous PGE_2 on cyclooxygenase expression in

fibroblasts from the NPs of patients with and without AIA compared to fibroblasts from nasal mucosa.

In general, COX-1 is considered a housekeeping gene, and the results obtained after the incubation with PGE_2 plus IL-1β for 4 h in Study 3 would support this notion. Some studies, however, have reported slight increases in expression under inflammatory conditions such as cystic fibrosis (Roca-Ferrer et al. 2006), indicating that COX-1 might be sensitive to inflammatory situations in the airways. In fact, in Studies 1 and 2, presented above, COX-1 increased its expression after incubation with IL-1β in control fibroblasts from NM. In accordance with these results,this observation is confirmed in Study 3 after incubation with PGE_2 plus IL-1β for 24 h. Moreover, when comparing COX-1 expression between the studied groups, the increase in NM fibroblasts was significantly higher compared to NP fibroblasts, especially those from AIA patients. In keeping with our findings, Pierzchalska and cols also demonstrated a lower presence of COX-1 mRNA transcripts in human bronchial epithelial cells and bronchial fibroblasts from AIA patients after stimulation with cytokine mix (Pierzchalska et al. 2003; Pierzchalska et al. 2007) .

As regards COX-2 expression, the measurements at 4 and 24 h showed a significant increase after PGE_2 plus IL-1β incubation in all the studied groups (NM, NP-AT and NP-AIA), compared to baseline and individual treatments. In line with these findings, the literature has reported positive COX-2 regulation by PGE_2, and also by other prostanoids, in various cellular models, such as mouse lung fibroblasts (Vichai et al. 2005) and U937 cells (Inoue et al. 2000). When the combined treatment effects were compared between groups, COX-2 expression was significantly reduced in NP-AIA fibroblasts compared to NM fibroblasts at 4 h. This observation concurs with the findings of some other works in the literature (Pujols et al. 2004; Perez-Novo et al. 2005) and with Studies 1 and 2, which demonstrated a reduced COX-2 induced expression, especially in patients with AIA.

The experimental results in Study 3 demonstrated a lack of response to PGE_2 in COX enzyme expression in NP fibroblasts, especially in those derived from AIA

patients. These observations, as well as those from Studies 1 and 2, suggest a general reduction in the COX pathway in these patients.

In keeping with the observations in Studies 1 and 2, several works have demonstrated a downregulation in the cyclooxygenase pathway, supporting the notion of arachidonic acid imbalance in aspirin intolerance. All in all, this dysregulation might be one of the features that could contribute to the establishment of the ethiopatological basis of NPs, asthma and AIA.

4. Mechanisms regulating COX-2 expression in nasal mucosa fibroblasts and nasal polyp fibroblasts from AIA patients.

The studies presented in this thesis have demonstrated that COX-2 is downregulated in NPs fibroblasts, especially those from AIA patients. However, the mechanisms involved in the COX-2 downregulation observed in AIA patients remains unclear. Accordingly, Study 3 presented in the current thesis was designed to describe some of the mechanisms regulating COX-2 expression under inflammatory conditions in NM fibroblasts and NP fibroblasts from AIA patients, who present the highest levels of AA acid abnormalities.

The MAPK activation observed in the study was similar to those previously reported in the literature, demonstrating the ability of IL-1β to activate these pathways (Wu et al. 2005; Ogata et al. 2007). To examine the involvement of MAPK pathways in COX-2 gene expression regulation, fibroblasts from NM and NP-AIA were incubated with selective MAPK inhibitors. The selective p38 MAPK inhibitor was the only one to significantly reduce the IL-1β-induced COX-2 expression in NM fibroblasts, while only a trend towards this was observed in NP fibroblasts from AIA patients. This observation can be explained by the difficulties in demonstrating inhibition in a downexpressed gene such as COX-2 in AIA. Although no differences were observed, these findings concur with some other studies demonstrating that the p38 MAPK pathway plays a critical role in COX-2 regulation (Chen et al. 2006).

Another mechanism that could explain the COX-2 downregulation previously described in AIA patients might be alterations in the nuclear translocation of some of the transcription factors involved in COX-2 regulation, such as NF-kB or C/EBP.

The participation of NF-kB and C/EBP in COX-2 regulation has been widely reported in the literature (Wu et al. 2005; Syeda et al. 2006). In this study we demonstrated the rapid nuclear translocation of the p50 and p65 subunits using two different methodologies such (TransAm and Western blot). No differences were found, however, between control NM and NP-AIA fibroblasts. with respect to C/EBPs, TransAM measurements did not reveal any changes after IL-1β incubation, suggesting that C/EBP translocation is not activated via this pathway. In keeping with this observation, some works in the literature demonstrate C/EBP activation using PMA or endotoxin as COX-2 inducers (Wadleigh et al. 2000; Saunders et al. 2001).

In summary, Study 3 demonstrated the role of p38 MAPK in COX-2 induction by IL-1β. Moreover, no differences in the nuclear translocation of NF-kB subunits and C/EBP transcription factors were observed between NM and NP-AIA fibroblasts.

Finally, although Study 3 did not succeed in finding the mechanisms responsible for the COX-2 downregulation described in aspirin intolerance, the information reported therein could contribute to furthering our knowledge of the aethiopathology of both NPs and AIA.

5. Conclusions

There is a downregulation of the arachidonic acid metabolism, particularly in the cyclooxygenase pathway (COX-1 and COX-2) and in the EP2 expression, in subjects suffering from nasal polyposis, especially aspirin-induced asthma patients.

The main signal transduction pathways and transcription factors regulating cyclooxygenase-2 expression remain unaltered in aspirin-induced asthma fibroblasts. This finding suggests that they are not involved in the abnormal regulation of this pathway described in asthma patients with and without aspirin sensitivity.

Further studies are needed to elucidate the mechanisms responsible for the abnormal regulation of the cyclooxygenase pathway in asthma patients with and without aspirin intolerance.

6. References

A

Adamjee, J., Y. J. Suh, H. S. Park, J. H. Choi, J. F. Penrose, B. K. Lam, K. F. Austen, A. M. Cazaly, S. J. Wilson and A. P. Sampson (2006). "Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma." J Pathol **209**(3): 392-399.

Alvarez, Y., J. V. Perez-Giron, R. Hernanz, A. M. Briones, A. Garcia-Redondo, A. Beltran, M. J. Alonso and M. Salaices (2007). "Losartan reduces the increased participation of cyclooxygenase-2-derived products in vascular responses of hypertensive rats." J Pharmacol Exp Ther **321**(1): 381-388.

Anselmi, A., A. Abbate, F. Girola, G. Nasso, G. G. Biondi-Zoccai, G. Possati and M. Gaudino (2004). "Myocardial ischemia, stunning, inflammation, and apoptosis during cardiac surgery: a review of evidence." Eur J Cardiothorac Surg **25**(3): 304-311.

Antczak, A., P. Montuschi, S. Kharitonov, P. Gorski and P. J. Barnes (2002). "Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma." Am J Respir Crit Care Med **166**(3): 301-306.

B

Bachert, C., K. Hormann, R. Mosges, G. Rasp, H. Riechelmann, R. Muller, H. Luckhaupt, B. A. Stuck and C. Rudack (2003). "An update on the diagnosis and treatment of sinusitis and nasal polyposis." Allergy **58**(3): 176-191.

Bachert, C., M. Wagenmann, C. Rudack, K. Hopken, M. Hillebrandt, D. Wang and P. van Cauwenberge (1998). "The role of cytokines in infectious sinusitis and nasal polyposis." Allergy **53**(1): 2-13.

Bachert, C., J. B. Watelet, P. Gevaert and P. Van Cauwenberge (2005). "Pharmacological management of nasal polyposis." Drugs **65**(11): 1537-1552.

Baigent, C. and C. Patrono (2003). "Selective cyclooxygenase 2 inhibitors, aspirin, and cardiovascular disease: a reappraisal." Arthritis Rheum **48**(1): 12-20.

Baldwin, A. S., Jr. (2001). "Series introduction: the transcription factor NF-kappaB and human disease." J Clin Invest **107**(1): 3-6.

Bauchau, V. and S. R. Durham (2004). "Prevalence and rate of diagnosis of allergic rhinitis in Europe." Eur Respir J **24**(5): 758-764.

Bauchau, V. and S. R. Durham (2005). "Epidemiological characterization of the intermittent and persistent types of allergic rhinitis." Allergy **60**(3): 350-353.

Beltran, A. E., A. M. Briones, A. B. Garcia-Redondo, C. Rodriguez, M. Miguel, Y. Alvarez, M. J. Alonso, J. Martinez-Gonzalez and M. Salaices (2009). "p38 MAPK contributes to angiotensin II-induced COX-2 expression in aortic fibroblasts from normotensive and hypertensive rats." J Hypertens **27**(1): 142-154.

Benitez, P., I. Alobid, J. de Haro, J. Berenguer, M. Bernal-Sprekelsen, L. Pujols, C. Picado and J. Mullol (2006). "A short course of oral prednisone followed by intranasal budesonide is an effective treatment of severe nasal polyps." Laryngoscope **116**(5): 770-775.

Benninger, M. S., B. J. Ferguson, J. A. Hadley, D. L. Hamilos, M. Jacobs, D. W. Kennedy, D. C. Lanza, B. F. Marple, J. D. Osguthorpe, J. A. Stankiewicz, J. Anon, J. Denneny, I. Emanuel and H. Levine (2003). "Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology." Otolaryngol Head Neck Surg **129**(3 Suppl): S1-32.

Benninger, M. S. and B. A. Senior (1997). "The development of the Rhinosinusitis Disability Index." Arch Otolaryngol Head Neck Surg **123**(11): 1175-1179.

Bilson, H. A., D. L. Mitchell and B. Ashby (2004). "Human prostaglandin EP3 receptor isoforms show different agonist-induced internalization patterns." FEBS Lett **572**(1-3): 271-275.

Blake, K. (2006). "Review of guidelines and the literature in the treatment of acute bronchospasm in asthma." Pharmacotherapy **26**(9 Pt 2): 148S-155S.

Bonazzi, A., M. Bolla, C. Buccellati, A. Hernandez, S. Zarini, T. Vigano, F. Fumagalli, S. Viappiani, S. Ravasi, P. Zannini, G. Chiesa, G. Folco and A. Sala (2000). "Effect of endogenous and exogenous prostaglandin E(2) on interleukin-1 beta-induced cyclooxygenase-2 expression in human airway smooth-muscle cells." Am J Respir Crit Care Med **162**(6): 2272-2277.

Bousquet, J., P. Van Cauwenberge and N. Khaltaev (2001). "Allergic rhinitis and its impact on asthma." J Allergy Clin Immunol **108**(5 Suppl): S147-334.

Braunstahl, G. J. (2011). "Chronic rhinosinusitis, nasal polyposis and asthma: the united airways concept reconsidered?" Clin Exp Allergy **41**(10): 1341-1343.

Breyer, M. D. and R. M. Breyer (2000). "Prostaglandin E receptors and the kidney." Am J Physiol Renal Physiol **279**(1): F12-23.

Briones, A. M., M. Salaices and E. Vila (2005). "Ageing alters the production of nitric oxide and prostanoids after IL-1beta exposure in mesenteric resistance arteries." Mech Ageing Dev **126**(6-7): 710-721.

Burgess, J. K., Q. Ge, S. Boustany, J. L. Black and P. R. Johnson (2004). "Increased sensitivity of asthmatic airway smooth muscle cells to prostaglandin E2 might be mediated by increased numbers of E-prostanoid receptors." J Allergy Clin Immunol **113**(5): 876-881.

C

Cao, J. J., P. Kurimoto, B. Boudignon, C. Rosen, F. Lima and B. P. Halloran (2007). "Aging impairs IGF-I receptor activation and induces skeletal resistance to IGF-I." J Bone Miner Res **22**(8): 1271-1279.

Caplin, I., J. T. Haynes and J. Spahn (1971). "Are nasal polyps an allergic phenomenon?" Ann Allergy **29**(12): 631-634.

Casadevall, J., P. J. Ventura, J. Mullol and C. Picado (2000). "Intranasal challenge with aspirin in the diagnosis of aspirin intolerant asthma: evaluation of nasal response by acoustic rhinometry." Thorax **55**(11): 921-924.

Cauna, N. (1970). "The fine structure of the arteriovenous anastomosis and its nerve supply in the human nasal respiratory mucosa." Anat Rec **168**(1): 9-21.

Cauna, N. and K. H. Hinderer (1969). "Fine structure of blood vessels of the human nasal respiratory mucosa." Ann Otol Rhinol Laryngol **78**(4): 865-879.

Cowburn, A. S., K. Sladek, J. Soja, L. Adamek, E. Nizankowska, A. Szczeklik, B. K. Lam, J. F. Penrose, F. K. Austen, S. T. Holgate and A. P. Sampson (1998). "Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma." J Clin Invest **101**(4): 834-846.

Chandrasekaran, C. and J. I. Gordon (1993). "Cell lineage-specific and differentiation-dependent patterns of CCAAT/enhancer binding protein alpha expression in the gut epithelium of normal and transgenic mice." Proc Natl Acad Sci U S A **90**(19): 8871-8875.

Chandrasekharan, N. V. and D. L. Simmons (2004). "The cyclooxygenases." Genome Biol **5**(9): 241.

Chen, F. and V. Castranova (2007). "Nuclear factor-kappaB, an unappreciated tumor suppressor." Cancer Res **67**(23): 11093-11098.

Chen, P., Y. Cai, Z. G. Yang, R. Zhou, G. S. Zhang, F. Domann and X. Fang (2006). "Involvement of PKC, p38 MAPK and AP-2 in IL-1beta-induced expression of cyclooxygenase-2 in human pulmonary epithelial cells." Respirology **11**(1): 18-23.

Chen, W., Q. Tang, M. S. Gonzales and G. T. Bowden (2001). "Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes." Oncogene **20**(29): 3921-3926.

D

Dahlen, B., A. Szczeklik and J. J. Murray (2001). "Celecoxib in patients with asthma and aspirin intolerance. The Celecoxib in Aspirin-Intolerant Asthma Study Group." N Engl J Med **344**(2): 142.

Dahlen, B. and O. Zetterstrom (1990). "Comparison of bronchial and per oral provocation with aspirin in aspirin-sensitive asthmatics." Eur Respir J **3**(5): 527-534.

Dalziel, K., K. Stein, A. Round, R. Garside and P. Royle (2003). "Systematic review of endoscopic sinus surgery for nasal polyps." Health Technol Assess **7**(17): iii, 1-159.

Demoly, P., L. Crampette, B. Lebel, A. M. Campbell, M. Mondain and J. Bousquet (1998). "Expression of cyclo-oxygenase 1 and 2 proteins in upper respiratory mucosa." Clin Exp Allergy **28**(3): 278-283.

Di Mari, J. F., J. I. Saada, R. C. Mifflin, J. D. Valentich and D. W. Powell (2007). "HETEs enhance IL-1-mediated COX-2 expression via augmentation of message stability in human colonic myofibroblasts." Am J Physiol Gastrointest Liver Physiol **293**(4): G719-728.

Diaz-Munoz, M. D., I. C. Osma-Garcia, C. Cacheiro-Llaguno, M. Fresno and M. A. Iniguez (2010). "Coordinated up-regulation of cyclooxygenase-2 and microsomal prostaglandin E synthase 1 transcription by nuclear factor kappa B and early growth response-1 in macrophages." Cell Signal **22**(10): 1427-1436.

Dong, C., R. J. Davis and R. A. Flavell (2002). "MAP kinases in the immune response." Annu Rev Immunol **20**: 55-72.

Duque, J., M. D. Diaz-Munoz, M. Fresno and M. A. Iniguez (2006). "Up-regulation of cyclooxygenase-2 by interleukin-1beta in colon carcinoma cells." Cell Signal **18**(8): 1262-1269.

E

El-Haroun, H., D. Bradbury, A. Clayton and A. J. Knox (2004). "Interleukin-1beta, transforming growth factor-beta1, and bradykinin attenuate cyclic AMP production by human pulmonary artery smooth muscle cells in response to prostacyclin analogues and prostaglandin E2 by cyclooxygenase-2 induction and downregulation of adenylyl cyclase isoforms 1, 2, and 4." Circ Res **94**(3): 353-361.

Eliopoulos, A. G., C. D. Dumitru, C. C. Wang, J. Cho and P. N. Tsichlis (2002). "Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals." EMBO J **21**(18): 4831-4840.

Eloy, P., A. L. Poirrier, C. De Dorlodot, T. Van Zele, J. B. Watelet and B. Bertrand (2011). "Actual concepts in rhinosinusitis: a review of clinical presentations, inflammatory pathways, cytokine profiles, remodeling, and management." Curr Allergy Asthma Rep **11**(2): 146-162.

F

Farooque, S. P. and T. H. Lee (2009). "Aspirin-sensitive respiratory disease." Annu Rev Physiol **71**: 465-487.

Ferguson, B. J. (2004). "Categorization of eosinophilic chronic rhinosinusitis." Curr Opin Otolaryngol Head Neck Surg **12**(3): 237-242.

Fireman, P. (2003). "Understanding asthma pathophysiology." Allergy Asthma Proc **24**(2): 79-83.

Fokkens, W. (2005). "Evidence based diagnosis and treatment of rhinosinusitis and nasal polyps." Rhinology **43**(1): 1.

Fokkens, W., V. Lund and J. Mullol (2007). "European position paper on rhinosinusitis and nasal polyps 2007." Rhinol Suppl(20): 1-136.

Fokkens, W. J. (2011). "Symptoms of rhinosinusitis." Rhinology **49**(2): 129-130.

Fokkens, W. J., V. J. Lund, J. Mullol, C. Bachert, I. Alobid, F. Baroody, N. Cohen, A. Cervin, R. Douglas, P. Gevaert, C. Georgalas, H. Goossens, R. Harvey, P. Hellings, C. Hopkins, N. Jones, G. Joos, L. Kalogjera, B. Kern, M. Kowalski, D. Price, H. Riechelmann, R. Schlosser, B. Senior, M. Thomas, E. Toskala, R. Voegels, Y. Wang de and P. J. Wormald (2012). "European Position Paper on Rhinosinusitis and Nasal Polyps 2012." Rhinol Suppl(23): 3 p preceding table of contents, 1-298.

Fujino, H. and J. W. Regan (2003). "Prostanoid receptors and phosphatidylinositol 3-kinase: a pathway to cancer?" Trends Pharmacol Sci **24**(7): 335-340.

G

Gauvreau, G. M., R. M. Watson and P. M. O'Byrne (1999). "Protective effects of inhaled PGE2 on allergen-induced airway responses and airway inflammation." Am J Respir Crit Care Med **159**(1): 31-36.

Gollapudi, R. R., P. S. Teirstein, D. D. Stevenson and R. A. Simon (2004). "Aspirin sensitivity: implications for patients with coronary artery disease." JAMA **292**(24): 3017-3023.

Gorgoni, B., D. Maritano, P. Marthyn, M. Righi and V. Poli (2002). "C/EBP beta gene inactivation causes both impaired and enhanced gene expression and inverse regulation of IL-12 p40 and p35 mRNAs in macrophages." J Immunol **168**(8): 4055-4062.

Grau, R., M. A. Iniguez and M. Fresno (2004). "Inhibition of activator protein 1 activation, vascular endothelial growth factor, and cyclooxygenase-2 expression by 15-deoxy-Delta12,14-prostaglandin J2 in colon carcinoma cells: evidence for a redox-sensitive peroxisome proliferator-activated receptor-gamma-independent mechanism." Cancer Res **64**(15): 5162-5171.

H

Hadfield, P. J., J. M. Rowe-Jones and I. S. Mackay (2000). "The prevalence of nasal polyps in adults with cystic fibrosis." Clin Otolaryngol Allied Sci **25**(1): 19-22.

Hallstrand, T. S. and W. R. Henderson, Jr. (2010). "An update on the role of leukotrienes in asthma." Curr Opin Allergy Clin Immunol **10**(1): 60-66.

Hamad, A. M., A. M. Sutcliffe and A. J. Knox (2004). "Aspirin-induced asthma: clinical aspects, pathogenesis and management." Drugs **64**(21): 2417-2432.

Hamid, Q. and M. K. Tulic (2007). "New insights into the pathophysiology of the small airways in asthma." Ann Thorac Med **2**(1): 28-33.

Hata, A. N. and R. M. Breyer (2004). "Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation." Pharmacol Ther **103**(2): 147-166.

Higashi, N., H. Mita, E. Ono, Y. Fukutomi, H. Yamaguchi, K. Kajiwara, H. Tanimoto, K. Sekiya, K. Akiyama and M. Taniguchi (2010). "Profile of eicosanoid generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers." J Allergy Clin Immunol **125**(5): 1084-1091 e1086.

I

Inoue, H., T. Tanabe and K. Umesono (2000). "Feedback control of cyclooxygenase-2 expression through PPARgamma." J Biol Chem **275**(36): 28028-28032.

J

Jaffar, Z., M. E. Ferrini, M. C. Buford, G. A. Fitzgerald and K. Roberts (2007). "Prostaglandin I2-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma." J Immunol **179**(9): 6193-6203.

Jarvis, D., R. Newson, J. Lotvall, D. Hastan, P. Tomassen, T. Keil, M. Gjomarkaj, B. Forsberg, M. Gunnbjornsdottir, J. Minov, G. Brozek, S. E. Dahlen, E. Toskala, M. L. Kowalski, H. Olze, P. Howarth, U. Kramer, J. Baelum, C. Loureiro, L. Kasper, P. J. Bousquet, J. Bousquet, C. Bachert, W. Fokkens and P. Burney (2012). "Asthma in adults and its association with chronic rhinosinusitis: the GA2LEN survey in Europe." Allergy **67**(1): 91-98.

Jones, N. S., A. S. Carney and A. Davis (1998). "The prevalence of allergic rhinosinusitis: a review." J Laryngol Otol **112**(11): 1019-1030.

K

Kay, L. J., W. W. Yeo and P. T. Peachell (2006). "Prostaglandin E2 activates EP2 receptors to inhibit human lung mast cell degranulation." Br J Pharmacol **147**(7): 707-713.

Klein, T., P. Shephard, H. Kleinert and M. Komhoff (2007). "Regulation of cyclooxygenase-2 expression by cyclic AMP." Biochim Biophys Acta **1773**(11): 1605-1618.

Klossek, J. M., F. Neukirch, C. Pribil, R. Jankowski, E. Serrano, I. Chanal and A. El Hasnaoui (2005). "Prevalence of nasal polyposis in France: a cross-sectional, case-control study." Allergy **60**(2): 233-237.

Kobayashi, T. and S. Narumiya (2002). "Prostanoids in health and disease; lessons from receptor-knockout mice." Adv Exp Med Biol **507**: 593-597.

Koga, T., Y. Oshita, T. Kamimura, H. Koga and H. Aizawa (2006). "Characterisation of patients with frequent exacerbation of asthma." Respir Med **100**(2): 273-278.

Kojima, M., T. Morisaki, K. Izuhara, A. Uchiyama, Y. Matsunari, M. Katano and M. Tanaka (2000). "Lipopolysaccharide increases cyclo-oxygenase-2 expression in a colon carcinoma cell line through nuclear factor-kappa B activation." Oncogene **19**(9): 1225-1231.

Kovell, L. C., J. Wang, S. L. Ishman, P. L. Zeitlin and E. F. Boss (2011). "Cystic fibrosis and sinusitis in children: outcomes and socioeconomic status." Otolaryngol Head Neck Surg **145**(1): 146-153.

Kowalski, M. L., R. Pawliczak, J. Wozniak, K. Siuda, M. Poniatowska, J. Iwaszkiewicz, T. Kornatowski and M. A. Kaliner (2000). "Differential metabolism of arachidonic acid in nasal polyp epithelial cells cultured from aspirin-sensitive and aspirin-tolerant patients." Am J Respir Crit Care Med **161**(2 Pt 1): 391-398.

Krause, H. F. (2003). "Allergy and chronic rhinosinusitis." Otolaryngol Head Neck Surg **128**(1): 14-16.

L

Levine, S. J. (1995). "Bronchial epithelial cell-cytokine interactions in airway inflammation." J Investig Med **43**(3): 241-249.

Libby, P. (2002). "Inflammation in atherosclerosis." Nature **420**(6917): 868-874.

Lo, C. J. (2003). "MAPK regulation of prostaglandin E2 production by lipopolysaccharide-stimulated macrophages is not dependent on nuclear factor kappaB." J Surg Res **113**(2): 189-194.

Loebinger, M. R., D. Bilton and R. Wilson (2009). "Upper airway 2: Bronchiectasis, cystic fibrosis and sinusitis." Thorax **64**(12): 1096-1101.

Lund, V. J., J. Flood, A. P. Sykes and D. H. Richards (1998). "Effect of fluticasone in severe polyposis." Arch Otolaryngol Head Neck Surg **124**(5): 513-518.

M

Marshak, T., Y. Rivlin, L. Bentur, O. Ronen and N. Uri (2011). "Prevalence of rhinosinusitis among atypical cystic fibrosis patients." Eur Arch Otorhinolaryngol **268**(4): 519-524.

Mascia, K., T. Haselkorn, Y. M. Deniz, D. P. Miller, E. R. Bleecker and L. Borish (2005). "Aspirin sensitivity and severity of asthma: evidence for irreversible airway obstruction in patients with severe or difficult-to-treat asthma." J Allergy Clin Immunol **116**(5): 970-975.

Mastalerz, L., M. Sanak, A. Gawlewicz-Mroczka, A. Gielicz, A. Cmiel and A. Szczeklik (2008). "Prostaglandin E2 systemic production in patients with asthma with and without aspirin hypersensitivity." Thorax **63**(1): 27-34.

May, A., D. Wagner, U. Langenbeck and A. Weber (2000). "[Family study of patients with aspirin intolerance and rhinosinusitis]." HNO **48**(9): 650-654.

McDonald, J. R., D. A. Mathison and D. D. Stevenson (1972). "Aspirin intolerance in asthma. Detection by oral challenge." J Allergy Clin Immunol **50**(4): 198-207.

Meltzer, E. O., D. L. Hamilos, J. A. Hadley, D. C. Lanza, B. F. Marple, R. A. Nicklas, C. Bachert, J. Baraniuk, F. M. Baroody, M. S. Benninger, I. Brook, B. A. Chowdhury,

H. M. Druce, S. Durham, B. Ferguson, J. M. Gwaltney, M. Kaliner, D. W. Kennedy, V. Lund, R. Naclerio, R. Pawankar, J. F. Piccirillo, P. Rohane, R. Simon, R. G. Slavin, A. Togias, E. R. Wald and S. J. Zinreich (2004). "Rhinosinusitis: establishing definitions for clinical research and patient care." J Allergy Clin Immunol **114**(6 Suppl): 155-212.

Mendelsohn, D., G. Jeremic, E. D. Wright and B. W. Rotenberg (2011). "Revision rates after endoscopic sinus surgery: a recurrence analysis." Ann Otol Rhinol Laryngol **120**(3): 162-166.

Metson, R. B. and R. E. Gliklich (2000). "Clinical outcomes in patients with chronic sinusitis." Laryngoscope **110**(3 Pt 3): 24-28.

Milewski, M., L. Mastalerz, E. Nizankowska and A. Szczeklik (1998). "Nasal provocation test with lysine-aspirin for diagnosis of aspirin-sensitive asthma." J Allergy Clin Immunol **101**(5): 581-586.

Moloney, J. R. (1977). "Nasal polyps, nasal polypectomy, asthma, and aspirin sensitivity. Their association in 445 cases of nasal polyps." J Laryngol Otol **91**(10): 837-846.

Moloney, J. R. and J. Collins (1977). "Nasal polyps and bronchial asthma." Br J Dis Chest **71**(1): 1-6.

Mori, A., S. Ito, M. Morioka, H. Aso, M. Kondo, M. Sokabe and Y. Hasegawa (2011). "Effects of specific prostanoid EP receptor agonists on cell proliferation and intracellular Ca(2+) concentrations in human airway smooth muscle cells." Eur J Pharmacol.

Morwood, K., D. Gillis, W. Smith and F. Kette (2005). "Aspirin-sensitive asthma." Intern Med J **35**(4): 240-246.

Mullol, J., J. C. Fernandez-Morata, J. Roca-Ferrer, L. Pujols, A. Xaubet, P. Benitez and C. Picado (2002). "Cyclooxygenase 1 and cyclooxygenase 2 expression is

abnormally regulated in human nasal polyps." J Allergy Clin Immunol **109**(5): 824-830.

Munoz-Lopez, F. (2010). "Prognostic bases of asthma. Natural history?" Allergol Immunopathol (Madr) **38**(6): 333-336.

Mygind, N. (1990). "Nasal polyposis." J Allergy Clin Immunol **86**(6 Pt 1): 827-829.

Mygind, N. (1999). "Advances in the medical treatment of nasal polyps." Allergy **54 Suppl 53**: 12-16.

N

Nieminen, R., S. Leinonen, A. Lahti, K. Vuolteenaho, U. Jalonen, H. Kankaanranta, M. B. Goldring and E. Moilanen (2005). "Inhibitors of mitogen-activated protein kinases downregulate COX-2 expression in human chondrocytes." Mediators Inflamm **2005**(5): 249-255.

Nizankowska-Mogilnicka, E., G. Bochenek, L. Mastalerz, M. Swierczynska, C. Picado, G. Scadding, M. L. Kowalski, M. Setkowicz, J. Ring, K. Brockow, C. Bachert, S. Wohrl, B. Dahlen and A. Szczeklik (2007). "EAACI/GA2LEN guideline: aspirin provocation tests for diagnosis of aspirin hypersensitivity." Allergy **62**(10): 1111-1118.

Nizankowska, E., A. Bestynska-Krypel, A. Cmiel and A. Szczeklik (2000). "Oral and bronchial provocation tests with aspirin for diagnosis of aspirin-induced asthma." Eur Respir J **15**(5): 863-869.

O

O'Byrne, P. M. (2008). "2007 update of the Global Initiative for Asthma management and prevention: what's new?" Pol Arch Med Wewn **118**(4): 179-180.

Ogata, S., Y. Kubota, T. Yamashiro, H. Takeuchi, T. Ninomiya, Y. Suyama and K. Shirasuna (2007). "Signaling pathways regulating IL-1alpha-induced COX-2 expression." J Dent Res **86**(2): 186-191.

Ogino, S., T. Harada, I. Okawachi, M. Irifune, T. Matsunaga and T. Nagano (1986). "Aspirin-induced asthma and nasal polyps." Acta Otolaryngol Suppl **430**: 21-27.

Ohnaka, K., K. Numaguchi, T. Yamakawa and T. Inagami (2000). "Induction of cyclooxygenase-2 by angiotensin II in cultured rat vascular smooth muscle cells." Hypertension **35**(1 Pt 1): 68-75.

Okano, M., T. Fujiwara, M. Yamamoto, Y. Sugata, R. Matsumoto, K. Fukushima, T. Yoshino, K. Shimizu, N. Eguchi, M. Kiniwa, Y. Urade and K. Nishizaki (2006). "Role of prostaglandin D2 and E2 terminal synthases in chronic rhinosinusitis." Clin Exp Allergy **36**(8): 1028-1038.

P

Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappaB transcription factors." Oncogene **18**(49): 6853-6866.

Paulsson, M. (1992). "Basement membrane proteins: structure, assembly, and cellular interactions." Crit Rev Biochem Mol Biol **27**(1-2): 93-127.

Pavord, I. D., R. Ward, G. Woltmann, A. J. Wardlaw, J. R. Sheller and R. Dworski (1999). "Induced sputum eicosanoid concentrations in asthma." Am J Respir Crit Care Med **160**(6): 1905-1909.

Pavord, I. D., A. Wisniewski, R. Mathur, I. Wahedna, A. J. Knox and A. E. Tattersfield (1991). "Effect of inhaled prostaglandin E2 on bronchial reactivity to sodium metabisulphite and methacholine in patients with asthma." Thorax **46**(9): 633-637.

Pawankar, R. and M. Nonaka (2007). "Inflammatory mechanisms and remodeling in chronic rhinosinusitis and nasal polyps." Curr Allergy Asthma Rep **7**(3): 202-208.

Peng, H., P. Chen, Y. Cai, Y. Chen, Q. H. Wu, Y. Li, R. Zhou and X. Fang (2008). "Endothelin-1 increases expression of cyclooxygenase-2 and production of interlukin-8 in hunan pulmonary epithelial cells." Peptides **29**(3): 419-424.

Perez-Novo, C. A., J. B. Watelet, C. Claeys, P. Van Cauwenberge and C. Bachert (2005). "Prostaglandin, leukotriene, and lipoxin balance in chronic rhinosinusitis with and without nasal polyposis." J Allergy Clin Immunol **115**(6): 1189-1196.

Petkova, D. K., C. Clelland, J. Ronan, L. Pang, J. M. Coulson, S. Lewis and A. J. Knox (2004). "Overexpression of cyclooxygenase-2 in non-small cell lung cancer." Respir Med **98**(2): 164-172.

Pfaar, O. and L. Klimek (2006). "Eicosanoids, aspirin-intolerance and the upper airways--current standards and recent improvements of the desensitization therapy." J Physiol Pharmacol **57 Suppl 12**: 5-13.

Picado, C. (2006). "Mechanisms of aspirin sensitivity." Curr Allergy Asthma Rep **6**(3): 198-202.

Picado, C., J. C. Fernandez-Morata, M. Juan, J. Roca-Ferrer, M. Fuentes, A. Xaubet and J. Mullol (1999). "Cyclooxygenase-2 mRNA is downexpressed in nasal polyps from aspirin-sensitive asthmatics." Am J Respir Crit Care Med **160**(1): 291-296.

Pierzchalska, M., J. Soja, M. Wos, Z. Szabo, E. Nizankowska-Mogielnicka, M. Sanak and A. Szczeklik (2007). "Deficiency of cyclooxygenases transcripts in cultured primary bronchial epithelial cells of aspirin-sensitive asthmatics." J Physiol Pharmacol **58**(2): 207-218.

Pierzchalska, M., Z. Szabo, M. Sanak, J. Soja and A. Szczeklik (2003). "Deficient prostaglandin E2 production by bronchial fibroblasts of asthmatic patients, with

special reference to aspirin-induced asthma." J Allergy Clin Immunol **111**(5): 1041-1048.

Pino, M. S., S. T. Nawrocki, F. Cognetti, J. L. Abruzzese, H. Q. Xiong and D. J. McConkey (2005). "Prostaglandin E2 drives cyclooxygenase-2 expression via cyclic AMP response element activation in human pancreatic cancer cells." Cancer Biol Ther **4**(11): 1263-1269.

Ponikau, J. U., D. A. Sherris, G. M. Kephart, E. B. Kern, T. A. Gaffey, J. E. Tarara and H. Kita (2003). "Features of airway remodeling and eosinophilic inflammation in chronic rhinosinusitis: is the histopathology similar to asthma?" J Allergy Clin Immunol **112**(5): 877-882.

Pujols, L., J. Mullol, I. Alobid, J. Roca-Ferrer, A. Xaubet and C. Picado (2004). "Dynamics of COX-2 in nasal mucosa and nasal polyps from aspirin-tolerant and aspirin-intolerant patients with asthma." J Allergy Clin Immunol **114**(4): 814-819.

Pujols, L., J. Mullol and C. Picado (2007). "Alpha and beta glucocorticoid receptors: relevance in airway diseases." Curr Allergy Asthma Rep **7**(2): 93-99.

Pujols, L., J. Mullol, A. Torrego and C. Picado (2004). "Glucocorticoid receptors in human airways." Allergy **59**(10): 1042-1052.

R

Ragab, S. M., V. J. Lund and G. Scadding (2004). "Evaluation of the medical and surgical treatment of chronic rhinosinusitis: a prospective, randomised, controlled trial." Laryngoscope **114**(5): 923-930.

Raman, M., W. Chen and M. H. Cobb (2007). "Differential regulation and properties of MAPKs." Oncogene **26**(22): 3100-3112.

Regan, J. W. (2003). "EP2 and EP4 prostanoid receptor signaling." Life Sci **74**(2-3): 143-153.

Ricciotti, E. and G. A. FitzGerald (2011). "Prostaglandins and inflammation." Arterioscler Thromb Vasc Biol **31**(5): 986-1000.

Ridley, S. H., J. L. Dean, S. J. Sarsfield, M. Brook, A. R. Clark and J. Saklatvala (1998). "A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA." FEBS Lett **439**(1-2): 75-80.

Roca-Ferrer, J., F. J. Garcia-Garcia, J. Pereda, M. Perez-Gonzalez, L. Pujols, I. Alobid, J. Mullol and C. Picado (2011). "Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma." J Allergy Clin Immunol **128**(1): 66-72 e61.

Roca-Ferrer, J., L. Pujols, S. Gartner, A. Moreno, F. Pumarola, J. Mullol, N. Cobos and C. Picado (2006). "Upregulation of COX-1 and COX-2 in nasal polyps in cystic fibrosis." Thorax **61**(7): 592-596.

Rocca, B. (2006). "Targeting PGE2 receptor subtypes rather than cyclooxygenases: a bridge over troubled water?" Mol Interv **6**(2): 68-73, 58.

S

Sakai, T., F. Kambe, H. Mitsuyama, N. Ishiguro, K. Kurokouchi, M. Takigawa, H. Iwata and H. Seo (2001). "Tumor necrosis factor alpha induces expression of genes for matrix degradation in human chondrocyte-like HCS-2/8 cells through activation of NF-kappaB: abrogation of the tumor necrosis factor alpha effect by proteasome inhibitors." J Bone Miner Res **16**(7): 1272-1280.

Samad, T. A., K. A. Moore, A. Sapirstein, S. Billet, A. Allchorne, S. Poole, J. V. Bonventre and C. J. Woolf (2001). "Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity." Nature **410**(6827): 471-475.

Samter, M. and R. F. Beers, Jr. (1968). "Intolerance to aspirin. Clinical studies and consideration of its pathogenesis." Ann Intern Med **68**(5): 975-983.

Sastre, B. and V. del Pozo (2012). "Role of PGE2 in asthma and nonasthmatic eosinophilic bronchitis." Mediators Inflamm **2012**: 645383.

Saunders, M. A., L. Sansores-Garcia, D. W. Gilroy and K. K. Wu (2001). "Selective suppression of CCAAT/enhancer-binding protein beta binding and cyclooxygenase-2 promoter activity by sodium salicylate in quiescent human fibroblasts." J Biol Chem **276**(22): 18897-18904.

Scadding, G. K., S. R. Durham, R. Mirakian, N. S. Jones, A. B. Drake-Lee, D. Ryan, T. A. Dixon, P. A. Huber and S. M. Nasser (2008). "BSACI guidelines for the management of rhinosinusitis and nasal polyposis." Clin Exp Allergy **38**(2): 260-275.

Scadding, G. K. and H. H. Kariyawasam (2009). "Airways disease: just nosing around?" Thorax **64**(11): 923-925.

Scherle, P. A., W. Ma, H. Lim, S. K. Dey and J. M. Trzaskos (2000). "Regulation of cyclooxygenase-2 induction in the mouse uterus during decidualization. An event of early pregnancy." J Biol Chem **275**(47): 37086-37092.

Schiavino, D., E. Nucera, A. Milani, M. Del Ninno, A. Buonomo, J. Sun and G. Patriarca (2000). "The aspirin disease." Thorax **55 Suppl 2**: S66-69.

Sestini, P., L. Armetti, G. Gambaro, M. G. Pieroni, R. M. Refini, A. Sala, A. Vaghi, G. C. Folco, S. Bianco and M. Robuschi (1996). "Inhaled PGE2 prevents aspirin-induced bronchoconstriction and urinary LTE4 excretion in aspirin-sensitive asthma." Am J Respir Crit Care Med **153**(2): 572-575.

Settipane, G. A. and F. H. Chafee (1977). "Nasal polyps in asthma and rhinitis. A review of 6,037 patients." J Allergy Clin Immunol **59**(1): 17-21.

Shishodia, S. and B. B. Aggarwal (2002). "Nuclear factor-kappaB activation: a question of life or death." J Biochem Mol Biol **35**(1): 28-40.

Simmons, D. L., R. M. Botting and T. Hla (2004). "Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition." Pharmacol Rev **56**(3): 387-437.

Slice, L. W., T. Chiu and E. Rozengurt (2005). "Angiotensin II and epidermal growth factor induce cyclooxygenase-2 expression in intestinal epithelial cells through small GTPases using distinct signaling pathways." J Biol Chem **280**(2): 1582-1593.

Smith, A. P. (1975). "Effect of indomethacin in asthma: evidence against a role for prostaglandins in its pathogenesis." Br J Clin Pharmacol **2**(4): 307-309.

Smyth, E. M., T. Grosser, M. Wang, Y. Yu and G. A. FitzGerald (2009). "Prostanoids in health and disease." J Lipid Res **50 Suppl**: S423-428.

Snipes, J. A., B. Kis, G. S. Shelness, J. A. Hewett and D. W. Busija (2005). "Cloning and characterization of cyclooxygenase-1b (putative cyclooxygenase-3) in rat." J Pharmacol Exp Ther **313**(2): 668-676.

Sousa, A., R. Pfister, P. E. Christie, S. J. Lane, S. M. Nasser, M. Schmitz-Schumann and T. H. Lee (1997). "Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma." Thorax **52**(11): 940-945.

Spector, S. L., C. H. Wangaard and R. S. Farr (1979). "Aspirin and concomitant idiosyncrasies in adult asthmatic patients." J Allergy Clin Immunol **64**(6 Pt 1): 500-506.

Stables, M. J. and D. W. Gilroy (2011). "Old and new generation lipid mediators in acute inflammation and resolution." Prog Lipid Res **50**(1): 35-51.

Stammberger, H. (1999). "Surgical treatment of nasal polyps: past, present, and future." Allergy **54 Suppl 53**: 7-11.

Stevenson, D. D. (2000). "Approach to the patient with a history of adverse reactions to aspirin or NSAIDs: diagnosis and treatment." Allergy Asthma Proc **21**(1): 25-31.

Stevenson, D. D. and R. A. Simon (2001). "Lack of cross-reactivity between rofecoxib and aspirin in aspirin-sensitive patients with asthma." J Allergy Clin Immunol **108**(1): 47-51.

Stevenson, D. D. and A. Szczeklik (2006). "Clinical and pathologic perspectives on aspirin sensitivity and asthma." J Allergy Clin Immunol **118**(4): 773-786; quiz 787-778.

Stock, J. L., K. Shinjo, J. Burkhardt, M. Roach, K. Taniguchi, T. Ishikawa, H. S. Kim, P. J. Flannery, T. M. Coffman, J. D. McNeish and L. P. Audoly (2001). "The prostaglandin E2 EP1 receptor mediates pain perception and regulates blood pressure." J Clin Invest **107**(3): 325-331.

Stratton, M. S. and D. S. Alberts (2002). "Current application of selective COX-2 inhibitors in cancer prevention and treatment." Oncology (Williston Park) **16**(5 Suppl 4): 37-51.

Sugimoto, Y. and S. Narumiya (2007). "Prostaglandin E receptors." J Biol Chem **282**(16): 11613-11617.

Syeda, F., J. Grosjean, R. A. Houliston, R. J. Keogh, T. D. Carter, E. Paleolog and C. P. Wheeler-Jones (2006). "Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-kappaB pathways." J Biol Chem **281**(17): 11792-11804.

Szczeklik, A., R. J. Gryglewski, E. Olszewski, A. Dembinska-Kiec and G. Czerniawska-Mysik (1977). "Aspirin-sensitive asthma: the effect of aspirin on the release of prostaglandins from nasal polyps." Pharmacol Res Commun **9**(5): 415-425.

Szczeklik, A., E. Nizankowska and M. Duplaga (2000). "Natural history of aspirin-induced asthma. AIANE Investigators. European Network on Aspirin-Induced Asthma." Eur Respir J **16**(3): 432-436.

Szczeklik, A. and M. Sanak (2006). "The broken balance in aspirin hypersensitivity." Eur J Pharmacol **533**(1-3): 145-155.

Szczeklik, A., K. Sladek, R. Dworski, E. Nizankowska, J. Soja, J. Sheller and J. Oates (1996). "Bronchial aspirin challenge causes specific eicosanoid response in aspirin-sensitive asthmatics." Am J Respir Crit Care Med **154**(6 Pt 1): 1608-1614.

Szczeklik, A. and D. D. Stevenson (1999). "Aspirin-induced asthma: advances in pathogenesis and management." J Allergy Clin Immunol **104**(1): 5-13.

Szczeklik, A. and D. D. Stevenson (2003). "Aspirin-induced asthma: advances in pathogenesis, diagnosis, and management." J Allergy Clin Immunol **111**(5): 913-921; quiz 922.

T

Takayama, K., G. Garcia-Cardena, G. K. Sukhova, J. Comander, M. A. Gimbrone, Jr. and P. Libby (2002). "Prostaglandin E2 suppresses chemokine production in human macrophages through the EP4 receptor." J Biol Chem **277**(46): 44147-44154.

Tian, M. and W. P. Schiemann (2010). "PGE2 receptor EP2 mediates the antagonistic effect of COX-2 on TGF-beta signaling during mammary tumorigenesis." FASEB J **24**(4): 1105-1116.

Togo, S., O. Holz, X. Liu, H. Sugiura, K. Kamio, X. Wang, S. Kawasaki, Y. Ahn, K. Fredriksson, C. M. Skold, K. C. Mueller, D. Branscheid, L. Welker, H. Watz, H.

Magnussen and S. I. Rennard (2008). "Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms." Am J Respir Crit Care Med **178**(3): 248-260.

Tos, M., F. Svendstrup, H. Arndal, S. Orntoft, J. Jakobsen, P. Borum, C. Schrewelius, P. L. Larsen, F. Clement, C. Barfoed, F. Romeling and T. Tvermosegaard (1998). "Efficacy of an aqueous and a powder formulation of nasal budesonide compared in patients with nasal polyps." Am J Rhinol **12**(3): 183-189.

Tsatsanis, C., A. Androulidaki, M. Venihaki and A. N. Margioris (2006). "Signalling networks regulating cyclooxygenase-2." Int J Biochem Cell Biol **38**(10): 1654-1661.

V

Van Dyke, T. E. and C. N. Serhan (2003). "Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases." J Dent Res **82**(2): 82-90.

Vancheri, C., C. Mastruzzo, M. A. Sortino and N. Crimi (2004). "The lung as a privileged site for the beneficial actions of PGE2." Trends Immunol **25**(1): 40-46.

Vento, S. I., L. O. Ertama, M. L. Hytonen, C. H. Wolff and C. H. Malmberg (2000). "Nasal polyposis: clinical course during 20 years." Ann Allergy Asthma Immunol **85**(3): 209-214.

Vichai, V., C. Suyarnsesthakorn, D. Pittayakhajonwut, K. Sriklung and K. Kirtikara (2005). "Positive feedback regulation of COX-2 expression by prostaglandin metabolites." Inflamm Res **54**(4): 163-172.

Vila-del Sol, V. and M. Fresno (2005). "Involvement of TNF and NF-kappa B in the transcriptional control of cyclooxygenase-2 expression by IFN-gamma in macrophages." J Immunol **174**(5): 2825-2833.

W

Wadleigh, D. J., S. T. Reddy, E. Kopp, S. Ghosh and H. R. Herschman (2000). "Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages." J Biol Chem **275**(9): 6259-6266.

Warner, T. D., I. Vojnovic, F. Giuliano, R. Jimenez, D. Bishop-Bailey and J. A. Mitchell (2004). "Cyclooxygenases 1, 2, and 3 and the production of prostaglandin I2: investigating the activities of acetaminophen and cyclooxygenase-2-selective inhibitors in rat tissues." J Pharmacol Exp Ther **310**(2): 642-647.

Watelet, J. B., T. Van Zele, M. Gjomarkaj, G. W. Canonica, S. E. Dahlen, W. Fokkens, V. J. Lund, G. K. Scadding, J. Mullol, N. Papadopoulos, S. Bonini, M. L. Kowalski, P. Van Cauwenberge and J. Bousquet (2006). "Tissue remodelling in upper airways: where is the link with lower airway remodelling?" Allergy **61**(11): 1249-1258.

Williams, S. C., N. D. Angerer and P. F. Johnson (1997). "C/EBP proteins contain nuclear localization signals imbedded in their basic regions." Gene Expr **6**(6): 371-385.

Wu, K. K., J. Y. Liou and K. Cieslik (2005). "Transcriptional Control of COX-2 via C/EBPbeta." Arterioscler Thromb Vasc Biol **25**(4): 679-685.

Wu, M. H., C. A. Wang, C. C. Lin, L. C. Chen, W. C. Chang and S. J. Tsai (2005). "Distinct regulation of cyclooxygenase-2 by interleukin-1beta in normal and endometriotic stromal cells." J Clin Endocrinol Metab **90**(1): 286-295.

Y

Yamaya, M., W. E. Finkbeiner and J. H. Widdicombe (1991). "Altered ion transport by tracheal glands in cystic fibrosis." Am J Physiol **261**(6 Pt 1): L491-494.

Yan, X., C. Wu Xiao, M. Sun, B. K. Tsang and W. Gibb (2002). "Nuclear factor kappa B activation and regulation of cyclooxygenase type-2 expression in human

amnion mesenchymal cells by interleukin-1beta." Biol Reprod **66**(6): 1667-1671.

Ying, S., Q. Meng, G. Scadding, A. Parikh, C. J. Corrigan and T. H. Lee (2006). "Aspirin-sensitive rhinosinusitis is associated with reduced E-prostanoid 2 receptor expression on nasal mucosal inflammatory cells." J Allergy Clin Immunol **117**(2): 312-318.

Yoshimura, T., M. Yoshikawa, N. Otori, S. Haruna and H. Moriyama (2008). "Correlation between the prostaglandin D(2)/E(2) ratio in nasal polyps and the recalcitrant pathophysiology of chronic rhinosinusitis associated with bronchial asthma." Allergol Int **57**(4): 429-436.

Z

Zakrzewski, J. T., N. C. Barnes, P. J. Piper and J. F. Costello (1987). "Detection of sputum eicosanoids in cystic fibrosis and in normal saliva by bioassay and radioimmunoassay." Br J Clin Pharmacol **23**(1): 19-27.

Appendix 1. Summary

INTRODUCCIÓ

1. Les vies respiratòries superiors.

El principal objectiu de les vies respiratòries es suplir al cos amb oxigen i eliminar el diòxid de carboni per promoure l'intercanvi gasós. Podem dividir les vies respiratòries en superiors i inferiors.

A les vies respiratòries superiors trobem el nas, un òrgan doble composat per dos cavitats nasals dividides per un septe. En aquestes cavitats existeixen tres estructures prominents anomenades cornets superiors, mitjos i inferiors. El septe nasal i els cornets es troben recoberts per la mucosa respiratòria. Aquesta organització prepara l'aire inhalat mitjançant filtració, escalfament, i humidificació abans d'arribar als pulmons, així es protegeixen les vies respiratòries d'agents irritants externs i patògens.

2. Rinosinusitis crònica amb poliposi nasal.

La rinosinusitis es un procés inflamatori que afecta la mucosa respiratòria. Aquesta patologia és un problema de salut que afecta al voltant del 15% de la població en els països occidentals. La rinosinusitis es pot classificar en aguda i crònica, d'acord amb la durada dels símptomes. La forma aguda dura fins a 12 setmanes amb una resolució completa dels símptomes. D'altra banda, la forma crònica persisteix més enllà de 12 setmanes i s'associa amb una morbiditat significativa i una menor qualitat de vida.

La rinosinusitis crònica (CRS) es pot dividir a la vegada en dos grups: CRS sense pòlips nasals i CRS amb pòlips nasals (CRSwNP). Aquesta segona forma, es defineix com una inflamació del nas i els sinus paranasals a llarg termini caracteritzada per bloqueig, congestió o secreció nasal i/o dolor facial, pressió i/o reducció o pèrdua de l'olfacte.

3. Epidemiologia i comorbiditats.

La prevalença exacta de la poliposi nasal és desconeguda, ja que existeixen pocs estudis epidemiològics i les estimacions són encara especulatives. Tot i això, amb les dades existents es pot estimar que la seva prevalença oscil·la entre el 2 i 5% en els

països occidentals. La incidència és major en homes i augmenta significativament després de l'edat de 40 anys. La CRSwNP és difícil de tractar, mentre que la seva etiologia i fisiopatologia encara no estan clares, i fins i tot després dels tractaments quirúrgics i mèdics, les recidives són freqüents. Els pacients amb CRSwNP presenten sovint altres malalties relacionades, com fibrosi quística, rinitis al·lèrgica, asma o intolerància a l'aspirina.

3.1. Asma. És una malaltia complexa de les vies respiratòries amb una estimació de 300 milions d'afectats. L'asma és un trastorn inflamatori crònic de les vies aèries. Aquesta inflamació s'associa amb hiperreactivitat de les vies respiratòries que porta a episodis recurrents de sibilàncies, dispnea, opressió toràcica i tos. Aquests episodis s'associen generalment amb obstrucció variable del flux aeri, dins el pulmó que és sovint reversible de forma espontània o amb tractament.

L'asma i la CRSwNP s'associen freqüentment, el 7% dels pacients amb asma manifesten CRSwNP en comparació amb la població no asmàtica. Aquest percentatge augmenta fins al 30% en la població de pacients amb poliposi nasal. Alternativament, fins al 60% dels pacients amb CRSwNP tenen una reducció de la funció respiratòria, avaluada per la història clínica i les proves de funció pulmonar.

3.2. Asma induïda per aspirina (AIA). Aquesta malaltia és una síndrome clínica caracteritzada per l'associació de CRSwNP, asma i episodis de broncoconstricció provocats per la ingesta de antiinflamatoris no esteroïdals (AINEs). Normalment, aquests fàrmacs són ben tolerats per a la majoria de les persones, però un subconjunt de pacients asmàtics són una excepció. En aquests individus, els AINEs són capaços de precipitar atacs d'asma. Cal tenir en compte que, l'AIA es reconeix com un fenotip agressiu de malaltia a les vies respiratòries que sovint presenta un curs prolongat. Quan es compara amb els subjectes tolerants a aspirina, els pacients amb AIA són més susceptibles a patir obstrucció irreversible del flux aeri, exacerbacions freqüents, així com a ser diagnosticats amb asma greu.

La intolerància a l'aspirina no és una resposta immunològica, donat que la patologia no

implica una reacció mitjançant immunoglobulina E. El mecanisme patològic responsable del desenvolupament d'AIA no ha estat completament dilucidat. No obstant això, diverses observacions suggereixen que anomalies en la regulació del metabolisme de l'àcid araquidònic (AA) poden estar implicades en la resposta adversa als AINEs.

Aquesta peculiar síndrome té una prevalença estimada d'un 1% en la població general i és present entre el 3 i el 20% de la població asmàtica adulta. Els pacients amb AIA sovint tenen una història subjacent d'asma, poliposi nasal, i/o CRS, encara que alguns poden no tenir cap malaltia de predisposició. La prevalença d'AIA s'ha descrit al voltant del 35-52% en pacients amb CRSwNP i al voltant del 65% en pacients que pateixen tant asma bronquial com CRSwNP (tríada de Samter).

4. Tractaments de la CRSwNP.

Els objectius del tractament són restablir la respiració nasal, reduir els símptomes, millorar l'olfacte, la qualitat de vida, així com prevenir complicacions. Hi ha diferents recomanacions, però els glucocorticoides tòpics i orals es consideren el tractament de primera línia en els pòlips nasals. El control de l'asma i la CRS en pacients amb AIA ha de seguir les pautes generals, però s'han de considerar l'educació del pacient i l'evitació acurada dels AINEs.

La cirurgia ha de ser considerada en cas de fallida, efectes secundaris o manca de disposició dels pacients per dur a terme el tractament. La cirurgia de sinus endoscòpica té com a objectius la restauració de la ventilació i la recuperació de les funcions mucociliars. No obstant això, cal tenir en compte que la poliposi nasal és una malaltia crònica amb una alta taxa de recurrència (40% després de 5 anys), malgrat haver fet un acurat tractament mèdic i quirúrgic. Els pacients amb AIA són més propensos a la recurrència i amb més freqüència s'han de sotmetre a cirurgia de revisió.

5. Metabolisme de l'àcid araquidònic.

L'àcid araquidònic (AA) és un àcid gras insaturat de 20 carbonis precursor dels eicosanoids. La major part d'AA s'emmagatzema en les membranes cel·lulars com a

part dels fosfolípids. La biosíntesi d'eicosanoids comença amb l'alliberament d'AA dels fosfolípids de membrana, per l'acció de les fosfolipases en resposta a diferents estímuls (físics, químics, hormonals, citocines, etc).

L'AA pot ser metabolitzat a través de diferents vies enzimàtiques:

• Via de la 5-lipoxigenasa (5-LOX): produeix leucotriens (LTs) i lipoxines. Els LTS participen en l'asma, la inflamació al·lèrgica i la immunitat innata jugant un paper clau en la resposta inflamatòria. Els LTs indueixen quimiotaxi, tenen un potent efecte broncoconstrictor i incrementen la permeabilitat vascular i la secreció mucosa durant la crisi asmàtica.

• Via de la ciclooxigenasa (COX): produeix els prostanoids que agrupa les prostaglandines (PGs), la prostaciclina (PGI_2) i el tromboxà A2 (TxA_2). Els prostanoids participen en funcions fisiològiques com la protecció de la mucosa gàstrica, el manteniment de la funció renal o l'hemostàsia. També tenen un paper important en condicions patològiques, com ara la modulació de la febre, el dolor o la inflamació, i s'ha descrit que tenen una producció exacerbada en malalties inflamatòries cròniques.

6. Via de la ciclooxigenasa (COX).

Les COXs són glicoproteïnes que catalitzen dues reaccions: ciclooxigenació, generant la PGG_2, metabòlit intermedi, que al seu torn es converteix en PGH_2 a través d'una peroxidació. Existeixen almenys 2 isoformes, la COX-1 i la COX-2 amb activitat catalítica similar. No obstant això, la regulació de la seva activitat i localització són diferents.

6.1. COX-1. La isoforma COX-1, es troba a la membrana del reticle endoplasmàtic i es considera un enzim constitutiu expressat en la major part de cèl·lules. Tot i això, s'ha descrit la seva inducció en determinades condicions inflamatòries.

6.2. COX-2. La COX-2 es troba a l'embolcall perinuclear i és considerada un gen induïble tot i que s'expressa constitutivament en algunes àrees del cervell i el ronyó. S'ha vist que l'expressió de la COX-2 es troba incrementada malalties inflamatòries cròniques.

7. Regulació transcripcional de la COX-2.

La regulació de la transcripció de la COX-2 implica mecanismes complexos com la senyalització per MAPKs o el reclutament al nucli de diferents factors de transcripció com el factor nuclear kB (NF-kB) o C/EBP (*CCAAT/enhancer-binding protein*) per tal d'induir l'activació del gen.

7.1 MAPKs. Aquestes proteïnes regulen activitats com l'expressió gènica, la mitosi, la circulació o el metabolisme. La seva fosforilació actua com un interruptor per encendre o apagar l'activitat de les seves proteïnes diana. Hi ha tres subfamílies de MAPKs ben caracteritzades que inclouen les ERK, JNK i p38. Existeixen evidències que demostren que aquestes proteïnes juguen un paper en la regulació de la COX-2.

7.2. NF-kB. És un factor de transcripció induïble i d'expressió ubiqua responsable de la regulació de gens implicats en la supervivència cel·lular, la inflamació o la diferenciació tenint un paper clau en processos inflamatoris i la resposta immune. La regió del promotor de la COX-2 conté dos llocs d'unió a NF-kb i s'ha demostrat que la seva activació regula positivament l'expressió de la COX-2.

7.3. C/EBP. Aquests factors de transcripció també regulen l'expressió de la COX-2. De fet, hi ha un lloc d'unió de C/EBP al promotor de la COX-2. Aquests factors de transcripció actuen en la regulació de gens implicats en la inflamació i la proliferació cel·lular i tendeixen a ser incrementats durant la resposta inflamatòria aguda.

8. La prostaglandina E2 (PGE_2).

La PGE_2 és la prostaglandina més abundant del cos i presenta gran varietat d'activitats biològiques. Entre els seus efectes s'inclouen el control de la secreció d'àcid gàstric, la regulació del flux sanguini renal, així com diversos aspectes de la funció reproductora femenina. A més, s'ha demostrat que la PGE_2 té un paper important en la regulació de processos inflamatoris ja que es troba activament involucrada en la patogènesi de diverses malalties com ara periodontitis o l'artritis reumatoide.

8.1. Receptors de prostaglandina E_2 (EP). L'activitat de la PGE_2 té lloc a través dels receptors de prostaglandina E_2 (EP1-EP4). Mitjançant aquests receptors, la PGE_2 desencadena diferents vies intracel·lulars amb diversos efectes finals, que a vegades semblen oposats en la mateixa cèl·lula o òrgan. Aquesta complexitat de respostes es deguda en part a que diferents poblacions de receptors sovint s'expressen en la mateixa cèl·lula o òrgan.

El receptor EP1 està involucrat en la percepció del dolor i la regulació de la pressió arterial. El receptor EP2 regula la vasodilatació, la proliferació tumoral i l'angiogènesi. El receptor EP4 actua regulant la funció renal i la osteoclastogènesi. Finalment, el receptor EP3 participa en la generació de febre, dolor i vasoconstricció.

8.2. Paper protector de PGE_2 en les vies respiratòries. S'ha descrit que a les vies respiratòries, al contrari que a la resta del cos, la PGE_2 té efectes protectors. Diversos estudis han observat que la PGE_2 atenua la broncoconstricció. També s'ha descrit que la PGE_2 inhibeix la síntesi de col·lagen, la transició fibroblasts-miofibroblasts d'aquesta manera modula la remodelació de les vies respiratòries en l'asma crònica i fibrosi pulmonar.

9. Alteracions del metabolisme de l'àcid araquidònic en CRSwNP i AIA.

9.1. Alteracions en la via 5-LOX en CRSwNP i AIA. La literatura ha demostrat àmpliament que la via de la 5-LOX és més activa en pacients amb AIA respecte a pacients amb tolerància a l'aspirina. Aquesta condició provocaria la síntesi de cis-LTS (LTC_4, LTD_4 i LTE_4) generant una acumulació d'aquestes substàncies amb propietats broncoconstrictores i vasoactives.

9.2. Alteracions en la via de la COX en CRSwNP i AIA. També s'han descrit alteracions en la via de la COX en pacients amb CRSwNP i AIA. No obstant això, la presència d'anomalies en aquesta via no s'ha trobat en tots els estudis. Per això, el paper que tindria la via de la COX en aquestes patologies és encara un tema de debat.

HIPÒTESI

Com hem dit prèviament, s'han descrit àmpliament anomalies en la via lipoxigenasa en pacients amb asma induïda per aspirina. No obstant això, i en contrast amb els resultats trobats en la via de lipoxigenasa, l'alteració en la regulació de la via de la ciclooxigenasa s'ha descrit en alguns però no tots els estudis, i per tant, el seu paper en la patogènesi de l'asma induïda per aspirina és encara tema de debat.

La nostra hipòtesi estableix que les discrepàncies de resultats obtingudes en els treballs centrats en la via de la ciclooxigenasa són degudes a l'ús de diferents tècniques i mètodes, així com a la manca d'estudis centrats en l'avaluació simultània dels múltiples factors que intervenen en aquesta via (prostaglandines, enzims i receptors de prostaglandina).

La nostra hipòtesi també estableix que per esclarir finalment la controvèrsia és necessari dur a terme un estudi exhaustiu utilitzant diferents metodologies que permetin avaluar la regulació de la via de la ciclooxigenasa en mostres obtingudes de vies respiratòries sanes i inflamades de pacients asmàtics amb intolerància a l'aspirina.

OBJECTIUS

- Estudiar la via de la ciclooxigenasa en fibroblasts de pòlips nasals de pacients amb o sense asma induïda per aspirina.
- Estudiar la via de la ciclooxigenasa en fibroblasts de mucosa nasal de pacients amb asma induïda per aspirina.
- Investigar el paper de vies de transducció de senyal (MAPKs, NF-kB, i C/EBP) en la regulació a la baixa de la COX-2 descrita en fibroblasts de pacients amb intolerància a l'aspirina.

RESULTATS

Anàlisi de les proteïnes implicades en el metabolisme de l'àcid araquidònic en cultius no estimulats. Després de 24 hores d'incubació en medi sense sèrum (SFM: sèrum free media), no és van trobar diferències significatives en l'expressió proteica de COX-1 (mesurada mitjançant western blot) ni en els nivells de secreció de PGE_2 (quantificada mitjançant ELISA) en els cultius estudiats. La proteïna COX-2 no va ser detectada a nivell basal (mesurada per Western blot).

Efecte de IL-1β sobre la producció de PGE_2. Cèl·lules quiescents van ser incubades en SFM en presència o absència d'IL-1β (10 ng/ml) durant 4 o 24 hores. La concentració de PGE_2 es va mesurar en els sobrenedants d'aquests cultius.

Comparat amb les cèl·lules incubades en medi SFM, la IL-1β va estimular significativament la ràtio PGE_2/proteïna total a les 4 i 24 hores en fibroblasts de (mucosa nasal) NM. Contràriament, la ràtio PGE_2/proteïna total no incrementava en els fibroblasts procedents de pòlips nasals de pacients tolerants a l'aspirina (NP-AT), NP-AIA i NM-AIA després de 4 hores d'incubació amb IL-1β i si ho feia lleugerament després de la incubació durant 24 hores en NP-AT i NP-AIA. Finalment, la ràtio PGE_2/proteïna va ser significativament inferior en els grups NM-AIA, NP-AT i NP-AIA comparat amb el grup NM.

Efecte de IL-1β sobre l'expressió de COX. Es va mesurar l'expressió de COX-1 i COX-2 en cèl·lules quiescents incubades en SFM en presència o absència de 10 ng/ml d'IL-1β. Estudis previs mostren varietat de resultats pel que fa a l'expressió de COX-2 en funció de la tècnica utilitzada, és per això que es van fer servir diversos mètodes per a quantificar l'expressió de COX-2: ELISA, Western blot i immunofluorescència.

Les mesures per ELISA mostraven increment de l'expressió de COX-2 després de 4 i 24 hores d'exposició a 10 ng/ml IL-1β al grup NM. En contrast, aquesta citocina presenta un efecte lleuger i no significatiu en la inducció de COX-2 en NP, NP-AIA i NM-AIA. Comparat amb les mostres NM, l'expressió de COX-2 va ser significativament inferior als fibroblasts NP a les 24 hores, als fibroblasts NP-AIA després de 4 i 24 hores i als

fibroblasts NM-AIA després de 4 hores. Finalment, destacar que la menor concentració de COX-2 va ser trobada als NP-AIA, sent significativament més baixa que als NP-AT a les 24 hores.

Les anàlisis mitjançant Western blot a les 24 hores d'exposició a 10 ng/ml IL-1β rebel·len increment estadísticament significatiu de l'expressió de COX-2 només en el grup de fibroblasts NM, comparat amb les cèl·lules no tractades. L'expressió induïda de COX-2 va ser significativament superior en els fibroblasts NM comparat amb els grups NP-AT, NP-AIA i NM-AIA.

Les anàlisis mitjançant immunofluorescència a les 24 hores d'exposició a 10 ng/ml d'IL-1β mostren un increment significatiu en el percentatge de fibroblasts COX-2 positius en tots els teixits, comparat amb aquells que van ser incubats en SFM. Tot i això, el percentatge de fibroblasts positius en el grup NM va ser significativament superior comparat amb els fibroblasts procedents de NP-AT, NP-AIA i NM-AIA.

Tot i que la COX-1 es considera un enzim constitutiu, la seva expressió pot incrementar de forma moderada en condicions inflamatòries. Es va mesurar l'expressió de COX-1 després d'incubar les cèl·lules amb IL-1β durant 24, 48 i 72 hores. En comparació amb les cèl·lules només incubades amb SFM, el grup NM expressa nivells significativament superiors de COX-1 a les 72 hores. En els fibroblasts procedents de NP-AT, NP-AIA i NM-AIA no varien els nivells d'expressió de COX-1 durant la incubació amb IL-1β.

Anàlisi dels receptors EP en cultius no estimulats. No va haver diferències a nivell d'expressió basal en cap dels receptors de PGE_2 (EP1, EP2, EP3 i EP4) en els teixits estudiats, NM, NP-AT i NP-AIA.

Efecte de la IL-1β en l'expressió dels receptors EP. Es va mesurar l'expressió dels receptors EP en cèl·lules quiescents incubades en SFM en presència o absència de 10ng/ml de IL-1β durant 24, 48 i 72 hores. La ràtio EP2/β-actina va incrementar en els fibroblasts NM a tots els temps estudiats. Contràriament, la ràtio EP2/β-actina no va incrementar en els fibroblasts procedents de NP-AT i NP-AIA. L'expressió dels

receptors EP1, EP3 i EP4 no va canviar en cap dels grups de fibroblasts durant la incubació amb IL-1β.

Efecte de la IL-1β en les dinàmiques de fosforilació de MAPKs. Donat que les diferències més grans pel que fa a l'expressió de COX-2 i la secreció de PGE_2 les trobem en la comparació entre els fibroblasts NM i el procedents de NP-AIA, aquests van ser els teixits escollits per l'estudi de MAPKs

Després de 5 minuts d'incubació amb IL-1β es van detectar increments en els ràtios de proteïna fosforilada/ no fosforilada de les MAPKs estudiades (p38 MAPK, JNK i ERK) en els fibroblasts procedents de NM i NP-AIA. En els fibroblasts NM, la cinètica de fosforilació assolia el seu màxim als 15 minuts en totes tres MAPKs. En Canvi, el nivell més alt de fosforilació en els fibroblasts procedents de NP-AIA el màxim s'assolia als 5 minuts tant per p38 MAPK com per ERK i als 15 minuts per JNK. Els nivells de fosforilació revertien fins a nivells propers als basals 60 minuts després d'incubar amb IL-1β tant en NM com en NP-AIA. La comparació de les ràtios entre la forma fosforilada/ no fosforilada de les proteïnes MAPK no va evidenciar diferències significatives entre els fibroblasts NM i els procedents de NP-AIA.

Efecte dels inhibidors de MAPKs en l'expressió de COX. Per tal d'estudiar quines de les vies MAPK es troben implicades en l'expressió de COX-2 es van fer servir inhibidors específics de les MAPKs. Cèl·lules quiescents van ser incubades 1 hora amb diferents concentracions (0,1 a 10 μM) dels inhibidors de MAPK p38 (SB203580), JNK (SP600125) o ERK (PD98059). A continuació les cèl·lules van ser incubades en SFM en presència o absència d'IL-1β (1 ng/ml) durant 24 hores. La mesura de la proteïna COX-2 es va fer mitjançant Western blot. El tractament amb 10 μM de l'inhibidor de la MAPK p38 va ser l'únic que va disminuir de forma significativa l'expressió de COX-2 en NM. Als fibroblasts procedents de NP-AIA el tractament a 1 i 10 μM amb l'inhibidor de la MAPK p38 també va disminuir l'expressió de COX-2 tot i que les diferències no van ser significatives.

Efecte de IL-1β en les dinàmiques de translocació de NF-kB (p65 i p50) i c/EBP. Fibroblasts aïllats de NM i NP-AIA van ser incubats en SFM en presència o absència

d'IL-1β (10 ng/ml) fins a 1 hora. Les mesures realitzades mitjançant TransAm van evidenciar un increment de la presència al nucli de p65 i p50 en els fibroblasts de NM i NP-AIA. Resultats similars es van obtenir mitjançant Western blot. En cap cas es van observar diferències significatives en les dinàmiques de translocació entre els fibroblasts de NM i NP-AIA. Pel que fa a les mesures de c/EBPα i β, no es van observar canvis en els nivells de translocació després del tractament amb IL-1β en els fibroblasts procedents de NM i NP-AIA.

DISCUSSIÓ

La via de la COX ha estat àmpliament estudiada en l'AIA fent servir diferents models. Malgrat això, els resultats obtinguts són contradictoris, probablement a causa de les diverses metodologies i protocols experimentals utilitzats en els diversos estudis.

En els estudis 1 i 2 presentats en la tesi actual hem demostrat que la via de la COX es troba alterada en fibroblasts de pòlips i mucosa nasal, especialment en aquelles mostres derivades de pacients amb AIA. Hem demostrat canvis a diferents nivells de la via, incloent: baixa producció de PGE_2, manca d'increment de la COX-2 en situació inflamatòria, i diferències en la regulació de la COX-1.

Com ja s'ha dit abans, la PGE_2 es considera un potent mediador de la inflamació. No obstant això, el paper inflamatori de la PGE_2 no sembla ser una regla general. Diversos estudis han demostrat efectes protectors de la PGE_2 i nivells baixos en pacients asmàtics.

El nostres resultats demostren que la secreció de PGE_2 es troba disminuïda en la CRSwNP, especialment en fibroblasts de mucosa i de pòlips nasal de pacients amb AIA. Aquests resultats són en concordança amb treballs que demostren una disminució en la producció de prostanoids i confirmen que la via de la COX es troba desregulada en poliposi nasal, asma i AIA.

Donat que la síntesi de PGE_2 està regulada, almenys en part, per l'activitat de les COXs, i s'espera que la seva expressió de COX es trobi incrementada en condicions inflamatòries, la pregunta que ens vam plantejar va ser: Com és l'expressió de les COXs en pacients amb CRSwNP, i particularment en aquells amb AIA?

Tot i que es considera un enzim constitutiu alguns estudis han demostrat que la COX-1 s'incrementa en algunes situacions inflamatòries. Pel contrari, en mostres de pacients amb asma i AIA, alguns treballs demostren una expressió reduïda. Aquests resultats coincideixen amb els obtinguts als estudis 1 i 2 on hem vist que fibroblasts procedents

de pacients AIA no responen a una situació inflamatòria en comparació amb els fibroblasts de NM.

Pel que fa a l'expressió de la COX-2, en general s'incrementa en condicions inflamatòries. De fet, en aquelles malalties caracteritzades per alts nivells de PGE_2 també té lloc l'augment d'expressió de la COX-2. A la literatura però, existeixen estudis on troben una expressió reduïda de COX-2 en CRSwNP i en particular en els pacients amb AIA. Aquestes observacions coincideixen amb les descrites als estudis 1 i 2, on els resultats es confirmen mitjançant 3 metodologies diferents: Western blot, ELISA i immunocitoquímica.

En conclusió podem dir que la via de la COX es troba regulada a la baixa, una troballa sorprenent si tenim en compte que tant la CRSwNP com l'AIA són malalties inflamatòries cròniques.

A l'estudi 1, també es va mesurar l'expressió de receptors de PGE_2 (EP1-4) després de 72 h d'inducció amb IL-1β. Els resultats obtinguts demostren que l'inducció de l'expressió del receptor EP2 és reduïda i absent en els fibroblasts de NP i NP-AIA, respectivament, en comparació amb els fibroblasts de NM, que si responen al tractament inflamatori. Aquesta desregulació del receptor EP2 en NPs i AIA pot tenir un paper important en l'AIA, ja que la major part dels efectes antiinflamatoris de PGE_2 són mediats per aquest receptor. Finalment, la disminució de l'alliberament de PGE_2 descrita als estudis 1 i 2 i la menor expressió del receptor EP2 poden augmentar el procés inflamatori present a les vies respiratòries dels pacients amb AIA.

En els estudis 1 i 2 d'aquesta tesi s'ha demostrat que la COX-2 és troba regulada a la baixa en fibroblasts aïllats de NPs especialment en subjectes amb AIA. Els mecanismes que regulen l'expressió de COX-2 en aquests pacients encara no es coneixen. D'acord amb això, l'estudi 3 es va dissenyar per analitzar l'activació de MAPKs i la translocació de NF-kB i C/EBP implicats en la regulació de COX-2 en fibroblasts de pacients amb CRSwNP i AIA.

L'activació de MAPK observada en l'estudi va ser similar als resultats publicats en la literatura demostrant la capacitat de la IL-1β per activar aquestes vies. Per examinar la implicació d'aquestes vies en la regulació COX-2, fibroblasts de NM i NP-AIA van ser incubats amb inhibidors selectius de MAPK. Els resultats obtinguts concorden amb els d'altres estudis que demostren que la MAPK p38 té un paper crític en la regulació de la COX-2. Tot i això no es van observar diferències significatives en els fibroblasts de pacients amb AIA comparats amb els controls.

Un altre mecanisme que podria explicar l'expressió disminuïda de COX-2 en pacients d'AIA podria ser alteracions en la translocació nuclear dels factors de transcripció reguladors de COX-2, NF-kB o C/EBP.

En aquest estudi hem demostrat la ràpida translocació nuclear de les subunitats p50 i p65 utilitzant dues metodologies diferents, TransAm i Western blot. No obstant això, no es van trobar diferències en la translocació en els fibroblasts de pacients AIA. En relació C/EBP, les mesures TransAm no van revelar canvis suggerint que la translocació d'aquest factor de transcripció no s'activa a través d'IL-1β.

Finalment, encara que aquest estudi no va aconseguir trobar els mecanismes responsables de la baixa expressió de COX-2 descrita en els intolerants a l'aspirina, aquesta informació podria contribuir a esclarir l'etiopatologia de l'AIA.

CONCLUSIONS

- Hi ha una regulació a la baixa del metabolisme de l'àcid araquidònic, especialment en la via de la ciclooxigenasa (COX-1 i COX-2) i en l'expressió del receptor EP2, en pacients que pateixen poliposi nasal i especialment en aquells que pateixen asma induïda per aspirina.

- Les principals vies de transducció de senyal i els factors de transcripció que regulen l'expressió de la ciclooxigenasa-2 no es troben alterats en els fibroblasts de pacients amb asma induïda per aspirina, aquesta troballa suggereix que aquests mecanismes no estan implicats en la regulació anormal d'aquesta via descrita en els pacients asmàtics amb i sense sensibilitat a l'aspirina.

- Es necessiten més estudis per esclarir els mecanismes responsables de la regulació anormal de la via de la ciclooxigenasa en els pacients asmàtics amb i sense intolerància a l'aspirina.

Appendix 2. Informe dels directors

Informe dels directors en relació a la Tesi Doctoral de Francesc Josep García García (publicacions i paper del doctorand).

En tant que directors de la Tesi doctoral de Francesc Josep García García, fem constar que, a la memòria de la Tesi, els resultats obtinguts s'estructuren en quatre articles científics que no s'han presentat explícita o implícitament en cap altra Tesi doctoral.

Articles científics que formen part de la Tesi:

1) Roca-Ferrer, J., **F. J. Garcia-Garcia**, J. Pereda, M. Perez-Gonzalez, L. Pujols, I. Alobid, J. Mullol and C. Picado (2011).

"Reduced expression of COXs and production of prostaglandin E(2) in patients with nasal polyps with or without aspirin-intolerant asthma."

J Allergy Clin Immunol. 2011; 128(1): 66-72 e61. IF: 11.003

Aquest primer article ha estat publicat a la revista Journal of Allergy and Clinical Immunology. Aquesta revista té un factor d'impacte, segons l'ISI, de 11.003, essent en el primer quartil de l'àrea "Allergy" i l'àrea "Immunology". El doctorand, segon autor d'aquesta publicació, ha participat de forma exhaustiva en el disseny i obtenció de les dades experimentals, així com en l'evolució i progressió dels experiments i també en la discussió dels resultats i la redacció de l'article.

2) Roca-Ferrer, J., M. Perez-Gonzalez, **F. J. Garcia-Garcia**, J. Pereda, L. Pujols, I. Alobid, J. Mullol and C. Picado (2013).

"Low Prostaglandin E2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics."

Respirology (accepted 10/12/2012). IF: 2.416

Aquest segon article ha estat recentment acceptat a la revista Respirology. Aquesta revista té un factor d'impacte, segons l'ISI, de 2.416, essent en el tercer quartil de l'àrea "Respiratory system". Aquesta publicació té una orientació experimental i metodològica semblant a l'anterior. En ella, el doctorand, tercer autor, ha participat fonamentalment en el disseny experimental, així com, donant suport experimental a les mesures realitzades, en els cultius cel·lulars realitzats i en la discussió dels resultats.

3) **Garcia-Garcia, F. J.** , J. Mullol, M. Perez-Gonzalez, L. Pujols , I. Alobid, J. Roca-Ferrer, C. Picado,

"Signal transduction pathways (MAPKs NF-KB and C/EBP) regulating COX-2 expression in nasal fibroblasts from asthma patients with aspirin intolerance."

PLOS ONE. IF: 4.092

Aquest ultim treball presentat en aquesta tesi doctoral ha estat recentment publicat a la revista PLOS one. Aquesta revista té un factor impacte, segons l'ISI, de 4.092, essent en el primer quartil de l'àrea "Biology". El doctorand, primer signant d'aquesta publicació ha tingut tot el protagonisme en el disseny experimental i en la realització de la bateria d'experiments i l'anàlilsi dels resultats obtinguts, així com en l'el·laboració de la discussió, les conclusions i la redacció d'aquest treball.

A Barcelona, el 15 de febrer de 2013

Signat:

Cèsar Picado Vallés — Director

Jordi Roca Ferrer — Director

Appendix 3. CV

CURRICULUM VITAE

PERSONAL DETAILS

Full Name: **Francesc Josep Garcia Garcia**
Id Card: 47637197 B
Date and place of Birth: March 7th, 1981. Vilanova i la Geltrú.
Home address: C/ Lepant 338 3º2º
City: Barcelona Zip Code: 08036
Phone: 34 699 225 992
E-mail address: fgarcia1@clinic.ub.es

WORK PLACE

Clinical and Experimental Respiratory Immunoallergy Department. (IRCE)
Institut d'investigacions Biomèdiques August Pi i Sunyer, IDIBAPS
C/ Villarroel 170, 08036 Barcelona (Spain)
Phone: 34 93 227 54 00 (#2906)
Fax: 34 93 227 98 13
E-mail address: fgarcia1@clinic.ub.es

EDUCATION

2008 University of Barcelona, **Master Degree in Biomedicine.**

2006 University of Barcelona, Department of Genetics**, Master Degree in Experimental Biology.**

2004 University of Barcelona, Faculty of Biology, **Bachelor Degree in Biological Sciences.**

CAREER/ EMPLOYEMENT

2007- up to now	Fellow Investigator	Fundació Clínic. Hospital Clínic. Barcelona, Spain.
2005-2006	Fellow Investigator	Departamento di Genetica Umana (Facoltà di Scienze), Padova, Italy.

LANGUAGES

English: First Certificate Level.
Italian: Intermediate Level.

ADDITIONAL EDUCATION

2010 **Formation course in risks and preventive measures.** NexAssistance prevención SL. Spain

2009 **I Setmana de la Recerca.** University of Barcelona, Spain.

2008 **Animal experimentation course**. Departament de Medi Ambient i Habitatge. Generalitat de Catalunya, Spain.

2007 **Ionic radiation course:** How to handle radioactive material in the Lab. Servicio de protección Radiológica. Hospital Clínic. Barcelona, Spain.

2005 **Certificate of pedagogic aptitude (CAP).** University of Barcelona, Spain.

STAYS IN FOREIGN CENTRES

Centre: Division of Respiratory Medicine, University of Nottingham.
Address: City Hospital, Hucknall Road, Nottingham NG5 1PB, UK
Duration: November 2010- July 2011
Topic: Study of chromatin structural changes in the regulation of COX-2 in aspirin-sensitive asthma.

Centre: Biology department, Facoltà di Scienze, University of Padova.
Address: via U.Bassi, 58/B - 35121 Padova.
Duration: October 2005- July 2006
Topic: Identification of Mutations involved in Brugada Syndrome.

RESEARCH PROJECTS

COLLABORATOR RESEARCHER

2007- 2010 **Research project:** "Inflammation and treatment in respiratory diseases." *Financing entity:* Agència de gestió d'ajuts universitaris i de recerca. Generalitat de Catalunya. *Principal Researcher*: César Picado Valles.

2010- up to now **Research project:** "Severe asthma: genetics, molecular and cellular aspects." *Financing entity:* Centro de Ingestigacion Biomedica en Red de Enfermedades Respiratorias (CIBERES). Instituto de Salud Carlos III. *Principal Researcher*: César Picado Valles.

2011 **Research project:** "Mechanisms involved in myocardic fibrosis associated to exercise." *Financing entity:* Societat Catalana de Cardiologia. *Principal Researcher:* Montserrat Batlle.

2010 **Research project:** "Mitochondrial respiratory chain function in sepsis. Modulator role of clinical evolution in humans." *Financing entity:* Fondo de Investigaciones Sanitarias-ISCIII. *Principal Researcher:* Jordi Casademont Pou.

PUBLICATIONS

AUTHOR

2013 Roca-Ferrer, J., M. Perez-Gonzalez, **F. J. Garcia-Garcia**, J. Pereda, L. Pujols, I. Alobid, J. Mullol and C. Picado (2013). **"**Low Prostaglandin E2 and Cyclooxygenase Expression in Nasal Mucosa Fibroblasts of Aspirin-Intolerant Asthmatics." *Respirology* (accepted 10/12/2012).

2012 **Garcia-Garcia F. J.**, Mullol J, Perez-Gonzalez M, Pujols L, Alobid I, Mullol J, Picado C (2012). Signal Transduction Pathways (MAPKs, NF-κB, and C/EBP) Regulating COX-2 Expression in Nasal Fibroblasts from Asthma Patients with Aspirin Intolerance. PLoS ONE 7(12): e51281. doi:10.1371/journal.pone.0051281

2011 Roca-Ferrer, J; **Garcia-Garcia, FJ**; Pereda, J; Perez-Gonzalez, M; Pujols, L; Alobid, I; Mullol, J; Picado, C. (2011) Nasal polyps and aspirin-intolerant patients produce little prostaglandin E2 and have reduced expression of cyclooxygenases. *J Allergy Clin Immunol,* 128 (1): 66-72.

COLLABORATOR

2012 Garrabou G; Morén C; López S; Tobías E; Cardellach F; Miró O; Casademont J.(2012) The effects of sepsis on mitochondria. *J Infect Dis* 205 (3): 392-400.

CONGRESS ATTENDANCE AND PRESENTATIONS

2012 The Epigenetic Regulation of Cellular Differentiation and Tissue Regeneration. II Barcelona Chromatin Club co-organized with the Molecular Biology Section of the Catalan Biology Society. Barcelona (Spain) 26th November. [Attendance].

2012 Campus Gutenberg. Scientific culture and communication. Universitat Pompeu Fabra. Barcelona (Spain) 17th-18th September, 2012. *Scientists and humanists: creative contaminations to communicate science.* [Workshop coordinator].

2012 II Meeting of Group of Rare Diseases in adult. Therapeutics aspects.. CIBERER, Hospital Clínic. Barcelona (Spain) 25th May, 2012. [Attendance].

2012 Global questions on advanced biology. Congrés Internacional de Biologia de Catalunya. 9-12 July, 2012. Societat Catalana de Biologia, Barcelona (Spain) *Cox-2 regulation by IL-1β through MAPKs: A comparison of nasal mucosa and nasal polyps fibroblasts from AIA patients.* [Abstract].

2012 V International Meeting of Art, Architecture and Digital Society group. Artistic innovations and new media: conservation, networks and technoscience. Barcelona (Spain) 8, 10 and 11th May, 2012. Workshop: "*Live biology, Bioart and experimentation in lab technologies."* [OP] [Workshop coordinator]

2010 Annual congress of the European Respiratory Society. Barcelona (Spain), 18-22 September, 2010. **Garcia-Garcia, FJ**; Roca-Ferrer, J; Pérez, M; Alobid, I**;** Mullol, J; Picado, C. *Cox-2 regulation by IL-1β through MAPKs: A comparison of nasal mucosa and nasal polyps fibroblasts from AIA patients.* [Abstract] [P].

2010 Inflammation 2010. Inflammatory cell signalling mechanisms as therapeutic targets. Luxembourg (Luxembourg), 27-30 January, 2010. [Attendance]

2009 60 Congreso Nacional de la Sociedad Española de Otorrinolaringología Y Patología Cérvico-Facial. Madrid (Spain), 13-17 November, 2009. Callejas, FB; Roca-Ferrer, J; Mendez, E; Alobid, I; **Garcia-Garcia, FJ**; Martinez, A; Guilemany, JM; Valero, A; Picado, C; Mullol, J. *Montelukast inhibe la supervivencia de eosinófilos inducida por las secreciones de células epiteliales de mucosa y pólipos nasales.* [Abstract] [OP].

2009 60 Congreso Nacional de la Sociedad Española de Otorrinolaringología Y Patología Cérvico-Facial. Madrid (Spain), 13-17 November, 2009. Callejas, FB; Roca-Ferrer, J; Mendez, E; Alobid, I; **Garcia-Garcia, FJ**; Martinez, A; Guilemany, JM; Valero, A; Picado, C; Mullol, J. *Montelukast inhibe la secreción de GM-CSF, IL-6 e IL-8 producida por células epiteliales de mucosa y pólipos nasales.* [Abstract] [OP].

2009 60 Congreso Nacional de la Sociedad Española de Otorrinolaringología Y Patología Cérvico-Facial. Madrid (Spain), 13-17 November, 2009. **Garcia-Garcia, FJ**; Roca-Ferrer, J; Pérez, M; Pujols, L; Alobid, I; Martínez, A; Mullol, J; Picado, C. *Expresión de Cox-1 y Cox-2 inducida por IL-1β y PGE_2 en*

fibroblastos de mucosa nasal y pólipos de pacientes con intolerancia a la aspirina. [Abstract] [OP].

2009 XVII Jornades de Biologia Molecular de la Societat Catalana de Biologia. Barcelona (Spain), 25-26 June, 2009. **Garcia-Garcia, FJ**; Roca-Ferrer, J; Pérez, M; Pujols, L; Alobid, I; Martínez, A; Mullol, J; Picado, C. *Expressió de Cox-1 i Cox-2 induïda per IL-1β i PGE_2 en els fibroblasts de mucosa i pòlips nasals de pacients amb asma induït per aspirina.* [Abstract] [OP].

2009 XXVIII Congress of the European Academy of Allergology and Clinical Immunology. Warsaw (Poland), 6-10 June, 2008. **Garcia-Garcia, F**; Roca-Ferrer, J; Perez, M; Pujols, L; Alobid, I; Pereda, J; Mullol, J; Picado, C. *Differential regulation of Cox-1 and Cox-2 expression by IL-1β and PGE_2 in nasal mucosa fibroblasts compared with nasal polyps from aspirin-sensitive asthma patients.* [Abstract] [P].

2009 XXVIII Congress of the European Academy of Allergology and Clinical Immunology. Warsaw (Poland), 6-10 June, 2008. Roca-Ferrer, J; Mendez, E; Callejas, F; Alobid, I; **Garcia-Garcia, F**; Martinez, A; Guilemany, J; Bartra, J; Picado, C; Mullol, J. *Montelukast inhibits eosinophil survival induced by epithelial cell secretions from nasal mucosa and nasal polyps.* [Abstract] [P].

2009 XXVIII Congress of the European Academy of Allergology and Clinical Immunology. Warsaw (Poland), 6-10 June, 2008. Roca-Ferrer, J; Mendez, E; Callejas, F; Alobid, I; **Garcia-Garcia, F**; Martinez, A; Guilemany, J; Valero, A; Picado, C; Mullol, J. *Montelukast inhibits GM-CSF, IL-6 and IL-8 secretion from nasal mucosa and nasal polyp epithelial cells.* [Abstract] [P].

2008 I Jornadas de Formación del Ciberes. Mallorca (Spain), 13-14 November, 2008. **Garcia, F**; Roca-Ferrer, J; Perez, M; Alobid, I; Luis M; Pereda, J; Martinez-Anton, A; Mullol, J; Picado, C. *Feedback positivo inducido por Il-1β y PGE_2 sobre la expresión de Cox-2 en fibroblastos de mucosa nasal.* [Abstract] [P].

2008 XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona (Spain), 7-11 June, 2008. **Garcia, F**; Roca-Ferrer, J; Perez, M; Alobid, I; Luis M; Pereda, J; Martinez-Anton, A; Mullol, J; Picado, C. *Positive feedback regulation of Cyclooxygenase-2 protein expression by Interleukin-1β and Prostaglandin E_2 in nasal mucosa fibroblasts.* [Abstract] [P].

2008 XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona (Spain), 7-11 June, 2008. Roca-Ferrer, J; **Garcia, F**; Perez, M; Alobid, I; Ayuso, M; Pereda, J; Pujols, L; Fernandez, L; Mullol, J; Picado, C. *Effect of Interleukin-1β, on Prostaglandin E_2 release, mPGEsynthase-1 and Cyclooxigenase protein expression in nasal mucosa fibroblasts from acetylsalicylic acid-tolerant and intolerant patients.* [Abstract] [OP].

2008 XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona (Spain), 7-11 June, 2008. Pereda, J; Molina-Molina, M; Uhal, B; Serrano-Mollar, A; Roca-Ferrer, J; **Garcia, F**; Mullol, J; Picado, C; Xaubet, A. *Effect of angiotensin receptor 1 antagonism on Prostaglandin E_2 and Cyclooxigenase-2 expression in experimental lung fibrosis.* [Abstract] [P].

AWARDS AND GRANTS

2012 **Doctoral Thesis last year Fellowship.** Universitat de Barcelona, Spain.

2011 **CIBERES Abroad Training Fellowship.** Project: "*Study of chromatin structural changes in the regulation of COX-2 in aspirin-sensitive asthma."* University of Nottingham, UK.

2010 **ERS Short-Term Research Training Fellowship.** Project: "*Study of chromatin structural changes in the regulation of COX-2 in aspirin-sensitive asthma."* University of Nottingham, UK.

2008 **Best Scientific Oral Communication:** Session: "*Inflammatory Mechanisms in Rhinosinusal Diseases"* Abstract: Roca-Ferrer, J; **Garcia, F**; Perez, M; Alobid, I; Ayuso, M; Pereda, J; Pujols, L; Fernandez, L; Mullol, J; Picado, C. *Effect of Interleukin-1, on Prostaglandin E_2 release, mPGEsynthase-1 and Cyclooxigenase protein expression in nasal mucosa fibroblasts from acetylsalicylic acid-tolerant and intolerant patients*. XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona (Spain), 7-11 June, 2008.

2008 **Best Scientific Poster Communication:** Session: "*Cells and Mediators of Allergy"* Abstract: Pereda, J; Molina-Molina, M; Uhal, B; Serrano-Mollar, A; Roca-Ferrer, J; **Garcia, F**; Mullol, J; Picado, C; Xaubet, A. *Effect of angiotensin receptor 1 antagonism on Prostaglandin E_2 and Cyclooxigenase-2 expression in experimental lung fibrosis.* XXVII Congress of the European Academy of Allergology and Clinical Immunology. Barcelona (Spain), 7-11 June, 2008.

2005 **Socrates Erasmus Grant.** Project: *"Detection of mutations involved in Brugada Syndrome."* Universita degli studi di Padova, "Il Bo", Italy.