SELECTIVE EP2 AGONISM ATTENUATES HDM-INDUCED MURINE AIRWAY PATHOLOGY AND MAST CELL ACTIVITY, AND TRIGGERS INTRACELLULAR INHIBITORY SIGNALING IN MAST CELLS

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Immunology Doctorate Program

Bellaterra, 2011

FACULTAT DE VETERINÀRIA DE BARCELONA

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Certifiquen:

Que la memòria titulada: ***"SELECTIVE EP2 AGONISM ATTENUATES HDM-INDUCED MURINE AIRWAY PATHOLOGY AND MAST CELL ACTIVITY, AND TRIGGERS INTRACELLULAR INHIBITORY SIGNALING IN MAST CELLS"*** presentada per **Mariona Serra Pagès** s'ha realitzat sota la seva direcció, i consideren que compleix totes les condicions exigides per optar al grau de Doctora en Immunologia per la Universitat Autònoma de Barcelona. Per tant, autoritzen la seva defensa pública per què sigui avaluada pel tribunal corresponent.

I perquè consti als efectes que corresponguin, firmen el present certificat a Bellaterra, 28 de Desembre 2011,

Fernando de Mora Pérez **Rosa Torres Blanch**

Agraïments

(Català)

Agraïments

Escollir el camí de la ciència no és fàcil. És una doctrina exigent i poc reconeguda. La passió per la ciència neix de la nostra inevitable voluntat de canviar les coses que ens desagraden del nostre voltant. Com si d'alguna manera, jugant a ser Déu, poguéssim trobar la fórmula màgica per saciar la gana al món, curar el patiment o evitar l'efecte hivernacle… Amb utopia al cap i anhels al cor un decideix començar el llarg viatge que suposa la tesi. Un procés de maduració professional i personal en què et converteixes en investigador i d'alguna manera et connectes amb el món que una vegada vas projectar.

Com ja sabeu la importància d'un viatge, recau en gran part sobre el camí que un traça per arribar al seu destí, tal i com rememora la cançó "Viatge a Ítaca".

"Quan surts per fer el viatge cap a Ítaca,
has de pregar que el camí sigui llarg,
ple d'aventures, ple de coneixences.
Has de pregar que el camí sigui llarg,
que siguin moltes les matinades
que entraràs en un port que els teus ulls ignoraven,
i vagis a ciutats per aprendre dels que saben.
Tingues sempre al cor la idea d'Ítaca." (…)

Avui, que arribo a un dels destins marcats, m'adono que he recorregut un costós camí, al llarg del qual he trobat persones que d'una manera o altra m'han ajudat a aconseguir la fita proposada. Són persones que considero la millor troballa, són persones a qui avui retré el meu sincer

homenatge d'agraïment. Totes elles han estat els meus companys de viatge en aquest incansable caminar pel món de la ciència.

-A Fernando de Mora i a Rosa Torres, perquè han estat els capitans del meu vaixell, agraeixo la seva brillant direcció. A Fernando, per la sàvia i precisa meticulositat en les seves directrius, que sempre m'han facilitat la comprensió dels conceptes més indispensables per aconseguir resultats i per ajudar a madurar el meu criteri científic amb tàctiques encoratjadores que moltes vegades han evitat el defalliment. A Rosa, per haver estat sempre al meu costat instruint-me rigorosament, ajudant-me a tirar endavant el projecte científic des de l'estímul més encomanadís. Sempre partint de la més sincera amistat i càlida proximitat. En tots dos penso com els meus principals referents professionals.

-A un fantàstic equip, la tripulació: A César Picado, pels seus eficients consells i dedicació en el projecte. A en Pere Losada per tota la paciència que ha tingut ensenyant-me des d'un bon principi tècniques de laboratori amenitzades sempre per l'art i el detall humà que transmet. A l'Aida Herrerias, per seva dedicació i afable suport en les meves primeres passes experimentals. A la Judith Plaza, per la seva gran l'ajuda en els experiments i per tots els moments divertits que m'ha regalat. A Toni Quesada, per tota l'ajuda acompanyada dels seus tocs d'humor. A la Cristina Costa i l'Alberto Marco, per tots els coneixements que m'han transmès durant la carrera i ara, d'una manera molt més propera i còmplice, en el doctorat. A Marcel

Jiménez, per la seva ajuda introduint-me en el món de la fisiologia del calci.

-En un llarg viatge sempre hi ha una parada, la meva ha estat Bethesda (MD, USA) al NIH, on he trobat persones tan dignes com Juan Rivera i Ana Olivera, a qui agraeixo la seva excel·lent mentorització i el càlid aixopluc amb què em van acollir. La gent del laboratori: Barbara, Jorg, Tiffany, Ryo Sun, Sara, Laurel, Jen, Sandra, Chao i Kevin a qui agraeixo haver compartit amb mi experiències inoblidables. A Asun, Aitor, Barbara, Jorg, Simone, Monica, Jose Manuel, Tanzer i Hicham per haver estat "la meva família" durant un any.

-A en Santi, a la Maria, a l'Anna i a la Laura, també viatgers en aquest camí, per la "penya" tan maca de becaris que hem format i pels moments compartits. A la resta del Departament de Farmacologia, Jose Luís, Sergi, Alhelí, Eva, Victòria, Gent del SAF, etc. per haver-me ajudat quan ho he necessitat.

-A tots els qui heu procurat que mai perdés el nord, heu estat el meu far. A Carlos pel seu amor i per sempre ajudar-me a treure el millor de mi. A la família pel seu incondicional suport, en especial la meva mare, Carme Pagès, per la seva dedicació en revisar la lingüística catalana de la Tesi. A Júlia, Laura, "Marrameus/es", "Les Nenes", "Les Trutxes", "Veterinaris/es" i "Immunonenes" per sempre haver confiat en mi i donar-me l'empenta quan l'he necessitada. A l'Àric per la fantàstica portada que m'ha regalat.

- A la meva Ítaca, que he materialitzat amb tots els qui pateixen asma, a qui dedico aquesta tesi, que apunta bons resultats, conscient, amb tot, que hem albirat un interessantíssim horitzó que caldrà perseguir en el llarg caminar.

-Em cal agrair el finançament de la beca FI de l'AGAUR (Agencia de Gestió d'Ajuts Universitaris i de Recerca), dels ajuts FIS PI060592 i PS0900171 durant els anys compresos entre gener 2009-gener 2012. També agreixo el finançament de la beca BE de l'AGAUR i el "National Institute of Health" (NIH) per l'estada a l'estranger.

A tots vosaltres, moltes gràcies!

“Research is to see what everybody else has seen, and to think what nobody else has thought”

Albert Szent-Györgyi

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Abbreviations

1. Abbreviations

-Ag: antigen

-AHR: airway hiperreactivity or airway hyperresponsiveness

-AKT: protein kinase of Fyn pathway

-AMPc/cAMP: cyclic AMP/*AMP cíclic*

-ANOVA: analysis of variance

-BAL: bronchoalveolar lavage

-BMMC: bone marrow mast cell

-C57: CI.MC/C57.1 mast cell line

-COX-2: cyclooxygenase-2

-Csk: C-terminal Scr kinase

-*CTHR2: receptor de la* PGD_2

-Cys-LTC: Cysteinyl-leukotriene

-DMSO: dimethylsulfoxide

-DP: *receptor de la* PGD_2

-ELISA: enzyme linked-immunosorbent assay

-EP: prostaglandin E2 receptor

-EPR: early phase response o *fase primerenca*

-FBS: fetal bovine serum

-FcεRI: high affinity receptor for IgE

-Fyn: proto-oncogene Src kinase

-GAB2: adaptor protein of Fyn pathway

-GM-CSF: granulocyte macrophage colony-stimulating factor

-GPCR: G protein coupled receptor

-HDM: house dust mite o *àcars de la pols*

-HLMC: human lung mast cell

-hMCs: human mast cell derived from cord blood

-HMC: human mast cell from adult peripheral blood progenitors

-HuMC: human mast cell derived from peripheral blood progenitors $CD34^+$

-IgE: immunoglobulin E

-IL: interleukin

-IBMX: 3-isobutyl-1-methylxanthine

-ITAM: immunoreceptor tyrosine-based activation motifs

-KO: knockout

-LAD2: LAD2 mast cell line, human mast cell line derived from a patient with untreated MC sarcoma

-Lck: lymphocyte-specific protein Src kinase

-LPR: late phase response o *fase tardana*

-Lyn: tyrosine protein kinase

-MC: mast cell

-MCP-1 (CCL2): monocyte chemotactic protein-1 o *proteïna quimiotàctica de monòcit -1*

-mMCP-1: mouse mast cell protease 1

-min: minutes

-OMS: *organització mundial de la salut*

-OVA: ovalbumin or *ovoalbúmina*

-PBS: phosphate buffered saline

-PCA: passive cutaneous anaphylaxis

-PDMC: peritoneum-derived mast cell

-PG: prostaglandin

-PGD_2: prostaglandin D_2

-PGE_2: prostaglandin E_2

-PI3: phosphathydilinositol-3

-RNA: ribonucleic acid

-sAv: streptavidin

-shRNA: small hairpin RNA

-SPMC: spleen mast cell

-Src: tyrosine protein kinase

-Syk: Spleen Src kinase

-Th_1: lymphocyte T helper type 1

-Th_2: lymphocyte T helper type 2

-TNF-α: tumor necrosis factor-α

-TRPC1: transient receptor potential channel type 1

-WBP: whole body plethysmography

-Wsh: Wsh/Wsh mice, mast cell deficient mice

Summary

2. Summary

Allergic asthma is a chronic respiratory disease with a high prevalence in developed countries. Current treatments do not halt the underlying allergic process and do not always control the symthomps of the disease. The most effective treatment is the use of glucocorticoids, which are based on chemical modifications of potent natural endogenous anti-inflammatory hormones. Studying endogenous anti-inflammatory pathways to explore new therapeutic targets is an efficient experimental strategy to uncover potential novel targets against asthma. One of such endogeneous pathways are cyclooxygenase (COX)-mediated. Prostaglandin (PG) PGE_2, a COX product, has been suggested to exert a protective effect in the lungs. Notably, experimental studies with asthma patients revealed that inhaled PGE_2 reduces airway hyperresponsiveness and inflammation. This protective PGE_2 effect has also been demonstrated, directly and indirectly, in mice sensitized to OVA or HDM. The mechanisms underlying the beneficial effect of PGE_2 in asthma are not understood. One of the most consistent features of PGE_2 is its ability to modulate mast cell activity *in vitro*. Our recent *in vivo* studies showed that PGE_2 also prevents mast cell activity in HDM sensitized mice and that this mast cell modulatory effect was paralleled by EP2 receptor overexpression. These results brought up the hypothesis that PGE_2 might interact with EP2 receptor on the bronchial mast cells surface to exert a protective action against allergen-driven airway pathology. The precise understanding of such mechanisms will certainly help uncover potential anti-asthma target molecules along the way.

The general objective of this thesis was to establish preclinically the relevance of the mast cell EP2 receptor to PGE_2 beneficial

effect in allergic asthma, and to uncover molecular mechanisms resulting from this receptor selective activation. To achieve this objective we have undertaken several *in vitro* and *in vivo* approaches.

We first determined the PGE2 EP receptors expression pattern on different human and murine mast cell population, and thereafter assessed (a) whether such differences in the relative expression of EP receptors 1 to 4 influenced the ability of PGE_2 to modulate mast cells degranulation and calcium mobilization, and (b) whether human mast cells behaved similarly to murine mast cells under different EP receptors expression scenarios. The results pointed at EP2 as the main contributor to mediate the inhibitory effect of PGE_2 on both murine and human mast cells. Once EP2 had been suggested to be the primary protective receptor, we addressed the relevance of selective EP2 activation to (a) protection against HDM-induced airway pathology in mice, and (b) correlation of such pathology to the ability of selective EP2 agonism to prevent mast cells activity *in vivo*. We showed that a selective EP2 agonist prevented AHR and inflammation from developing, and that such effect was linked to the ability of such selective agonistic action to attenuate airway mast cell activity. We then studied potential inhibitory signaling mechanisms involved in such EP2-mediated blocking effect. We observed that EP2 agonism inhibited *in vivo* and *in vitro,* mast cell activity. We described that the PGE_2-EP2 interaction on mast cells inhibiting mast cell degranulation through the supression of calcium influxes mediated by an inhibition of the Src-Fyn pathway, and cAMP/PKA.

Our observations highlight that the "PGE_2"-"mast cells EP2"-"airway" axis is an endogeneous pathway leading to natural protection against aeroallergens-induced airway pathology, and

helps elucidate the precise mechanisms that will uncover clue molecules to be targeted by potential novel antiasthma treatments.

Introducció

(Català)

3. Introducció

3.1. Asma al·lèrgica i tractament

L'asma és una malaltia crònica de les vies aèries que es manifesta com una dificultat respiratòria ocasionada per una obstrucció dels bronquis, i que cursa amb una inflamació broncovascular (Bousquet et al., 2000). Els factors desencadenants de l'asma poden ser diversos. Els principals són l'exercici, els antiinflamatoris no esteroïdals, les infeccions virals i els aeroal·lergens. Quan l'asma és ocasionada per un al·lergè es parla d'asma al·lèrgica, atòpica o extrínseca. Actualment, l'asma al·lèrgica és el tipus d'asma més prevalent. L'asma al·lèrgica és resultat de l'exposició als aeroal·lergens de persones genèticament predisposades. Aquesta modalitat d'asma consta d'un quadre clínic amb fase aguda ocasionada per la contracció espasmòdica dels músculs bronquials, desencadenada per un conjunt de reaccions immunològiques que es produeixen davant l'exposició a l'aeroal·lergè (broncoespasme). A més a més, la musculatura bronquial dels pacients asmàtics reacciona exageradament constrenyent-se davant estímuls inespecífics, el que s'anomena hiperreactivitat bronquial (AHR); i les vies respiratòries inferiors pateixen inflamació (Torres et al., 2005). En la fase crònica, l'obstrucció dels bronquis també queda agreujada per la hipersecreció de moc i per l'alteració de la microestructura de les vies aèries afectades (remodelat o reestructuració tissular). Aquest seguit de fenòmens desencadenen simptomatològicament accessos de dispnea, tos, raneres, opressió pectoral, etc. que en alguns casos aguts poden causar la mort del pacient (*National Asthma Education and Prevention Program,* 2002).

L'asma al·lèrgica té una major incidència en països desenvolupats (Borchers et al., 2005). A Europa, l'asma afecta el 70-75% de la

població. En el 80% dels casos l'asma és d'origen al·lèrgica i a Espanya un 40% dels casos d'asma en adults joves (20-44 anys) té un component al·lèrgic, segons dades del subestudi de l'enquesta de Salut Respiratòria-Comissió Europea (Basagana et al., 2000; Martinez-Moratalla et al., 1999). Actualment, l'asma afecta 300 milions de persones en el món i causa 250.000 morts a l'any (Akinbami et al., 2011). L'evolució d'aquests pacients cap a l'asma crònica dificulta el seu tractament, ja que l'abordatge terapèutic actual no resol la malaltia i s'encamina fonamentalment a contrarestar els episodis de broncospasme i controlar la inflamació subjacent en el procés crònic. Aquesta malaltia genera un problema econòmic de primera magnitud, per absentisme laboral, costos farmacèutics i disminució de la productivitat (Nieto et al., 2000). A més a més, existeix un risc de mort en atacs aguts, que arriba a una comptabilització de 18 casos per cada milió d'habitants (Office of Press and Public Relations WHO: Bronchial asthma, 2000). Segons l'Organització Mundial de la Salut (OMS), les morts per asma s'incrementaran en els próxims 10 anys. Per aquests motius, cada vegada més, es fa palesa la necessitat de trobar un tractament que no vagi destinat solament a pal·liar els símptomes de la patologia respiratòria, sinó a impedir la seva evolució.

Les recomanacions terapèutiques actuals per a l'asma al·lèrgica suggereixen incrementar el número, freqüència i dosis de medicació d'acord amb la severitat de l'asma, fins que la malaltia quedi sota control (Taylor et al., 2008). Per a l'asma intermitent s'administren β2-agonistes d'acció curta. Per a la resta de formes d'asma s'utilitzen tractaments amb corticosteroids a dosis creixents en funció de la severitat de l'asma. Finalment, per a l'asma severa i moderada, s'aplica una combinació de β2-agonistes

d'acció prolongada i corticosteroids. Altres combinacions poden incloure antagonistes del receptor dels leucotriens (CysLT1) com el montelukast o antagonistes de receptors muscarínics, com el tiotròpium o la teofilina (Adcock et al., 2008; Mullane et al., 2011).

Tot i la importància dels corticosteroids en el tractament de l'asma, no s'aconsegueix erradicar la malaltia. A més a més, d'un 5 a un 10% dels pacients que pateixen asma severa no responen adequadament als tractaments convencionals (Adcock et al., 2008). Aquesta problemàtica s'evidencia en nombrosos estudis, com per exemple l'estudi "Gaining Optimal Asthma control" (GOAL) on es va comparar el tractament de corticoids amb el tractament combinat de β2-agonistes d'acció prolongada i corticosteroids en 3.400 pacients que presentaven diverses formes d'asma. Tot i utilitzar dosis escalades i tractaments per un any, el 41% (corticosteroids sols) i el 29% (β2-agonistes d'acció prolongada i corticosteroids) dels pacients no van aconseguir controlar la malaltia adequadament (Bateman et al., 2004). A això cal afegir que l'asma crònica suposa un repte terapèutic a causa de la irreversibilitat del procés de remodelat.

Davant d'aquest escenari en què, tot i els avenços terapèutics, la incidència de la malaltia és creixent: només 4 fàrmacs amb abordatges terapèutics innovadors han aconseguit sortir al mercat aquests últims 30 anys (Mullane et al., 2011). Tres d'aquests fàrmacs s'inclouen al grup terapèutic dels antileucotriens. Es tracta dels antagonistes CysLT1, zafirlukast i el montelukast, i l'inhibidor de la síntesi de leucotriens, zileuton. L'altre és l'omalizumab, un anticòs monoclonal anti-IgE. Altres productes han sigut llençats al mercat, però en general són versions millorades de fàrmacs ja existents com corticosteroids inhalats, β2-agonistes, o

anticol.linèrgics, als quals s'ha prolongat la durada d'acció, millorat l'especificitat o canviat la via d'administració. Altres tractaments com altres tipus d'anticossos monoclonals, o inhibidors de citocines com la IL-4, la IL-5, la IL-13 i el TNF-α i enzims repressors *(DNA zymes)* de factors de transcripció com el GATA-3, entre d'altres, encara es troben en fase de desenvolupament. No obstant això, cap d'aquests medicaments s'utilitzen com a tractaments únics, sinó de forma combinada en els casos d'asma moderada i severa, i en el cas dels anticossos monoclonals anti-IgE són tractaments cars. Aquests nous abordatges terapèutics demostren l'esforç que s'està fent en les dues últimes dècades per desenvolupar nous agents dirigits selectivament contra molècules específiques rellevants en la patogènia de l'asma. L'èxit d'aquestes teràpies ha estat limitat per 3 motius principals: la dificultat per poder trobar un model animal adequat que permeti una valoració clínica que pugui ser translacional a pacients asmàtics; la gran varietat de factors, cèl·lules i molècules que participen en la patogènia de l'asma; i la dificultat, risc i cost que suposa triar el grup de pacients asmàtics adients per un assaig clínic (Mullane et al., 2011). Així doncs, els corticoids es mantenen com a tractament d'elecció (Sin et al., 2004).

Els corticoids són molècules endògenes amb una activitat pleiotròpica antiinflamatòria i immunorreguladora sobre diferents mecanismes implicats en la resposta asmàtica. L'efectivitat única d'aquests compostos remarca l'interès i el potencial impacte terapèutic derivat de l'estudi de les molècules i els mecanismes endògens reguladors de la resposta asmàtica, com a eina identificadora de possibles noves dianes farmacològiques.

Una d'aquestes molècules és la PGE2. Existeixen precedents sobre l'efecte protector de la PGE_2, una molècula endògena a la qual s'atribueixen activitats diverses en les vies implicades en la resposta asmàtica.

3.2. Eix PGE_2-MC-Asma

3.2.1. PGE_2 i asma

Tot i les dificultats per al desenvolupament de noves estratègies terapèutiques antiasmàtiques, a causa del desconeixement i la complexitat de la patogènia de l'asma, se sap que poques famílies de mediadors poden incidir tant i de manera tan variada en la inducció i l'evolució de l'asma com les prostaglandines (PG). Per aquest motiu, l'anàlisi dels mecanismes, pels quals les prostaglandines actuen, poden ajudar a desemmascarar noves dianes i, per tant, nous abordatges terapèutics.

Les prostaglandines són molècules lipídiques derivades del metabolisme de l'àcid araquidònic per la via de la ciclooxigenasa (COX). La COX-2, enzim induïble, augmenta la seva activitat sota estímuls proinflamatoris i dóna lloc a diferents PG. Algunes PG tenen efectes perjudicials/proinflamatoris, i altres de beneficiosos/antiinflamatoris, en l'asma. Per exemple, s'ha vist que la PGD_2 indueix la broncoconstricció i l'AHR (Oguma et al., 2008). Assaigs clínics basats en antagonistes dels receptors de PGD_2, com CRTH2 i DP, han donat bons resultats (Ulven & Kostenis E, 2010). Per contra, en alguns d'estudis amb models murins, s'ha observat que si s'administren agonistes dels receptors de la PGD_2 (DP i CRTH2), es pot obtenir un efecte antiinflamatori: inhibint la migració i degranulació dels basòfils (Yoshimura-Uchiyama et al., 2004), incrementant el nombre de cèl·lules reguladores T $Foxp3^+$

$CD4^+$, la IL-10 i suprimint la inflamació de les vies aèries (Hammad et al., 2007). La PGI2 s'ha associat a la prevenció del desenvolupament de la inflamació pulmonar en un model murí d'asma (Jaffar et al., 2007). Una observació rellevant dels últims anys és el possible efecte protector de la prostaglandina E_2 (PGE_2), una mol·lècula endògena amb efectes antiinflamatòris i que té nombroses funcions en el pulmó (Jaffar et al., 2007; Vancheri et al., 2004). La PGE_2 és el producte predominant de la COX-2 i és produïda, majoritàriament, per macròfags, cèl·lules epitelials i cèl·lules musculars en les vies aèries. Des dels anys 70, ja es parla de l'efecte antiasmàtic de la PGE_2 administrada a pacients (Szczeklik et al., 1977; Smith et al., 1975). Estudis clínics experimentals demostren que l'administració de la PGE_2 en forma d'aerosol a pacients asmàtics prevé el desenvolupament d'inflamació de les vies aèries i l'AHR, tant en l'asma al·lèrgica (Gauvreau et al., 1999; Pavord et al., 1993), com en la no al·lèrgica (Sestini et al., 1996; Melillo et al., 1994). Tot i l'aparició d'alguns efectes secundaris en el tractament, tals com tos, s'ha observat una disminució de l'eosinofília en les vies aèries després de l'exposició a l'al·lergè, en pacients asmàtics que havien estat localment exposats a PGE_2 (Hartert et al., 2000; Gauvreau et al., 1999). D'acord amb aquests efectes s'ha descrit també una disminució en l'alliberació de PGE_2 en cèl.lules epitelials de les vies aèries aïlllades de pacients asmàtics (Roca-Ferrer et al., 2011; Pierzchalska et al., 2003; Picado et al., 1999) i s'ha vist que la PGE_2 modula processos implicats en el remodelat, en l'asma crònica i en la fibrosis pulmonar. La PGE_2 és capaç d'inhibir la proliferació de fibroblasts i cèl·lules de la musculatura llisa procedents de pacients asmàtics, la síntesis de col·lagen, la transició de fibroblast a miofibroblast i la regulació de la reparació

de l'epiteli de les vies aèries (Burgess et al., 2006; Burgess et al., 2004; Vancheri et al. 2004; Petkova et al., 2004; Kolodsick et al., 2003; Ricupero et al., 1999; Wilborn et al., 1995; Saltzman et al., 1982).

L'efecte protector de la PGE_2 ha estat evidenciat indirecta i directament també en estudis preclínics. Nosaltres i altres grups hem vist que en un model murí de sensibilització a ovoalbúmina (OVA), la inhibició de la COX-2 causa un empitjorament de la inflamació de les vies respiratòries, així com de l'AHR (Torres et al., 2009; Nakata et al., 2005; Hashimoto et al., 2005; Peebles et al., 2005; Peebles et al., 2002). Posteriorment vàrem estendre aquesta observació a un model murí de sensibilització a àcars de la pols (House Dust Mite, HDM), en el que es va inhibir localment l'expressió de COX-2 mitjançant un oligonucleòtid antisentit (Torres et al., 2008). De manera directa, s'ha demostrat la resposta beneficiosa de la PGE_2 en estudis preclínics amb rates i ratolins sensibilitzats a OVA. Martin et al. (2002) van observar que l'administració intratraqueal de la PGE_2 era capaç de reduir el broncoespasme i la eosinofília en rates sensibilitzades a OVA. De Campo & Henry (2005) van demostrar un efecte antiinflamatori de la PGE_2 endògena en ratolins sensibilitzats a OVA. El nostre grup va testar l'efecte de la PGE_2 exògena administrada per via subcutània en ratolins sensibilitzats a HDM i va observar una clara protecció enfront el desenvolupament de la resposta induïda per HDM en les vies respiratòries (Herrerias et al., 2009). Paral·lelament als estudis en pacients, en models animals es va revelar el possible efecte preservador de la PGE_2 en el remodelat de la vies aèries, és a dir, a la fase crònica de la patogènia de l'asma (Moore et al., 2000; Ogushi et al. 1999).

Malgrat moltes veus discrepants, totes aquestes dades palesen que la PGE_2 i/o la COX-2 poden exercir un efecte protector en l'asma, ja que atenuen la inflamació i l'AHR en pacients i en models experimentals. Tot i això, el mecanisme de la PGE_2 encara es desconeix, i indubtablement el seu estudi permetrà identificar molècules clau en aquest efecte beneficiós. És per aquest motiu que es requereix de més estudis bàsics per descriure el mecanisme implicat en l'efecte beneficiós de la PGE_2.

3.2.2. PGE_2 i MC in vitro

Talment com els glucocorticoids, la PGE_2 sembla exercir efectes diversos sobre una gran diversitat de cèl·lules i molècules implicades en la resposta asmàtica.

In vitro, a la PGE_2 se li atribueixen propietats antiinflamatòries i immunoreguladores però es desconeixen els mecanismes responsables del seu efecte protector en l'asma al·lèrgica.

Entre d'altres accions, s'ha descrit que la PGE_2 exerceix un efecte antiasmàtic mitjançant vies d'acció antiinflamatòries: la modulació de l'activitat mastocitària (Brown et al., 2011; Feng et al., 2006; Duffy et al., 2006) i l'alteració de la producció de citocines i quimiocines proinflamatòries en el mastòcit tals com: inhibició de citocines Th_2 típiques de la resposta asmàtica (Brown et al., 2011; Martin et al., 2002; Peebles et al., 2002); inhibició de la síntesis de quimiocines proinflamatòries com IL-8 i GM-CSF (Clarke et al., 2004; Vancheri et al., 2004; Lazzeri et al. 2001, Standiford et al. 1992); i també indueix a les Treg a la síntesis d'IL-10, la qual té un efecte anti-inflamatori (Harizi et al., 2002; Demeure et al., 1997; Strassman et al., 1994) i a la migració i diferenciació dels limfòcits T (Vancheri et al., 2004). Part de l'efecte protector de la PGE_2 podria ser mediat per algunes propietats immunoreguladores

com ara la inhibició de les cèl·lules dendrítiques en la fase primerenca de la instauració de la malaltia (Shiraishi et al., 2008; Son et al., 2006; Jing et al., 2003; Vassiliou et al., 2003; Harizi et al., 2002) i la modulació de la producció de la IgE (Pène et al., 1988).

Aquests mecanismes inhibitoris neixen de la interacció de la PGE_2 amb una o diverses poblacions cel·lulars rellevants en la resposta al·lèrgica, capaces de desencandenar la patologia asmàtica; entre d'altres, possiblement els mastòcits.

El mastòcits han estat àmpliament reconeguts com a cèl·lules amb un paper central en la mediació de processos al·lèrgics d'hipersensibilitat de tipus I, com l'asma al·lèrgica (Rao et al., 2008; Marone et al., 2005; Brightling et al. 2003; Holgate et al., 1986). Es considera que són cèl·lules efectores que participen tant en la fase primerenca (EPR), com en la fase tardana (LPR) de la resposta asmàtica. L'activació del mastòcit, per l'al.lergè o altres estímuls, porta a la generació i/o alliberació d'una gran varietat de mediadors de la inflamació com histamina, eicosanoids, citocines i enzims que desencadenen i regulen la resposta al·lèrgica (Williams & Galli, 2000). Aquests compostos preformats i de nova síntesis contribueixen a la broncoconstricció i a la inflamació per la capacitat que tenen de reclutar i activar cèl·lules inflamatòries com eosinòfils, i de promoure la proliferació de fibres musculars llises (Brightling et al., 2003). Hi ha evidències del fet que els mastòcits també poden tenir un paper regulador en la resposta immune (Schröder & Maurer, 2007), i que poden participar en la fase de sensibilització com a cèl·lules presentadores d'antigen, promoure l'activació i migració de cèl·lules dendrítiques o bé desviar la resposta immunitària cap a Th_2. S'ha descrit també que algunes de les molècules alliberades pels mastòcits podrien contribuir al

procés de remodelat (Okayama et al., 2007). La participació dels mastòcits en l'asma es demostra, en part, per l'observació de l'augment del número de mastòcits en les vies aèries de pacients. Estudis recents apunten que aquest augment és especialment evident en la musculatura llisa bronquial de pacients amb asma crònica (Marone et al., 2005; Brightling et al., 2003; Brightling et al., 2002). En un treball realitzat per Yu et al. (2006) en un model d'asma crònica d'OVA, en ratolí, es va descriure també la presència de mastòcits en l'epiteli de les vies aèries. Una altra observació que revela la probable contribució dels mastòcits en la patogènia de l'asma és la detecció, durant la fase primerenca de la resposta asmàtica, d'un clar increment de productes derivats de l'activitat mastocitària en el rentat broncoalveolar (BAL) de pacients asmàtics, com la histamina, els leucotriens, la PGD_2 i la triptasa (Boyce et al., 2003; Brightling et al., 2003).

Així doncs, el mastòcit és una cèl·lula efectora que participa en la majoria de processos en què la PGE_2 sembla exercir el seu efecte protector (Taula 1): la regulació de l'activitat mastocitària, de citocines i quimiocines, l'activació i la diferenciació de cèl·lules dendrítiques i l'activació de limfòcits B i la posterior producció d'IgE (Tkaczyc et al., 1996). Aquests indicis apunten que l'efecte protector de la PGE_2 en l'asma pugui relacionar-se amb la capacitat inhibitoria dels mastòcits.

Funcions immunològiques i inflamatòries	Mastòcits	PGE_2
Inflamació i broncoconstricció	+	-
Citocines i quimiocines Th_2	+	-
Activació i migració de cèl·lules dendrítiques	+	-

Taula 1. Efectes oposats in vitro dels mastòcits i la PGE_2 en l'asma al·lèrgica.

En estudis *in vitro* s'ha testat l'efecte de la PGE_2 en diferents poblacions mastocitàries. S'ha mostrat que la PGE_2 és capaç de modular: en primer lloc, l'alliberació de mediadors proinflamatoris, en segon lloc, la síntesis de citocines i, finalment, la migració dels mastòcits. En funció de la procedència i població mastocitària utilitzada per avaluar la PGE_2, s'obtenen efectes dispars. Pel que fa a poblacions mastocitàries procedents de ratolins, s'ha observat que en mastòctis procedents de moll de l'ós (BMMC), o de la melsa (SPMC) la PGE_2 incrementa l'alliberació de mediadors inflamatoris com histamina, IL-6 i GM-CSF (Wang & Lau et al., 2006; Nguyen et al., 2002; Gomi et al., 2002); i de quimiotàctics com MCP-1 o CCL2 dels mastòcits (Kuehn et al., 2011; Kuehn et al., 2010; Weller et al., 2007). Per contra, en algunes poblacions mastocitàries humanes com les procedents de cordó umbilical (hMCs), s'ha comprovat que quan les cèl·lules són estimulades amb IgE-al·lergè i tractades amb PGE_2 hi ha una inhibició de

l'alliberament d'ecosanoids i TNF-α (Feng et al., 2006). El mateix s'observa en mastòcits procedents de progenitors sanguinis perifèrics (HMC), és a dir: la PGE_2 inhibeix l'alliberació d'histamina i la producció de Cys-LTs dependent d'IgE (Wang et al., 2007). En mastòcits humans procedents de pulmó (HLMC) s'observa que la PGE_2 causa una inhibició de la degranulació i de la migració dels mastòcits (Duffy et al., 2008; Kay et al., 2006). Aquest efecte dispar també ha estat detectat dins d'una mateixa població de mastòcits humans $CD34^+$ procedent de progenitors perifèrics de sang (HuMC) en funció del donant del qual procedien. És a dir, la PGE_2 regulava l'activitat mastocitària tant negativa com positiviament en aquesta mateixa població procedent de diferents donants (Kuehn et al., 2011).

Pel que fa a les dades obtingudes d'experiments *in vivo*, hi ha alguns estudis que demostren l'efecte protector de la PGE_2 en models animals d'anafilaxi utilitzats per a l'estudi de la resposta al·lèrgica, on els mastòcits són clau. S'observa que la PGE_2 és capaç de disminuir l'alliberació de mediadors mastocitaris com la histamina i de reduir la resposta al·lèrgica en ratolins (Raud et al., 1996; Hedgvist et al., 1989; Raud et al., 1988; Lake et al., 1984).

La PGE_2 és doncs capaç de modular l'activitat mastocitària. Aquesta modulació, a vegades, resulta ambivalent en funció de la població mastocitària diana. Cal no oblidar, però, la finalitat de la investigació que en molts casos no és altra que la cerca d'una diana terapèutica curativa per a pacients asmàtics. És per això, que tant estudis *in vitro*, com *in vivo*, amb animals, ens poden ajudar a entendre els mecanismes de la PGE_2 i estudiar com dirigir el seu efecte beneficiós. També és important considerar el valor afegit que suposen els estudis *in vitro* amb poblacions

mastocitàries humanes, ja que ens permeten extrapolar possibles estudis translacionals a pacients.

3.2.3. Relació entre la PGE_2, MC i asma

Analitzant els resultats esmentats anteriorment (Taula 1), podrem comprovar com es defineix la relació que hi ha entre la PGE_2 i l'asma, i la PGE_2 i els mastòcits, i ens adonarem de com es fa notòria la relació que hi pot haver entre aquests 3 elements, és a dir, entre l'efecte de la PGE_2 sobre els mastòcits i la seva repercussió en l'asma al·lèrgica.

Estudis portats a terme en el nostre laboratori ens han permés demostrar que l'administració local de la PGE_2 en un model de sensibilització a HDM causa una millora de la patologia de les vies respiratòries. En conjunció amb aquests resultats, també hi hem observat una disminució de l'activitat mastocitària (Herrerias et al., 2009). D'acord amb aquestes dades, el fet de tractar ratolins sensibilitzats a OVA amb un inhibidor de l'activitat COX-2, i com a conseqüència doncs d'una disminució de la PGE_2 endògena, vam advertir un empitjorament de la funció respiratòria acompanyada d'un increment de l'activitat mastocitària (Torres et al., 2009).

En concordança amb aquesta relació PGE_2-MC-asma suggerida pels models animals també cal citar que Hartert et al. (2000) van determinar que pacients tractats amb PGE_2 presentaven una reducció de la PGD_2, mediador mastocitari, en el BAL, malgrat que els nivells d'histamina i triptasa, mediadors mastocitaris, no estaven afectats. Cal ressaltar també que aquests autors van detectar, en alguns pacients, efectes secundàris del tractament amb PGE_2, com vòmits o tos. Aquesta polifacètica vessant de la

PGE_2 va en consonància amb els contrastats efectes de la PGE_2 en les diferents poblacions mastocitàries.

Per tal de valorar la PGE_2 com una possible via per explorar dianes terapèutiques dirigides a combatre l'asma, és de vital importància aprofundir en aquest eix PGE_2-MC-asma, és a dir: en com la PGE_2 interactua amb els mastòcits i provoca una millora en la patologia respiratòria en els asmàtics. Un aspecte essencial, desconegut fins els anys 90, era conèixer els receptors involucrats en l'acció de la PGE_2. Conèixer la naturalesa i la funció de cada un d'ells és fonamental per entendre el controvers efecte de la PGE_2.

3.3. El receptor EP2

3.3.1. Receptors EP1 a EP4: descripció

La PGE_2 actua a través de 4 receptors acoblats a proteïna G (GPCR) anomenats EP1, EP2, EP3 i EP4 (Chung et al., 2005). A cada un dels receptors, se'ls atribueixen funcions molt divergents ja que estimulen vies diferents. EP2 i EP4 activen les vies unint-se a G_s, EP3 normalment s'uneix a G_i però té dues isoformes i EP1 s'uneix a G_q (Sugimoto et al., 2007; Narumiya et al., 2007). Es coneix que tan el receptor EP2 com el receptor EP4 activen l'adenilat ciclasa i indueixen un increment de l'AMP cíclic (AMPc) (Fujino et al., 2003; Breyer et al., 2001; An et al., 1994; Irie et al., 1993). EP1 i EP3, en canvi, s'ha vist que disminueixen l'activitat de l'adenil ciclasa (Nguyen et al., 2002; McGraw et al., 2006; Sakanaka et al., 2008). Així doncs, la PGE_2 presentarà activitats biològiques molt divergents en funció del receptor amb el que interactuï i segons la cèl·lula que expressi aquest receptor. Per exemple, en cèl·lules de musculatura llisa que expressen els receptors EP1, EP2 i EP3, s'observa que quan la PGE_2 interacciona

amb els receptors EP1 i EP3, s'indueix contracció i quan la PGE_2 interacciona amb EP2, s'indueix relaxació (Botella t al., 1993).

Tant en cèl·lules musculars com en mastòcits, el receptor que es relaciona amb una funció preventiva de les accions proinflamatòries, de broncoconstricció i remodelat en l'asma és el receptor EP2. En cèl·lules musculars s'ha vist que quan la PGE_2 interacciona amb el receptor EP2 a part d'induir relaxació també redueix la proliferació muscular llisa de les vies respiratòries implicada en l'AHR i en remodelat (Mori et al., 2011). En els mastòcits, quan s'estimula selectivament EP2 s'inhibeix l'alliberació dels mediadors inflamatoris en hMC i HLMC, i la migració mastocitària en HLMC (Feng et al., 2006; Kay et al., 2006; Duffy et al., 2008).

Encara, però, es desconeix el receptor específic implicat en l'efecte protector de la PGE_2 en la resposta asmàtica. Alguns estudis *in vivo* atribueixen el receptor EP3 a aquesta resposta. Kunikata et al. (2005), van mostrar com ratolins *knockout* (KO) per EP3 presentaven un resposta inflamatòria a OVA major, en comparació amb ratolins *Wild type* (WT), o els KO per a d'altres receptors EP. Aquests mateixos autors van observar una disminució de la resposta inflamatòria a OVA en els ratolins WT que havien estat tractats amb un agonista selectiu per EP3. Aquesta dismunició no va ser observada quan els ratolins van ser tractats amb agonistes dels altres receptors EP. D'altra banda, també hi ha evidències del fet que el receptor EP3 es troba alterat en pacients asmàtics (Park et al., 2007).

La transcendència dels estudis *in vitro i in vivo* citats subratllen l'ambivalència de l'efecte de la PGE_2 i la divergència de funcions dels receptors EP en l'asma. Es fa palesa la necessitat d'un estudi detallat de la presència de receptors EP en el mastòcits i la funció

que compleixen per tal de descriure el mecanisme beneficiós de la $PGE_{2,}$ i identificar molècules clau en aquest efecte.

3.3.2. Efecte del receptor EP2 en mastòcits i asma: què se'n sap?

Pocs investigadors han estudiat l'expressió dels receptors EP en els mastòcits. La presència dels receptors EP ha quedat determinada mitjançant l'ús de la PGE_2, antagonistes o agonistes selectius pels receptors EP, i mitjançant l'avaluació de la seva modulació de l'activitat mastocitària.

S'ha descrit la presència dels 4 receptors, tant en mastòcits humans, com en murins. En BMMC, ha quedat establert que expressen el receptor EP1, EP3 i EP4 (Weller et al., 2007; Nguyen et al., 2002), i en PDMC s'ha detectat la presència d'EP3 (Sakanaka et al., 2008). Feng et al. (2006) van descriure que els mastòcits procedents de cordó umbilical (hMcs) expressen EP2, EP3 i EP4 majoritàriament.

En estudis *in vitro* es va descriure que la PGE_2, a través dels receptors EP1 i EP3 presents en la superfície mastocitària, estimula la degranulació i la producció de IL-6, GM-CSF, i histamina (Wang & Lau et al., 2006; Nguyen et al., 2002; Gomi et al., 2000). Weller et al. (2007), en un estudi *in vitro*, van observar que la PGE_2 via receptor EP3 exercia una acció quimiotàctica en mastòcits de ratolí. És a dir, que la PGE_2, quan actua sobre els receptors EP1/EP3, tendeix a activar el mastòcit.

Pel que fa als receptors EP2 i EP4, s'ha demostrat que són receptors que actuen per la mateixa via, i se'ls hi associa un efecte inhibitori de l'activitat mastocitària, especialment, al receptor EP2. La majoria d'estudis conclouen que EP4 no té un impacte important en la modulació de la funció mastocitària (Duffy et al., 2008; Kay et al., 2006; Feng et al., 2006). Només en estudis *in*

vitro amb BMMC –mastòcits que no expressen el receptor EP2– s'associa, al receptor EP4, una acció inhibitòria sobre el mastòcit (Brown et al., 2011). Feng et al. (2006) van descriure que la PGE_2 podia suprimir, via receptor EP2, l'alliberació de mediadors mastocitaris en mastòcits procedents de cordó umbilical. Kay et al. (2006) van determinar que la PGE_2 a través del receptor EP2 estabilitzava l'activitat de mastòcits pulmonars humans (HLMC), i Duffy et al. (2008) van demostrar que la PGE_2 atenuava la migració dels HLMC. D'acord amb aquestes dades es suggereix que la PGE_2 actuant sobre el receptor EP2 present en la superfície mastocitària inhibeix la seva activitat *in vitro*. Així doncs, aquests resultats suggereixen que l'efecte protector observat en la PGE_2 en la resposta asmàtica podria ser mediat pel receptor EP2. Aquesta hipòtesis és reforçada per dades recents obtingudes pel nostre grup; dades que palesen que ratolins sensibilitzats a HDM presenten un increment en l'expressió del receptor EP2 acompanyat d'un increment de la PGE_2 endògena. Quan a aquests ratolins sensibilitzats se'ls administra la PGE_2 de forma exògena l'increment d'EP2 es manté, però la PGE_2 endògena no augmenta (Herrerias et al., 2009). Aquests fets indiquen que l'eix COX-2/PGE_2/EP2 pot ser una via per la qual la PGE_2 actua i que aquest eix és autoregulat com a mecanisme compensatori per tal d'atenuar la inflamació de les vies respiratòries induïda per l'al·lergè.

Per aquest motiu cal definir el paper de l'EP2 en l'asma i descriure les vies per les quals l'eix PGE_2-EP2 modula l'activació mastòcitària dependent d'IgE.

Receptors EP	Subunitat acoblada	AMPc	Efecte en l'activitat mastocitària
EP1	Gq	↓	+
EP2	Gs	↑	-
EP3	Gi (and Gs)	↓	+
EP4	Gs	↑	-

Taula 2. Resum del presumpte funcionament dels receptors de Prostaglandina E2 i el seu efecte en els mastòcits.

3.3.3. Mecanismes intracel·lulars d'activació mastocitària i EP2

Els mastòcits s'activen mitjançant l'entrecreuament (*cross-linking*) de les molècules d'immunoglobulines E (IgE), que es dóna quan l'al·lergè s'uneix a aquestes immunoglobulines. Aquests *cross-linking* provoca l'activació del receptor d'IgE d'alta afinitat (FcεRI) que es troba a la superfície dels mastòcits desencadenant-se un seguit de reaccions intracel·lulars que culminen en l'alliberació de mediadors preformats i la producció de citocines i quimiocines. El FcεRI en mastòcits és un tetràmer (Blank & Rivera et al., 2004; Nadler et al., 2000) compost per una cadena α on s'uneix la IgE, una cadena β transmembranal i una cadena γ homodímer disulfídic (Nalder et al., 2000). Tant la cadena β, com la γ, contenen immunoreceptors basats en motius d'activació de tirosina (ITAMs), els quals són essencials per a la senyalització del receptor. FcεRI, per si sol, està desproveït d'activitat tirosina quinasa, per aquest motiu s'associa a la família de proteïnes no receptores Src que posseeïxen activitat tirosina quinasa, per tal de fosforilar els residus de tirosina dels ITAMs (Pribluda et al., 1994). Hi ha 3 Src

principals: Lyn, Fyn i Syk. Lyn i Fyn es troben unides a la cadena β, i Syk, a la cadena γ. Lyn és la que es troba preactivada. Un cop el conjunt al·lergè-IgE interactua amb el FcεRI s'incrementa la seva activació i promou l'activació de Fyn, un pas essencial perquè es doni la degranulació i la producció de citocines (Gomez et al., 2005), i Syk, essencial per la propagació dels senyals procedents de Lyn (Benhamou et al., 1993). Així doncs, existeixen dues vies fonamentals de senyalització per tal que es dugui a terme l'activació del mastòcit: Lyn-Syk i Fyn (Figura 1). Aquestes generen una cascada de lípids, tals com missatgers secundaris i proteïnes adpatadores que activaran els processos necessaris desencadenants de la degranulació i producció de citocines i quimiocines.

Per tal que es doni la degranulació un dels mecanismes requerits és l'increment en la concentració de calci citosòlic (Vig et al., 2009; Habara et al., 1996; Putney et al., 1993; Janiszewkski et al. 1992). Lyn es troba principalment controlant l'activació dels canals de calci de les mitocòndries que aporten un increment de calci en el citosol procedent de les reserves de calci intracel·lulars. Fyn controla, mitjançant Gab2, AKT, PI3K i PLCγ, els canals de calci extracel·lulars que aporten calci en el citosol (Suzuki et al., 2010).

Fins ara no hi ha cap evidència de com l'estimulació del receptor EP2 exactament modula la degranulació, ni de quin mecanisme i via de senyalització dependent de IgE afecta en el mastòcit.

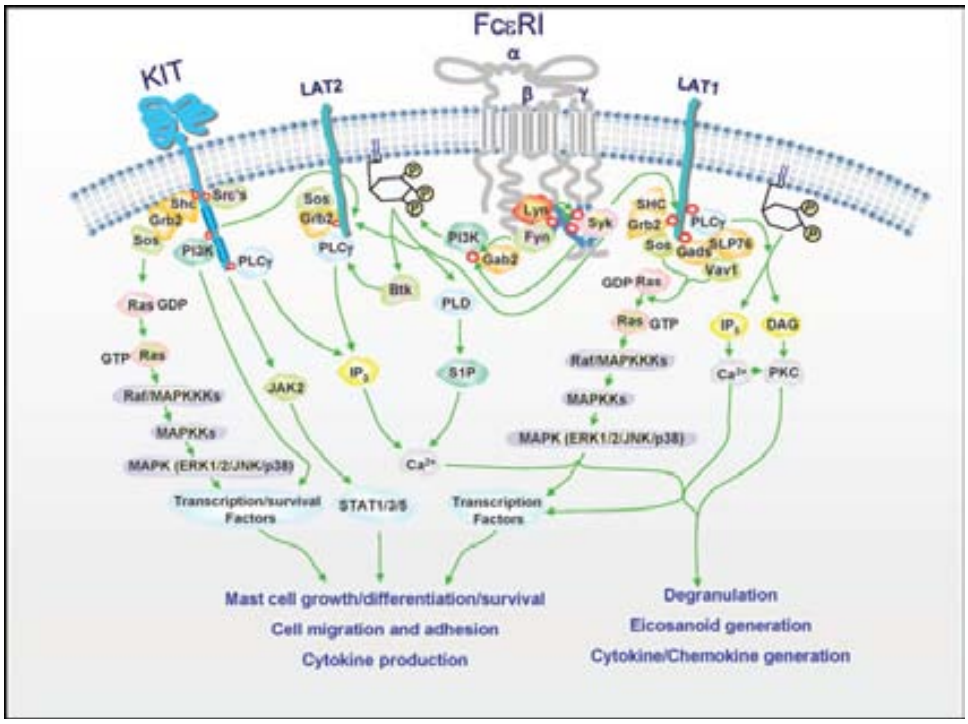

Figura 1. Vies de senyalització implicades en l'activació del mastòcits (Gilfillan & Rivera, 2009)

És conegut que la PGE_2, paral·lelament a la modulació de la degranulació, també modula la mobilització de calci. En mastòctis humans procedents de cordó umbilical (hMcs), s'ha demostrat com la PGE_2 actuant via EP3 indueix un augment del calci citosòlic en el mastòcit (Feng et al., 2006). Duffy et al. (2008) han vist en HLMC que l'estimulació selectiva d'EP2 provoca el tancament del canals de potassi tipus KCa3.1. implicats en l'influx de calci extracel·lular. Aquest fet suggereix que el receptor EP2 podria causa una inhibició

en la mobilització de calci. Al nostre parer, encara no hi ha dades que ho demostrin clarament.

El receptor EP2 activa l'adenilat ciclasa i produeix un increment de AMP cíclic (AMPc) (Fujino et al., 2003; Bryer et al., 2001; An et al., 1994; Irie et al., 1993). La funcionalitat del receptor EP2 ha quedat palesada en mastòctis humans procedents de cordó umbilical (hMCs) i HLMC, on l'estimulació selectiva del receptor EP2 va lligada a un increment d'AMPc i una inhibició de la degranulació (Feng et al., 2006; Kay et al., 2006). També s'ha observat que AMPc en hMCs activa PKA per tal d'inhibir la producció de citocines (Feng et al., 2006). En HLMC, s'ha demostrat que PGE_2 via receptor EP2 inhibeix la quimiotaxis i la degranulació del mastòcit (Duffy et al., 2008).
En BMMC, que expressen majoritàriament EP3, s'ha observat com PGE_2 indueix la quimiotaxis dels mastòcits augmentant la fosforilació d'AKT (Kuehn et al., 2011). En altres tipus cel·lulars, com cèl·lules tumorals de mama o condròcits, s'ha demostrat que el receptor EP2 estimulat per la PGE_2 inhibeix la via de la PI3K/AKT induint l'increment d'AMPc i l'activació de PKA (Han et al., 2010; Wang et al., 2010). En eosinòfils, en canvi, s'observa que l'estimulació selectiva d'EP2 causa una inhibició de la migració dependent de l'activació de PI3K i PKC però no de AMPc (Sturm et al., 2008). La funcionalitat i les vies d'acció del receptor EP2 en els mastòcits encara estan per descriure. L'estudi d'aquestes poden aportar la millor comprensió de les propietats antiasmàtiques de la PGE_2 i l'exploració de possibles dianes terapèutiques en l'asma.

3.4. El model murí d'HDM

La investigació en pacients asmàtics és fonamental per a l'avenç del coneixement de la patogènia de l'asma i per la identificació o recerca de noves dianes. Malgrat això hi ha obstacles científics i ètics en la recerca clínica que impedeixen abordar determinades qüestions o verificar certes hipótesis. Per això sorgeix la necessitat d'utilitzar models animals d'asma experimental en recerca translacional. Aquesta necessitat es fa també palesa en la recerca de l'efecte protector de l'eix PGE2-EP2-mastòcit en l'asma. S'han utilitzat nombroses espècies animals, de les quals la més utilitzada és el ratolí, ja que pot oferir avantatges científiques, econòmiques i de maneig. A més, aquesta espècie ofereix l'opció de manipular genèticament i/o immunològicament l'animal. El creixement exponencial, en els últims anys, de nombrosos models d'asma experimental al·lèrgica induïda en ratolí han impulsat la investigació en aquest terreny ja que ofereix un valor afegit als estudis amb pacients asmàtics, als estudis *in vivo* en altres models animals i als experiments *in vitro* i *ex vivo* (Torres et al., 2005).

Existeixen varis models d'asma experimental en ratolí. La major diferència entre ells rau en l'al·lergè emprat, via d'administració, dosis i utilització, o no, d'adjuvant. En base l'al·lergè utilitzat es distingeixen principalment dos tipus de models: el d'OVA i el d'aeroal·lergens naturals.

El model d'OVA és el que s'ha fet servir clàssicament i segueix essent un model útil per a l'estudi de mecanismes implicats en l'asma al·lèrgica i en les funcions de la via de la COX-2/PGE_2 *in vivo*. Hi ha estudis on s'ha demostrat el rol que juga la COX-2 i la producció de PGE_2 endògena com a mecanisme beneficiós per atenuar la inflamació i l'AHR induïda per OVA, o en la seva absència, l'empitjorament de la resposta asmàtica (Torres et al.,

2009; Nakata et al., 2005; Hashimoto et al., 2005; Peebles et al., 2002; Peebles et al., 2000; Gavett et al., 1999). En altres estudis amb rates i ratolins sensibilitzats a OVA, es va administrar la PGE_2 exògenament i es va veure que aquests presentaven una atenuació en la resposta de les vies aèries (De campo & Henry et al., 2005; Martin et al., 2002).

Tot i això, hem de considerar la importància de l'estructura antigènica i la via d'immunització en aquest tipus de resposta immunitària. Per aquest motiu, en ocasions pot ser preferible decantar-se per utilitzar models d'asma en què s'empri al·lèrgens naturals com els àcars de la pols (HDM).

La inducció d'asma experimental mitjançant OVA presenta una sèrie de limitacions pel que fa a la inducció mitjançant aeroal·lergens:

	MODEL OVA	MODEL HDM
Al·lergè	no produeix asma al·lèrgica en humans	al·lergè natural responsable de gran part de l'asma al·lèrgica en humans
Fase de sensibilització (Via d'administració)	via intraperitoneal	via intranasal
Fase de sensibilització (Adjuvant)	sí (Alum)	no
Protocol d'administració	discontinuat/per fases	continuat
Asma crònica per estudi de remodelat	de vegades, problemes de tolerància	s'observen els canvis típics de remodelat

Taula 3. Comparació dels models de sensibilització a OVA i HDM.

En la Taula 3 veiem que el model on s'utilitzen aeroal·lergens naturals per sensibilitzar, com els àcars de la pols (HDM), cobreixen moltes de les limitacions que es plantegen davant el model d'OVA. Així doncs, el protocol d'inducció d'asma experimental per HDM mimetitza millor la forma d'exposició natural dels pacients asmàtics als al·lergens, que és un contacte per via aèria, diari i sense adjuvant. Per tant, s'aconsegueix

possiblement un model molt més semblant a la inducció espontània d'asma en les persones. Per això, en el nostre laboratori hem adoptat els models d'asma experimental aguda i crònica per exposició de ratolins a HDM (Cates et al., 2004; Jonhson et al., 2004).

En el model d'asma experimental aguda induït per exposició a HDM, hem obtingut resultats que corroboren la importància de la COX-2 i l'efecte beneficiós de la PGE_2 en la resposta asmàtica. Per un costat, vam inhibir l'expressió gènica de la COX-2 mitjançant un oligonucleòtid anitsentit instil·lat per via intranasal contra aquest enzim (Torres et al., 2008). Vam observar que paral·lelament a la disminució de l'expressió de COX-2, la resposta asmàtica va empitjorar només l'AHR. En aquest mateix model, vam avaluar l'efecte de la PGE_2 administrada per via intranasal, abans d'iniciar el procés de sensiblització i vam poder comprovar que la PGE_2 era capaç d'inhibir la inflamació de les vies respiratòries i AHR (Herrerias et al., 2009). Aquest mateix model va ser utilitzat per estudiar el paper de la COX-2, PGE_2 endògena i els receptors EP. Vam poder evidenciar que l'eix COX-2/PGE_2/EP2 és fonamental per tal que l'efecte preventiu de la PGE_2 exògena o endògena en la sensibilització a HDM es doni (Herrerias et al., 2009).

L'activitat mastocitària induïda per HDM és rellevant. Els nostres estudis amb models de sensibilització a HDM en ratolins també suggereixen que els mastòcits juguen un paper important, tant en el desenvolupament de l'asma experimental, com en l'efecte inhibitori de la PGE_2 (Herrerias et al., 2009, Torres et al. 2008). Medim l'activitat mastocitària mitjançant mMCP-1 en pulmó, una

proteasa alliberada específicament per mastòcits activats de la mucosa. Vam observar que l'exposició intranasal de ratolins a HDM indueix un notable increment dels nivells de mMCP-1 en pulmó, però no un augment de mastòcits pulmonars. El no augment del nombre de mastòcits va en conjunció a les observacions fetes en pacients i en altres models murins d'asma aguda. Aquest increment s'ha determinat en la musculatura llisa bronquial de pacients amb asma crònica (Brightling et al., 2011; Marone et al., 2005; Brightling et al., 2003; Brightling et al., 2002) i també en l'epiteli de les vies aèries en un model d'asma experimental crònica d'OVA en ratolí (Yu et al., 2006). De manera que l'augment de proteasa reflecteix l'augment de l'alliberació de mediadors per part dels mastòcits o l'acumulació de la proteasa en els seus grànuls. El nostres resultats conflueixen amb els de diferents estudis realitzats en pacients on es demostra la important participació dels mastòcits en la patogènia de l'asma (Marone et al., 2005; Boyce et al., 2003; Brightling et al., 2003; Brightling et al., 2002). D'altra banda, alguns treballs realitzats en models d'asma experimental en ratolí no induït per HDM, sinó per OVA, o *Dermatophagoides farinae*, també descriuen un increment en els nivells de mMCP-1 en animals sensibilitzats vers als no sensibilitzats.
Indiscutiblement, aquests resultats permeten corroborar l'especial interès d'utilitzar el model d'HDM i la seva idoneïtat per definir el mecanisme principal implicat en l'efecte de PGE_2 en la resposta asmàtica.

No existeixen dades *in vivo* ni *in vitro* que determinin aquest mecanisme, els resultats en models d'asma experimental en ratolí i en pacients de forma *in vitro* suggereixen l'important paper del mastòcits en la protecció exercida per la PGE_2 en asma

possiblement intervinguda per EP2. En base a aquests antecedents, formulem la hipòtesis que es descriu en el següent apartat.

Hypothesis & Objectives

4. Hypothesis and objectives

Hypothesis

Based on the background data, we formulate the following general hypothesis:

"The protective effect induced by PGE_2 in patients with allergic asthma is a consequence, at least in part, of its agonistic action on the airway mast cells EP2 receptor "

In order to confirm such general statement, we have sub-divided the above hypothesis into 3 *proofs-of-concept* to be checked under 3 sub-projects:

Hypothesis 1: PGE_2 effect on mast cells activity is a result of a direct interaction with this cell population, and its nature is dependent on the relative expression of E prostanoid (EP) receptors.

Hypothesis 2: The PGE_2-induced beneficial effect in aeroallergen-sensitized mice is attributable to the EP2 receptor, and the selective action on this specific receptor is also linked to a diminished mast cell activity.

Hypothesis 3: The selective agonism on EP2, triggers molecular inhibitory signals in mast cells that may explain the PGE_2-induced protective effect in vivo.

Objectives

The current thesis is part of a long-standing research project aimed at underlying the relevance of molecules within the "PGE_2"-"mast cell EP2 receptor"-"airways" axis for the identification of new pharmacological targets against allergic asthma.

The General Objective is to "*establish preclinically the relevance of the mast cell EP2 receptor to the observed PGE_2 beneficial effect in allergic asthma*".

In order to contribute to meet this General Objective, we have established 10 specific objectives.

Hypothesis 1

Objective 1:

- To determine the relative expression of EP receptors in murine and human mast cell systems *in vitro*

Objective 2

- To assess the effect of PGE_2 on the activity of murine and human mast cells expressing different receptor profiles

Objective 3

- To assess the effect of PGE_2 on calcium mobilization of murine and human mast cells expressing different receptor profiles

Hypothesis 2

Objective 4:

- To compare the effect of EP non selective (PGE_2), and EP2 selective-agonism in HDM-induced airway pathology in mice

Objective 5

- To compare the effect of EP non selective (PGE_2) and EP2 selective-agonism in HDM-triggered airway mast cells activity *in vivo*

Objective 6

- To determine the ability of mast cell mediators to induce airway pathology, and of EP2 receptor activation to modulate such effect

Hypothesis 3

Objective 7:

- To establish a correlation between the effect of EP2 agonism on mast cells and the EP2 receptor expression pattern

Objetive 8:

- To assess cAMP and Ca^{2+} fluctuation, in IgE-stimulated mast cells incubated with an EP2 agonist

Objective 9:

- To determine the relevance of the Fyn-PKA signalling pathway in EP2 agonism mediated-inhibition in IgE-stimulated mast cells

Objective 10

- To assess the relevance of the mast cell EP2 receptor to antigen-IgE triggered pathology

Sub-Projects

5. Sub-Projects

5.1. Outline of Experimental Approach

In this chapter the three Sub-Projects addressing the above objectives are thoroughly described. Each Sub-Project includes a specific Introduction and Material & Methods, Results and Discussion section.

Under Sub-Project 1 we determined the EP receptors expression pattern in different human and murine mast cell population, and assessed (a) whether such differences in the relative expression of EP receptors 1 to 4 influenced the ability of PGE_2 to modulate mast cells degranulation and calcium mobilization, and (b) whether human mast cells behaved similarly to murine mast cells under different EP receptors expression scenarios. The data pointed at EP2 as the main contributor to mediate the inhibitory effect of PGE_2 on both murine and human mast cells.

Under Sub-Project 2 we addressed the relevance of selective EP2 activation to (a) protection against HDM-induced airway pathology in mice, and (b) correlation of such pathology to the ability of selective EP2 agonism to prevent mast cells activity. We showed that the EP2 agonist prevented AHR and inflammation from developing and that such effect was linked to the ability of such selective agonist to attenuate mast cell activity.

Under Sub-Project 3 we attempted to uncover the intracellular mechanisms involved in the signaling pathway of IgE-dependent mast cell activation under EP2 selective agonism. We observed that EP2 agonism inhibits, *in vivo* and *in vitro,* mast cell activity. We observed that PGE_2-EP2 mediated inhibition of mast cell

degranulation is linked to the supression of the calcium influx, in turn mediated by an inhibition of the Src-Fyn pathway and cAMP/PKA.

5.2. Sub-Project 1:

Inhibition of human and murine mast cells activity by PGE_2 correlates with EP2, but not EP3, receptor expression

ABSTRACT

Prostaglandin (PG) E2 has proved experimentally to prevent development of airway pathology in allergic asthma patients, and more recently in house dust mite (HDM)-induced airway pathology in mice, while diminishing airway lung mast cell activity *in vivo*. In order to uncover the likely role of PGE_2 receptors (EP) in PGE_2-mast cells interaction, we evaluated the effect of the PG on human and murine mast cells degranulation, and intracellular calcium mobilization, and correlated such data with the relative expression of the 4 receptors on the cells surface.

We showed that human mast cells from $CD34^+$peripheral blood progenitors (HuMC) matured *in vitro*, expressed primarily EP2, while EP3 predominated in the LAD2 human mast cell line. PGE_2 attenuated HuMC degranulation, but enhanced LAD2's. This finding was consistent with cytoplasmatic Ca^{2+} fluxes. PGE_2 decreased calcium mobilization in HuMC. Regarding the murine cells peritoneum derived (PDMC), bone marrow (BMMC), and CI.M/C57.1 (C57) mast cells were studied. The highest level of expression of EP2 was found on C57. As a result, this mast cell population was the most sensitive to PGE_2's inhibitory effect. PDMC and BMMC mainly expressed EP3, and consequently PGE_2 exerted a degranulatory effect, and mobilized calcium fluxes in these populations.

These data point at the relevance of EP2, versus EP3, in PGE_2-driven inhibition of human and murine mast cells activity. In addition, they show that the inhibitory effect exerted by PGE_2 on mast cells is associated with intracellular calcium mobilization. It therefore suggests that the protective effect observed in human asthmatics and murine models of asthma might be mediated

primarily by PGE_2 agonism on EP2 receptors on the lung mast cells surface.

INTRODUCTION

Despite the advances in asthma therapy, current treatments of allergic asthma do not halt the underlying processes, and corticosteroids remain the most efficient management approach (1-2). Based on the experience with corticosteroids, we hypothesized that the identification of endogenous mechanisms that spontaneously counteract the development of aeroallergens-induced airway pathology may be an efficient strategy in search for new antiasthma targets (3).

PGE_2 is a natural molecule that had been shown to exert beneficial effects in asthma patients in experimental settings (4-6). Such PGE_2-associated protective effect has been also described in experimental models of asthma *in vivo* (7-9). We showed recently that inhibiting endogenous COX-2, hence inhibiting PGE_2 and other PG production, aggravated antigen-driven airway reactivity *in vivo* (10-11) in OVA and HDM-induced murine models of allergic asthma. Accordingly, we demonstrated that exogenously administered PGE_2 could attenuate such airway reactivity to HDM extracts in mice (12). These protective effects of PGE_2 against airway pathology were associated with opposing effects on mast cells activity *in vivo* (10, 12). PGE_2 is known to exert anti-allergic and anti-inflammatory actions; notably, to modulate human airway mast cells activity (13-15), but little is known about the precise protective mechanisms involved. Understanding the nature of the PGE_2-mast cell interaction will provide clues as to the relevant endogenous inhibitory mechanisms and molecules within the PGE_2-MC-asthma axis.

PGE_2's immunomodulatory ability is likely exerted at various levels (16), one of them being its capacity to modify mast cell activity (14-15, 17). It appears likely that the PGE_2-driven

beneficial effect in allergic asthma (18-20) is mediated, at least in part, by constraining mast cells activity. PGE_2 has opposing effects *in vitro* on different mast cell populations. On the one hand, PGE_2 is able to stimulate the release of mediators such as IL-6 and GM-CSF, and to induce mast cell chemotaxis in BMMC (21-23); on the other hand, PGE_2 stabilizes human lung mast cells, abrogates mast cell degranulation and cytokine production, and inhibits mast cell migration (14-15). PGE_2 has also an inhibitory effect *in vivo* in animal models of anaphylaxis, where mast cells play a key role (24-25). This dual PGE_2 effect is probably related to the presence of four divergent G protein-coupled receptors (GPCRs) on the mast cell surface (17, 20-22, 26-27): EP1, EP2, EP3 and EP4. There are still many uncertainties as to which one of the specific EP receptor is responsible for the potentiating or suppressor effect on mast cells. The understanding of the relevance of EP receptors to mast cells stimulatory or inhibitory outcomes will probably provide many clues on the fundamental molecules involved in PGE_2 protective effect. *In vitro* data suggest that PGE_2 interaction with EP1 and EP3 is associated with mast cell activation and migration (20-22, 26). On the contrary, EP2 seems to be related to inhibition of both effects (14-15, 17). Some of our recent data suggested that PGE_2 acting through EP2, prevented (HDM)-induced allergic response, and this effect appeared to be mediated by the inhibition of lung mast cell activity (28). However, others point at EP3 as the relevant receptor (29). To characterize the PGE_2-MC-asthma axis it is crucial to determinate which EP receptor, but also which mast cell-associated mechanisms are involved. One potentially mechanisms is the mobilization of intracytosolic calcium. Mast cell activation is directly conditioned by a peak of cytoplasmatic calcium (30-31). It is known that PGE_2

can also modulate calcium fluxes. In HuMC, PGE_2 acting trough EP3 was able to enhance calcium mobilization (17). Additional studies are needed to provide insights on the role of calcium mechanisms, and its modulation, in PGE_2's driven beneficial effect. PGE_2 effect on mast cells results from the simultaneous activation of the 4 EP receptors. Stimulating mast cells with different EP receptor expression patterns with the non specific ligand such as PGE_2 will help to uncover the function associated to each individual receptor. We have therefore assessed the effect of non-selective EP stimulation (PGE_2) on various human and murine mast cell populations, and have investigated it in the light of the relative level of expression of receptors EP1 to 4. We have also studied the link between PGE_2 interaction with mast cell populations and total intracellular calcium mobilization. We found that the PGE_2 inhibitory effect on mast cells *in vitro* is linked to a dominant EP2 expression.

MATERIAL AND METHODS

Reagents and Antibodies

DNP-specific mouse IgE was produced as previously described (32). DNP human serum albumin (DNP-HSA; Ag; Sigma-Aldrich, Sant Louis, MO, USA) was diluted in Phosphate Buffer Saline (PBS) before use. Biotin anti-human IgE was from ABBIOTEC (San Diego, CA, USA). Streptavidin (SAv, Ag) was purchased from BD Biosciences (San Jose, CA, USA). PGE_2 was purchased from Cayman (Ann Arbor, MI, USA). Dimethylsulfoxide (DMSO) was obtained from Sigma-Aldrich (Sant Louis, MO, USA).

Mice

Mast cell populations were obtained from female 5- to 6-week-old C57/BL6 mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and bred in Taconic (Rockville, MD, USA). The mice were maintained and used in accordance with National Institutes of Health (NIH) guidelines and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)-approved animal study proposal A010-0403.

Murine and human mast cell cultures

Bone marrow mast cells (BMMC) were extracted from the mice's femurs and tibias, and cultured in the presence of IL-3 (20 ng/ml) and SCF (20 ng/ml) in RPMI medium with 10% FBS for 4 to 6 weeks as previously described (33). Peritoneal lavage mast cells were harvested from mice by using 3 ml of RPMI medium injected into the peritoneal cavity. Cells were then cultured for 3-4 weeks

in the presence of IL-3 (20 ng/ml) and SCF (20 ng/ml) in RPMI medium with 20% FBS to obtain an expanded population of peritoneal-derived MC (PDMC) (34). Recovered BMMC and PDMC were analyzed for the mast cells content and FcεRI and cKit expression by FACS as previously described (33). Cells were rested overnight in the absence of SCF before the stimulatory assay. PDMC and BMMC from EP2 knockout mice (-/-) (35) were kindly provided by Dr. M. Kovarova and Dr. B.H. Koller from the University of North Carolina-Chapel Hill, NC, USA. The CI.M/C57.1 (C57) mast cell line, kindly provided by Dr. Stephen J. Galli, from Stanford University, was originally derived from BALB/c mice (36-37). C57 mast cells were grown in DMEM supplemented with 10% FBS, L-glutamine and 2-mercaptoethanol.

LAD2 MCs (38) (kindly provided by Dr. A. Gilfillan from the National Institutes of Health) is a human MC line derived from a patient with untreated MC sarcoma. LAD2 mast cells were cultured in serum-free media (StemPro-34 SFM, Life Technologies, Gaithersburg, MD, USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml SCF. Human peripheral blood-derived $CD34^+$ mast cells (HuMC) (kindly provided by Dr. A. Gilfillan from the National Institutes of Health) were also cultured in StemPro-34 SFM, supplemented with 2 mM L-glutamine, 50 µg/ml streptomycin, 100 IU/ml penicillin, 100 ng/ml SCF to which 100 ng/ml of rhIL-6 (PeproTech, Inc., Rocky Hill, NJ, USA). rhIL-3 (30 ng/ml) was also added from the first week. Half of the culture medium was replaced every 7 days. Cultures on weeks 8 to 10 consisted of over 99% HuMC (39-40). Cells were rested in the absence of SCF o.n. before stimulation.

Prostaglandin E2 receptors (EP) mRNA expression

EP 1 to 4 receptors mRNA expression in murine and human mast cells was determined by real time PCR using TaqMan® Gene Expression Assays with 2 unlabeled primers and one 6-FAM™ dye-labeled TaqMan® MGB probe (Applied Biosystems, Foster City, CA, USA) mouse EP1 (Mm00443097_m1), mouse EP2 (Mm00436051_m1), mouse EP3 (Mm0.1316856_m1), mouse EP4 (Mm00436053_m1), human EP1 (Hs00168752_m1), human EP2 (Hs00168754_m1), human EP3 (Hs00168755_m1) and human EP4 (Hs00168761_m1). mRNA were extracted from $2x10^6$ cells using the Rneasy Mini Kit with One column DNAse treatment (Qiagen, Valencia, CA, USA). The reverse transcription to convert mRNA to DNA and the DNA amplification were done by qScript One-step qRT-PCR Kit, Low ROX cocktail (Quanta, Santa Clara, CA, USA). This cocktail contains the reverse transcriptase enzyme, nucleotides and the DNA polymerase. 50 ng of total mRNA for each sample were added to 20 µl qScript One-step qRT-PCR Kit, Low ROX cocktail. The reaction was then run in Applied Biosystems 7500 system. Mouse GAPDH and human GAPDH were used as endogenous controls. Expression was calculated as the comparative threshold method normalized to GAPDH expression as described by Applied Biosystems (Foster City, CA, USA), and it is expressed and represented in terms of relative units (EP expression to GAPDH ratio).

Mast cell degranulation: β- Hexosaminidase release assay

Murine mast cells were sensitized with 1 µg/ml of DNP specific IgE for 2 h in free SCF media. After sensitization, cells were washed and resuspended with HEPES buffer (10 mM HEPES [pH7.4], 137

mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4_7H2O, 5.6 mM glucose, 1.8 mM CaCl2_2H2O, and 1.3 mM MgSO4_7H2O) with 0.04% BSA (Sigma-Aldrich, Sant Louis, MO, USA). Cells were seeded in a V-bottom 96-well plate with 50.000 cells in a final volume of 100 µl. Cells were treated with 10^{-5} M PGE_2 or vehicle (PBS with 0.1% DMSO) for 15 minutes at 37ºC with 5% v/v of CO_2. Cells were stimulated with 50 ng/ml Ag (DNP-HSA) for 30 minutes at 37ºC with 5% v/v of CO_2. The degranulation was stopped in ice. Cells were centrifuged for 10 min at 4ºC at 1500 rpm.

Human mast cells were sensitized with 100 ng/ml biotinylated human IgE for 2 h in free SCF media. After sensitization, cells were washed and resuspended with HEPES buffer with 0.04% BSA. Cells were seeded in a V-bottom 96-well plate with 30.000 cells in a final volume of 100 µl. Cells were treated with 10^{-5} M PGE_2 or vehicle (PBS with 0.1% DMSO) for 15 minutes at 37ºC with 5% v/v of CO_2. Cells were stimulated with 10 ng/ml Ag (SA) for 30 minutes at 37ºC with 5% v/v of CO_2. The degranulation was stopped in ice. Cells were centrifuged for 10 min at 4°C at 1500 rpm.

The enzymatic activity of the granule marker, β-hexosaminidase, released to the extracellular media was measured as described in murine and human mast cell from the supernatants and pellets of cells challenged (Percentage degranulation = 100 X (supernatant content)/(supernatant + lysate content)) (33).

The β-hexosaminidase fold release from Fig 3 was calculated as the percentage of increase or decrease that cause PGE_2 on the different stimulated mast cell and treated with vehicle (%= 100x (%hexo-PGE_2 treated and stimulated mast cells)-(%hexo-vehicle treated and stimulated mast cells)/(%hexo-vehicle treated and stimulated mast cells)).

Total intracytosolic calcium mobilization measurement

For intracytosolic calcium (Ca^{2+}) measurements, cells were sensitized overnight (o.n.) and loaded with 1mM FURA-2AM (Invitrogen, Carlsbad, CA, USA) for 30 minutes, washed, resuspended in Tyroids buffer and aliquoted in 96-well plates (30.000 cells/well). After 10 minutes, cells were treated for 15 minutes with 10^{-5} M PGE_2 and vehicle (0,1%DMSO+PBS). Cells were then challenged with Ag (25 ng/ml for PDMC or BMMC and 20 ng/ml for HuMC) for 10 minutes in order to measure calcium mobilization. Changes in intracellular calcium were monitored with a microplate fluorescence reader Wallac Victor2 1420 Multilabel Counter" (PerkinElmer Life Sciences, Waltham, MA, USA). FURA-2 emission at 510 nm during fast excitation between 340 and 380 nm at 37ºC was measured, and background fluorescence was determined with non-stained. The ratio of the fluorescence at 340 nm and 380 nm (R) after subtracting the respective background values was calculated for each measurement.

Statistics

Statistical significance was determined using a 2-tailed Student's *t* test, and statistical differences among the treatments were evaluated by a 2-way ANOVA, as indicated in the figure legends. A *P* value of less than 0.05 was considered significant. Data are shown as mean.

RESULTS

EP receptors expression on murine and human mast cells

Fig 1 (A, B, C, D and E) show the relative expression of EP receptors mRNA in the studied human and murine mast cell populations. As depicted, C57 (Fig 1A) express high levels of EP2 mRNA relative to GAPDH (34318.2 relative units), followed by EP3 and then by EP4. On the other hand, PDMC (Fig 1B) do mostly express EP3 mRNA relative to GAPDH (0.045 relative units), followed by EP2, and then by EP4. The levels of EP1 in both murine mast cell systems are virtually null. Interestingly, when PDMC are extracted from EP2 knockout (-/-) mice, mRNA expression of EP3 and EP4 is upregulated (51.1% and 114% of fold increase, respectively), but not EP1. Wild Type BMMC (Fig 1C) do express mainly EP3 mRNA relative to GAPDH expression (0.179 relative units); followed by EP4>EP2>EP1. The relative expression of EP receptors in the BMMC from EP2 KO also is upregulated in EP3 and EP4 (47% and 67% of fold increase, respectively), but not in EP1.

Human mast cells, HuMC (Fig 1D) express primarily EP2 mRNA (0.034 relative units) followed by EP3 and EP4. The level of expression of EP1 was the lowest ($1.29x10^{-4}$ relative units). LAD2 (Fig 1E) express basically EP3 ($9.37x10^{-3}$ relative units) and EP4 ($2.91x10^{-4}$ relative units) with almost no mRNA expression for EP2 and EP1.

Modulation of mast cell degranulation by PGE_2

The effect of PGE_2 on human and murine mast cells is shown in Fig 2 (A, B, C, D and E). β-hexosaminidase release was inhibited by 22.85% in C57 murine mast cells when incubated with PGE2 as

shown in Fig 2A. Contrarily, PGE_2 triggered degranulation in PDMC and BMMC (55.24% and 302.9% increase, respectively). PGE_2 also increased β-hexosaminidase release in PDMC and BMMC from EP2 KO (-/-) mice up 24.75% and 180%, respectively (Fig 2B and 2C).

In HuMC (Fig 2D), PGE_2 significantly inhibited β-hexosaminidase release by 21.46%. On the other hand, PGE_2 significantly enhances mast cells degranulation by 19.28% in LAD2 mast cells (Fig 2E).

PGE_2 and EP2 deletion effect on mast cell degranulation relative to EP2/EP3 expression ratio

In Fig 3A we have represented the impact of PGE_2 on mast cells in relation to the EP2/EP3 expression rate. The EP2/EP3 ratio is below 1 for LAD2, BMMC and PDMC ($4.75x10^{-4}$, $6.10x10^{-4}$ and 0.31 respectively) because an overexpression of EP3 versus EP2 is observed in these cells. The ratio is above 1 in HuMC and C57 mast cells (1,492 and 262006 respectively) reflecting that in these populations EP2 expression predominates. As shown in the Fig 3A a ratio above 1 is associated with an increased release of β-hexosaminidase, while a ratio below 1 is linked to a diminished release of this mediator. The maximum increase (57.6%) of β-hexosaminidase release is seen in BMMC with EP2/EP3 ratio of $6.1x10^{-4}$. The maximum inhibitory PGE_2 effect is shown in C57 (EP2/EP3 ratio=262000).

Fig 3 (B and C) show β-hexosaminidase release versus EP2/EP3 ratio on WT PDMC and WT BMMC compared to EP2 KO (-/-) PDMC and EP2 KO (-/-) BMMC, that is to say they show the impact of

deleting EP2 receptor on mast cell degranulation. Interestingly, EP2-deficient PDMC, which expressed about 1.6 fold higher EP3 versus GAPDH than the WT counterparts (71.29% of EP2/EP3 ratio decrease), showed a 1.4 fold higher degranulation response to IgE/Ag than WT PDMCs (17% increase). In EP2 KO (-/-) BMMC, the EP2/EP3 ratio is reduced down to 52.5%. This change of EP2/EP3 is linked to an enhanced β-hexosaminidase release (6.7% increase).

Total intracytosolic calcium mobilization

Total intracytosolic calcium mobilization is represented in Fig 4 (A, B and C). When DNP (Ag) is added to PDMC, Ca^{2+} flux rises (Fig 4 A). The rise in Ca^{2+} is more pronounced if the murine mast cells are incubated the presence of external PGE_2 (350% increase), than when no PGE_2 is added (122.7%). Such difference is statistically significant. Similarly, in BMMC (Fig 4B) total intracytosolic calcium mobilization increases whether PGE_2 is present or not after Ag stimulation. However, such increase is higher in the presence of the prostaglandin (95.7% versus 56% respectively). When Ag is added to HuMC (Fig 4C) calcium mobilization also increases. However, this increase is more pronounced in cellular preparations where no PGE_2 is added (170% versus 134.7% in preparation without PGE_2).

DISCUSSION

We have demonstrated that the relative expression of PGE_2 receptors EP1 to 4 on different human and murine mast cell systems vary widely. In spite of this variation, the EP2 and EP3 subtypes do commonly predominate, while EP4 expression is minor, and EP1 is virtually null in each and every mast cell preparation whether human or murine. Such diverse mast cells EP phenotype provided an ideal basis for studying the relevance of a specific EP expression profile to the mast cell function in the presence of PGE_2. Notably, a predominance of EP2 versus EP3 correlated with an inhibitory effect of PGE_2 on both degranulation and Ca^{2+} mobilization. On the contrary, when EP3 was overexpressed, PGE_2 addition resulted in a stimulatory outcome. Interestingly, such behavior was equivalent in human and murine mast cell systems.

In the present study it has been shown that murine and human mast cells express EP receptors. We observe that among the different murine and human mast cell populations there is variability on the EP receptor expression pattern. The dominant receptors are EP2 and EP3. The cell systems expressing mainly EP2, are C57 and HuMC. Those expressing mostly EP3 are PDMC, BMMC and LAD2. Other author's reports on BMMC (22, 24), LAD2 (41) and HuMC (17, 41) support our data. Sakanaka et al. (2008) found EP3 expression in PDMC but did not find either EP2 or EP4. The lack of EP2 and EP4 receptors expression could be attributable to the utilization of the mast cells right after isolation in these reports, while we cultured the cells extracted from the peritoneum lavage at least for 3 weeks with medium complemented with cytokines and SCF.

The differential expression of this divergent EP receptors

influences in the PGE_2 effect on mast cell activity, since PGE_2 exerts a direct interaction with mast cells. Probably, mast cells have the capacity to modulate their own receptor expression as a way to modulate the effect resulting from exposure to PGE2. Indeed, it is important to determine the EP expression profile when assessing the effect of EP agonist or antagonist on mast cells. There is also a need to underline the similarity between human and murine mast cells. In both mast cell types the expression of EP1 is null, EP4 is minor, and EP2 and EP3 is major. This, in fact, contributes to propose murine mast cells as a useful model to investigate the nature of PGE_2 to EP interaction.
In this study we have also determined the compensatory upregulation of EP receptors expression in mast cells. When EP2 gene is deleted, PDMC and BMMC have the ability to upregulate EP3 and EP4, but not EP1. Nguyen et al. (2002) did not observe the upregulation of EP receptors expression at mRNA level in EP2 KO (-/-) BMMC. This observation could be associated to the low sensitivity of the technique that they used.

A compelling observation is that there is a correlation between EP expression profile and the functional outcome in mast cells exposed to PGE_2. EP2 and EP3 are the crucial players. When EP2 is overexpressed mast cell degranulation and calcium mobilization are inhibited by PGE_2 as seen in C57 and HuMC. Oppositely, when EP3 is overexpressed PGE_2 enhances mast cell degranulation and calcium mobilization as observed in PDMC, BMMC and LAD2. Interestingly, these functional consequences are equivalent in murine and human mast cells.
The EP2-mediated inhibitory effect had been observed under similar experimental circumstances by others (17, 22, 24, 41).

Kuehn et al. (2011) also observed a divergent PGE_2 effect depending on the EP2/EP3 expression ratio, but not in the disparate effect on HuMC from different donors. The nature of the donor appeared to be critical for the outcome resulting from stimulation of the mast cells with PGE_2. We have not observed this uneven PGE_2 effect in HuMC from different donors. The differences between these studies could be explained by the higher doses of PGE_2 used in our study (10^{-5}M compared to 10^{-7} M). The dose of PGE_2 used in our study was shown to induce maximal inhibitory effects in human lung mast cells (14), in keeping with the lower affinity of the EP2 receptors (Kd>10nM) as compared to EP3 receptors (Kd<1 nM) (42). Thus it is possible that opposing effects for PGE2 are evident at different doses reflecting which receptors may be engaged. So, contrastingly, we have determined EP2/EP3 correlation with PGE_2 effect.

The fact that the use of a non selective ligand such as PGE_2 can exert different effects depending on the EP that predominates, might be a mechanism used *in vivo*: a predominant mast cell EP2 expression would explain the inhibitory effect of PGE_2 in some circumstances, while EP3-mediates a triggering effect. It also suggests that since in murine experimental asthma we primarily observe a blocking effect by PGE_2 on mast cells (12), it is most probable that this is a result of direct interaction with EP2 receptor on the mast cell surface. Such constraining mechanism on mast cells might also explain the beneficial effect of PGE_2 observed in humans (3-5, 13).

In this article, we therefore point at EP2 receptor as the clue receptor in mediating the inhibitory effect exerted by PGE_2 on mast cells. We also show that this inhibitory outcome is linked to a decrease on intracytosolic calcium mobilization. Further studies

need to be perform to further uncover the relevance of the mast cell EP2 in murine models of asthma *in vivo* and, to investigate whether this is a relevant mechanism in human asthma patients.

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2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk. Res.* 2003. 27,677-82.

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Figure 1

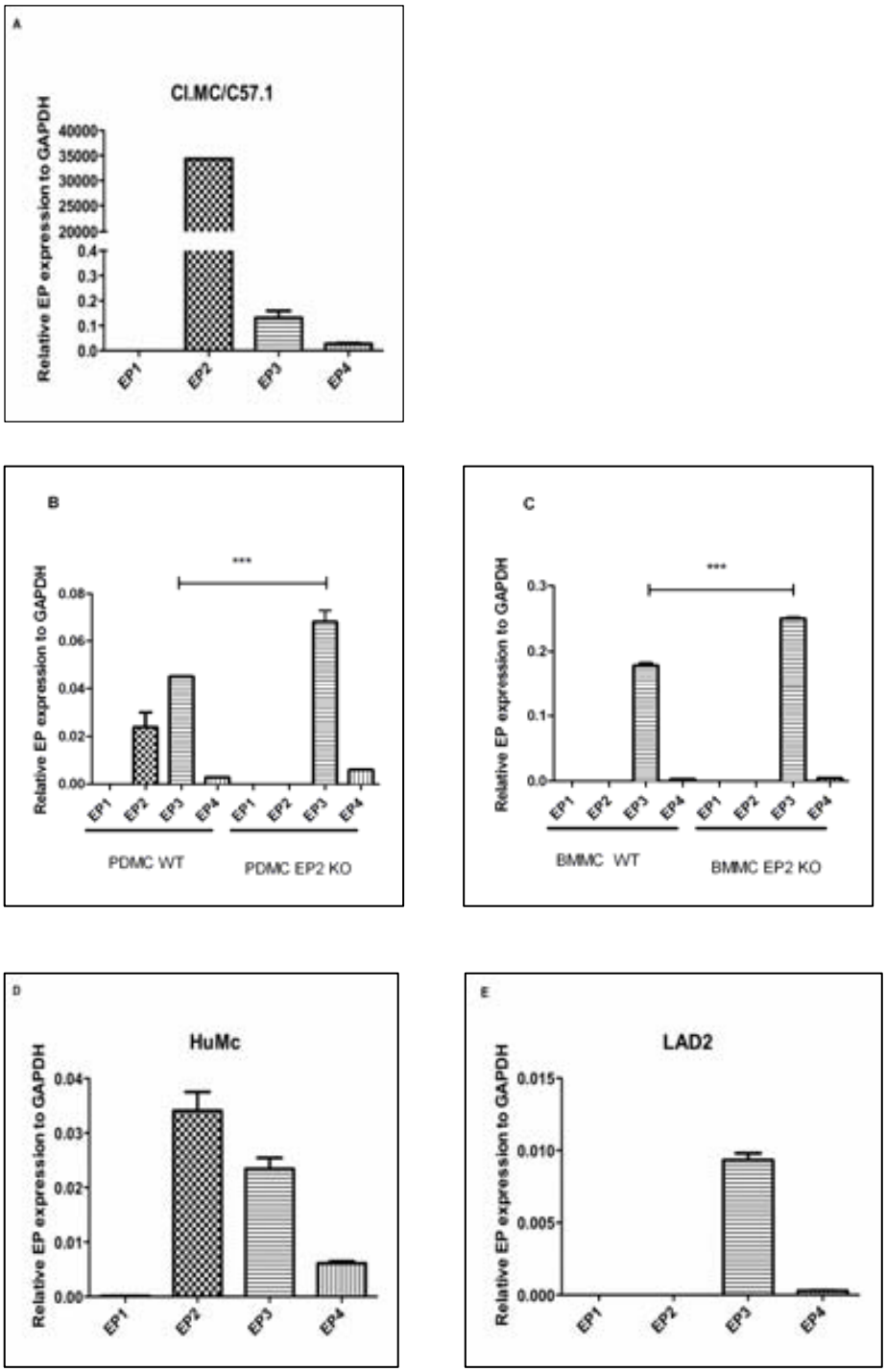

A
Cl.MC/C57.1
Relative EP expression to GAPDH
40000
35000
30000
25000
20000
0.4
0.3
0.2
0.1
0.0
EP1
EP2
EP3
EP4
B
***
Relative EP expression to GAPDH
0.08
0.06
0.04
0.02
0.00
EP1 EP2 EP3 EP4 EP1 EP2 EP3 EP4
PDMC WT
PDMC EP2 KO
C
***
Relative EP expression to GAPDH
0.3
0.2
0.1
0.0
EP1 EP2 EP3 EP4 EP1 EP2 EP3 EP4
BMMC WT
BMMC EP2 KO
D
HuMc
Relative EP expression to GAPDH
0.04
0.03
0.02
0.01
0.00
EP1
EP2
EP3
EP4
E
LAD2
Relative EP expression to GAPDH
0.015
0.010
0.005
0.000
EP1
EP2
EP3
EP4

Figure 1. *Expression of EP(1 to 4) receptors in C57, WT PDMC, EP2 KO (-/-) PDMC, WT BMMC, EP2 KO (-/-) BMMC, HuMC and LAD2 (A-E).* EP2 and EP3 are the primarily expressed receptors in all mast cell types (n=8). (*** Pvalue<0.001)

Figure 2

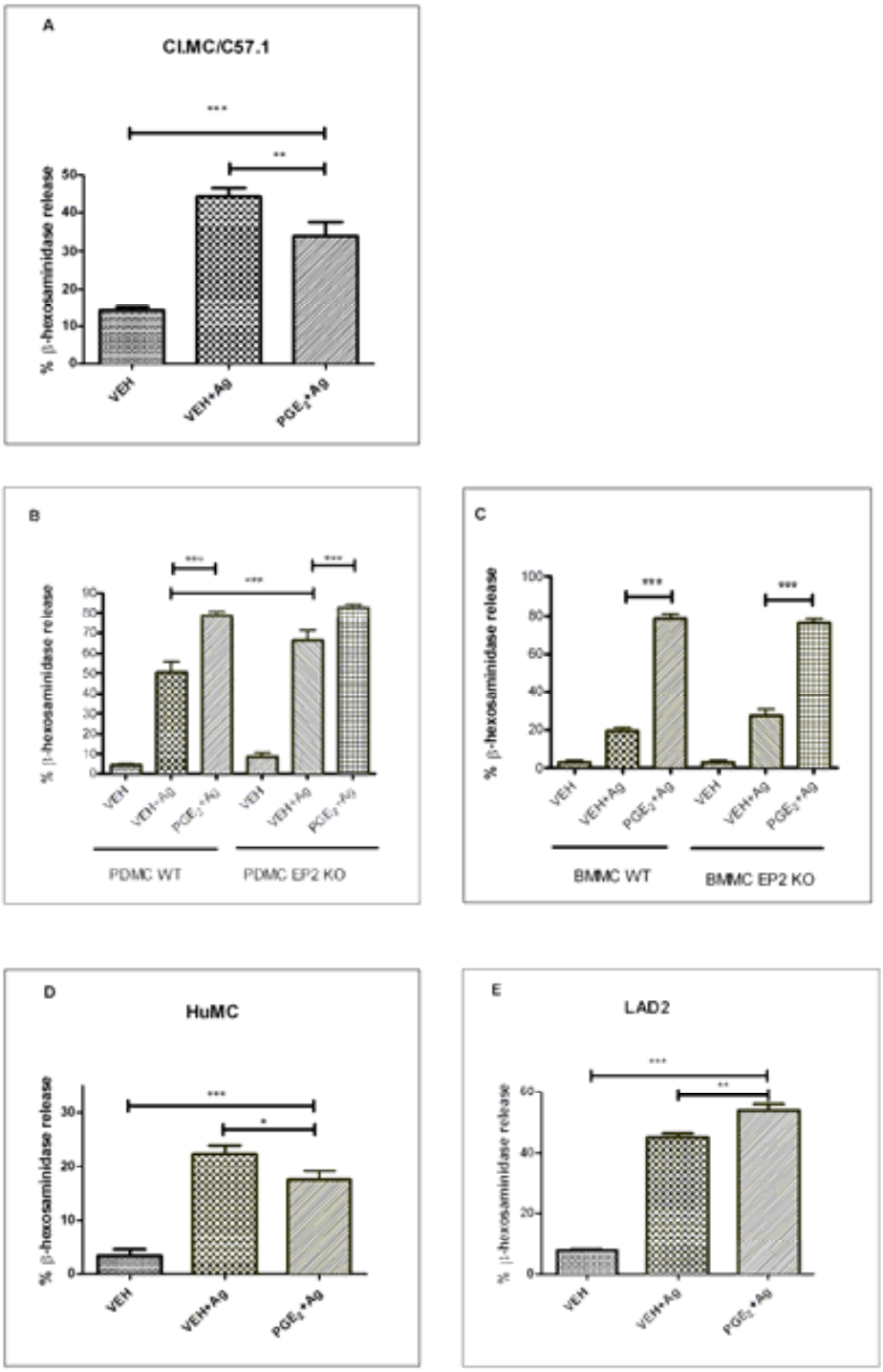
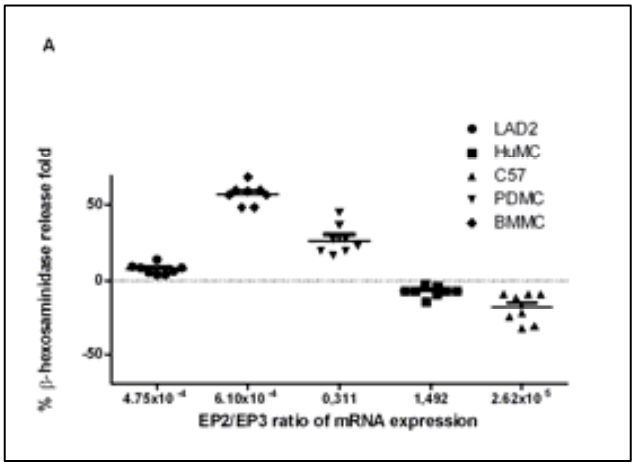
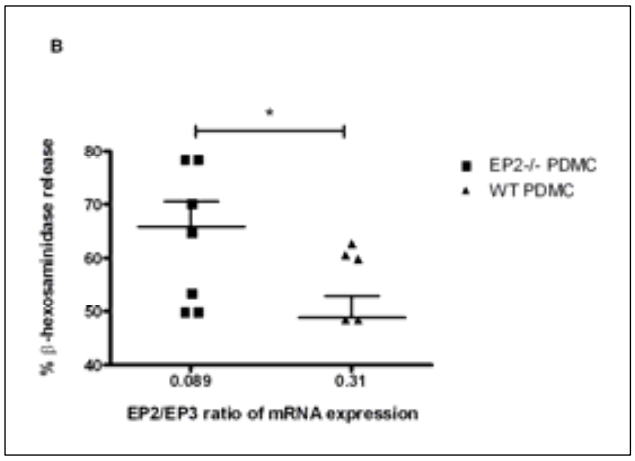

A
CLMC/C57.1
% β-hexosaminidase release
VEH
VEH+Ag
PGE2+Ag
B
PDMC WT
PDMC EP2 KO
C
BMMC WT
BMMC EP2 KO
D
HuMC
E
LAD2

Figure 2. *Effect of PGE_2 on mast cell degranulation in wild type mice, murine EP2 -/- and human mast cells.* The effect of PGE_2 treatment was tested in different mast cells types (A-E). PGE_2 was able to inhibit mast cell degranulation in CI.MC/C57.1 and HuMC (n=8-10), the cells populations with a predominant EP2 expression.
(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001)

Figure 3


A
% β-hexosaminidase release fold
50
0
-50
LAD2
HuMC
C57
PDMC
BMMC
4.75x10 -4
6.10x10 -4
0,311
1,492
2.62x10 -5
EP2/EP3 ratio of mRNA expression


B
*
% β-hexosaminidase release
80
70
60
50
40
EP2-/- PDMC
WT PDMC
0.089
0.31
EP2/EP3 ratio of mRNA expression

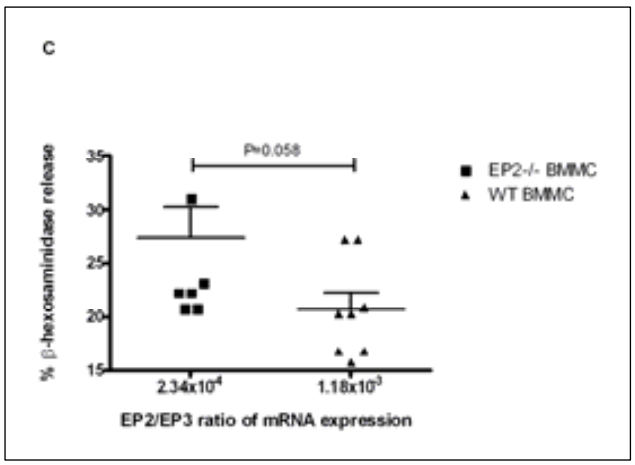
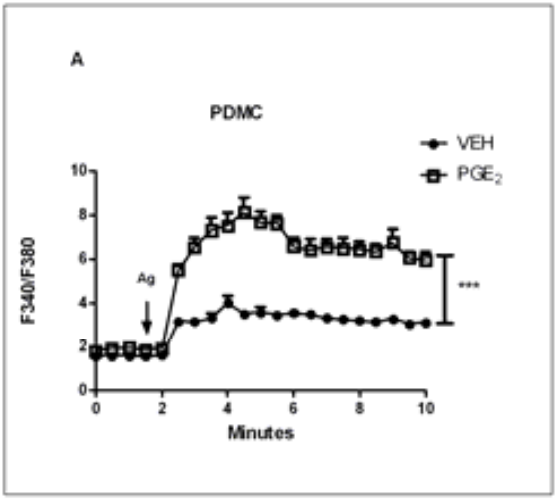
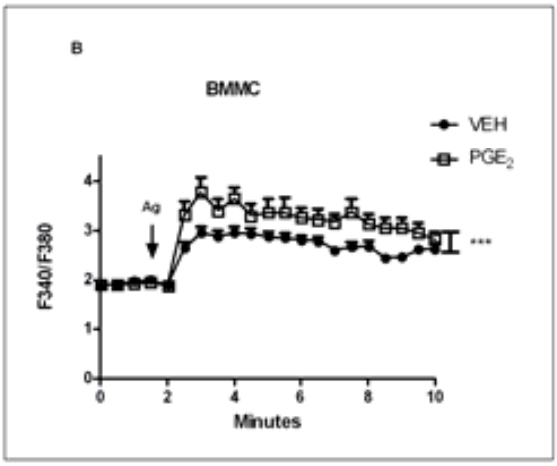

C
P=0.058
% β-hexosaminidase release
35
30
25
20
15
EP2-/- BMMC
WT BMMC
2.34x10^4
1.18x10^3
EP2/EP3 ratio of mRNA expression

Figure 3. *PGE_2 and EP2 deletion effect on mast cell degranulation according to EP2/EP3 receptor expression.* (A) We represent the % β-hexosaminidase fold release caused by PGE_2 effect in different conditions of EP2/EP3 expression. We see a proportional effect of PGE_2 mast cell enhancement or inhibition according to EP2 expression (n=8-10). (B and C) We see that in the absence of the EP2 in the murine mast cells and in EP3 dominant scenery, the β-hexosaminidase release of EP 2KO is increased (n=8).
(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001)

Figure 4


A
PDMC
VEH
PGE2
F340/F380
Ag
***
Minutes


B
BMMC
VEH
PGE2
F340/F380
Ag
***
Minutes

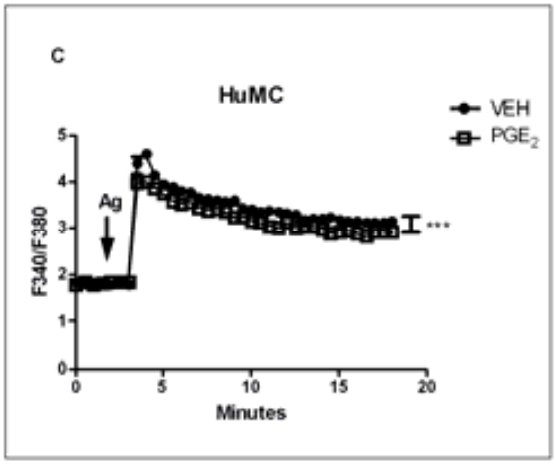

C
HuMC
VEH
PGE2
F340/F380
Ag
***
Minutes

Figure 4. *PGE_2 effect on total intracytosolic calcium mobilitzation.* PGE_2 significantly increased the total intracytosolic calcium mobilization in PDMC (A) and BMMC (B). In HuMC (C) PGE_2 significantly decreased calcium mobilization (n=8).
(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001)

5.3. Sub-Project 2:

Activation of the PGE_2 receptor EP2 prevents HDM-induced sensitization and murine mast cells activation

ABSTRACT

PGE_2 has been shown to exert a beneficial effect in asthmatic patients. Despite the likely impact on the identification of pharmacological targets of understanding this endogeneous protective pathway, there is scarce information about the mechanisms, and the primary PGE_2 receptor involved. Our study aimed at assessing the relevance of EP2 agonism, and its connection to airway mast cells, in PGE_2-induced protection. BALB/c mice were sensitized with HDM extracts for 10 days, and either treated with intranasal Butaprost, an EP2 agonist, AH-6809, an EP1/EP2 antagonist, or PGE_2. Butaprost effect on the murine C57 mast cell line was also studied, and the supernatants from these *in vitro* experiments were delivered to naive mice. Butaprost inhibited HDM-induced airways hyperresponsiveness and inflammation *in vivo*. This effect correlated with a diminished activity of lung mast cells. In contrast, a trend towards a worsening of the airway response was observed under AH-6809; a phenomenon that was neutralised by a mast cell stabiliser. Accordingly, EP2 activation was shown to directly prevent degranulation of C57 mast cells. Finally, only supernatants from C57 cells not incubated with Butaprost, had the ability to increase smooth muscle reactivity to methacholine in naive mice. The protective effect of PGE_2 in aeroallergens-sensitive mice is associated with EP2 agonism. Such effect is possibly the result of a direct activation of lung mast cells EP2 receptors, which in turn do release less pathogenic mediators. Our data open a path for the identification of new molecular targets along the EP2-mast cell-airways axis.

INTRODUCTION

Despite great efforts have been made to uncover novel therapeutic strategies; treatment of asthma has still many limitations. To identify pharmacological targets, creative hypothesis that may even challenge our current understanding, need to be explored. One of such assumptions is the role of prostaglandins (PG) in asthma. Although cyclooxygenase (COX) products have been generally viewed as pathogenic in inflammatory diseases, there is now enough evidence to support the protective nature of PG such as PGE_2 (1). We know that locally administered PGE_2 prevents airway hyperresponsiveness (AHR) and inflammation in human allergic and non-allergic asthma (2-4). Accordingly, a diminished PGE_2 release has been detected in airway cells isolated from asthmatic patients (5-7). However, data regarding the mechanisms underlying this PGE_2 beneficial action are scarce, and preclinical studies are needed to elucidate the phenomena involved.

Peebles et al. (8-9) showed that a COX inhibitor worsened OVA-induced airway inflammation in mice. Others and us demonstrated that inhibition of COX-2 in OVA-sensitized animals aggravates allergic inflammation and/or airway AHR (10-13). We extended this observation to the house dust mite (HDM)-sensitized mouse model, in which a genetic approach to downregulate the activation of COX-2 led to a partial beneficial effect (14). Accordingly, when exogeneous PGE_2 was administered, we observed a clear-cut protection against HDM-induced harm (15). Some authors have reported intervention of PGE_2 at immunological and inflammatory levels such as the regulation of T-cell differentiation and lymphocyte trafficking (1), and the production of pro-inflammatory cytokines (16-17). Interestingly, PGE_2 stimulates the release of IL-

10, an anti-inflammatory cytokine (18), and contributes to the airway epithelium integrity (19). These multiple effects are to some extent similar to those exerted by glucocorticoids, whose efficacy in asthma is attributable to their broad spectrum of action.

A detailed understanding of how an endogenous molecule such as PGE_2 generates a beneficial outcome, will uncover many potential targets along the way. One of such targets might be in mast cells (MC) (20). The presence of four PGE_2 receptors (EP1-EP4) with a broad range of functions (reviewed in 21) in such a key cell type in allergic diseases (22) may mask a unique MC-related PGE_2 protective effect. A major debate is which one of those receptors is primarily involved in the PGE_2-driven improvement. Our recent data suggested that EP3 aggravates HDM-induced pathology in mice (15). EP2 appears as a more likely candidate. For instance, there is a wide consensus about its ability to attenuate human MC degranulation (23-24). We have consistently observed in aeroallergens-sensitized mice that PGE_2 exerts an inhibitory activity on lung MC *in vivo*, and we hypothesized that EP2 is responsible for such effect (15, 25).

To help investigate the protective nature of PGE_2 in allergic asthma, we studied the impact of a selective EP2 agonist in preventing the development of AHR and inflammation in aeroallergen-sensitized mice. We also assessed whether such specific action modulated airway MC activity *in vitro* and/or *in vivo*.

MATERIAL AND METHODS

We designed four studies to investigate the mechanisms involved in the anti-asthmatic effect of PGE_2: Two *in vivo,* one *in vitro* and a combined in *vitro*/*in vivo* study.

In vivo studies

Experimental animals

Eight-week-old female BALB/c mice (Harlan, Spain) housed under a 12-hour light-dark cycle were used. All animal procedures were approved by the Ethics Committee for Animal Research of the Universitat Autònoma de Barcelona (Spain).

Sensitization to House Dust Mite Aeroallergens

Sensitization to HDM was induced according to Cates et al. (26). Briefly, the mice were exposed to a purified HDM extract (Alk-Abelló, Madrid, Spain) with a low lipopolysaccharide (LPS) content (<0.5 EU/dose). The aeroallergens were administered intranasally under light isoflurane anesthesia at a dose of 25 µg/mouse for 10 consecutive days. Non-sensitized (control) animals received intranasal saline solution.

Treatments

The mice were treated with either PGE_2, the EP2 agonist Butaprost (Cayman, Ann Arbor, MI, USA), or the EP1/EP2 antagonist AH-6809 (Sigma-Aldrich, Sant Louis, MO, USA). Untreated mice received PBS in 0.1% DMSO. PGE_2 and Butaprost were administered intranasally (0.3 mg/kg) 1 hour before the exposure to the HDM extract, starting from 1 day before initiating sensitization (day -1) to day 4. AH-6809 was given at a dose of 1

mg/kg intraperitonally one hour prior to HDM administration from day 0 until the last day of sensitization.

Assessment of Airway Hyperresponsiveness

Twenty four hours after exposure to HDM, airway reactivity to increasing doses of methacholine was assessed. Lung resistance was measured with the Buxco FinePointe plethysmograph system (Buxco, Troy, NY) in ketamine and xylazine anesthetized mice. The trachea was cannulated with an 18-gauge needle, and mice were ventilated with a pump ventilator (tidal volume, 12,5ml/kg; frequency, 120 breaths/min; positive end-respiratory pressure, 2.5–3.0 cm H2O). Changes from baseline airway reactivity (R_L) were assessed after 1 min of intratracheal nebulization of methacholine from Sigma-Aldrich (Sant Louis, MO, USA). A non-invasive whole body plethysmography procedure (WBP Buxco Europe Ltd, Winchester, UK) was also used to screen the respiratory function under different experimental conditions (27, 28). The response to methacholine was averaged and expressed as Penh (Enhanced Pause), a bronchoconstiction index that has been shown to correlate with lung mechanics (29).

Assessment of Airway inflammation

HDM-induced airway inflammation in treated and untreated mice was assessed 24 hours after exposure to the aeroallergens. Bronchoalveolar lavage (BAL) was performed by slowly infusing 0.3 ml of PBS + 2% FBS twice, and recovering it by gentle aspiration. BAL was stained with Turk solution (0.01% crystal violet in 1% acetic acid). Total airway cellularity and differential cell count (Diff-Quick) were performed in BAL cytospins.

Assessment of mast cell activity

The right lung lobe was collected for protein extraction using a lysis buffer (Mini complete tablet, Roche Diagnostics, Barcelona, Spain). 250 µl of the buffer were added to the tissue sample and the resulting solution was homogenized. The samples were centrifuged and the supernatants were collected to measure mMCP-1 (mouse mat cell protease-1) levels with a sandwich ELISA (Moredun Scientific Ltd.,Scotland, UK). A group of mice receiving the EP1/EP2 antagonist AH-6809 was also given the MC inhibitor sodium cromoglycate (SGC, Sigma-Aldrich, Sant Louis, MO, USA). SGC was administered daily at 25 mg/ml in PBS by intraperitoneal injection one hour prior to AH-6809.

***In Vitro* study**

C57 mast cell supernatant production

Cl.MC/C57.1 (C57) mast cells (kindly provided by Dr. Stephen J. Galli, Stanford University, California) were sensitized with 1µg/ml of mouse IgE anti-DNP (Sigma-Aldrich, Sigma-Aldrich, Sant Louis, MO, USA). After a 15 min pre-incubation of the murine MC with increasing concentrations of Butaprost, the cells at the concentration of $3x10^6$ cell/ml were challenged for 6 hours with 50 ng/ml DNP at 37C in a shaker. MC supernatants were collected and stored at -20C for further use.

Determination of C57 mast cells activity

Histamine and IL-4 release were measured in C57 cells supernatant by ELISA (IBL-International and Biosource, respectively). The cell viability was checked by trypan blue dye

exclusion, and was shown to be the same for each and every condition.

In vitro/In vivo study

Airway hyperreactivity induced by C57 mast cell supernatant

Supernatants from stimulated C57 murine MC were delivered with an intranasal administration to naïve mice (a single 35µl administration per mouse). For this *in vitro/in vivo* study, four groups of mice were settled under the conditions summarized in Fig 1. Twenty four hours after the local delivery of MC supernatants, airway reactivity and inflammation were recorded.

Statistical analysis

Statistical significance was assessed using a 2-tailed Student's *t* test, and differences among the treatments were evaluated by 2-way ANOVA with Bonferroni posthoc. A Pvalue of less than 0.05 was considered significant. Data are represented as mean ± SEM.

RESULTS

In vivo studies

Effect of EP2 agonism and antagonism on HDM-induced airway hyperreactivity (AHR)

Fig 2A and B depict the airway reactivity to methacholine of mice sensitized to HDM aeroallergens, and exposed intranasally to the EP2 selective agonist Butaprost. Mice sensitized to HDM displayed a robust airway response. Local administration of Butaprost significantly prevented the aeroallergen-driven increased airway resistance (R_L), approximately down to a half of the reactivity measured in non-treated mice (Fig 2A). The use of a non-invasive method (i.e. WBP) showed a very similar outcome (Fig 2B). A methacholine concentration as low as 2.5 mg/ml under invasive conditions, and 12.5mg/ml with WBP, already unravelled a significant Butaprost-effect. Such attenuated smooth muscle reactivity was also observed in a parallel group of mice exposed to the non-selective EP agonist PGE_2. In an additional experiment, the EP1/EP2 antagonist AH-6809 was administered to both sensitized and non-sensitized mice to uncover the relevance of the endogeneous PGE_2-EP2 pathway (Fig 2C). In contrast to the protective effect of the EP2 agonism, and despite the fact that no statistical significance was detected, blockade of the EP2 receptor with AH-6809 tended to worsen AHR. Such worsening was prevented by pre-treating the mice with the MC inhibitor, SCG (Fig 2C).

Effect of the EP2 agonist on HDM-induced inflammation

The differential inflammatory cell recruitment to the airways was also assessed in mice exposed to Butaprost (Fig 3). A strong eosinophilic recruitment was induced in HDM sensitized mice. Such eosinophilia was significantly prevented by local activation of the EP2 receptor. Butaprost reduced eosinophilic, but not lymphocytic or neutrophilic, infiltration, by up to 84% in mice exposed to HDM aeroallergens.

Effect of the EP2 agonist Butaprost on mast cells activity

The *in vivo* lung MC activity was evaluated by measuring mMCP-1 protein concentration in the airways (Fig 4). mMCP-1 was clearly overexpressed locally by a factor of 20 in HDM-exposed versus non-exposed mice. Butaprost administered i.n. at a dose of 0.3 mg/kg attenuated *in vivo* MC activity by 40% (Fig 4A).

***In vitro* Study**

In order to unravel the potential for Butaprost to directly interact with the MC EP2 receptor, C57 murine MC were stimulated immunologically and incubated with different concentrations of the EP2 agonist (Fig 4B). Histamine release was increased upon IgE/DNP activation. At a concentration of $3x10^{-8}$M, the EP2 agonist Butaprost already significantly prevented MC degranulation. IL-4 was also produced by stimulated C57 MC, but Butaprost did not inhibit the release of this cytokine (Fig 4C).

***In vivo / In vitro* Study**

Effect of the EP2 agonist Butaprost on mast cells-driven AHR in vivo

Supernatants from stimulated C57 MC that had been checked for activation were selected (Table 1), and delivered into the airways of naive mice. Twenty hours after the local exposure to mediators released by immunologically activated MC, the mice developed AHR, but not inflammation. When C57 MC had been incubated with Butaprost prior to stimulation, airway reactivity to methacholine of mice exposed to the supernatant reached back baseline values (Fig 5).

DISCUSSION

We have shown that Butaprost, a selective EP2 agonist, administered in the airways of mice sensitized to HDM aeroallergens, prevents hyperresponsiveness and inflammation. Such protective effect was paralleled by a diminished lung MC activity *in vivo*, a phenomenon possibly attributable to the ability of Butaprost to directly interact with MC, as revealed using the C57 murine MC line. We have also uncovered an association between modulation of the MC EP2 receptor and changes in airway pathology.

Both invasive and non-invasive procedures were used to demonstrate the protective effect on AHR of the EP2 agonist, Butaprost. Accordingly, in spite of the lack of statistical significance, the use of an EP1/EP2 receptor antagonist (AH-6809) that blocks endogeneous PGE_2 activity, does seemingly aggravate AHR. It is noteworthy that both the EP2 agonist (Butaprost), and the non-selective EP (PGE_2), agonist did prevent development of AHR and inflammation to a similar extent. This similarity may be surprising in the light of our recent observation that activation of EP3, a receptor bound by PGE_2, worsens HDM-induced airway pathology (15), and of the well known opposing effects of different EP receptors (18). However, fluctuations in the level of expression of EP3 versus EP2 may well explain why EP3 does not seem to counteract the EP2-driven effect in the presence of PGE_2. Our *in vivo* data seem to contradict Kunikata et al (30) observations in an OVA model, were EP3 was suggested to be the main beneficial receptor. This discrepancy could be due to procedural differences, mainly the use of OVA, rather than aeroallergens to sensitize the mice. Irrespective of the reasons underlying the discrepancy, in our view, the HDM-sensitive murine model provides ideal

conditions to address PGE_2-driven antiinflammatory mechanisms (reviewed in 31). With respect to inflammation, eosinophil recruitment was significantly lower under Butaprost's treatment. This supports Sturm et al (32) demonstration of an over 50% reduction of eosinophilia due to endogenous PGE_2. Finally, it is worthwhile underlining the ability to modulate HDM-driven airway pathology through a local pharmacological intervention (intranasal), when searching for antiasthma targets with a high safety ratio.

In vivo, selective EP2 agonism significantly reduced, by approximately 50%, airway MC ability to release mMCP-1, a MC specific protease, *in vivo*. At the same time, at a very low concentration ($3x10^{-8}$ M), Butaprost, prevents histamine release from murine C57 MC *in vitro*, causing up to a 75% reduction. The observed *in vivo* Butaprost blocking effect on lung MC is thus presumably the result, at least in part, of a direct action on the MC receptor. We know that human airway MC bear EP receptors, and that, specifically EP2 activation, leads to an inhibitory effect (22-24). Hence, the potential relevance of this MC's receptor in the course of airway pathology. Interestingly, in our experiments IL-4 release is not attenuated by the EP2 agonist. This result supports the fact that PGE_2 does not have an influence on IL-4 production in MC after 6h of *in vitro* incubation with PGE_2 and antigen (33).

Is the beneficial effect of Butaprost a result of its action on MC? We have undertaken two experiments that point towards an answer. We first have looked at the effect of C57 MC supernatants, either incubated or not with Butaprost, on the *in vivo* airway reactivity in naive mice. The delivery of a single shot of MC mediators (i.e. MC supernatants) into the airways, increased the

bronchial response to metacholine. When the C57 cells undergoing stimulation had been incubated with Butaprost, the *in vivo* smooth muscle reactivity to metacholine was significantly attenuated. This is, in our view, a unique set of data that reveals that the MC population by itself has the potential to predispose murine bronchial smooth muscle to hyperreactivity. Hence, the observed over-activity of MC *in vivo* triggered by HDM aeroallergens is likely contributing to the increased reactivity to methacholine in the areoallergens-sensitized mice. On the other hand, we showed that activation of EP2 receptors on the C57 MC surface has the capacity to prevent development of AHR *in vivo*. This has been further suggested by the use of the MC stabiliser SCG in sensitive mice exposed to the EP1/EP2 antagonist. The administration of AH-6809 increased HDM-induced AHR. Although this increase was mild and not statistically significant, the observation that it could be neutralized by pre-treating the mice with SCG, supports the hypothesis that the EP1/EP2 antagonist-mediated worsening of airway reactivity is a result of an increased MC releasability. An experimental approach that could help to further elucidate the relevance of the MC EP2 receptors in mediating PGE_2's beneficial effect is the use of MC deficient mice. Therefore the MC deficient mice do not provide an appropriate system for investigating the relevance of MC blockade to PGE_2's protective effect. The limited interest of such strategic approach for our purpose is further supported by recent observations on the minor role of MC in antigen sensitized C57/BL6 mice, the genetic background of mice strains lacking MC (34-35).

We have thus shown that, among the EP receptors of PGE_2, EP2 seems to be exerting a protective effect in mice exposed to

aeroallergens. Such beneficial effect is achieved through a local EP2 activation, and is possibly the result of a direct interaction of PGE_2 with the airways MC. Although the actual *in vivo* consequences of the EP2 agonistic action on MC need to be further studied, our data reveal a correlation between such action and the improvement of airway pathology. All in all, this points at the likely existence of relevant antiasthma target molecules in the EP2-MC-airway axis.

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Figure 1

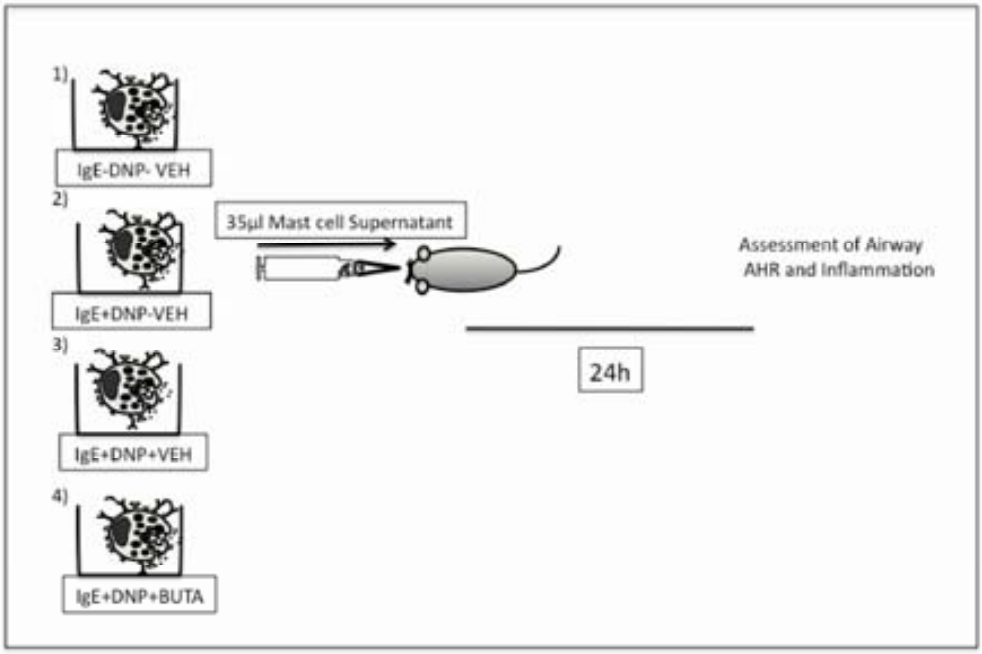

Figure 1. *In vitro/in vivo model of C57 mast cell mediators induced airway reactivity*. 35 µl of the supernatants from the selected C57 experiments (see Table 1) were delivered intranasally to naïve BALB/c mice in a single bolus. Twenty four hours after the *in vivo* mice's exposure to the supernatant, both, airway reactivity to methacholine, and inflammation were assessed.

Figure 2

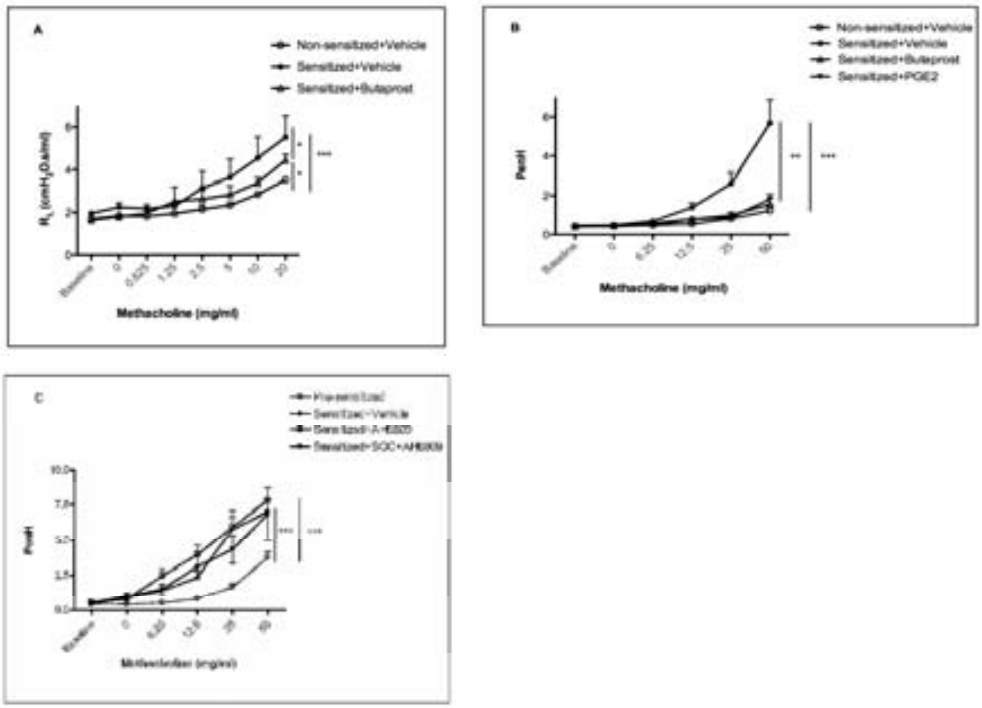

Figure 2. *Airway hyperresponsiveness in HDM-exposed mice treated with EP2 receptor modulators.* Butaprost, an EP2 agonist, given intranasally prevents development of airway hyperreactivity (AHR) assessed through an invasive (A) and non invasive (B) procedure. Both, the EP2 selective agonist, and PGE_2, the non selective ligand, bring airway reactivity back to baseline levels (B). The effect of both agonists is statistically significant when compared with sensitized-non treated mice. Despite no statistical significance was uncovered, the EP1/EP2 antagonist, AH-6809 slightly worsened airway reactivity when pre-administered to HDM-sensitized mice. Mice subject to the treatment with the EP1/EP2 antagonist and treated with SCG recovered from the deleterious

effect of AH-6809 (C). (n=8-13)(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001)

Figure 3

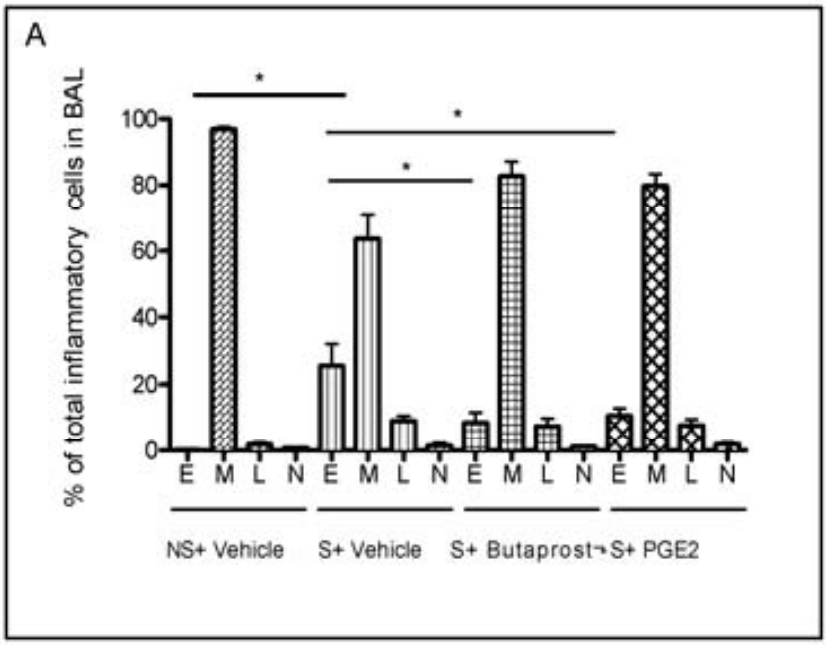

Figure 3. *Inflammatory cells in HDM-exposed, EP2 modulators-treated mice.* Cells from bronchoalveolar lavage (BAL) were stained and counted as described under Material and Methods. The percentatge of inflammatory cell subtypes was determined from counting at least 300 cells (E-eosinophils, M-macrophages, L-lymphocytes, N-neutrophils). Butaprost dampens HDM-driven airway inflammation to the same extent as PGE_2 (n=8-13).

(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001)

Figure 4

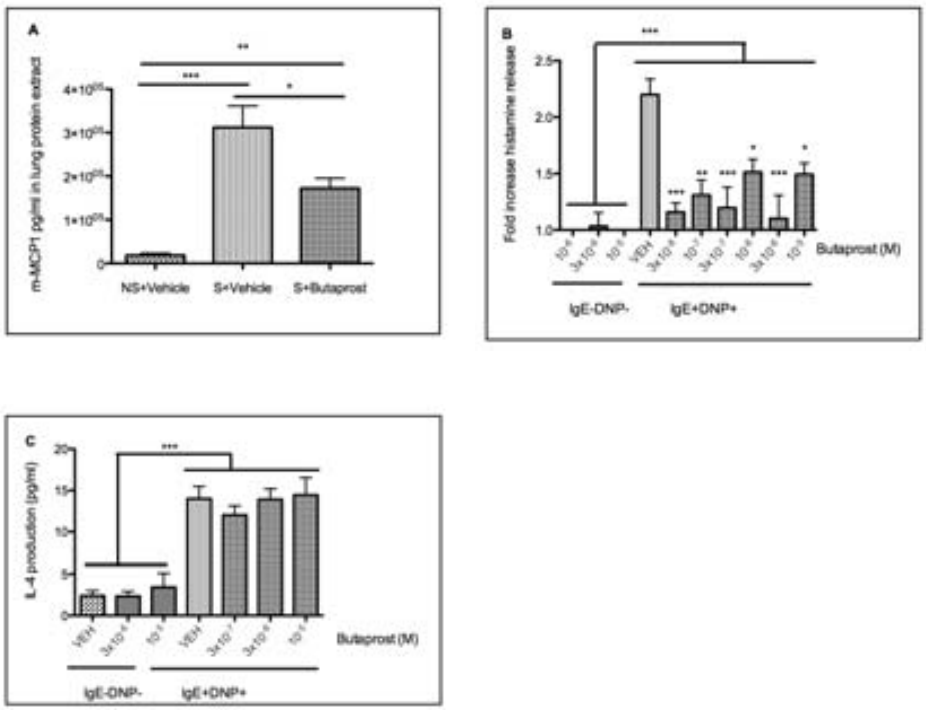

Figure 4. *Mast cell activity under Butaprost treatment in vivo and in vitro***.** Airway mast cell activity was assessed in the lungs of sensitized, treated and untreated, mice by measuring the local protein production of m-MCP1. Butaprost neutralises HDM-induced mast cells overactivity *in vivo* (A). In an *in vitro* experiment, the ability of Butaprost to prevent mast cell activity through a direct action was assessed on the C57 murine mast cell line (B and C). Fig 4B depicts the inhibitory effect of Butaprost on mast cell degranulation, i.e. histamine release. Fig 4C shows that the EP2 agonist did not prevent the release of IL-4. (n=8)

(*Pvalue<0.05, **PValue<0.01 and ***Pvalue<0.001)

MC sp	IgE	DNP	Butaprost*	Histamine (ng/ml)	IL-4 (pg/ml)
1	-	-	-	560 (±113.3)	2.34 (±1.8)
2	+	-	-	1100 (±178.8)	2.39 (±1.6)
3	+	+	-	3295 (±263.3)	13.97 (±4.7)
4	+	+	+	1280 (±100.8)	13.90 (±4.1)

*In the treatment control condition the cells were incubated with vehicle (i.e. PBS+0.1%DMSO)

Table 1. *Histamine and IL-4 concentration in C57 mast cell supernatants (MC sp) selected for the in vitro/in vivo study*. The cells had been stimulated with IgE-DNP, and either treated or not with the EP2 agonist Butaprost under the conditions stated uner Material and Methods. Four samples were selected on the basis of the widest mediators differences detected between the experimental conditions. These supernatants were administrated intranasally to the mice as described in Material and Methods.

Figure 5

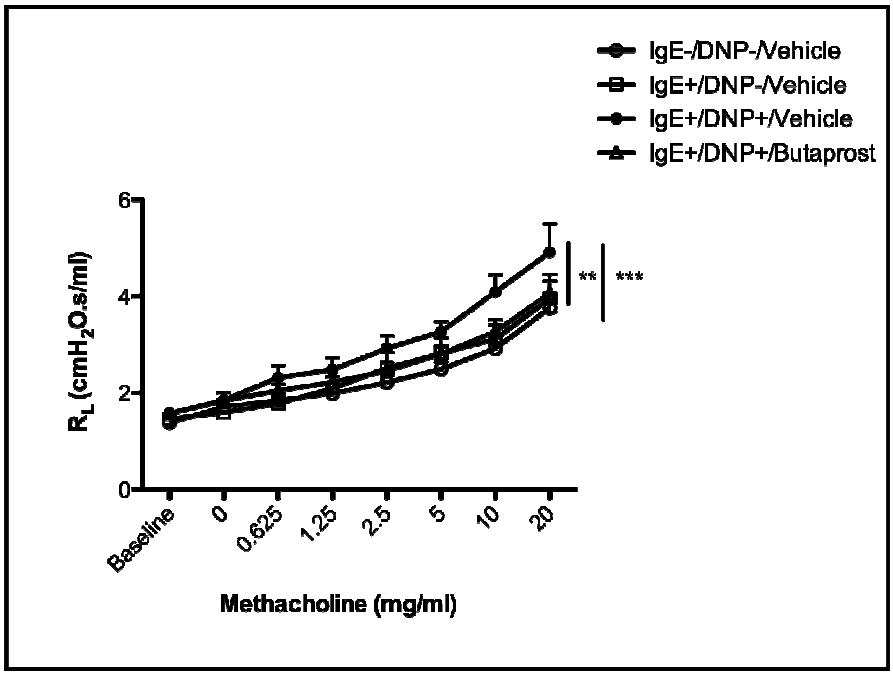

Figure 5. *In vitro / in vivo study of the effect of mast cell mediators on airway reactivity.* Selected supernatants from stimulated and non-stimulated C57 mast cells (see Table 1) were tested for their ability to induce changes of airway reactivity in naïve mice *in vivo*. One single intranasal administration of mast cell mediators from immunologically stimulated, but not from unstimulated, cells induced airway hyperreactivity to methacholine. Such effect was not observed when the C57 cells had been pretreated *in vitro* with the EP2 agonist Butaprost. (n=7).

(*Pvalue<0.05, **Pvalue<0.01 and ***Pvalue<0.001.)

5.4. Sub-Project 3:

Activation of the PGE_2 receptor EP2 on mast cells suppresses FcεRI-induced degranulation and immediate hypersensitivity reactions by mechanisms involving cAMP/PKA-mediated inhibition of the Fyn pathway

ABSTRACT

PGE_2 has been proven beneficial in asthma patients experimentally exposed to the PG, and in antigen-induced airway hyperreactivity and inflammation in rodent models. Mast cells are important effectors cells in allergy and are known to express PGE_2 receptors. We have found that the regulation by PGE_2 of mast cell immediate responses to antigen vary amongst various types of connective-tissue mast cells, both murine and human, ranging from activation to suppression depending primarily on the EP2 to EP3 receptor expression ratio. In this study, we determine that specific activation of the EP2 receptor by the selective agonist Butaprost mediates suppression of mast cell degranulation in all human and murine mast cell types that express detectable amounts of this receptor. Correlating with this suppression *in vitro*, Butaprost inhibited IgE-mediated immediate hypersensitivity reactions in a model of passive cutaneous anaphylaxis both in WT mice and mast cell-deficient mice engrafted subcutaneously with mast cells, but not in the mast cell-deficient mice. We show that the EP2 mediated signaling pathways in mast cells involved an increase in cyclate adenyl monophosphate (cAMP) production and an inhibition of FcεRI-mediated calcium (Ca^{2+}) influx. Activation of EP2 also decreased FcεRI-induced Fyn kinase activation, Fyn-mediated Gab2 and AKT phosphorylation. Treatment with an antagonist of cAMP or downregulation of PKA by lentiviral transduction of PKA-Cα-specific shRNA, reverted the inhibition of Fyn pathways and the degranulation response while partially preventing the inhibition of calcium influx induced by Butaprost. Thus, our results demonstrate a suppressive role for EP2 on mast cell immediate responses that may be the basis for the preventive effect of PGE_2 in asthma, delineate cAMP/PKA/Fyn as the signaling

pathway involved in this process, and suggest that selective agonism of EP2 may be more effective than PGE_2 in preventing allergic diseases or uncovering molecular targets.

INTRODUCTION

The prevalence of allergic diseases has almost doubled over the last 25 years, especially in western countries (1). Current treatments are still limited, particularly for complex diseases such as allergic asthma, which present multiple phenotypes and multiple features that may respond differentially to therapies (2). Approved drugs are effective in relieving the symptoms of certain allergic diseases; however they may only provide limited or no relief to a number of patients. Research efforts are being made to understand the cellular components and the mechanisms behind the development of allergies in search for new viable therapies.

Recently, experimental clinical studies have indicated that treatment with Prostaglandin E_2 (PGE_2), a cyclooxigenase-2 (COX-2) product, prevented airway inflammation and airway hyperreactivity in allergic asthma patients (3-6). Some studies have delineated a correlation between asthmatic patients and low levels of PGE_2 in isolated airway cells, suggesting that PGE_2 protect from allergen induced airway pathology (7-9). Furthermore, we found that subcutaneous injection of PGE_2 reduced airway hyperresponsiveness in the House Dust Mite (HDM) mouse model of allergic airway hypersensitivity (10). Additional studies suggesting a potential anti-inflammatory role for PGE_2 come from the observations that inhibition of COX-2, which resulted in the decrease of PGE_2 levels, worsens the airway response in OVA and HDM sensitized mice (11-17). While the preventive effect of PGE_2 in airway inflammation has been related to its stimulatory effect on the production of anti-inflammatory cytokines such as IL-10 in dendritic cells (18), other results point towards an effect of PGE_2 in regulating mast cell activity (19-21) and thus, reducing the allergic responses. Mast cells have been

shown to play a key role in IgE-mediated allergic inflammation (Type I hypersensitivity reaction) such as allergic asthma (22-23). The activation of mast cell by allergens or other stimuli leads to the release of a wide variety of mediators such as histamine, eicosanoids, cytokines and enzymes that contribute to the initiation and regulation of the allergic response (24). Thus exploring the regulation of mast cell activation may provide interesting therapeutic strategies to prevent the allergic response.

PGE_2, acts at 4 divergent G protein–coupled receptors (GPCRs), named the EP1, EP2, EP3, and EP4 receptors (reviewed in 25), which signal through different signaling pathways and trigger diverse cellular responses. PGE_2 receptors are expressed in cultured mast cells. PGE_2 has been described to increase mast cell reactivity (26-27) and trigger AKT by increasing its phosphorylation (28). This contrasts with the previous observations of PGE_2 as an anti-inflammatory mediator (18-21), and our recent data suggesting that PGE_2 reduces (HDM)-induced allergic response by inhibiting lung mast cell activity through Prostaglandin E2 receptor type 2 (EP2) (10,29,30). We also demonstrated *in vitro* that the inhibitory effect of PGE_2 on mast cell activity is mediated by EP2 (30-31); however, this effect may be masked by the expression of other PGE_2 receptors, particularly EP3, in various types of mast cells, making a specific EP2 agonist a better candidate for therapy.

Based on our recent studies suggesting mast cells as targets for the suppressive effects of PGE_2 in the lung acting through EP2 (29-31), here we study the effect of a specific agonist of the EP2 receptor on immediate mast cell responses in various human and murine connective tissue mast cells populations. We show that

EP2 mediates the inhibitory effect of PGE_2 on mast cells activity and that such effect on mast cells has effector consequences in a murine model of cutaneous anaphylaxis. We describe that the EP2 intracellular signaling pathways regulating FcεRI-induced mast cell activity involve cAMP interference with the Src kinase Fyn and calcium influx via PKA activation.

MATERIALS AND METHODS

Reagents and Antibodies

DNP-specific mouse IgE was produced as previously described (32). Biotinylated anti-human IgE was from ABBIOTEC (San Diego, CA, USA) and Streptavidin (SAv, Ag) was purchased from BD Biosciences (San Jose, CA, USA). DNP human serum albumin (DNP-HSA; Ag), Forskolin, 3-Isobutyl-1-methylxanthine (IBMX), dimethylsulfoxide (DMSO), Formamide, Formaline and Evans Blue were from Sigma-Aldrich (Sant Louis, MO, USA). Butaprost (EP2 agonist) was purchased in Cayman (Ann Arbor, MI, USA). The Rp-Isomer adenosine 3', 5'-cyclic Monophosphorothioate Triethylammonium Salt was obtained from Calbiochem (San Diego, CA, USA). Rabbit anti-EP2 antibody was purchased from Santa Cruz; rabbit anti-phospoAKT (ser473), anti-phosphoSrcY416 (pY416), anti-phosphoSrcY527 (pY527), anti-phosphoGab2 (pGab2) and anti-PKA-Cα antibodies were from Cell Signaling (Boston, MA, USA). Mouse anti-AKT antibody and mouse anti-β-actin were from BD Biosciences (Sparks, MD, USA). Mouse anti-phosphotyrosine antibody (4G10) from Millipore (Billerica, MA, USA).

Mice

All mice were maintained and used in accordance with National Institutes of Health (NIH) guidelines and National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)-approved animal study proposal A010-0403. Mice used for the Passive Cutaneous Anaphylaxis (PCA) were 6-8-week-old female BALB/c or C57/BL6 Kit $^{Wsh/Wsh}$ (Wsh) from The Jackson Laboratory (Bar Harbor, Maine, USA).

Mast cell populations were obtained from female 5- to 6-week-old C57/BL6 and Lyn KO mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and bred in Taconic (Rockville, MD, USA).

Passive Cutaneous Anaphylaxis (PCA)

Mice were passively sensitized with an intradermal injection of 75 ng of DNP-specific mouse IgE (20 µl) into the right ear, while the contralateral ear was injected with 20 µl of Phosphate Buffered Saline (PBS) as negative control. After 24 h, mice were treated with an intravenous injection of 0.3 mg/kg EP2 agonist (Butaprost) or vehicle (100 µl PBS with 0.1% DMSO). Thirty min later, mice were challenged intravenously with 200 µg of antigen (Ag, DNP-HAS) in PBS containing 1% Evans blue (100 µl). Mice were euthanized with CO_2, 30 minutes after the Ag injection. The right and left ears were cut, minced and the Evans blue dye extracted with 700 µl of Formamide at 55ºC for 2 h. The absorbance of Evans blue in the extract was determined at 620 nm. Some of the mice used for these experiments were mast cell deficient mice (Wsh) or Wsh mice reconstituted or not intradermally with PDMC. In brief, the left and the right ears of 5-week-old Wsh mice were injected with $5x10^6$ PDMC (>98% positive for FcεRI and cKit receptor expression) that had been washed and resuspended in PBS (maximum volume of 100 µl per ear). After 6 weeks engrafting of mast cells in the dermis is evident (33) and mice were subjected to PCA.

Murine and human mast cell cultures

Bone marrow mast cells (BMMC) were extracted from the mice's femurs and tibias, and cultured in the presence of IL-3 (20 ng/ml) and SCF (20 ng/ml) in RPMI medium with 10% FBS for 4 to 6 weeks as previously described (34). Peritoneal lavage mast cells were harvested from mice by using 3 ml of RPMI medium injected into the peritoneal cavity. Cells were then cultured for 3-4 weeks in the presence of IL-3 (20 ng/ml) and SCF (20 ng/ml) in RPMI medium with 20% FBS to obtain an expanded population of peritoneal-derived MC (PDMC) (35). Recovered BMMC and PDMC were analyzed for the mast cells content and FcεRI and cKit expression by FACS as previously described (34). Cells were rested overnight in the absence of SCF before the stimulation assay. PDMC and BMMC from EP2 knockout mice (-/-) (36) were kindly provided by Dr. M. Kovarova and Dr. B.H. Koller from the University of North Carolina-Chapel Hill, NC, USA. The CI.M/C57.1 (C57) mast cell line, kindly provided by Dr. Stephen J. Galli, from Stanford University, was originally derived from BALB/c mice (37-38). C57 mast cells were grown in DMEM supplemented with 10% FBS, L-glutamine and 2-mercaptoethanol.

LAD2 MCs (39) (kindly provided by Dr. A. Gilfillan from the National Institutes of Health) is a human MC line derived from a patient with untreated MC sarcoma. LAD2 mast cells were cultured in serum-free media (StemPro-34 SFM, Life Technologies, Gaithersburg, MD, USA), supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, and 100 ng/ml SCF. Human peripheral blood-derived $CD34^+$ mast cells (HuMC) (kindly provided by Dr. A. Gilfillan from the National Institutes of Health) were also cultured in StemPro-34 SFM, supplemented with 2 mM

L-glutamine, 50 µg/ml streptomycin, 100 IU/ml penicillin, 100 ng/ml SCF to which 100 ng/ml of rhIL-6 (PeproTech, Inc., Rocky Hill, NJ, USA). rhIL-3 (30 ng/ml) was also added from the first week. Half of the culture medium was replaced every 7 days. Cultures on weeks 8 to 10 consisted of over 99% HuMC (40-41). Cells were rested in the absence of SCF o.n. before stimulation.

293LTV cells used for viral production (Cells Biolabs, San Diego, CA, USA) were cultured in DMEM media supplemented with 10% fetal calf serum (Invitrogen Life Technologies, Gaithersburg, MD, USA).

mRNA expression of the Prostaglandin E2 receptor (EP) by quantitative real-time PCR

mRNA was extracted from the various types of murine and human mast cell lines ($2x10^6$ cells) using the Rneasy Mini Kit with On column DNAse treatment (Qiagen, Valencia, CA, USA). The reverse transcription to convert mRNA to DNA and the DNA amplification were done by qScript One-step qRT-PCR Kit, Low ROX cocktail (Quanta, Santa Clara, CA, USA). This cocktail contains the reverse transcriptase enzyme, nucleotides and the DNA polymerase. 50 ng of total mRNA for each sample were added to 20 µl qScript One-step qRT-PCR Kit, Low ROX cocktail. Expression of EP 1, 2, 3, 4 mRNA in these samples was determined by real time PCR using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The gene expression assays for EP 1, 2, 3 and 4 receptors mRNA expression in murine and human mast cells were: mouse EP1 (Mm00443097_m1), mouse EP2 (Mm00436051_m1), mouse EP3

(Mm0.1316856_m1), mouse EP4 (Mm00436053_m1), human EP1 (Hs00168752_m1), human EP2 (Hs00168754_m1), human EP3 (Hs00168755_m1) and human EP4 (Hs00168761_m1). As an endogenous control we used the expression of mGAPDH and hGAPDH, respectively. Expression was calculated as the comparative threshold method normalized to GAPDH expression as described by Applied Biosystems (Foster City, CA, USA), and it is expressed and represented in terms of relative units (EP expression to GAPDH ratio).

Hexosaminidase release

Murine mast cells were sensitized with 1 μg/ml of DNP specific IgE for 2 h in free SCF media. After sensitization, cells were washed and resuspended with HEPES buffer (10 mM HEPES [pH7.4], 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4_7H2O, 5.6 mM glucose, 1.8 mM CaCl2_2H2O, and 1.3 mM MgSO4_7H2O) with 0.04% BSA (Sigma-Aldrich, Sant Louis, MO, USA). Cells were seeded in a V-bottom 96-well plate with 50.000 cells in a final volume of 100 µl. Cells were treated with 10^{-5} M Butaprost or vehicle (PBS with 0.1% DMSO) for 15 minutes at 37ºC with 5% v/v of CO_2. Where indicated, cells were pretreated at 37ºC with 1 mM Rp-cAMP (a cAMP antagonist) or 30 μm of Forskoline as positive control for 1 hour prior to the addition of Butaprost. Cells were stimulated with 50 ng/ml Ag (DNP-HSA) for 30 minutes at 37ºC with 5% v/v of CO_2. The degranulation was stopped by ice. Cells were centrifuged for 10 min at 4ºC at 1500 rpm.

Human mast cells were sensitized with 100 ng/ml biotinylated human IgE for 2 h in free SCF media. After sensitization, cells were washed and resuspended with HEPES buffer with 0.04%

BSA. Cells were seeded in a V-bottom 96-well plate with 30.000 cells in a final volume of 100 μl. Cells were treated with 10^{-5} M Butaprost or vehicle (PBS with 0.1% DMSO) for 15 minutes at 37ºC with 5% v/v of CO_2. Cells were stimulated with 10 ng/ml Ag (SA) for 30 minutes at 37ºC with 5% v/v of CO_2. The degranulation was stopped in ice. Cells were centrifuged for 10 min at 4°C at 1500 rpm.

The enzymatic activity of the granule marker, β-hexosaminidase, released to the extracellular media was measured as described in murine and human mast cell from the supernatants and pellets of cells challenged (Percentage degranulation = 100 X (supernatant content)/(supernatant + lysate content) (34).

Immunoprecitipitation and Immunoblots

To determine expression of EP2 receptor, murine mast cells ($2x10^6$ cells) were lysed in lysis buffer (Borate buffer saline containing 60 mM octyglucoside, 1% v/v Triton X-100, 1% v/v Thermo Halt Protease and Phosphatase inhibitor cocktail 100X, 5 mg/ml Pepstatin A, 2mM PMSF,) and incubated on ice for 20 minutes. Lysates were cleared by centrifugation at 14000 rpm for 20 min at 4ºC and protein concentration determined by BCA protein Assay (Thermo Fisher, Waltham, MA, USA). Tris-glycine sample buffer (Invitrogen, Life Technologies, Gaithersburg, MD, USA) was added 1:1 to the lysates containing equal protein amounts and boiled for 3 minutes. Lysates from human mast cells ($1x10^6$ cells) were prepared as described (40,42). Proteins were separated by electrophoresis on 12% NuPAGE Tris-Glycine gels (Invitrogen, Life Technologies, Gaithersburg, MD, USA) and

transferred to nitrocellulose membranes. In experiments testing the effects of Butaprost on the induction of total tyrosine, AKT phosphorylation or Gab2 phosphorylation by IgE/antigen, mast cells were placed in SCF-free media overnight. Cells ($3X10^7$ cells per condition) were then sensitized with 1 μg/ml DNP specific mouse IgE in HEPES buffer containing 0.04% BSA for 2 h. Cells were washed and treated with 10^{-5} M Butaprost, and vehicle (PBS with 0.1%DMSO) for 15 minutes a 37ºC and then stimulated with 50 ng/ml Ag (DNP-HSA) for 1, 3, 9 minutes. The reaction was stopped by placing the tubes on ice. Cell lysates were prepared, proteins separated in Tris-Glycine gels and transferred to nitrocellulose membranes as described above. The membranes were probed with the corresponding primary antibodies or, anti-AKT or anti-β-actin as a loading control, then washed and immunostained with corresponding infrared-labeled secondary antibodies. Membranes were analyzed using an Infrared Imaging System (Odyssey, Li-COR Biosciences, Lincon, NE, USA). The quantification of western blots were the intensity analysis (by infrared analysis) of the band corresponding to the EP2 receptor, pAKT or pGab2 was corrected to that of β-actin or total AKT used as an internal control.

For Fyn immunoprecipitations, $25x10^6$ PDMC were sensitized and challenged and the cell pellets lysed as described above. Equal amount of protein in the cell lysates were incubated with anti-Fyn antibody (8 μg/sample) pre-bound overnight to G-sepharose beads (Amersham Biosciences, Piscataway, NJ, USA). After 3h incubation at 4ºC, immunocomplexes were washed 5 times with lysis buffer and recovered by boiling in Tris-glycine SDS sample buffer that contained 1% 2-Mercaptoethanol and 1 mM orthovanadate. Proteins in the immunoprecipitates were resolved

by SDS-PAGE and analyzed by western blot using as primary antibody anti-phosphoSrcY416 and anti-phosphoSrcY527, and anti-Fyn for loading control.

Calcium mobilization measurements

For intracellular calcium (Ca^{2+}) measurements, cells were sensitized overnight (o.n.) and loaded with 1mM FURA-2AM (Invitrogen, Carlsbad, CA, USA) for 30 minutes, washed, resuspended in Tyroids buffer (10 mM HEPES [pH7.4], 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4_7H2O, 5.6 mM glucose, 1.8 mM CaCl2, and 1.3 mM MgSO4) with 0.04% BSA) and aliquoted in 96-well plates (30.000 cells/well). After 10 minutes, cells were treated for 15 minutes with 10^{-5} M Butaprost and vehicle (0,1%DMSO+PBS). Cells were then challenged with Ag (25 ng/ml for PDMC or BMMC and 20 ng/ml for HuMC) for 10 minutes in order to measure calcium mobilization. Changes in intracellular calcium were monitored with a microplate fluorescence reader Wallac Victor2 1420 Multilabel Counter" (PerkinElmer Life Sciences, Waltham, MA, USA). FURA-2 emission at 510 nm during fast excitation between 340 and 380 nm at 37ºC was measured, and background fluorescence was determined with non-stained. In most experiments, calcium was omitted from the incubation buffer and after challenge with antigen for 5 min, 1.8 mM of $CaCl_2$ was added to cells to assess the rate of extracellular calcium influx. Where indicated, cells were pretreated at 37ºC with 1 mM Rp-cAMP (a cAMP antagonist) for 1 hour prior to the addition of Butaprost. Background fluorescence was determined in cells non-labelled with FURA-2. The ratio of the fluorescence at 340 nm and

380 nm (R) after subtracting the respective background values was calculated for each measurement.

cAMP determination

PDMC were resuspended in HEPES buffer at the concentration of 10^6 cells/ml and pretreated with 1mM of an inhibitor of the cAMP phosphodiesterase (IBMX) for 1 hour to prevent degradation of cAMP. Afterwards they were treated for 15 minutes with 10^{-5}M Butaprost or vehicle (0.1%DMSO+PBS). As positive control, cells were incubated for 1 hour with 30 μm Forskoline. Cells were then washed and the cell pellets lysed by resuspension in 0.1M HC/0.1% Triton X-100 (10^7 cells/ml) plus 2 freeze-thaw cycles. cAMP in the cleared lysates was measured by ELISA (Sigma-Aldrich, Sant Louis, MO, USA).

shRNA PKA construction and gene transduction

A lentiviral based transduction system was used for shRNA-mediated gene knockdown of the α catalytic side of PKA (PKA-Cα) in PDMC. Bacterial glycerol stocks of shRNA clone for mouse PKA-Cα were purchased from Sigma-Aldrich (Sant Louis, MO, USA). The shRNA sequence used was TRCN0000012460. Nontarget shRNA (SHC002) was used as a negative control. 293LTV cells (Cell Biolabs, San Diego, CA, USA) were cotransfected with 3.9μg shRNA vector and 39μl lentiviral packaging mix (Sigma-Aldrich, Sant Louis, MO, USA) using Lipofectamine 2000 (Invitrogen, Life Technologies, Gaithersburg, MD, USA). The viral supernatants were collected 72-hours post-

transfection and concentrated by centrifugation at 20,000 x g for 2 hours. The viral pellet was resuspended in 1 ml of culture medium and used to transduce $1x10^7$ PDMC. Two days post-transduction, cells were changed to virus-free medium and following an additional two-day recovery period, selection was started using 1.5 µg/ml puromycin (Sigma-Aldrich, Sant Louis, MO, USA) (43). Cells were kept in selection media for a week at the end of which 98 % of non-transduced cells were not viable. After at least 2 days recovery in media without puromycin, cells were used for the experiments. Downregulation of the PKA-Cα expression was demonstrated by western blot.

Statistics

Statistical significance was determined using a 2-tailed Student's *t* test, and statistical differences among the treatments were evaluated by 2-way ANOVA, as indicated in the figure legends. A *P* value of less than 0.05 was considered significant. Data are shown as mean ± SEM.

RESULTS

Differential EP2 expression in murine or human mast cell populations

In Fig 1 (A, B, C and D), we represent the relative expression of EP2 mRNA and protein in the studied murine and human mast cell populations.

In the murine mast cells, as for EP2 mRNA expression (Fig 1A) C57 showed the highest expression of EP2 (34318.2 relative units) followed by PDMC (0.023 relative units). BMMC had the lowest EP2 mRNA expression ($2x10^{-4}$ relative units). In HuMC, EP2 mRNA expression was of 0.034 relative units, while in LAD2 EP2 was barely expressed.

Comparative EP2 receptor protein level of expression (Fig 1B) was associated to the observed in mRNA expression. C57 and PDMC had a higher EP2 protein expression than BMMC's. The EP2/AKT ratio of the band intensity determined by infrared analysis in the western blot was 1.46, 1.155 and 0.127 respectively. In human mast cells populations, HuMC (EP2/AKT ratio=0.819) expressed more EP2 receptor than LAD2 (EP2/AKT ratio=0.819).

Minimal or no expression of EP2 was detected either at mRNA or at protein level in EP2 KO PDMC (Fig 1C and D) indicating the suitability of the probes and antibody to specifically determine EP2 expression.

The effect of selective EP2 agonist, Butaprost, on FcεRI-induced mast cell degranulation vary depending on the mast cell type

Fig 2 (A, B, C, D and E) shows the effect of specific activation of EP2 using the selective agonist Butaprost on the murine and human mast cells populations immunological degranulation.

Butaprost significantly reduced FcεRI-induced mast cell degranulation in PDMC, and in C57 (49% and 38.9%, respectively). However, Butaprost had no effect on BMMC's exocytosis. We observed no effect of the selective EP2 agonist neither in EP2 KO PDMC nor in EP2 KO BMMC (Fig 2B and C). Similarly, Butaprost reduced degranulation by more than 64% in $CD34^{+}$-derived human mast cells (HuMC) (Fig 2D) but had no effect on LAD2 cells (Fig 2E).

cAMP production induced by EP2 agonism suppresses mast cell degranulation

Butaprost induced a 50% increase in the levels of intracellular cAMP in PDMC as compared to non-treated cells treated (Fig 3A). Butaprost did not induce cAMP production in PDMC derived from the EP2 KO mice (Fig 3B).

We found that the increase of cAMP induced in PDMC by the pharmacological activator of adenyl cyclase, Forskolin (Fig 3A), prevented FcεRI-induced mast cell degranulation by 50% (Fig 3C). When PDMC were pretreated with the antagonist of cAMP (Rp-cAMP) the inhibitory effect of Butaprost in mast cell degranulation was abrogated (Fig 3C).

Butaprost induces a reduction in extracellular calcium influx partially through cAMP production

EP2 agonism significantly inhibited the total intracytosolic calcium response induced by IgE/Ag in PDMC (Fig 4A), but not in BMMC (Fig 4B). We evaluated the effect of Butaprost on calcium influx from extracellular media and on calcium release from intracellular sources (in calcium free media). As it is shown in Fig 4C, Butaprost only reduced calcium influx, but not calcium release, in PDMC. Similar results, although not as pronounced, were obtained in HuMC (Fig 4D). When PDMC were treated with the antagonist of cAMP (Rp-CAMP) prior to Butaprost and Ag stimulation, Rp-cAMP did not fully restore calcium influx (Fig 4E).

Butaprost inhibits FcεRI-induced tyrosine phosphorylation and Fyn activation

We tested Butaprost effect on general phosphorylation tyrosine sites in IgE activation in PDMC. EP2 agonism reduced by 50% the overall FcεRI-induced phosphotyrosine responses within 3 min, and specifically inhibited the phosphorylation of the Src kinase tyrosine 416 site (Fig 5A). Then, we evaluated Butaprost effect in PDMC from Lyn-deficient mice. We found that Butaprost still reduced by 20% the phosphotyrosine responses in Lyn-deficient PDMC. We observed that Butaprost treatment induced a reduction within 3 min in the phosphorylation of the Src kinase tyrosine 416 Lyn KO PDMC (Fig 5A).

To demonstrate Src-Fyn involvement, Fyn was immunoprecipitated from PDMC pretreated with Butraprost or VEH

and stimulated for 1 or 3 min with antigen. EP2 selective agonism significantly ($p<0.05$) inhibited Fyn phosphorylation in tyrosine 416 (Fig 5B) but had no effect in the phosphorylation of the inhibitory tyrosine 527 (data not shown).

Then we determined Butaprost effect on phosphorylation of the targets of Fyn, Gab2 and AKT. In Fig 5C and D we show that Butaprost induced a reduction by 42% and 80%, respectively, in the phosphorylation of Gab2 and AKT with in 9 min.

Butaprost inhibits Fyn signaling pathway through cAMP/PKA

To determine the role of cAMP on the Butaprost inhibition of Fyn, the expression of cAMP-dependent protein PKA-Cα was downregulated by shRNA using a lentiviral system as described in Materials and Methods. shRNA-mediated silencing of PKA-Cα resulted in an 84% reduction in protein expression (Fig 6A). Butaprost effect on β-hexosaminidase release was completely abrogated in PDMC where the PKA-Cα was silenced (Fig 6B). Furthermore, we found that the inhibition of AKT phosphorylation by Butaprost was abrogated by downregulation of PKA-Cα expression in PDMC (Fig 6C).

Butaprost inhibits mast cell-mediated skin reactivity in vivo in a mouse model of passive cutaneous anaphylaxis

Animals locally sensitized with IgE in the ear skin and stimulated with DNP developed a significant increase (about 3 fold) in vascular permeability (as evidenced by extravasation of systemic

Evans blue into the ear) due to mast cell-mediated release of vasoactive mediators (Fig 7A). When WT mice were treated with the EP2 agonist Butaprost prior to Ag challenge, *in vivo* mast cell activation and thus local Evans blue extravasation, was significantly ($p<0.01$) decreased by 30% (Fig 7A). No significant effects were observed in the non-sensitized ears.

Mast cell deficient mice (Wsh) did not develop any reaction due to IgE/Ag stimulation (Fig 7B). When Wsh mice were intradermally reconstituted with WT PDMC and subjected to passive cutaneous anaphylaxis; they developed an inflammatory reaction (Fig 7B). This reaction was reduced by more than 60% when the Wsh mice reconstituted with PDMC were treated with Butaprost prior to Ag challenge (Fig 7B).

DISCUSSION

We have demonstrated that the inhibitory EP2 agonism on mast cell activity changes according to EP2 expression of mast cell type. We also describe that EP2-induced effect on mast cell activity is mediated by an increase of cAMP and activation of PKA that exerts an inhibition of Src-Fyn intracellular signaling pathway.

In agreement with our previous findings suggesting that EP2 mediates a PGE_2 negative regulation of mast cell release (31), we find that the relative expression of EP2 at the mRNA or protein levels in the various mast cell types is to some extent predictive of Butaprost effect on mast cell degranulation and calcium mobilization. BMMC and LAD2 showed basically no expression of EP2, correlating with the lack of Butaprost effect on degranulation and calcium mobilization in BMMC. However, mast cells with higher expression of EP2 at the message or protein levels exhibited reduced IgE/Ag-induced degranulation and calcium mobilization when treated with Butaprost. It is important to point that the Butaprost inhibition in HuMC was greater than C57 (which present more levels of EP2 at mRNA levels). This may suggest differential coupling efficiency to signaling components between these cell types as has been previously proposed (28). We also observed that, in general, the murine and human mast cell types presented an amount of EP2 mRNA corresponding to EP2 protein. Although, C57 showed the highest expression of EP2 at mRNA level, at the protein level the relative expression of this receptor was not dramatically different to that in PDMC suggesting differences in its protein turnover or its mRNA turnover depending on the type of mast cell. According to the *in vitro* EP2 inhibitory effect on mast cell activity, we demonstrate *in vivo* that specific engagement of the EP2 receptor also prevents mast cell mediated hypersensitivity

allergic reactions in a model of passive cutaneous anaphylaxis. Our data further support the notion that specific agonism of EP2 instead of PGE_2 may prove beneficial for the treatment of allergic diseases where mast cells are key effectors cells.

EP2 receptors couple to Gs, regulating intracellular cAMP production while EP3 receptors couple predominantly to Gi, regulating PLCβ and PI3Kγ activities (42, 44) EP2-mediated inhibition of mast cell late responses (19), T cell and other immune cells responses (45-47) have been linked to cAMP production. Elevations in the intracellular levels of cAMP induced by other stimuli, including Adenosine, β-adrenoreceptor agonists (48) and Ox40-Ox40L (49) have also been involved in the down-regulation of early mast cell responses, although the mechanisms are not well understood. In agreement, we demonstrate that cAMP induced by EP2 activation is a negative regulator of allergen-induced mast cell immediate responses in PDMC. cAMP exerts these effects via PKA since either antagonism of cAMP by an inactive cAMP analog or down-regulation of the catalytic subunit of PKA by shRNA completely reverted the suppressive effects of Butaprost in PDMC.

In T cells, cAMP inhibits IL-2 production and T cell proliferation and this negative regulation is also mediated by PKA (50). PKA is recruited to the immunological synapse in proximity with the TCR receptor. In this site PKA phosphorylates the C-terminal Scr kinase (Csk) which in turn phosphorylates the C-terminal inhibitory tyrosine residue in the Src kinases Lck and Fyn (Tyr-527). These phosphorylations prevent the activation of these Src kinases and T cell responses (50-51). In mast cells, two non-receptor Src family tyrosine kinases, Lyn and Fyn associate with the FcεRIβ subunit and are key for receptor phosphorylation and

the initiation of phosphorylation cascades that mediate mast cell responses (52-53). We find that activation of the EP2 receptor with Butaprost resulted in an overall reduction in the tyrosine phosphorylation of multiple proteins induced by engagement of the FcεRI. This fact suggests an interference with an initial step in the signaling cascade after mast cell FcεRI activation. Indeed, Butaprost prevented tyrosine phosphorylation of the activation loop of Src kinases (PTyr-416) induced by antigen and, specifically, Fyn phosphorylation. Consequently Butaprost also affected Fyn's ability to activate its downstream targets AKT and Gab2. In particular, the adaptor protein Gab2 once phosphorylated recruits and enables the activation of PI3K (54). This Butaprost effect was PKA-dependent because the inhibition of the phosphorylation of AKT was prevented upon downregulation of PKA-Cα in PDMC. However, unlike in T cells, we found no evidence for an involvement of the negative regulator Csk since treatment with Butaprost did not increase the phosphorylation of C-terminal inhibitory tyrosine residue Fyn (PTyr-527), a reflection of Csk activation (55). In Lyn-deficient mast cells Csk is not properly targeted and activated. As a consequence Fyn phosphorylation at PTyr-527 is reduced and Fyn activity increased (56). However, the negative effect of Butaprost on Fyn phosphorylation at PTyr-416 was still apparent in Lyn KO PDMC indicating a direct or indirect effect on the phosphorylation of this residue. Fyn has multiple serine phosphorylation sites that could affect its tyrosine kinase activity. For example, it has been reported that serine 21 in Fyn can be phosphorylated by PKA enhancing its activity (57). However, to the best of our knowledge, it is not known whether or not PKA could phosphorylate other residues that would down-regulate Fyn activity. Our study does not distinguish between a

possible direct effect by PKA on Fyn or for example, a disruption by PKA of the proper signaling complexes necessary for targeting and/or activation of Fyn in mast cells. But our results suggest a PKA-dependent, Csk-independent mode of regulation of Fyn by selective EP2 stimulation.

Another interesting aspect of this study is the finding that EP2 activation decreased intracytosolic calcium mobilization in PDMC and HuMC cultures (which highly express EP2) in front of BMMC (barely express EP2), where EP2 agonism had no effect on calcium mobilization. We also demonstrate that this decrease came from that EP2 agonism reduced antigen-induced calcium influx, without affecting calcium mobilization from intracellular stores in PDMC and HuMC. This contrasted with the positive effects of PGE_2 in calcium mobilization in mast cell types expressing more abundantly the EP3 receptor (31), which as described previously may regulate PLCβ and thus, release of calcium from intracellular stores. Calcium is an important regulator of mast cell function, particularly degranulation (58-59), so this process may be relevant to the suppressive effects of EP2. The Butaprost inhibition of calcium entry after antigen stimulation appears to be partially mediated by cAMP in PDMC, however the mechanism is unclear. Since EP2 reduced Fyn activation via cAMP, and Fyn has been involved in the regulation of calcium entry from the extracellular media via transient receptor potential channel type 1 (TRPC1) (54), we can speculate that the inhibition of Fyn activity may be a contributory factor to the regulation of mast cell calcium channels by Butaprost. Although a Fyn-independent channel regulation cannot be excluded. However, an additional component in the inhibition of calcium entry by Butaprost appears to be cAMP-independent since it was not completely abrogated by cAMP

antagonism in PDMC. In agreement, EP2 receptors were found to close the intermediate conductance Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ in human lung mast cells (21) by a Gs-mediated mechanism but cAMP-independent mechanism. This channel is known to affect directly IgE-dependent calcium influx and degranulation (60-62). The closure of this channel by EP2 agonism is related to an attenuation of migration and inhibition of degranulation in human lung mast cells.

In this article, we showed the EP2 inhibitory effect on mast cell activity *in vitro* and *in vivo* allergic response, and we described its main mechanism. We demonstrated that the specific mechanism involved in the EP2 beneficial effect is inhibiting mast cell degranulation through a cAMP via PKA impairing the activation of the IgE dependent Src-Fyn pathway.

In summary, our data contributes to the understanding of the mechanism by which the EP2 receptor attenuates mast cell responses. The effects of this mast cell prostanoid receptor in the suppression of cutaneous anaphylaxis as well as airway pathology (30) make EP2 a potential target for future therapies in allergic disease. The reproducibility of this effect in the human lung mast cells (20-21) and human mast cells reinforces its consideration for translational studies.

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Figure 1

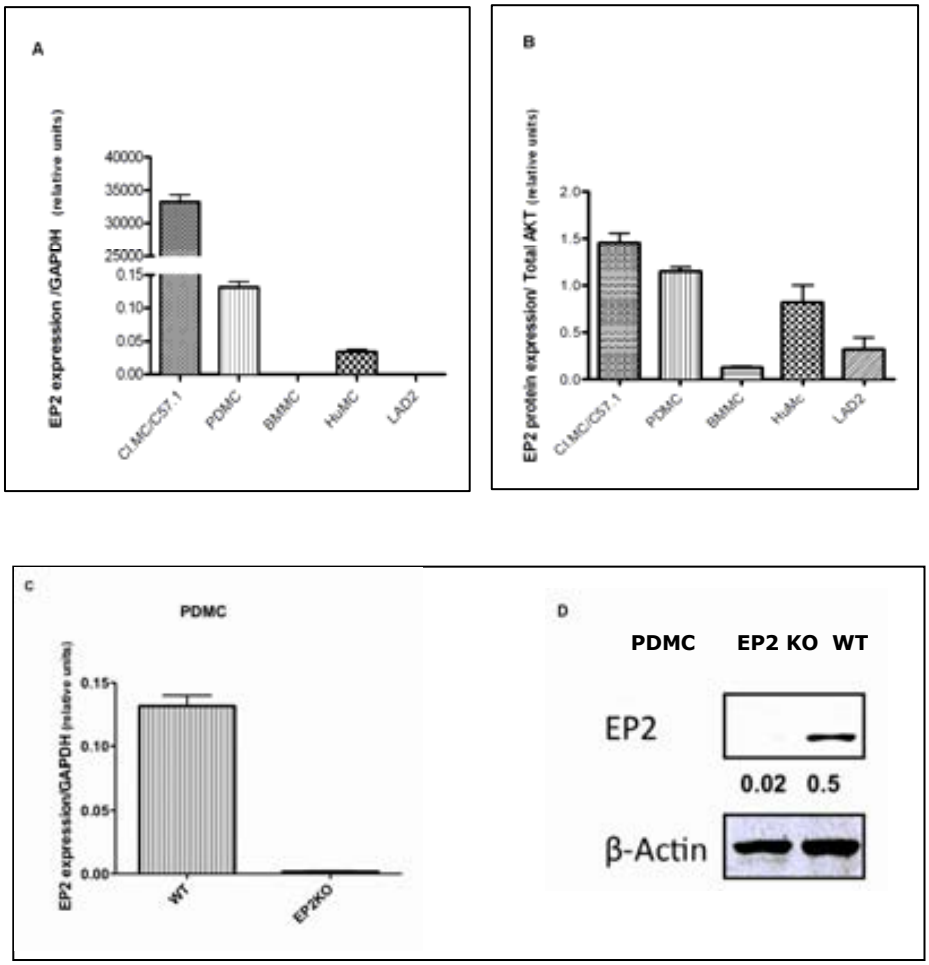
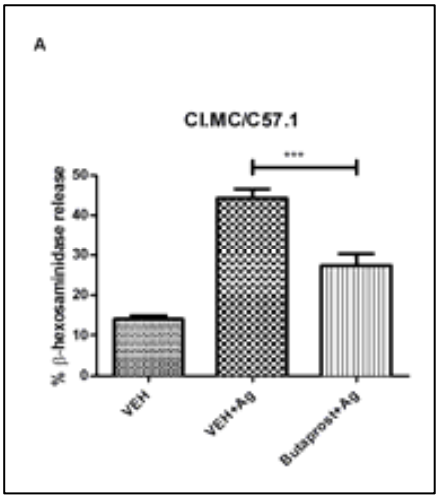


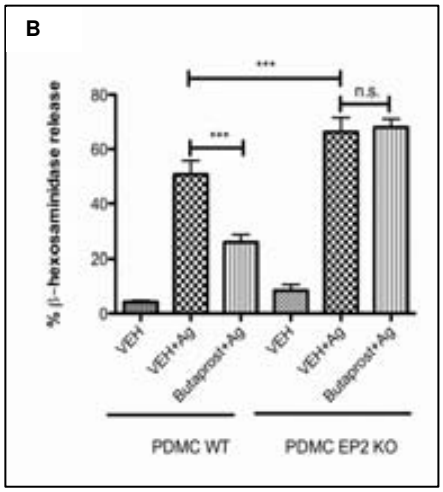
Figure 1. *Expression of EP2 in murine and human mast cells.* A) EP2 expression at mRNA level in the different murine (C57, PDMC, BMMC) and human mast cell (HuMC and LAD2) populations. Expression is represented as relative units of the comparative threshold method normalized to GAPDH expression (EP2 expression/GAPDH). Average results from 4 different experiments done in duplicate. B) EP2 expression at protein level in the different murine and human mast cell populations. Expression is quantified from 3 independent experiments where the intensity analysis (by infrared analysis) of the band corresponding to EP2

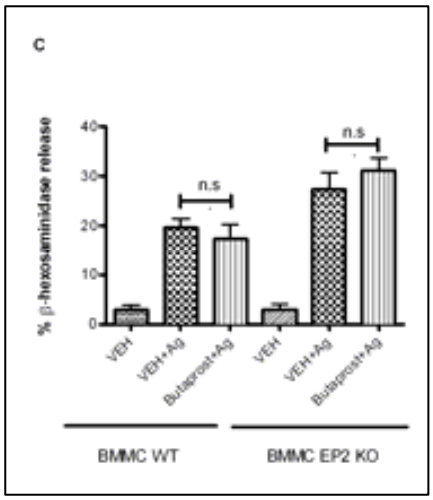
receptor was corrected to that of total AKT used as an internal control. In (C) and (B) it is determined the EP2 expression in WT and EP2 KO PDMC, respectively, at mRNA level (n=8) and protein level (n=3). In this case protein EP2 expression was corrected to β-actin internal control.

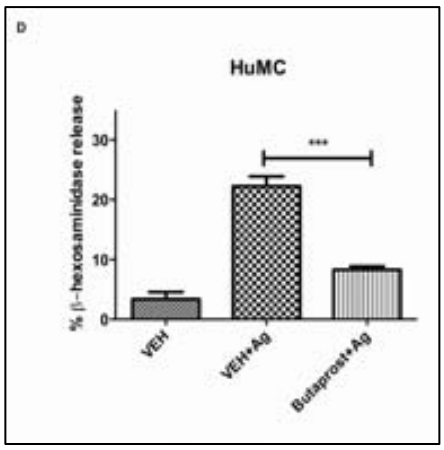
2

Figure 2


A
CLMC/C57.1
% β-hexosaminidase release
***
VEH
VEH+Ag
Butaprost+Ag


B
% β-hexosaminidase release
***
n.s.
VEH
VEH+Ag
Butaprost+Ag
PDMC WT
PDMC EP2 KO


C
% β-hexosaminidase release
n.s
n.s
VEH
VEH+Ag
Butaprost+Ag
BMMC WT
BMMC EP2 KO


D
HuMC
% β-hexosaminidase release
***
VEH
VEH+Ag
Butaprost+Ag

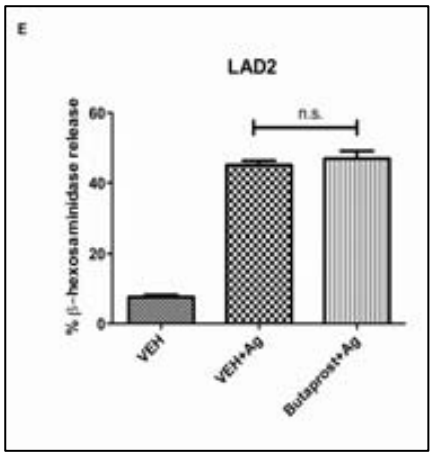

E
LAD2
% β-hexosaminidase release
n.s.
VEH
VEH+Ag
Butaprost+Ag

Figure 2. *Effect of the selective EP2 agonist, Butaprost, on FcεRI-induced mast cell degranulation.* In these figures it is shown the effect of Butaprost in β-hexosaminidase release in murine mast cells: C57 (A), PDMC (B) and BMMC (C) and in human mast cells: HuMC (D) and LAD2 (E) and PDMC and BMMC derived from the EP2 KO mouse (B and C, respectively). Results are representative of at least 4 independent experiments done in triplicate and are expressed as mean±SD.

(***Pvalue<0.001)

Figure 3

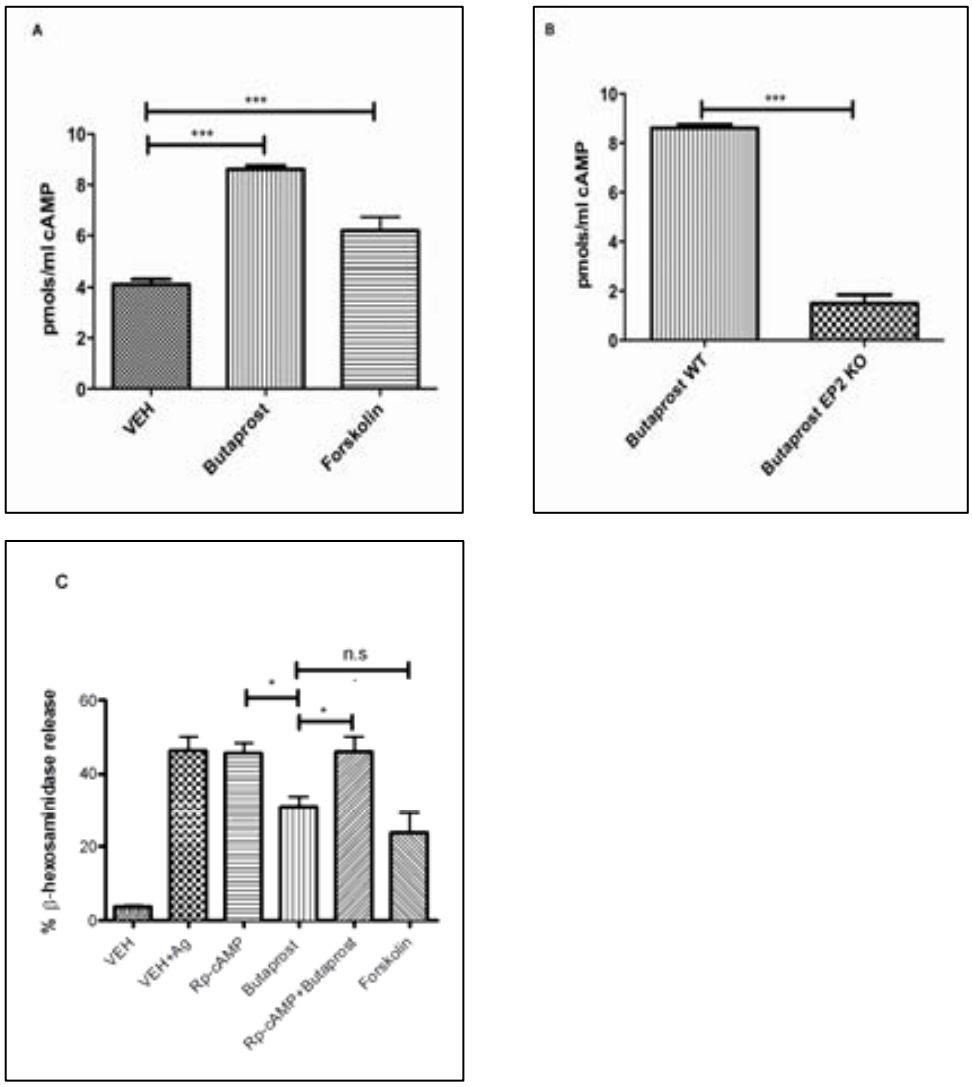
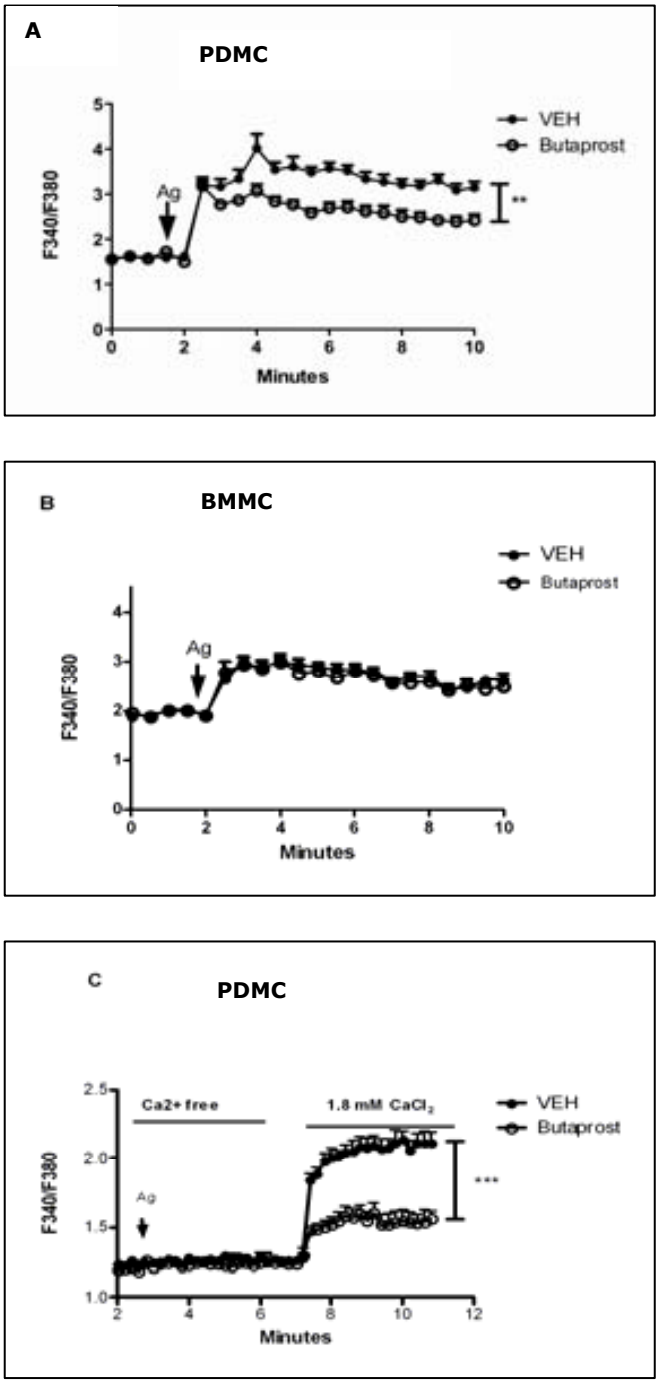


Figure 3. *Relation of cAMP and Butaprost effect on PDMC degranulation.* cAMP accumulated in the cells was measured by ELISA in WT PDMC (A) and EP2 KO PDMC (B) pretreated with VEH, Butaprost or Forskolin. Results are an average of 2 independent

experiments done in duplicates and are expressed as mean±SEM. C) The relation of cAMP with Butaprost effect on degranulation was determined on β-hexosaminidase release of PDMC pretreated with an antagonist of cAMP. Pretreatment with Forskolin was used as positive control of cAMP activity. Results are an average of 2-4 independent experiments done in duplicate and are expressed as mean±SEM.

(*P value<0.05, ***P value<0.001)

Figure 4



PDMC

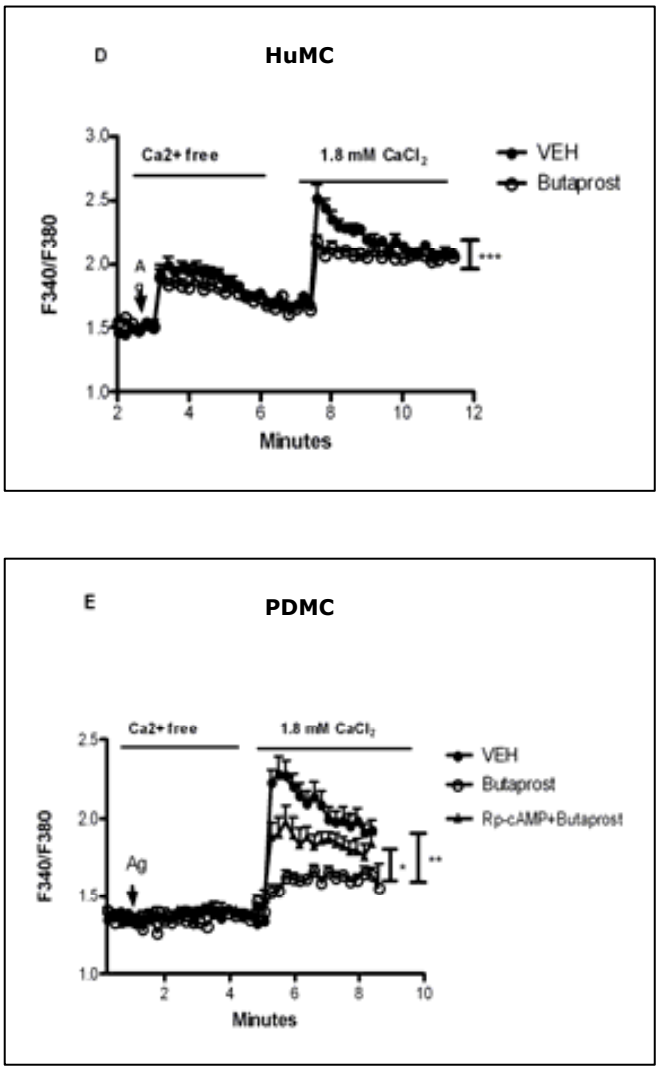
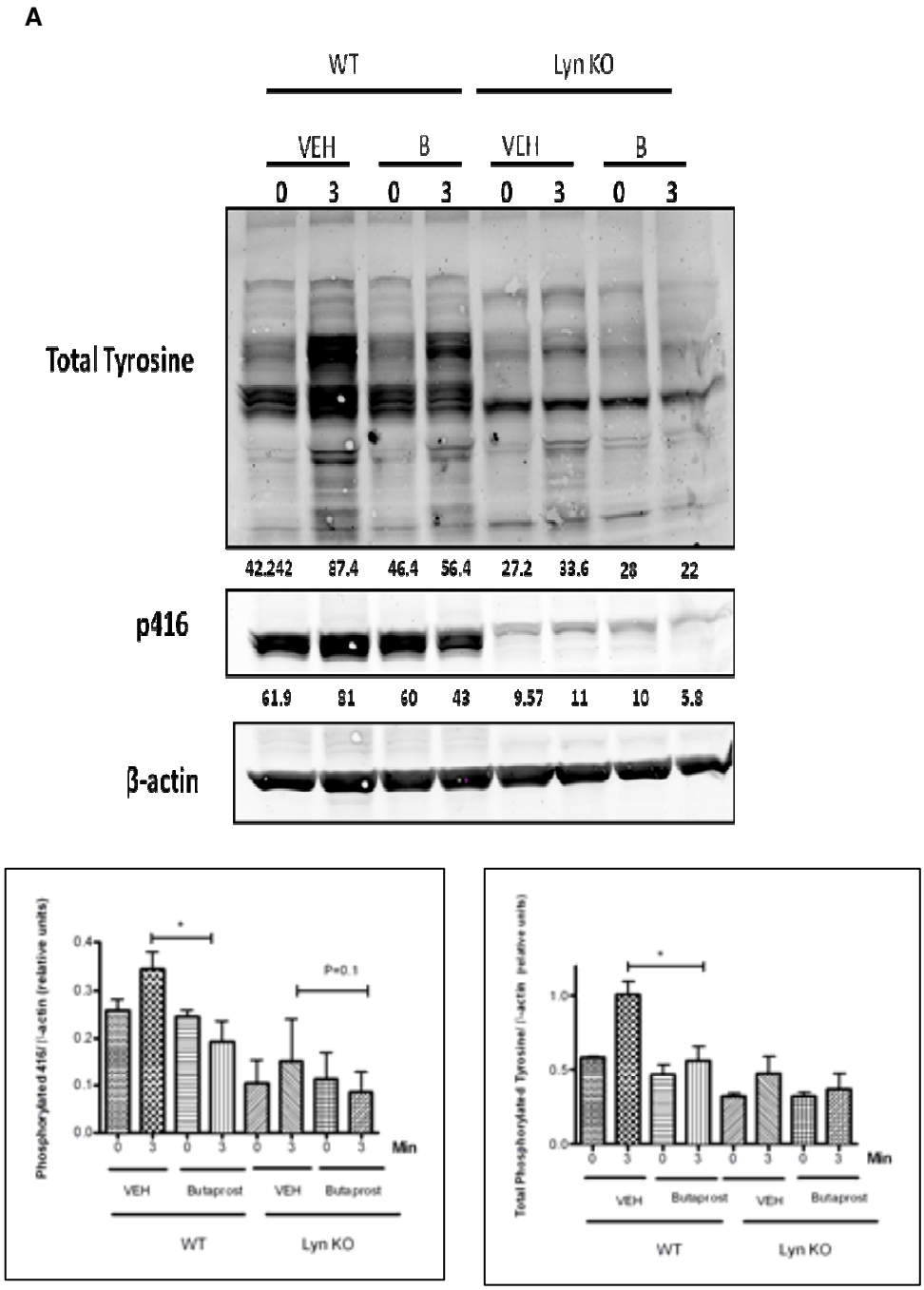


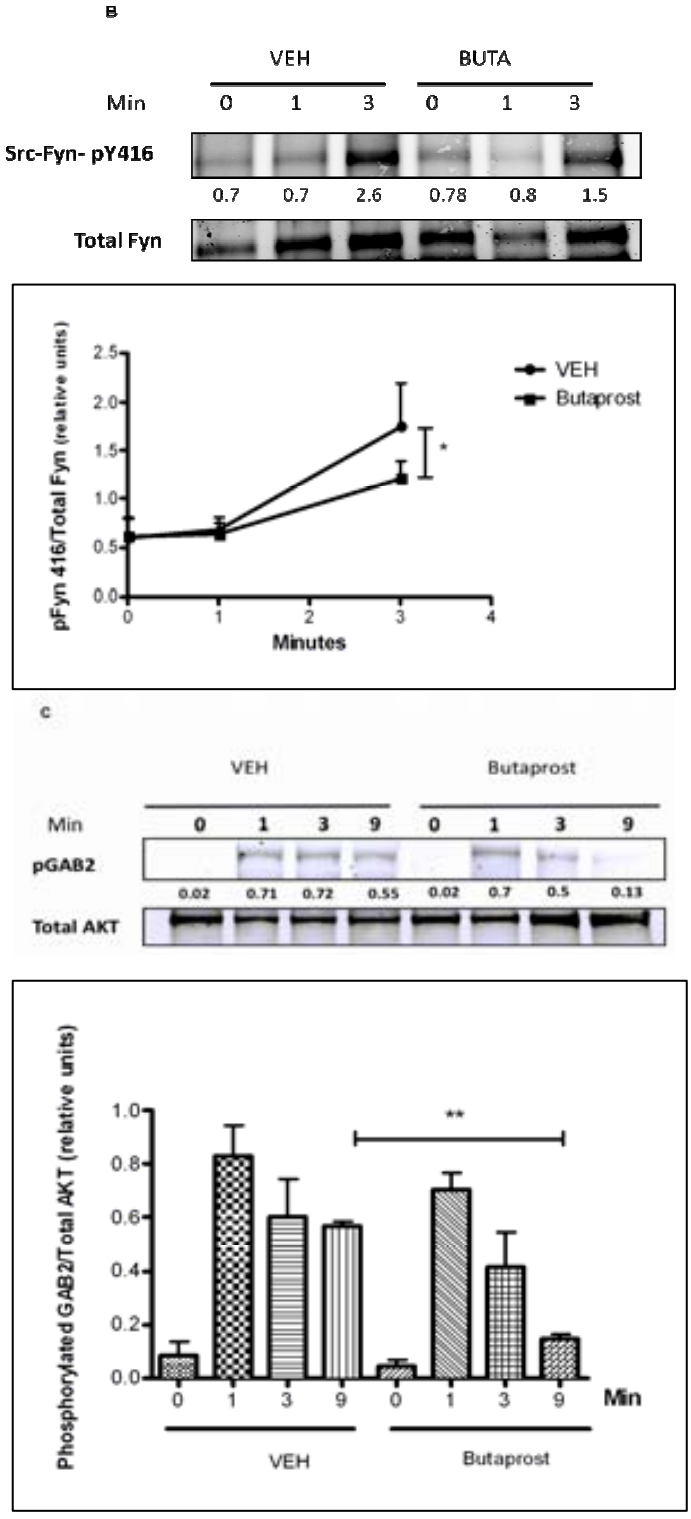
Figure 4. *Effect of Butaprost on calcium mobilization and their relation with cAMP increase.* Effect of Butaprost in intracytosolic calcium mobilization after stimulation with IgE/Ag in FURA2-loaded PDMC (A) and BMMC (B) were monitored as emission at 510 nm and 340 nm excitation compared to emission at 510 nm at 380 nm excitation for each time point. FURA2-loaded PDMC (C) and HuMC (D) were stimulated with Ag in the absence of

extracellular calcium to measure calcium release. When indicated, $CaCl_2$ was added to the media to promote calcium influx. Changes in intracytosolic calcium concentration were monitored as in A. E) The role of cAMP in Butaprost effect on calcium influx was determined in PDMC pretreated with Rp-cAMP for 1 h prior to Ag stimulation. Results are the average of 6-8 independent experiments.

(*P value<0.05, **P value<0.01 and ***P value<0.001 using a ANOVA-2 test).

Figure 5


A
WT
Lyn KO
VEH
B
VEH
B
0 3 0 3 0 3 0 3
Total Tyrosine
42.242 87.4 46.4 56.4 27.2 33.6 28 22
p416
61.9 81 60 43 9.57 11 10 5.8
β-actin
Phosphorylated 416/ β-actin (relative units)
0.4
0.3
0.2
0.1
0.0
*
P=0.1
0 3 0 3 0 3 0 3
Min
VEH
Butaprost
VEH
Butaprost
WT
Lyn KO
Total Phosphorylated Tyrosine/ β-actin (relative units)
1.0
0.5
0.0
*
0 3 0 3 0 3 0 3
Min
VEH
Butaprost
VEH
Butaprost
WT
Lyn KO


B
VEH
BUTA
Min
0 1 3 0 1 3
Src-Fyn- pY416
0.7 0.7 2.6 0.78 0.8 1.5
Total Fyn
pFyn 416/Total Fyn (relative units)
VEH
Butaprost
*
Minutes
C
VEH
Butaprost
Min
0 1 3 9 0 1 3 9
pGAB2
0.02 0.71 0.72 0.55 0.02 0.7 0.5 0.13
Total AKT
Phosphorylated GAB2/Total AKT (relative units)
**
Min
VEH
Butaprost

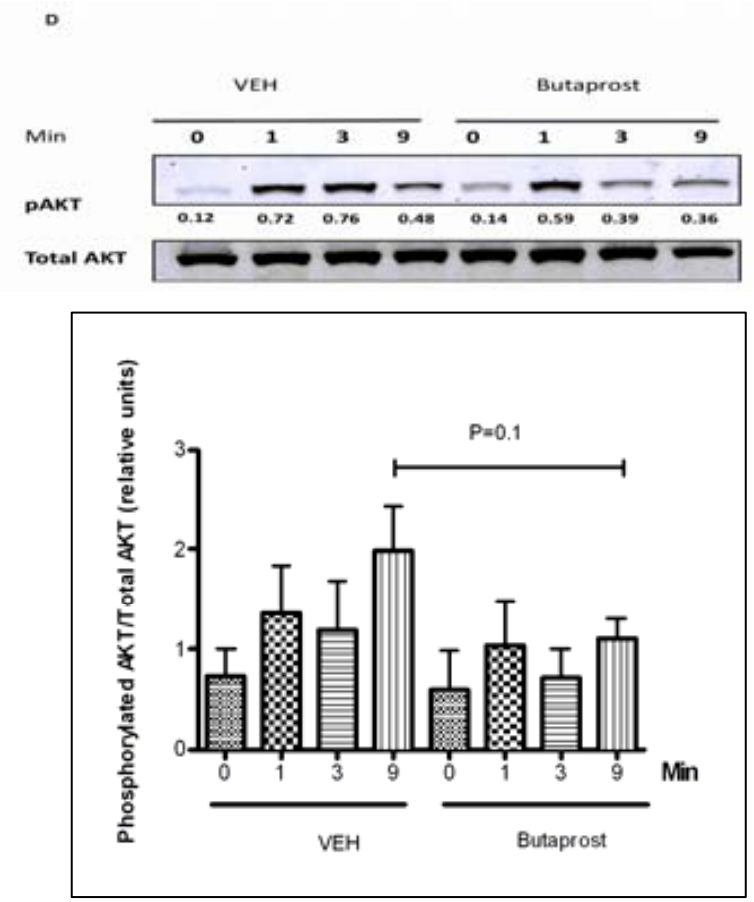


Figure 5. *Effect of Butaprost in Src-Fyn kinase signaling by IgE/Ag.* A) Butaprost effect on the phosphorylation of general tyrosine activity and Src kinase tyrosine 416 from IgE/Ag stimulated WT and Lyn KO PDMC at 3 min. Panels below show the average intensity of the indicated band corrected to β-actin internal control in 2 independent experiments. B) Src-Fyn was immunoprecipitated from IgE/Ag stimulated PDMCs treated or not with Butaprost. The immunoprecipites were probed with anti-PY416-Src to determine Fyn activation in 3 min. Right panel shows the average changes in the intensity of PY416-Src corrected to total Fyn internal control in 3 independent experiments (*P value<0.05 using ANOVA-2 test). Phosphorylation of Gab2 (C) and

AKT (D) at the indicated times after stimulation with IgE/Ag in PDMC treated or not with Butaprost. Right panel shows the average changes in the intensity of indicated band corrected to total AKT internal control in 3 independent experiments.

(*P value<0.05, **P value<0.01)

Figure 6

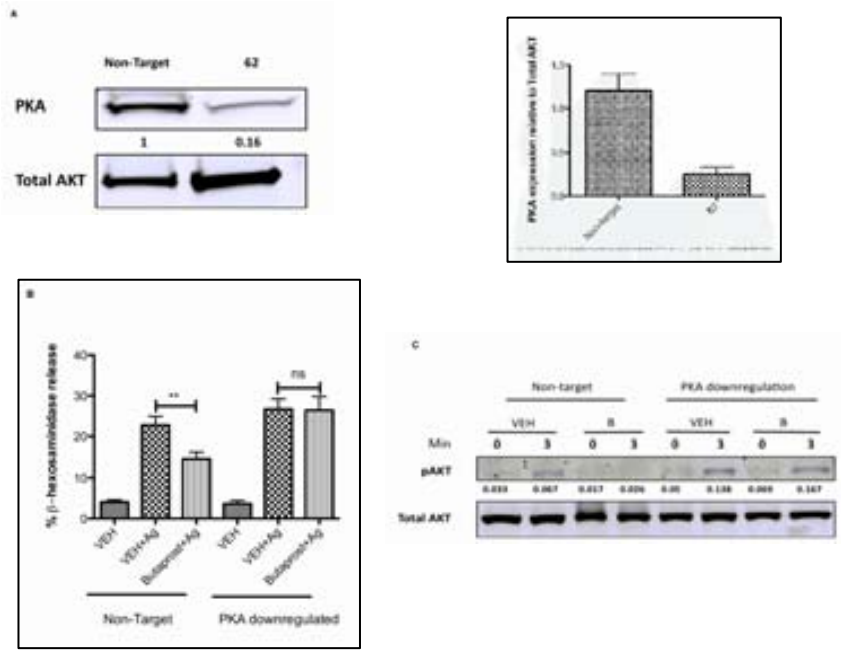


Figure 6. *Impact of PKA-Cα downregulation on Butaprost effect on mast cell degranulation and Src-Fyn kinase signaling pathway.* A) PKA-Cα protein expression was measured by western blot after lentiviral shRNA downregulation. Right panel shows the average changes in the intensity of PKA-Cα corrected to total AKT internal control in 3 independent experiments. B) Effect of Butaprost on IgE/Ag degranulation from cells where PKA-Cα was downregulated. Data represent an average of 2 independent experiments done with n=8. C) Effect of PKA-Cα downregulation on the suppression of AKT phosphorylation by Butaprost. Quantification of expression is calculated from the intensity of PKA-Cα corrected to total AKT internal control in an experiment.

(**P value<0.01)

Figure 7

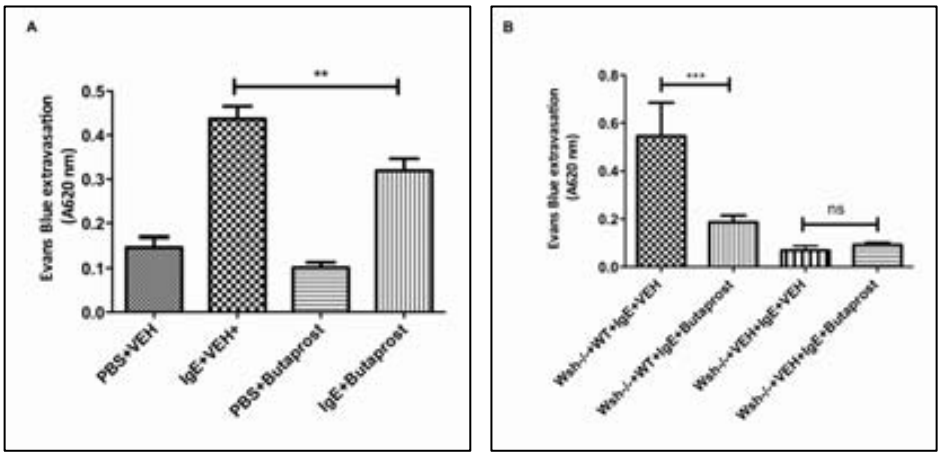


Figure 7. Effect of *Butaprost on FcεRI-induced mast cell immediate responses in vivo.* A) It is shown the absorbance measured from the extravasation of Evans Blue into the ears, which represents the skin hypersensitivity reaction induced by local sensitization with IgE/DNP (passive cutaneous anaphylaxis, PCA) from mice that has been intravenously treated with Vehicle or Butaprost (n=10). B) Wsh mice were reconstituted with PDMC intradermally 6 weeks prior to the induction of PCA. Mice were then treated as in A (n=4-5).

(**Pvalue<0.01 and ***Pvalue<0.001)

Discussió general

(Català)

6. Discussió general

En aquesta tesi doctoral s'han fet 3 observacions principals:

-La PGE_2, *in vitro*, és capaç d'interactuar directament amb diverses poblacions mastocitàries (humanes i de ratolí). L'efecte resultant és la inhibició de l'activitat mastocitària si el receptor predominant en la superfície mastocitària és l'EP2.

-La PGE_2, a través del receptor EP2, té un efecte protector *in vivo* en el desenvolupament de la resposta asmàtica en ratolins sensibilitzats a HDM acompanyat d'una disminució de l'activitat mastocitària.

-L'activació del receptor EP2 dels mastòcits inhibeix la mobilització de calci i la via de senyalització de la Src-Fyn i provoca un augment d'AMPc/PKA, en mastòcits activats via IgE.

La conjectura d'aquests resultats permet suggerir que l'efecte protector de la PGE_2 en models animals d'asma, i possiblement en pacients asmàtics, ve donat pel bloqueig de les vies de senyalització implicades en l'activació dependent d'IgE dels mastòcits bronquials, originada per la interacció directa de la PGE_2 amb els receptors EP2 mastocitaris. En aquesta discussió analitzarem amb més detall els resultats que ens han conduït a formular aquesta afirmació.

6.1. L'estimulació selectiva d'EP2 inhibeix la sensibilització a HDM, en ratolins

Hem demostrat que l'administració d'un agonista selectiu EP2 (Butaprost) per via intranasal és capaç d'inhibir l'AHR i la

inflamació desenvolupades com a conseqüència de la sensibilització a aeroal.lergens d'àcars de la pols (HDM). Butaprost causa una reducció significativa de l'AHR, tant en la medició invasiva, com en la no invasiva. Aquesta observació reprodueix la que altres grups i nosaltres vam fer amb l'administració subcutània de PGE_2 en ratolins sensibilitzats a HDM (Herrerias et al.,2009), i a OVA (De Campo & Henry 2005). El paper d'EP2 en prevenir el desenvolupament de l'AHR induïda per l'al·lergè també es veu reflectit en l'efecte resultant de l'administració de l'antagonista EP1/EP2. Quan l'EP2 és bloquejat, s'aprecia un empitjorament de l'AHR en els animals sensibilitzats a HDM. D'altra banda, Butaprost causa una disminució significativa del total de les cèl·lules inflamatòries en les vies respiratòries, deguda principalment a la disminució de la eosinofília, similar a la que s'observa en administrar la PGE_2. Aquests resultats coincideixen amb els observats per Sturm et al. (2008), el quals van veure que –en animals sensibilitzats a OVA– tant PGE_2, com Butaprost, causava una reducció de l'eosinofília en BAL. Així doncs, el paral·lisme d'efectes de la PGE_2 i de l'agonista EP2, en els models d'asma, suggereix que l'efecte beneficiós de la PGE_2 *in vivo* observat en ratolins, i probablement, també l'observat en pacients asmàtics, és mediat principalment pel receptor EP2. Aquests indicis són recolzats per dades aportades pel nostre grup de recerca, atès que hem observat que, en el model murí de sensibilització a HDM, sembla haver-hi un mecanisme de regulació immunitària basat en un augment de la PGE_2 endògena, associat a un augment de l'expressió dels receptor EP2 en els pulmons accentuat per l'adminstració exògena de PGE_2 (Herrerias et al., 2009). En concordança amb aquestes observacions, Sturm et al. (2008) també van detectar un augment en l'expressió d'EP2 en

l'epiteli de les vies aèries i en l'infiltrat peribronquial, després del *challenge* en ratolins sensibilitzats a OVA.

Aquests resultats contradiuen els publicats per Kunikata et al. (2005), que van atribuir l'activitat protectora de la PGE2 al receptor EP3. Aquests autors van descriure que, en ratolins sensibilitzats a OVA, l'adminstració d'un agonista EP3 tres hores després de l'exposició a l'al·lergè, causa una reducció de l'AHR. Cal considerar que l'al·lergè emprat i el protocol d'administració del fàrmac disten de les condicions experimentals utilitzades per nosaltres, cosa que pot justificar la diferència en els resultats. Aquesta explicació a les discrepàncies es veu reforçada per les nostres observacions (Herrerias et al., 2009), vam avaluar l'efecte d'un agonista EP1/EP3 en un model murí de sensibilització a àcars de la pols i no vam observar cap efecte beneficiós, ni en l'AHR, ni en la inflamació de les vies aèries.

En conseqüència, es demostra en un model de sensibilització als principals aeroal·lergens responsables de l'asma en humans, que el receptor EP2 és probablement el receptor de la PGE_2 principalment implicat en l'efecte protector de la PGE_2 observat en l'asma.

6.2. Cèl·lules implicades en la resposta protectora exercida per l'eix PGE_2-EP2: els mastòcits

6.2.1. L'efecte de la PGE_2 en la resposta asmàtica és mediat per l'EP2 mastocitari

És important elucidar quina és la cèl·lula efectora que expressa el receptor EP2 on la PGE_2 exerceix principalment el seu efecte

protector *in vivo.* El receptor EP2 es troba àmpliament distribuït en cèl·lules efectores de la inflamació: com macròfags, eosinòfils, limfòcits Th_1, Th_2, Th_{17} (Sakata et al., 2010) i mastòcits (Honda et al., 1993; Nishigaki et al., 1993; Chan et al., 2000; Nguyen et al., 2002; Feng et al., 2006; Kay et al., 2006). També es troba en altres cèl·lules com epitelials, fibroblasts i musculars, les quals intervenen en la patologia respiratòria (Lee et al., 2010; Ben-Av et al., 1995; Kasugai et al., 1995; Nakata et al., 1992). Per aquest motiu, no resulta fàcil identificar quina és la cèl.lula amb la que interactua principalment la PGE_2 per produir l'efecte protector. En els resultats obtinguts en la present tesi hi ha 3 tipus de dades que indiquen que la població candidata que expressa el receptor EP2 i responsable de l'efecte observat, tant amb l'agonista EP2, com amb la PGE_2, són els mastòcits.

El primer resultat que apunta cap als mastòcits com a cèl·lula diana de la PGE_2 és: la fluctuació de l'activitat mastocitària de les vies respiratòries observada en els ratolins sensibilitzats a àcars de la pols i tractats. Tant en els ratolins pre-tractats amb PGE_2, com en els pre-tractats amb Butaprost, s'aprecia una millora de la patologia respiratòria, acompanyada d'una reducció significativa de la mMCP-1 en pulmó, un medidador específic de mastòcits. La disminució d'aquesta proteasa reflecteix una disminució en l'alliberament d'altres mediadors dels mastòcits bronquials, és a dir, en l'activitat d'aquesta població cel·lular. Aquests resultats estan en consonància amb els observats anteriorment al nostre laboratori, que indiquen que la PGE_2 administrada subcutàniament actua en la resposta de sensibilització a HDM acompanyada també d'una disminució d'activitat mastocitària (Herrerias et al., 2009). Recentment, vam publicar que en inhibir la COX-2, és a dir, la producció de PGE_2 endògena entre d'altres, els ratolins

sensiblitzats a OVA presentaven un augment en l'AHR i la inflamació acompanyat d'un increment de la mMCP-1 al pulmó (Torres et al., 2009). Tot i que existeixen estudis que relacionen *in vitro* l'activació d'EP2 amb l'activitat mastocitària, i l'efecte de la PGE_2 en asma (Duffy et al., 2008; Kay et al., 2006; Feng, et al., 2006), els nostres resultats involucren, per primer cop, els mastòcits pulmonars i el seu receptor EP2 com a elements clau en la protecció exercida per PGE_2 en la resposta asmàtica *in vivo.* D'altra banda, en aquest treball s'ha determinat com la PGE_2 és capaç d'actuar directament sobre mastòcits tant humans com murins en cultiu i exercir un efecte inhibitori només en els mastòcits que sobreexpressen EP2. En contraposició, també hem vist que, quan les cèl·lules expressen amb predominança EP3, enlloc d'EP2, PGE_2 no exerceix efecte inhibitori, sinó que magnifica l'activitat mastocitària. Weller et al. (2007) i Nguyen et al. (2002) també han observat aquesta resposta *in vitro* en BMMC; mastòcits que expressen únicament: EP1, EP3 i EP4. Això reforça el paper atribuït al receptor EP2 en la protecció exercida per la PGE_2. La capacitat inhibitòria de la PGE_2, a través d'EP2 sobre l'activitat mastocitària, també ha estat determinada en diferents estudis en HLMC i hMCs (Duffy et al., 2008; Kay et al., 2006; Feng, et al., 2006).

Per últim, els nostres resultats *in vitro*, amb mastòcits aïllats de ratolins mancats del receptor EP2, mostren que la PGE_2 no és capaç d'inhibir l'activitat mastocitària. És a dir, confirmen que l'efecte bloquejant de la PGE_2 en els mastòcits és totalment dependent del receptor EP2.

Amb aquests resultats es constata el rol predominant del receptor EP2 en l'efecte de la PGE_2 i s'evidencia la probable importància de

la seva presència en una cèl·lula efectora clau en l'asma, com és el mastòcit (Williams & Galli, 2000). Aquestes 3 observacions no ens permeten afirmar categòricament que el receptor EP2 mastocitari estigui directament involucrat en l'efecte beneficiós de la PGE_2 observat *in vivo*, però sí que ens permet considerar-lo com a molècula candidata o target, per al futur desenvolupament d'estratègies terapèutiques antiasmàtiques.

6.2.2. La modulació de l'activitat mastocitària mitjançant PGE_2-EP2 modula la resposta a aeroal·lergens

Un altre aspecte important –fins i tot més rellevant– a considerar és en quina mesura el receptor EP2 mastocitari és responsable directe de la millora de la patologia respiratòria atribuïda a la PGE_2. És a dir, detectar si és la inhibició de l'activitat mastocitària explica la millora d'AHR i la inflamació de les vies aèries observada en els ratolins exposats a HDM i tractats amb PGE_2. Vam abordar aquesta qüestió en 3 experiments.

En primer lloc, vam establir un model on vam veure com mediadors inflamatoris procedents de mastòcits cultivats *in vitro,* administrats intranasalment, són capaços de desenvolupar AHR en ratolins naïve (no sensibilitzats). Altres autors també han demostrat de manera més indirecta l'important paper dels mastòcits en el desenvolupament d'AHR, per exemple, Nakae et al. (2007) han demostrat que ratolins desproveïts de mastòcits disminueixen significativament l'AHR i la inflamació de les vies aèires al sensibilitzar amb OVA. Fattouh et al. (2011) han comprovat com en un model murí crònic de sensibilització a HDM, quan es suprimeixen els eosinòfils i es mantenen els nivells d'IgE,

tant l'AHR, com la resposta immunitària corresponent, es desenvolupen normalment. Mayr et al. (2002) han percebut que ratolins sensibilitzats a OVA i tractats amb l'anticòs IL-5, per tal de suprimir l'eosinifília, mantenen l'AHR. Quan aquests ratolins són desproveïts del receptor d'IgE (FcεRI -/-), l'hiperreactivitat bronquial és suprimida. Aquestes determinacions situen als mastòcits com a cèl·lula clau en la patogènia de l'asma. En el nostre model *in vitro/in vivo* hem demostrat com l'administració a ratolins naïve de mediadors inflamatoris procedents de mastòcits tractats amb Butaprost (agonista EP2) reverteix l'AHR. Aquesta observació ens suggereix com la modulació d'EP2 mastocitari repercuteix en el desenvolupament i la intensitat de l'AHR.

El segon experiment on ens mostra com l'efecte protector *in vivo* de l'estimulació selectiva del receptor EP2 en la resposta al·lèrgica és atribuïble al receptor EP2 mastocitari, és el del model d'anafilaxi cutània. En aquest model és conegut que la resposta tissular cutània és conseqüència únicament de l'activitat mastocitària. Per un costat, hem vist com l'agonista EP2 Butaprost inhibeix significativament la resposta al·lèrgica cutània principalment mediada per l'activació de mastòcits a través d'IgE. D'altra banda, hem vist com l'efecte de Butaprost, en aquesta resposta al·lèrgica, és directament dependent de la presència de mastòcits en ratolins desproveïts de mastòcits (Wsh) reconstituïts amb PDMC. Aquest efecte de l'agonista EP2, observat en models d'anafilaxi, reprodueix l'efecte preventiu de la PGE_2 observat en models d'anafilaxi en ratolins (Raud et al., 1996; hedgvist et al., 1989; Raud et al., 1988; Lake et al., 1984). Així, queda palesa la relació entre la modulació de l'activitat mastocitària a través del receptor EP2 i la seva implicació en la resposta al·lèrgica.

Vam verificar la rellevància del mastòcits en l'efecte preventiu de PGE_2 en l'asma es fa patent en experiments recents (no inclosos en els subprojectes) que demostren que la PGE_2 no exerceix el seu efecte protector en un model de sensibilització a HDM en ratolins C57/BL6 on el mecanisme de desenvolupament de la patologia respiratòria no implica el mastòcit (Pae et al., 2010; Becker et al., 2011). En contraposició, es mostra com en el model de sensibilització a HDM en BALB/c, on consta una implicació dels mastòcits en la resposta de les vies aèries (Pae et al., 2010; Becker et al., 2011), la PGE_2 exerceix el seu efecte protector.

Totes aquestes dades apunten que l'efecte protector de la PGE_2 *in vivo* ve donat per la capacitat de la PGE_2 de contenir l'activitat dels mastòcits a través de la seva interacció directa amb el seu receptor EP2. Aquests resultats també indiquen que aquesta contenció redueix l'alliberament de mediadors que, si més no, participen en l'AHR.

Per tal de conèixer els mecanismes moleculars que porten a l'efecte inhibitori de l'activitat mastocitària via PGE_2-EP2 ens vam plantejar estudiar amb detall les molècules intracel·lulars involucrades en l'activació del mastòcit dependent d'IgE i la seva modulació per part de l'agonista EP2. L'exploració d'aquestes vies és un pas rellevant en la identificació de noves possibles dianes terapèutiques.

6.3. L'efecte inhibitori d'EP2 en l'activació mastocitària dependent d'IgE és mediat pel bloqueig de la via de senyalització de la Src-Fyn mitjançant AMPc/PKA

6.3.1. Via de Src-Fyn: principal via de senyalització implicada en l'efecte inhibitori d'EP2

Tres experiments ens han permès identificar la via de senyalització principalment implicada en la inhibició per agonisme EP2 de la degranulació induïda per FcεRI.

El primer indicador de la via de senyalització implicada en el mecanisme d'EP2 és la mobilització de calci; indispensable perquè es doni la degranulació (Vig et al., 2009; Habara et al., 1996; Putney et al., 1993; Janiszewkski et al., 1992). Hem observat que tant en mastòcits murins, com en mastòcits humans que expressen EP2, Butaprost disminueix de manera significativa la mobilització de calci citosòlic mitjançant, únicament, el bloqueig dels canals de calci extracel·lular, i no els canals de calci intracel·lulars. Aquests resultats són coincidents amb els determinats per Duffy et al. (2008), que observaven com l'estimulació selectiva del receptor EP2 tanca els canals $K_{Ca}3.1$. Aquests canals de potassi dependents de calci es localitzen extracel·lularment i s'associen majoritàriament a la migració dels mastòcits i amb la degranulació (Cruse et al., 2006; Duffy et al., 2004; Duffy et al., 2001).

Suzuki et al. (2010) van demostrar la implicació de Fyn en la regulació de l'entrada de calci extracel·lular pel canals TRPC1. D'acord amb aquestes dades la inhibició dels canals de calci extracel·lulars per part d'EP2 és una prova indirecta de la impossibilitat de la via de senyalització de Fyn d'activar aquests canals.

També observem que tot i l'absència de la Src-Lyn en PDMC obtinguts de ratolins Lyn KO (Lyn -/-), una de les proteïnes senyalitzadores protagonista en la degranulació mastocitària, l'agonista EP2, és capaç d'inhibir la fosforilació de les tirosines implicades en l'activació mastocitària dependent d'IgE, tals com la fosforilació de la tirosina 416 total. Aquests fets indiquen que Lyn no és necessària perquè es doni la inhibició de la degranulació mastocitària per EP2; i connota la possible implicació de Fyn en l'efecte bloquejant d'EP2, ja que aquesta proteïna conté activitat tirosina 416 (Parravicini et al., 2002).

Aquests indicis es confirmen en comprovar que Butaprost inhibeix significativament la fosforilació de la tirosina 416, específicament de Fyn, i de Gab2 i AKT, proteïnes que es troben en la cascada de senyalització de Fyn (Suzuki et al., 2010), en PDMCs Ag-IgE estimulades.

En aquesta tesi, intentem demostrar com la inhibició de la via de senyalització de Fyn, i la conseqüent disminució del calci dels canals extracel·lulars, és un mecanisme bàsic de l'efecte inhibitori de l'eix PGE_2-EP2 en el mastòcit. La versemblança dels resultats, pel que fa al bloqueig dels canals de calci extracel·lular per part d'EP2 en HuMC, suggereix que el Butaprost en mastòcits humans també actua mitjançant aquesta via de senyalització. La determinació de les molècules intracel·lulars mediadores d'aquest procés és vital per a la comprensió completa del mecanisme protector de PGE_2-EP2 en l'asma i en el potencial avantatge que ofereix des del punt de vista terapèutic.

6.3.2. AMPc/PKA intervenen en la inhibició de la via de la Src-Fyn exercida per EP2

Es coneix que l'estimulació selectiva d'EP2 en hMCs indueix l'augment de AMPc i, en aquesta via, l'activació de PKA inhibeix la producció de citocines (Feng et al., 2006). Però encara és desconegut a quina via de senyalització està afectant.

En aquest estudi s'ha demostrat que el paper d'AMPc/PKA és inhibir la via de senyalització Fyn, crucial perquè l'efecte de l'EP2, en l'activitat mastocitària, es doni. Principalment, 2 experiments donen suport a aquesta conclusió.

A l'inici, veiem com el pretractament del mastòcits amb un antagonista d'AMPc bloqueja completament l'efecte inhibitori de l'agonista EP2 sobre la degranulació mastocitària IgE-Ag i bloqueja parcialment la disminució de l'influx de calci extracel·lular causada per l'estimulació d'EP2. L'acció d'EP2 mitjançant AMPc sobre l'activitat mastocitària ha quedat demostrada en diferents estudis (Feng et al., 2006; Kay et al., 2006). Però la seva essencialitat en l'efecte d'EP2, en l'activitat mastocitària, encara no s'havia comprovat. Només en altres cèl·lules, com els limfòcits T, on s'observava que la PGE_2 via EP2 prevé la producció de citocines mitjançant un increment de AMPc i activació de PKA (Su et al., 2011). Aquestes dades constaten, l'indispensable paper de l'AMPc induït per tal que es doni l'efecte inhibitori d'EP2. D'altra banda, el bloqueig parcial de l'efecte de Butaprost sobre els canals de calci extracel·lular per l'antagonista d'AMPc ens suggereix que l'AMPc es troba regulant de manera parcial la mobilització de calci. Fet que pot anar lligat a la presència de canals extracel·lulars, també regulats per EP2, que contribueixen en al influx de calci

extracel·lular i actuen de manera independent de Fyn i de AMPc, tals com els K_{Ca}3.1. descrits per Duffy et al. (2008).

Per últim, el fet de reprimir l'expressió de PKA en PDMC fa que l'AMPc no pugui actuar sobre ell en estimular l'EP2. Així que, en aquestes cèl·lules que no expressen PKA, l'agonista EP2 no aconsegueix inhibir la degranulació ni la fosforilació d'AKT en l'activació mastocitària dependent d'IgE. Aquest fets demostren una vegada més el paper essencial d'AMPc en l'efecte inhibitori d'EP2 en l'activitat mastocitària; i evidencien, d'una manera més directa, com l'activació de PKA induïda per AMPc és una peça clau per a la inhibició de la via de Fyn. En cèl·lules de micròglia de ratolins s'ha fet un observació molt semblant a la nostra. Han vist que l'estimulació selectiva d'EP2 produeix un increment d'AMPc i, per via PKA, causa una inhibició d'AKT (Kawashita et al., 2011). Aquesta apreciació també s'ha determinat en altres cèl·lules com queratinòcits (Chun et al., 2010) i " *lymphokine activated killer (LAK) cells"* (Su et al., 2008).

Per tant, aquests experiments ens demostren que l'estimulació selectiva d'EP2 suprimeix l'activació mastocitària dependent d'IgE mitjançant la inhibició de la via de senyalització de Src-Fyn, mitjançant l'increment d'AMPc i l'activació de PKA. La qual cosa reforça la potència de la via PGE_2-EP2-MC com a eina d'exploració de noves dianes terapèutiques per l'asma.

Conclusions

7. Conclusions

(each conclusion is linked to each specific objective)

1. The relative expression of the PGE_2 receptors EP 1 to 4 is different among the studied murine and human mast cell populations. However, in all of them the expression of EP1 on the mast cells surface is virtually null. (objective 1)

2. The capacity of PGE_2 to inhibit mast cell activity correlates positively with the EP2/EP3 expression ratio in murine and human mast cells. (objective 2)

3. PGE_2 modulates human and murine mast cell activity *in vitro* through the regulation of total calcium mobilization. (objective 3)

4. Selective EP2 agonism improves AHR and inflammation in a murine model of HDM-induced sensitization. (objective 4)

5. Selective EP2 agonism inhibits lung mast cell overeactivity *in vivo* to the same extent as PGE_2, in HDM-sensitized mice. (objective 5)

6. Mast cell mediators are able to induce the development of AHR in naïve mice, and EP2-mediated blockade of mast cell function prevents such effect. (objective 6)

7. Selective *in vitro* stimulation of EP2 receptor inhibits mast cell degranulation of murine and humans mast cells, and such inhibition correlates with the level of expression of the EP2 receptor. (objective 7)

8. EP2 receptor mediated inhibited degranulation of human and murine mast cells activity is associated with inhibition of extracellular calcium channels and increased production of intracellular cAMP. (objective 8)

9. PGE_2 interaction with EP2 decreases murine mast cells activity by inhibiting Src-Fyn signaling pathways through cAMP/PKA. (objective 9)

10. Selective EP2 stimulation *in vivo* attenuates allergic responses which are known to be attributable uniquely to mast cells activity. (objective 10)

General conclusion

The "PGE_2"-"mast cells EP2"-"airway" axis is probably contributing to endogeneous protection against aeroallergens-induced airway pathology. The elucidation of the precise inhibitory mechanisms involved will uncover clue molecules to be proposed as potential novel antiasthma targets.

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