Regulation of Proteinase-Activated Receptor-2 (PAR-2) in Airway Epithelium

by

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Abstract

Proteinase-activated receptor-2 (PAR-2), a pro-inflammatory G-coupled receptor, is activated by exogenous or endogenous serine proteinases. PAR-2 is involved in the pathogenesis of inflammatory diseases including asthma. PAR-2 activation on airway epithelial cells (AECs) results in the release of inflammatory mediators that play an important role in the development of asthma. PAR-2 expression is upregulated on AECs in asthma and upregulated PAR-2 expression augments airway inflammation. Hence it is important to inhibit PAR-2 upregulation, but mechanisms involved in PAR-2 regulation are poorly studied.

Using *in vivo* and *in vitro* models, we showed that insulin regulates PAR-2 expression and upregulated PAR-2 increased the pro-inflammatory potential of AECs. We further showed that insulin regulates PAR-2 expression through PI3K-Akt-FOXO-1 pathway. This observation led to the hypothesis for future studies that inflammation-induced localized insulin resistance in the airway epithelium in asthma may result in PAR-2 upregulation on AECs.

PAR-2 expression is increased in inflammatory diseases, thus we studied the effect of stress stimuli, which are associated with inflammation, on PAR-2 expression in AECs. Our preliminary results suggest that oxidative stress and hypoxia do not modulate PAR-2 expression. We found that withdrawal of media supplements containing growth factors and hormones resulted in PAR-2 upregulation, and again PAR-2 upregulation increased the pro-inflammatory potential of AECs. We also showed that, in addition to insulin, hydrocortisone, bovine pituitary extract and retinoic acid regulate PAR-2 expression in AECs.

We cultured AECs obtained from asthmatic patients. We found that PAR-2 expression is increased in AECs from asthmatic compared to healthy individuals and this upregulated PAR-2 expression is maintained when cells were cultured *ex vivo* for multiple passages. Asthmatic AECs also showed similar media supplement deprivation-mediated PAR-2 regulation.

Our study indicates the possibility that inflammatory microenvironments such as insulin resistance and decreased cell growth nutrients may play a role in regulation of expression of pro-inflammatory PAR-2 receptors. Since our study highlights the role of inflammatory microenvironment in PAR-2 expression, our observations can be applied to inflammatory diseases of other organs such as colon and skin, where localized inflammation is present and PAR-2 expression is increased. Similar studies can be performed which may identify common dysregulatory mechanisms involved in increased PAR-2 expression in different inflammatory conditions and may lead to the development of novel therapeutic strategies to inhibit PAR-2 upregulation and control PAR-2-mediated inflammation.

Preface

Proteinase-activated receptor-2 (PAR-2) is a pro-inflammatory receptor, which is activated by serine proteinases. These serine proteinases can be exogenous such as allergens from house dust mite, cockroach or endogenous such as mast cell tryptase. PAR-2 activation on airway epithelial cells (AECs) results in the release of mediators that play an important role in the pathogenesis of asthma. In asthma, PAR-2 expression is increased on AECs but the underlying mechanisms and consequences of PAR-2 upregulation are not known. This thesis represents the work that was performed to understand the regulation of PAR-2 expression on AECs.

This is an original work by Vivek Gandhi. I was responsible for performing all the experiments, data collection and analysis as well as manuscript preparation. I have mentioned below the experiments which were done by our collaborators.

Chapter 1 is the introduction. Here I describe the role of airway epithelium and PAR-2 in asthma pathogenesis, which leads to the rationale for studying PAR-2 expression regulation on AECs.

Chapter 2 of this thesis is published as **Gandhi VD**, Shrestha Palikhe N, Hamza SM, Dyck JRB, Buteau J, Vliagoftis H., "Insulin decreases expression of the proinflammatory receptor proteinase-activated receptor-2 on human airway epithelial cells", *J Allergy Clin Immunol*. 2018 Jun 8. doi: 10.1016/j.jaci.2018.04.040. Shrestha Palikhe N performed transfection experiments. Hamza SM provided lung tissues and blood plasma from mice.

Chapter 3 contains preliminary work aiming at understanding role of insulin on PAR-2 expression in mice lungs *in vivo*. Ethics approval to perform these animal experiments was received from the University of Alberta Research Ethics Board, Project Name "Serine protease receptors (PARs) in airway inflammation and allergic sensitization", No. AUP00000353, 19th December 2017.

Chapter 4 contains preliminary work aiming at understanding role of insulin on PAR-2 expression in airway smooth muscle cells (SMCs). The experiments with SMCs were performed in collaboration with Dr. Andrew Halayko, University of Manitoba. Dr. Halayko's lab treated the SMCs as per our discussion and sent us cell lysate to evaluate PAR-2 expression.

Chapter 5 is a manuscript in preparation, where we studied the effect of media supplement deprivation on PAR-2 expression in AECs obtained from healthy and asthmatic individuals. Ethics approval to obtain AECs from asthma patients was received from the University of Alberta Research Ethics Board, Project Name "Airway epithelial cells", No. Pro00001190, 12th March 2018.

Chapter 6 shows data from experiments that were performed to understand the effect of different stress stimuli on PAR-2 expression in AECs. One of the stress stimuli was mechanical stretch and human rhino virus infection; these experiments were done in collaboration with the laboratories of Dr. Richard Leigh and Dr. David Proud, University of Calgary. The lab in Calgary treated the AECs as per our discussion and sent us cell lysate to evaluate PAR-2 expression.

Chapter 7 presents the results of experiments performed to determine if media supplement deprivation regulates expression of G protein-coupled receptors, other than PAR-2, in AECs.

Chapter 8 is a general discussion about the findings presented in this thesis, which leads to the conceptual model for regulation of PAR-2 expression in AECs.

Dedication

I dedicate my Ph.D. to my grandparents Mr. Manilal Gordhandas Gandhi and Mrs. Kusum Manilal Gandhi, and my parents Mr. Dipak Manilal Gandhi and Mrs. Rekha Dipak Gandhi. My words fall short to describe the effort and energy that you have invested in nurturing me and my character, which have made me the person that I am today. Your blessings, foresight and trust in me made this feat possible.

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I would also like to thank my committee members Dr. Paige Lacy and Dr. Allan Murray for their support, suggestions and valuable feedback that helped further the research projects.

I would also like to extend my sincere gratitude towards Dr. Dean Befus. I am fortunate to have you as my mentor. Thank you very much for always listening to my queries and investing time for discussion, feedback and guidance, which have contributed positively to my training as a researcher.

A special thanks to all the collaborators Dr. Jean Buteau, Dr. Jason Dyck, Dr. Shereen Hamza, Dr. James Martin and Dr. Alice Panariti for their help, support and guidance in their area of expertise.

I also thank funding agencies: The Lung Association of Alberta and NWT and University of Alberta for their studentship support.

I pay a special thanks to AllerGen for providing support to go to different national and international conferences. A heartily thanks to AllerGen and the late Michelle for their role in my professional development, by allowing me to be part of the trainee symposiums and AllerGen

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List of Abbreviations

ABECs - asthmatic bronchial epithelial cells

ADRB2 - Beta-2 adrenergic receptor

AECs - airway epithelial cells

AHR - airway hyperresponsiveness

ALI - air-liquid interface

BEBM - bronchial epithelium basal media

BECs - bronchial epithelial cells

BEGM - bronchial epithelial growth media

BPE - bovine pituitary extract

DCs - dendritic cells

ECM - extra-cellular matrix

EMT - epithelial-mesenchymal transition

ER - endoplasmic reticulum

ERK1/2 - extracellular signal-regulated kinase 1/2

FOXO-1 CA - FOXO-1 constitutively active

FOXO-1 - forkhead box protein O1

GAPDH - glyceraldehydes 3-phosphate dehydrogenase

GPCRs - G- protein coupled receptors

HDM - house dust mite

HFD - high fat diet

HPRT -   hypoxanthine-guanine phosphoribosyltransferase

IRS-1 -   insulin receptor substrate-1

JNK -   c-Jun N-terminal kinase

LPS -   lipopolysaccharide

NBECs -   normal bronchial epithelial cells

P2RY2 -   P2Y purinoceptor 2

PAR-1 -   proteinase-activated receptor-1

PAR-2 AP -   PAR-2 activating peptide

PAR-2 CP -   PAR-2 control peptide

PAR-2 -   proteinase-activated receptor-2

PARs -   proteinase-activated receptors

PI3K -   phosphoinositide 3-kinase

RA -   retinoic acid

SAPK -   stress-activated protein kinase

SMCs -   smooth muscle cells

TEER -   Trans Epithelial Electrical Resistance

Th2 -   type 2 helper T

TI -   Alveolar type I cells

TII -   Alveolar type II cells

TLR-3 -   toll-like receptor 3

TNF -   tumor necrosis factor

Chapter 1: Introduction

1.1 Inflammation

Inflammation is an initial protective immune response of tissue against injury and infection. Aulus Celsus first defined inflammation, by a combination of clinical sign and symptoms, as *rubor et tumor cum calore et delore* (redness and swelling with heat and pain) (1). Rudolf Virchow later added the fifth sign of inflammation: *function laesa* (loss of function) (1). The basic roles of inflammation are (i) to eliminate the injurious agent (ii) if elimination is not possible, then wall off the injurious agent and (iii) repair or replace the damaged tissue. With advances in cell biology and microscopy, we now have a better understanding of different types of inflammation, namely acute inflammation and chronic inflammation, which do not only differ by the time of onset or by the duration they last but also by the different types of cells involved. The defence mechanisms that the involved cells employ for the elimination of injurious agents also damage the body tissues. Thus, the longer the inflammation persists, the more damage is caused to the tissue which is observed in chronic inflammatory diseases of different tissues. So it is equally important to resolve the inflammation in a timely manner. During the resolution process, synthesis of pro-inflammatory mediators stops, which ceases the recruitment of inflammatory cells at the site of inflammation. Also the already recruited cells either go back to systemic circulation or undergo apoptosis; apoptotic cells are later cleared by phagocytes. If this homeostatic process is dysregulated, it results in increased levels of inflammatory mediators at the site of inflammation which recruits high number of inflammatory cells ultimately resulting in "tissue-damaging" chronic inflammation. We will now focus on asthma, a chronic inflammatory disease of the airways of the lung tissue.

1.2 Asthma

Asthma was first defined by Henry Salter in 1860 as "Paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks" (2). Today asthma is defined as an airway disease characterized by episodes of shortness of breath and wheezing due to reversible airway obstruction that develops as a result of airway inflammation and airway hyperresponsiveness (AHR). According to the World Health Organization over 300 million

people suffer from asthma, with 250,000 deaths every year (http:/ /www.who.int /gard/publicat ions/GARD%20Book%202007.pdf). According to a 2014 report from Statistics Canada, close to 2.5 million Canadians suffer from asthma (http://www.statcan.gc.ca/tables-tableaux/sum-som/l01/cst01/health50a-eng.htm) with a total burden of over $4 billion in direct and indirect costs annually (http://www.health.gov.on.ca/en/common/ministry/publications/reports/asthma /asthma2.aspx).

Asthma is a complex disease involving interactions between genetics and environment (3). We now understand that the majority of asthma is atopic, meaning that an asthma attack occurs in allergic individuals upon contact with an allergen. There is genetic predisposition to atopy but the development of allergic sensitization also requires environmental factors such as exposure to a particular allergen. The inhaled air is one of the main sources for exposure to pathogens, allergens (aeroallergens) and pollutants (4). Airway epithelium acts as a physical barrier to prevent the invasion of the inhaled air constituents but also acts as an innate immune cell. The innate immune responses of airway epithelium play an important role in the development of allergic sensitization (5). Moreover, genome wide association studies, aiming at identifying genes that increase susceptibility to asthma development, have revealed genes that are primarily expressed in airway epithelium (6). These results have now set the airway epithelium as a prime interest in the pathogenesis of asthma.

1.3 Airway Epithelium

Throughout the body, epithelium lines the surface of organs and cavities; hence acts as a mechanical barrier against the external environment, for example airway epithelium against inhaled air, gut epithelium against ingested food and skin epithelium against atmospheric air. The type of cells, namely squamous, cuboidal and columnar defines the epithelium. Further epithelium can be comprised of a single layer (simple epithelium) or multiple layers (stratified epithelium) of cells. An additional type of epithelium is called pseudostratified epithelium which in fact is a single layer of cells but appears as multiple layers due to presence of cell nuclei at different heights.

The airway epithelium belongs to the pseudostratified columnar epithelium, where each cell of single layer of columnar epithelial cells is in contact with basal lamina. There are a variety of specialized cells within the airway epithelium and their distribution varies throughout the airways. For example, the most abundant cells in the bronchial epithelium of the large airways are basal cells, ciliated cells and secretory goblet cells. These cells gradually decrease as we approach the smaller airways. The secretory club cells, also known as Clara cells, are more abundant in the smaller airways. The alveolar epithelium is made up of type I and type II alveolar epithelial cells.

1.3.1 Bronchial Epithelium

Basal cells

Basal cells are present in large and smaller airways (7), however their numbers decrease in the smaller airways (8). They are relatively undifferentiated and can be identified by expression of the transcription factor p63 and cytokeratin 5 and 13 (9). These cells are considered as the main progenitor cells in the airways that possess stem cell-like self-renewal properties and also differentiate into goblet and ciliated epithelial cells (10). Further, these are the only cells in the airways that express hemidesmosomes and are thus tightly attached to the basement membrane via integrins.

Ciliated cells

Ciliated cells are present in high numbers accounting for over 50% of airway epithelial cells. These cells are characterized by the presence of cilia on apical side (up to 300 cilia/ cell) and a high number of mitochondria below the apical membrane (11) reflective of the high need of energy for the constant ciliary beating. Basal cells, goblet cells and club cells differentiate into ciliated cells (12).

Goblet cells

As secretory cells of the large airways goblet cells secrete mucus, a mixture of highly glycosylated mucin proteins. The mucus layer is mainly present in the upper airways and it is replaced by the surfactant in the lower airways as the number of goblet cells decreases and club

cells increases. Goblet cells also have self-renewal capacity and can also differentiate into ciliated cells (8).

Club cells

Club cells are surfactant-secreting cells that are present in the smaller airways and can be characterized by electron-dense granules (11). These cells also possess stem cell-like self-renewal property and can differentiate into ciliated and goblet cells (13). Recent evidence suggest that Club cells can dedifferentiate into basal-like cells (14) or can also give rise to type I and type II alveolar epithelial cells (15).

1.3.2 Alveolar Epithelium

Type I cells (T1)

These very thin cells cover over 95% of the internal surface of alveoli. Their morphology helps create a thin barrier between air and blood and thus enables gas exchange (16).

Type II cells (TII)

The presence of secretory granules known as lamellar bodies, that store surfactant, is a characteristic of these cuboidal shaped cells (16). TII are secretory cells of alveoli region of the lung that synthesize and secrete surfactant and also maintain the levels of surfactant by reuptake (17). Upon injury, these cells act as progenitor cells to reconstitute alveolar epithelium (18).

1.3.3 Functions of Airway Epithelium

Since the airway epithelium is at an interface between external environment and lung tissue, it is the first site of interaction with the constituents of inhaled air including pathogens, aeroallergens, and pollutants. This interaction with external environment demands several important functions for epithelium. To execute these functions, epithelium is equipped with different types of specialized cells, as described above, which differ based on the type of tissue, for example types of specialized cells present in airway epithelium vs. gut epithelium are different.

Physical barrier property

The barrier property of epithelium is important for preventing entry of pathogens or particles into the subepithelial mucosa and thus limiting unnecessary activation of the immune system. The barrier property is attributed to the apical-junctional complex (AJC), which includes tight junctions, adherens junctions and desmosomes. The tight junctions are the AJC made up of tight junction proteins such as occludin, claudin and zonula occludens, which create a semipermeable barrier that regulates paracellular transport of solute across the epithelium (11). The tight junctions and adherens junctions also help polarize the epithelium (19). Adherens junctions and desmosomes, through cell-cell adhesion, maintain structural integrity of epithelium (20, 21). Club cells also help maintain integrity of epithelium by secreting antiproteinases such as secretory leukocyte protease inhibitor that neutralizes harmful proteinases (22). Finally interaction of epithelial cells, through integrins, with underlying extracellular matrix not only helps in maintaining mucosal barrier but also provides signals for epithelial cell survival and proliferation (23).

Mucocilliary escalator

Goblet cells and ciliated cells work in collaboration to provide this defense mechanism. The mucus released by goblet cells traps the inhaled pathogen or particles, which is then cleared by the highly coordinated ciliary beating.

Gas exchange

The thin layer of surfactant maintained by TII cells at the alveolar epithelium regulates surface tension and facilitates gas exchange by preventing alveoli from collapse (11). Surfactant secreted by club cells reduces surface tension of the bronchioles and helps them prevent their collapse during expiration. Interestingly, Club cells are shown to produce p450 mono-oxygenases which can metabolize xenobiotic compounds such as aromatic hydrocarbons (24) dissolved in surfactant fluid, thus maintains the purity of surfactant fluid.

Epithelial restitution

Airway epithelial cells (AECs) exhibit a rapid rate of turnover. Moreover, since they are exposed to external environment they are more prone to encounter environmental insults and experience epithelial tissue damage. It is very important to re-establish the homeostasis as soon as possible by reconstruction of normal airway. The current literature does not indicate the presence of a single stem cell type that can differentiate into all the other types of lung epithelial cells. However, as described above, multiple cell types in the airways have stem cell-like properties that undergo differentiation, transdifferentiation or dedifferentiation (epithelial-mesenchymal transition) to give rise to the different cell types in the airway to repair the wound and establish the homeostasis.

Airway epithelium: an immunologically active barrier

Being the front line soldiers, it is very important that AECs sense invading pathogens and mount an immune response against them. Moreover, it is equally important that AECs alert the immune system about the danger that they have encountered. The activation of the receptors on AECs not only results in the release of anti-microbial or anti-viral mediators but immune mediators such as cytokines and chemokines that recruit and activate other immune cells (25).

There is increasing evidence implicating the airway epithelium as a critical regulator of allergic sensitization and allergic airway inflammation. Allergic sensitization and allergic airway inflammation are involved in the pathogenesis of asthma. The above mentioned functions of airway epithelium are dysregulated in asthma, which puts airway epithelium at the focal point for the pathogenesis of asthma. It is still not clear that the observed dysregulation in structure and functions of airway epithelium is the result of genetics or airway-environment interactions.

1.3.4 Airway Epithelium in Asthma: Genetics

Studies aiming at understanding the genetics of asthma have shown a variety of genes associated with asthma susceptibility; many of these asthma susceptibility genes are expressed in airway epithelium (Table. 1.1).

Table 1.1: Asthma susceptibility genes identified in AECs, the method of identification and their functions

Gene	Method	Relevant function in asthma	Reference
ADRB2		Bronchial smooth muscle relaxation (26)	(27)
TNF	Candidate gene approach	Allergic sensitization, airway inflammation and hyperresponsiveness (28-30)	(27)
ADAM33		Associated with bronchial hyperresponsiveness (31)	(31)
PCDH1		Epithelial differentiation and barrier function (32)	(33)
DPP10		Not known	(34)
HLA-G	Positional cloning	Associated with bronchial hyperresponsiveness (35)	(35)
GPRA		Cell homeostasis (36)	(37)
SPINK5		Serine proteinase inhibitor (38)	(39)
IRAK-M		Inhibition of TLR signaling and inflammation (40)	(41)

IL1RL1/IL18R1		Receptor for IL-33 and IL-18 involved in the development of Th2 inflammation (42, 43)	(44-48)
IL-33		Th2 inflammation (43) and impaired tolerance (49)	(44-48)
HLA-DQ	Genome-wide analysis study	Not known	(45, 47, 48)
SAMD3		Signaling molecule for TGFβ and plays role in TGFβ-induced EMT (50)	(45, 47, 48)
ORMDL3/GSDMB		Release of asthma related mediators and activation of unfolded protein response pathway (51), Sphingolipid metabolism (52)	(45-47)
TSLP		Th2 cell polarization and Th2 inflammation (53)	(46, 48)

As summarized in the Table 1.1, many of the identified genes are involved in the regulation of airway epithelial structure and functions, indicating that dysregulation of these epithelial properties could play a central role in asthma pathogenesis.

These studies focusing on understanding genetic component of asthma are association studies. Gene polymorphisms are shown to affect gene expression in tissue-specific manner (54) and expression of many of these asthma susceptibility genes, for example ADAM33 (55), IRAK-M (40), TSLP (56), is not limited to airway epithelium. Thus it is important to understand the effect

of the identified polymorphisms on gene expression and function specifically in airway epithelium. Efforts are already initiated in these directions by correlating the polymorphism and gene expression in AECs with disease phenotype (57, 58), which give more strength to the idea that airway epithelium is an important player in asthma pathogenesis.

1.3.5 Airway Epithelium in Asthma: Dysregulated Functions

There is evidence that secretory functions of airway epithelium are altered in asthma. Asthmatic AECs are shown to express higher levels of inflammatory mediators constitutively (59-63), and also upon injury and infection (64, 65) or allergenic stimulation (66, 67), which reflects the increased pro-inflammatory potential of airway epithelium in asthma. The other properties of airway epithelium which are altered in asthma are cellular differentiation and regeneration. Studies have shown that ciliated cells are decreased in asthmatic airway epithelium (68), which may result in inefficient removal of inhaled pathogen or particles from the airways. On the other hand, the number of undifferentiated basal cells (63, 64) and mucus producing goblet cells (68, 69) are increased. The latter is associated with altered composition of mucus (70) and also increased levels of mucus (69) in asthma, which could contribute to airway obstruction and airway hyperresponsiveness (71), the characteristic features of asthma. The regeneration capacity of airway epithelium is important for wound healing after an injury. *In vivo* wound healing requires cell proliferation and epithelial-mesenchymal transition (EMT). During EMT, differentiated AECs undergo dedifferentiation and secrete extra-cellular matrix (ECM) to cover the wounded area, which facilitates the tissue repair process. However, if not regulated, this process could result in increased deposition of ECM and airway remodeling, another characteristic feature of asthma. Asthmatic AECs are less efficient at proliferation (65) and wound healing (65, 72) upon mechanical wounding. Moreover, *in vitro* cultures have shown that more number of asthmatic AECs, compared to non-asthmatic AECs, undergo EMT upon stimulation (50) indicating dysregulated epithelial repair process in asthma.

Many of these studies including (73) demonstrate that asthmatic AECs retain these altered characteristics when cultured *in vitro* suggesting a possibility of asthma-induced epigenetic changes in airway epithelium. This idea is supported by the studies showing differences in the

expression of epigenetic modifying enzymes (74) and epigenetic changes, such as DNA methylation, in AECs obtained from asthmatic individuals compared to healthy individuals (75).

Dysregulated functions of airway epithelium also increase epithelial interactions with allergens. There is evidence that mucocilliary clearance is not effective in the airways of asthma patients (76, 77), which may result in prolonged presence of the allergens in the airways allowing them to have protracted effects. In addition, decreased expression of anti-proteinases, for example, secretory leukocyte protease inhibitor (SLPI) by airway epithelium in asthma (78) renders barrier property of the epithelium more susceptible to the allergens containing proteinase activity (79-82). Finally, barrier property of airway epithelium is compromised in asthma as observed by decreased expression of proteins involved in the formation of tight junctions (83, 84) and adherens junctions (64, 84, 85) resulting in increased epithelial permeability. However, it is still not clear that the observed barrier dysfunction in asthmatic airway epithelium is the cause for asthma or an outcome of the inflammatory mediators present in asthma since the mediators released by AECs, such as tumor necrosis factor (TNF) (86), vascular endothelial growth factor (VEGF) (87) and thymic stromal lymphopoietin (TSLP) (88), or by other cells such as IL-13 (85) and IL-4 (89), are shown to affect the barrier property of airway epithelium by decreasing the expression of junction proteins. Ultimately, inefficient mucocilliary clearance and increased epithelial permeability facilitate allergen access to subepithelial mucosa. Allergens interact with a variety of immune cells, present in subepithelial mucosa, resulting in a strong immune response, which otherwise would not have occurred if the epithelial functions were optimal.

1.3.6 Airway Epithelium in Asthma: Interactions with Allergens

Being a first line of defense, immune responses generated by epithelium upon interactions with allergens play a crucial role in deciding the fate of the interactions. The first step of the interaction is the recognition of allergen by AECs. AECs express an array of pattern recognition receptors (PRRs) that sense the invading allergens. On top of the dysregulated epithelial functions, the other factor that affects the outcomes of epithelial-allergen interactions is the properties of allergens. For example, many of the asthma-relevant allergens from house dust mite (HDM), cockroach and fungi contain proteinase activity that activates pro-inflammatory proteinase-activated receptor-2 (PAR-2), which will be discussed in detail in the next section.

These airway epithelium-allergen interactions result in increased epithelial permeability and release of inflammatory mediators from epithelium, which activate downstream innate immune system. This immune response leads to the development of tolerance or allergic sensitization, however the factors that make the decision of sensitization development over tolerance is under investigation. Also interactions of airway epithelium with allergens after the development of sensitization result in the release of mediators that recruit immune cells resulting in allergic inflammation.
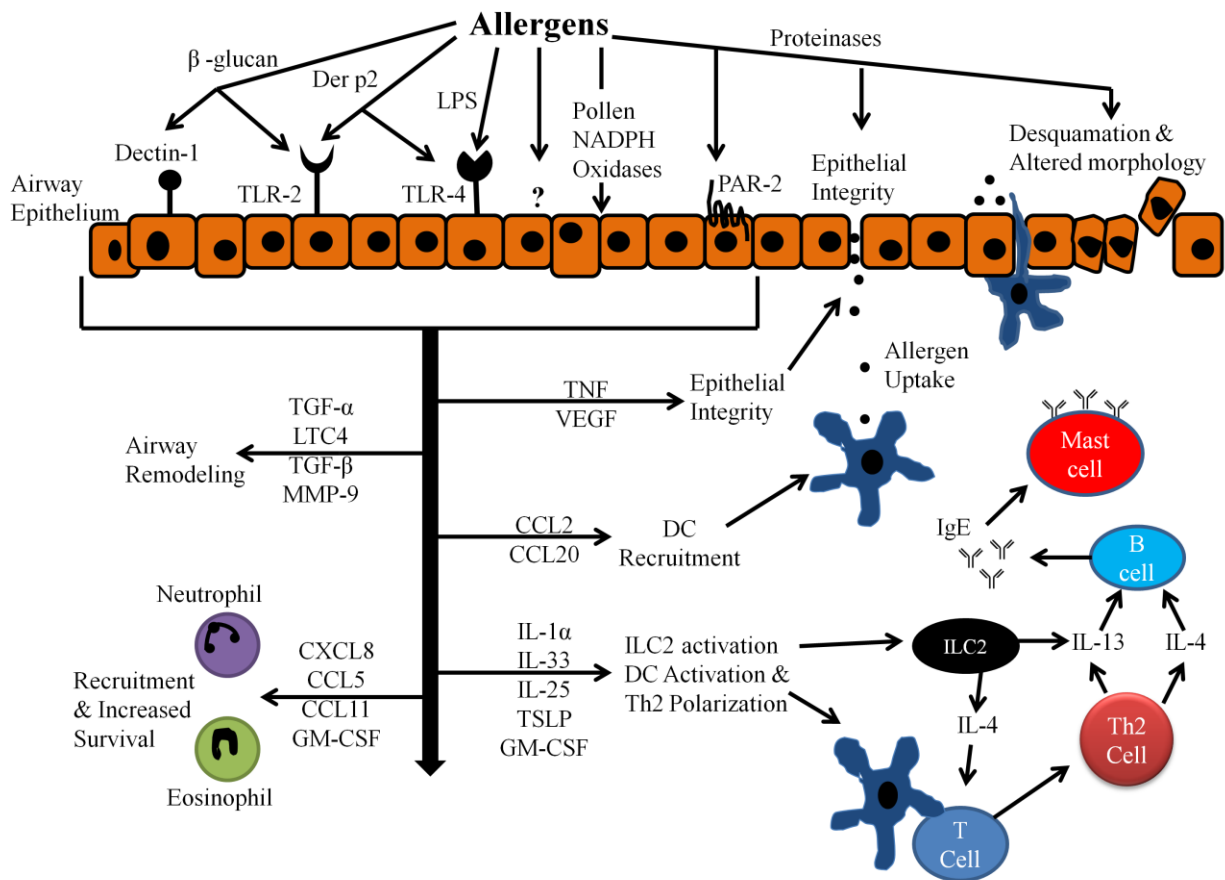
Fig. 1.1: Airway epithelium interaction with allergen: innate and adaptive immune responses leading to allergic sensitization and allergic airway inflammation. Modified from *Gandhi VD and Vliagoftis H (2015) Front. Immunol. 6:147*

AECs express PRRs (90) and PAR-2 (91); activation of these receptors by allergens results in the release of inflammatory mediators from AECs that shape the innate and adaptive immune responses towards the development of allergic sensitization and allergic airway inflammation (Fig. 1.1).

As depicted in Fig. 1.1, interactions of AECs with allergens decrease epithelial integrity resulting in allergens' entry across the epithelium. Simultaneously AECs also recruit DCs to the airways by releasing CCL2 and CCL20, where dendritic cells (DCs) take up the antigen and migrate to lymph nodes where they interact with and influence differentiation of T cells. Differentiation of T cells into type 2 helper T (Th2) cells is a vital step in the development of allergic sensitization. The mediators that affect this DC-T cell interaction and play important roles in the development Th2 cells are IL-33, TSLP, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-25; AECs release these mediators upon interactions with allergens. Recent evidence suggests that IL-33 dysregulates lung Treg cells and impairs the development of tolerance to inhaled allergens (49). IL-33 (92) and TSLP (53) influence the DC-T cell interaction by inducing expression of OX40 ligand on DCs, which results in the development of T cell into Th2 cells (93). Airway epithelium-induced GM-CSF is also shown to play a vital role in the development of Th2 cells (94, 95). Th2 cells and type 2 innate lymphoid cells (ILC-2) cells, which are activated by IL-33 and IL-25, release Th2 mediators such as IL-4, IL-5 and IL-13 (96, 97). The released IL-4 and IL-13 act on B cells to produce allergen specific antibodies, which bind to mast cells resulting in the allergic sensitization (98). IL-4 (99-101) and IL-13 (102) also act on AECs to induce the release of mucus and other inflammatory mediators resulting in the development of the vicious cycle of inflammation.

Eosinophilic infiltration of the airways is one of the cardinal features of allergic airway inflammation (103). The mediators released by airway epithelium IL-33, IL-25 and by Th2 cells IL-5 also induce eosinophilia (104-106). Finally, AEC-allergen interactions release chemoattractants for the innate immune cells eosinophils and neutrophils, such as CXCL8, CCL5 and CCL11, and also release GM-CSF, which increases production and survival of granulocytes resulting in airway inflammation.

Apart from the mediators involved in allergic sensitization and allergic inflammation, airway epithelium releases mediators involved in airway remodeling, a prominent feature of asthma. Transforming growth factor-β (TGF-β) (107, 108) and leukotriene C4 (LTC4) (108, 109) play a vital role in airway remodeling by inducing proliferation of airway smooth muscle cells (SMCs) and fibroblasts. Moreover TGF-β induces differentiation of AECs (110) and fibroblasts (111) to myofibroblasts, a cell type involved in the production of ECM; uncontrolled deposition of ECM is a feature of airway remodeling. Matrix metalloproteinase-9 (MMP-9) also plays a role in airway remodeling not only by degrading ECM (112) but also by activating TGF-β (113), which is released as a latent form.

1.4 Proteinase-Activated Receptor-2 (PAR-2)

Proteinase-activated receptors (PARs) are a family of 7-transmembrane G protein-coupled receptors (GPCRs) that belong to the A rhodopsin-like GPCR subfamily (114, 115). Four members of this family have been identified so far, PAR-1 to PAR-4. All members of the family are activated by serine proteinases and have a unique activation mechanism; serine proteinases cleave the extracellular N-terminal of the receptor and reveal the tethered ligand (TL), which lies within the extracellular N-terminus of the receptors. The revealed TL (new N-terminal of the receptor) then folds and binds to the second extracellular loop of the cleaved receptors initiating conformation changes that activate downstream signaling pathways (116-119) (Fig. 1.2).

PAR-2 was first cloned and identified as a receptor for the serine proteinase trypsin in 1994 (120). Trypsin causes PAR-2 hydrolysis at position $R^{36/S37}$ to reveal the TL SLIGKV in humans and at position $R^{38/S39}$ to reveal the TL SLIGRL in mice and rats. Examples of serine proteinases that activate PAR-2 signaling are trypsins I, II, and IV, mast cell tryptase, coagulation factors VIIa and Xa, acrosin, granzyme A and kallikreins 2, 4, 6 and 14 (120-128).

Synthetic ligands, called activating peptides (AP), mimic the amino acid sequence of the TL of PAR-2 and activate PAR-2 without the requirement for proteolysis (Fig. 1.2); control peptides (CP), containing same amino acids as AP but in reverse order, are used as control during the experiments. PAR-2 is activated by APs mimicking TLs revealed by trypsin such as SLIGKV-NH_2 for human PAR-2 and SLIGRL-NH_2 for mouse or rat PAR-2 (129). Various other APs have

also been used to activate PAR-2. These correlate to TLs generated by proteinase-dependent PAR-2 activation in different species or are substituted peptides with better affinity and/or potency than the native TLs (129). AP-mediated PAR-2 activation mimics the effects of proteinases and has been used widely as an alternative to proteinase-induced activation.

Sequence of peptides used in our studies

PAR-2-Activating peptide:

SLIGRL-NH$_2$

PAR-2-Control peptides:

LRGILS-NH$_2$ (Reversed sequence)

LSIGRL-NH$_2$ (First 2 amino acids reversed)
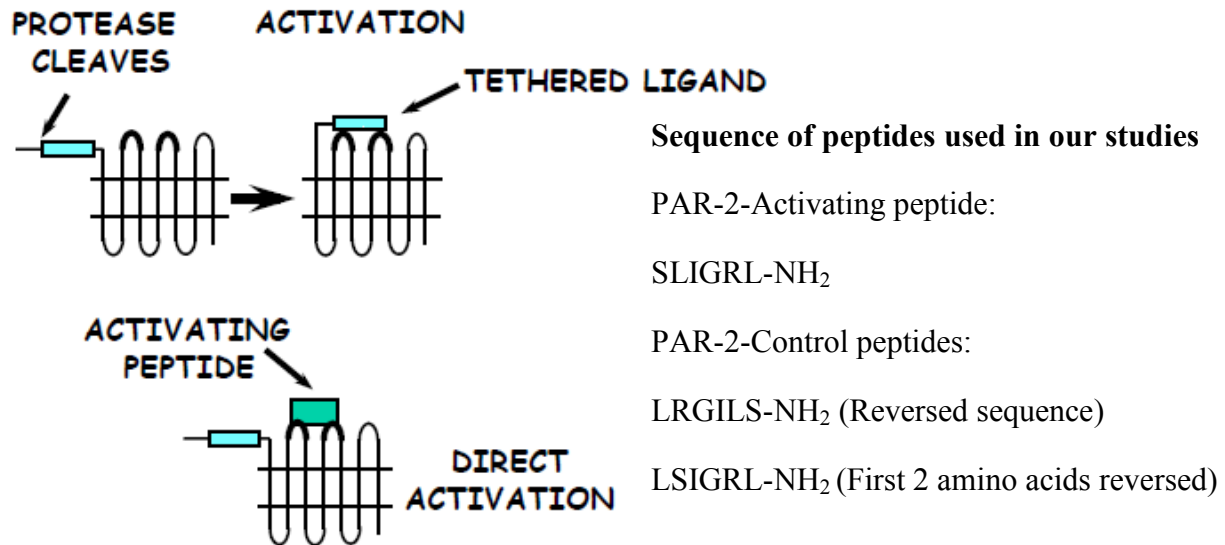
Fig. 1.2: Model for PAR-2 activation

PAR-2 activation results in the activation of multiple G protein-dependent and independent signaling pathways. PAR-2 activation leads to Gα_s-dependent formation of cAMP (130), G$\alpha_{12/13}$-mediated increase in Rho-kinase activity (130) and Gα_q mediated Ca^{++} mobilization (121). Further, PAR-2 activation results in the recruitment of β–arrestin-1 and -2 (131) to the receptor followed by ERK1/2 phosphorylation (132, 133) and receptor internalization and degradation (121). Thus activation of PAR-2 is an irreversible proteolytic phenomenon mediated by serine proteinases.

PAR-2 has been studied extensively over the last 15 years because it was identified as a major player in inflammatory reactions in many tissues and organs. PAR-2 expression is widespread on cells in different tissues where PAR-2 activation is shown to induce inflammation, such as, colon (134, 135), skin (136, 137), liver (138) and joints (139) and also shown to play a role in

inflammatory pain sensing (140). PAR-2 is implicated in the pathogenesis of inflammatory respiratory diseases such as allergic asthma (141, 142), idiopathic pulmonary fibrosis (IPF) (143) and pulmonary arterial hypertension (PAH) (144).

1.4.1 PAR-2 in Atopy and Asthma

In the airways PAR-2 recognizes both endogenous and exogenous "danger" signals; these signals are serine proteinases released from inflammatory and other cells or inhaled through the air, respectively. In this respect PAR-2 has functional similarities to pattern recognition receptors. These proteinase danger signals, through PAR-2, activate the innate immune system and lead to pro-inflammatory effects. In many cases the same danger signals act through PAR-2 to activate the adaptive immune system. Animal models have shown that PAR-2 activation by inhaled exogenous allergens with proteinase activity from HDM (145), cockroach (146) and fungi (147, 148) play a vital role in the development of allergic asthma.

The first evidence for an association between PAR-2 activation and asthma came in 1999 when Cocks et al., showed that PAR-2 activation causes relaxation of trachea preparations from many species, including mice and humans, and also protects anesthetised mice from bronchoconstriction *in vivo*; these effects were mediated by PAR-2-induced release of prostaglandin E2 (PGE2) from AECs (149). However, soon after that observation a series of studies suggested that PAR-2 activation in the airways may have significant pro-inflammatory effects. Using PAR-2 knock out (KO) mice it was shown that PAR-2 activation results in expression of P-selectin and leukocyte rolling on endothelial cells, which is the initial step in leukocyte recruitment to the site of inflammation (150). A detrimental role of PAR-2 in the development of allergic airway inflammation *in vivo* was first reported in 2002 when Schmidlin et al., showed that PAR-2 KO mice were protected from the development of airway inflammation (141). The role of PAR-2 in allergic airway inflammation was confirmed in a study showing decreased accumulation of eosinophils and eosinophil chemotactic factors in the airways and lungs of PAR-2 KO mice (151). Since then the role of PAR-2 is reproduced using PAR-2 KO (152) or PAR-2 blockade (153, 154) models and using intranasal administration of biologically relevant allergens.

PAR-2 KO mice generated less IgE (141, 152) compared to wild type mice after the exposure to allergen, indicating that PAR-2 may be involved in the development of allergic sensitization. Using ovalbumin (OVA) as an innocuous antigen, our group showed that immune tolerance develops when an innocuous antigen comes in contact with the nasal mucosa but simultaneous PAR-2-mediated activation of the nasal and/or airway mucosa in the presence of an innocuous antigen acts as an adjuvant to develop allergic sensitization against the innocuous antigen (28). Inhaled allergens are taken up by DC in the airways to be presented to the immune system and in the absence of other danger signals this process leads primarily to immune tolerance and not allergic sensitization. In the same study, we also showed that PAR-2 activation increases capacity of lung DC to carry antigen and increases their ability to migrate to lymph nodes (28). Thus PAR-2 activation can be a danger signal that biases the immune response to the inhaled innocuous antigen / allergen towards allergic sensitization.

OVA does not contain proteinase activity hence cannot activate PAR-2 but these observations raised an interesting hypothesis that when innocuous antigen / allergens have intrinsic proteinase activity to activate PAR-2 they induce local inflammation and release inflammatory mediators or danger signals to bias the immune response to the inhaled allergen towards allergic sensitization. Our lab has shown, using allergens containing proteinase activity that PAR-2 activation leads to the development of allergic sensitization (153, 154). Our group has also shown that PAR-2 blockade, during allergen challenge of sensitized animals, prevents the development of allergic airway inflammation and AHR (142) and airway remodeling (155). These results indicate that PAR-2 is involved in the development of allergic sensitization and allergic airway inflammation.

In summary, PAR-2 KO and PAR-2 blockade studies have confirmed the involvement of airway PAR-2 in the development of allergic sensitization and allergic inflammation. In the airways, PAR-2 is expressed on bronchial SMCs (156, 157), fibroblasts (158) and lung microvascular endothelial cells (157, 159, 160) and AECs (157). Also, PAR-2 is expressed on tissue resident cells such as mast cells (161), DC (162), and macrophages (163) and on the cells which are recruited to the airways during inflammatory reactions such as eosinophils (164) and monocytes (165). The *in vivo* studies discussed have not shown the specific PAR-2-expressing cell types involved in these PAR-2 effects. It is also possible that PAR-2 activation on different cell types

is primarily responsible for different stages of the development of PAR-2-mediated allergic sensitization and allergic inflammation. To identify this, cell specific PAR-2-knock out models, where PAR-2 expression is eliminated in the airways from a particular type of structural cell or eliminated from a particular type of immune cell, can be used. Until then the only studies that give us clues on the role of PAR-2 on specific cells are *in vitro* studies with isolated or cultured cells, which suggest an important role of PAR-2 activation on airway epithelium for the asthma pathogenesis.

1.4.2 PAR-2 and Airway Epithelium in Asthma

The outcomes of PAR-2 activation on AECs could play an important role in the development of allergic sensitization (Fig. 1.3). PAR-2 activation in AECs decreases the expression of gap junction protein connexin 26 (166) and also destabilizes a junction protein E-cadherin by transactivation of epidermal growth factor receptor (EGFR) (167). This result in decreased epithelial integrity and increased allergen access to underlying immune cells including DCs. DCs are the main antigen presenting cells in the body and are required for development of allergic sensitization (168). PAR-2 activation on AECs releases chemotactic factors for DCs airways (169) resulting in DCs recruitment to the airways. Once allergen gets captured and processed by the DCs, DCs then migrate to lymph nodes where they interact with and influence T cells differentiation. PAR-2 activation on AECs releases GM-CSF (95, 170, 171), TSLP (172) and IL-25 (173) that provide a favorable microenvironment for the development of allergic sensitization by supporting DC maturation and activation which in turn influence DC-T cell interaction and result in T cell differentiation into Th2 cells, the T cell phenotype that plays a role in the development of allergic sensitization. The mechanisms by which these mediators facilitate the development of allergic sensitization are depicted in Fig. 1.1.

Increased in the numbers of eosinophils in the airways is one of the cardinal features of allergic airway inflammation (103). PAR-2 could contribute to this by inducing the release of CCL11, which acts as a eosinophil chemotactic factor, and GM-CSF, which increases the survival of eosinophils (170). Further, PAR-2 activation on AECs also releases mediators that attract cells involved in innate and adaptive immune responses in the airways (91, 148, 169-171, 174-176) resulting in airway inflammation (Fig. 1.3).

Finally, airway remodeling is a characteristic feature of asthma, where airways undergo structural changes including increase in airway smooth muscle, subepithelial fibrosis and changes in the cartilage (177). PAR-2 activation on AECs releases leukotriene 4 (LTC4) (108, 109) and matrix metalloproteinase-9 (113, 178), which could play direct or indirect roles in airway remodeling by inducing proliferation of bronchial SMCs and fibroblasts and also by degrading ECM.
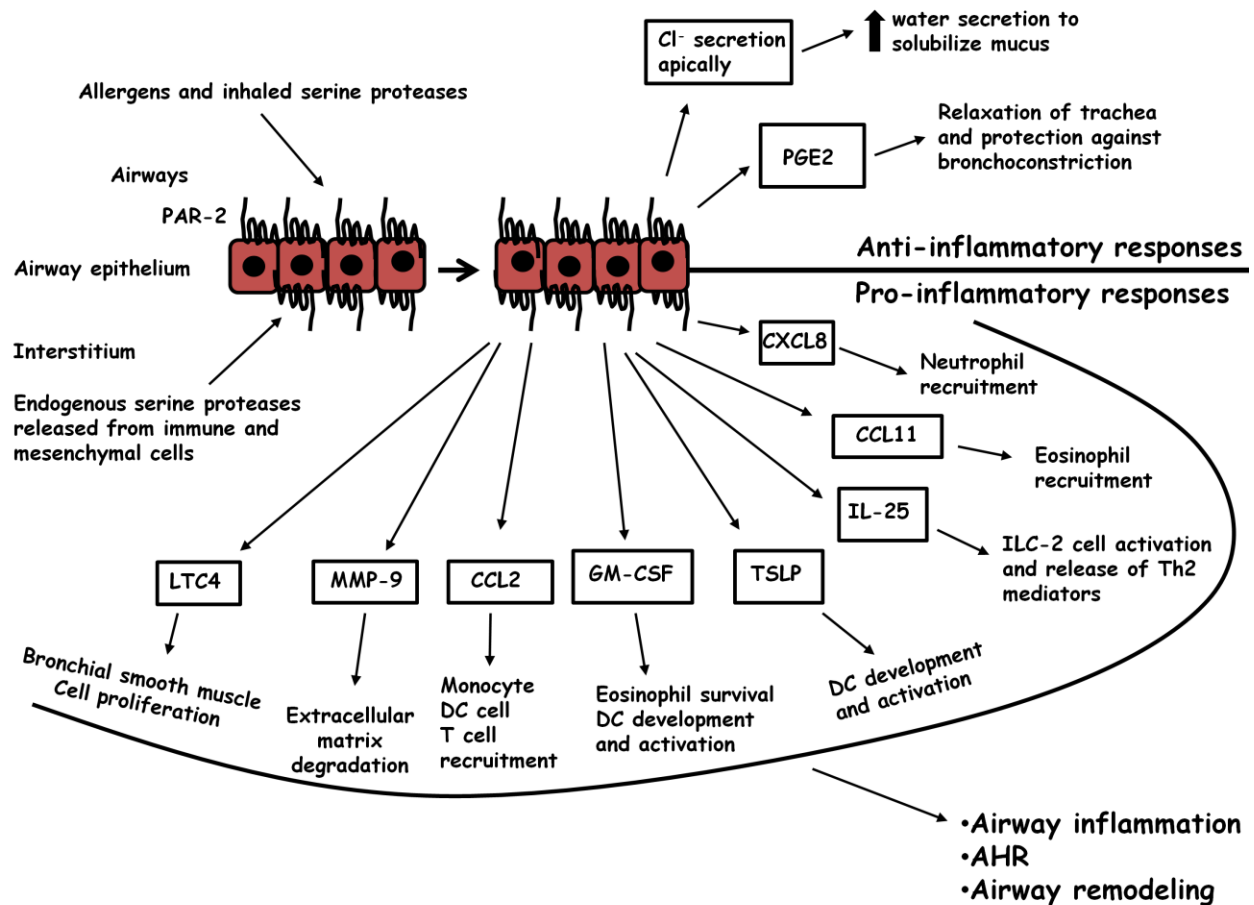
Fig. 1.3: Biological functions of PAR-2-mediated activation of AECs

The other mechanism by which PAR-2 activation can perpetuate inflammation is by exhibiting synergy with other inflammatory mediators for AEC activation. Asthmatic airways are characterized by the presence of chronic inflammation; interestingly, inflammatory mediators

present in the airways, such as IL-4 and TNF, could synergize with allergens to amplify the PAR-2-mediated release of inflammatory mediators (172, 179, 180).

The *in vivo* and *in vitro* approaches give complementary and very important results allowing us to reach a unifying consensus about the role of PAR-2 activation on AECs in the pathogenesis of asthma. These *in vitro* studies to understand the effect of PAR-2 activation on AECs have been carried out using both alveolar and bronchial AECs. A549 (human lung carcinoma cells with properties of TII alveolar epithelial cells, (181)) is the most utilized cell line to study the effect of PAR-2 activation on alveolar epithelium, while various cell lines, such as BEAS-2B (virus transformed AECs (182)), 16HBE14o-, Calu-3, NCI-H292, and primary airway epithelial cultures have been used to study the effect of PAR-2 activation on the bronchial epithelium. In addition, both immersed cultures and air-liquid interface (ALI) cultures have been used in studies performed with bronchial epithelial cells (BECs). ALI cultures mimic a physiological airway epithelium and facilitate *in vitro* study of epithelial functions such as barrier property, mucus secretion and mucociliary escalator, which is not possible with immersed cultures. ALI cultures also provide a unique opportunity to study cellular interactions between epithelial cells and other immune cells by using co-culture systems. There is no clear published evidence to indicate that AECs from different locations in the airways respond differently to PAR-2 activation, although this would not be unexpected. Studies to compare cells from different location in the same experimental model will be very useful to better define the role of PAR-2 in different compartments of the airways.

In conclusion, PAR-2-mediated activation of the epithelium leads to the release of pro-inflammatory mediators including chemotactic mediators for immune cells (neutrophils, eosinophils, DC, T cells), mediators that activate immune cells (survival factors and factors leading to production of pro-inflammatory mediators) and mediators involved in tissue remodeling. These mediators then, directly or indirectly, lead to allergic sensitization, allergic airway inflammation and tissue remodeling.

1.4.3 Regulation of PAR-2 Expression in AECs

PAR-2 expression is increased in inflammatory diseases such as asthma (183-185), atopic dermatitis (137), arthritis (165, 186), idiopathic pulmonary fibrosis (143) and pulmonary hypertension (187), diseases that evidence suggest also depend on PAR-2 activation. In all these cases the reasons for increased PAR-2 expression are not clear, but the consequences may be enhanced inflammation as has been shown in the case of asthma using PAR-2 overexpressing animals (141). Increased PAR-2 expression may also predispose the individuals to development of allergic sensitization, since we have shown that PAR-2 activation can deviate the immune response from tolerance to allergic sensitization (28). Therefore, understanding the mechanism of regulation of PAR-2 expression may allow us to device strategies to decrease PAR-2 expression and PAR-2-mediated inflammation in these conditions.

We have put a lot of effort in our lab to understand the role of PAR-2 in asthma, and especially on AECs, the first line of defense against inhaled inflammatory triggers and an important participant in the pathogenesis of inflammatory lung diseases. In this project my efforts have focused in understanding the regulation of PAR-2 expression on AECs, since there is very little information in this area.

There is evidence that PAR-2 expression is increased on airway SMCs (183, 184) and AECs (185) in patients with asthma. It is however, not known whether PAR-2 upregulation is a cause of the disease or an effect of the inflammatory environment in the airways. The literature indicates that a variety of inflammatory mediator upregulate PAR-2 in other cell types and other systems (summarized on Table 1.2 and Table 1.3). Many of these mediators are increased in the airways of patients with asthma and they may be the cause for PAR-2 upregulation in these patients. Inflamed and edematous airways in asthmatic individuals may also promote hypoxic environment around the epithelium, and hypoxia can also regulate PAR-2 expression in other systems. Changes in asthmatic airways may also promote epigenetic changes that may be responsible for altered PAR-2 expression. Finally genetic variations may also be the cause of altered PAR-2 expression in asthma, which would mean that the increased expression may precede the development of the disease. A PAR-2 coding region SNP has been associated with

atopy in Korean children, a SNP that increases mRNA stability leading to increased PAR-2 expression in peripheral blood mononuclear cells (PBMCs) (188).

Although there is no specific information about PAR-2 regulation on AECs by inflammatory mediators, there is evidence that HDM (166), cockroach (175) and mold (148) allergen may upregulate PAR-2 expression on airway epithelial cells, but the studies lack mechanistic components that will allow us to better understand this regulation. PAR-2 activation, by proteinases, results in rapid internalization of the receptor and decreased PAR-2 expression on the cell surface (189), but the studies mentioned above show increased expression using activators with similar proteolytic activity. More detailed studies will be needed to address these disparities. In addition, LPS can also upregulate PAR-2 expression on AECs (190), and the effect of allergens may be mediated, at least in part, through LPS contaminating the allergen preparation.

Table 1.2: Regulation of PAR-2 expression by different stimuli in cells other than AECs

Stimuli	Cell culture model	Upregulation	Downregulation	Reference
IL-1β, Trypsin, LPS, PAR-2 AP, IL-6, GM-CSF	Human umbilical vain endothelial cells, P815 mast cells	mRNA		(191-193)
PDGF-BB, TGFβ	Skin fibroblast cells, Lung fibroblast cells	Protein		(194)
IL-1α, IL-1β, TNF, LPS, PDGF-BB, Recombinant cockroach allergen Per	Airway smooth muscle cells, Chondrocytes, Synovial cells, Human umbilical vain endothelial cells, Pulmonary arterial smooth muscle cells,	mRNA & protein		(144, 195-201)

a 7, Recombinant cockroach allergen Per a 1.0101	P815 mast cells			
Hypoxia	Pulmonary arterial smooth muscle cells, Human umbilical vain endothelial cells	mRNA & protein		(144), (202)
IL-1β, TNF, LPS	Human coronary arteries, Primary rat astrocytes	mRNA & functional response		(203, 204)
TNF	Human umbilical vain endothelial cells	mRNA, protein & functional response		(191)
Extracellular matrix	Skin fibroblast cells, Lung fibroblast cells		Protein	(194)
TGF β, IL-12	Chondrocytes, P815 mast cells		mRNA & protein	(196, 205)
IL-1β	Primary rat astrocytes		mRNA & functional response	(204)

Table 1.3: Regulation of PAR-2 expression by different stimuli in AECs

Stimuli	Cell culture model	Upregulation	Downregulation	Reference
PAR-2 AP, LPS, Cockroach allergens, Neutrophil elastase, House dust mite allergen Der p 1	A549 cells, Calu-3 cells, Bronchial epithelial cell line, Human nasal epithelial cells	mRNA		(166, 175, 190, 206)
Purified mold allergen Pen c 13	A549 cells	mRNA & protein		(148)

Thus the available literature supports the notion that inflammatory microenvironment is crucial for PAR-2 regulation, but the mechanisms involved are poorly studied.

1.5 Insulin – An Anti-inflammatory Hormone

The β cells of Langerhans islets in the pancreas secrete insulin. Insulin was identified, for its role in glucose homeostasis, and purified by the researchers Banting, Best and Collip, under the guidance of McLeod at the University of Toronto in 1921-1922 (207). Further research has now established the role of insulin in energy metabolism by regulating metabolism of carbohydrate, lipid and protein (208, 209).

Early animal studies also supported an anti-inflammatory role for insulin. Researchers in the 1970s showed that carrageenan-induced rat paw edema was blunted by insulin administration and was augmented in insulin deficient animals (210). However, it was thought that the anti-inflammatory effect of insulin was mediated by lowering blood glucose since hyperglycemia is pro-inflammatory (211). Studies during the last 15 years have shown that anti-inflammatory

properties of insulin can be attributed to its ability to modulate key inflammatory molecules (212).

1.5.1 Biological effects of insulin

Insulin exerts its effect by activating multiple signaling pathways upon binding to the insulin receptor. Insulin is also shown to activate another structurally similar receptor insulin-like growth factor-1 receptor (213) albeit with low affinity than the insulin receptor (214). Insulin receptor consists of two extracellular α-subunits and two intracellular β-subunits and belongs to a class of receptors called receptor tyrosine kinases (RTK). Binding of insulin to the α-subunits results in receptor dimerization and activation of intrinsic tyrosine-kinase activity of the β-subunits, which then trans-phosphorylate tyrosine residues in β-subunits. These phosphorylated tyrosine residues act as a docking site for adaptor proteins that possess phosphotyrosine-binding (PTB) domains or Src homology 2 (SH2) domains such as insulin receptor substrate (IRS) and Src homology 2 domain containing transforming protein (Shc). It has been shown that these two adaptor proteins compete for the same binding site of the insulin receptor (215, 216). Based on the adapter protein that binds the insulin receptor, different signaling pathways are activated and mediate the regulatory functions of insulin (Fig. 1.4).

1.5.1.1 Metabolic Regulation: PI3K-Akt Pathway

Recruitment of IRS to the insulin receptor through the PTB domain (215) activates the PI3K-Akt signaling pathway. Once recruited, tyrosine residues of IRS are phosphorylated by intrinsic tyrosine-kinase activity of the insulin receptor. Tyrosine-phosphorylated IRS binds to phosphatidylinositol 3-kinase (PI3K) through the SH2 domain. There are three classes of PI3K according to their structural characteristics and substrate specificity; class I PI3K are the most commonly studied for insulin signaling as there are no good data for insulin-mediated activation of other classes of PI3K. Class I PI3K are further divided in to Class IA, activated by RTKs, GPCRs and G-protein Ras and Class IB, activated by only GPCRs. The literature suggests that Class IA (217), but not class IB (218, 219) are the main enzymes responding to insulin. PI3K IA contain two subunits: regulatory p85, which has SH2 domain, thus binds and recruits PI3K to IRS (220), and catalytic p110. Activated PI3K acts on the plasma membrane

phosphatidylinositol 4,5-bisphosphate (PIP2) and generates phosphatidylinositol 3,4,5-trisphosphate (PIP3) (221), which acts as a docking site for proteins containing PH domain. This results in the recruitment of phosphoinositide-dependent protein kinase 1 (PDK-1) and Akt. PDK-1 phosphorylates Thr308 on Akt, while PDK-2 (mTORC2) phosphorylates Ser473 on Akt. These two phosphorylations activate Akt (serine/threonine kinase) (222-225), which will further regulate the downstream signaling involved in inhibition of apoptosis, uptake of glucose, synthesis of glycogen, fat and protein as energy reserves and prevent glucose production by inhibiting gluconeogenesis (glucose synthesis from non-carbohydrate precursors) and glycogenolysis (breakdown of glycogen to glucose) (226). Thus insulin, through PI3K-Akt pathway regulates cell survival, metabolism and protein synthesis.

1.5.1.2 Mitogenic Regulation: Ras-ERK1/2 Pathway

The binding of IRS or Shc to the insulin receptor activate the Ras-ERK1/2 pathway. Phospho-IRS or Phospho-Shc recruit adaptor/guanine nucleotide exchange factor complex Grb2/Sos (227). Sos mediates GDP/GTP exchange on Ras and activates Ras (227, 228), which then activates Raf (229). Raf activates mitogen-activated protein kinase kinase 1/2 (MEK1/2) by phosphorylating it on two serine residues (230). MEK1/2 is a dual specificity kinase that activates extracellular signal-regulated kinase 1/2 (ERK1/2) by tyrosine and threonine phosphorylation (231). Active ERK1/2 (serine/threonine kinase) further phosphorylates many substrates, which are localized in the cytoplasm or nucleus (232). This pathway results in the expression of genes and synthesis of proteins involved in cell growth, differentiation, survival and cell migration (233, 234).

1.5.1.3 Regulation of Inflammation

In addition of decreasing pro-inflammatory effects of hyperglycemia, by decreasing glucose levels, insulin has also anti-inflammatory properties by directly modulating expression or activity of pro-inflammatory molecules. Insulin decreases the recruitment of leukocytes by downregulating expression of chemokines (235), chemokine receptors (235) and cell adhesion molecules (236). TLRs are a group of innate immune receptors that identify a range of pathogen-associated molecular patterns to initiate the inflammatory cascade (237). Insulin

downregulates expression of TLRs (238), including TLR-4 (239), which in turn decreases TLR-4-mediated inflammation (240, 241). Insulin can also modulate the activity of transcription factors rather than affecting their expression, for example, insulin suppresses activity of nuclear factor kappa B (NF-κB) transcription factor by preventing its nuclear localization (242, 243); NF-κB upon activation induces inflammation by increasing expression of pro-inflammatory cytokine and cell adhesion molecules (244). In addition to NF-κB, a class of transcription factors, whose functions are directly under the negative regulation of insulin signaling, are the Forkhead box O (FOXO) transcription factors.
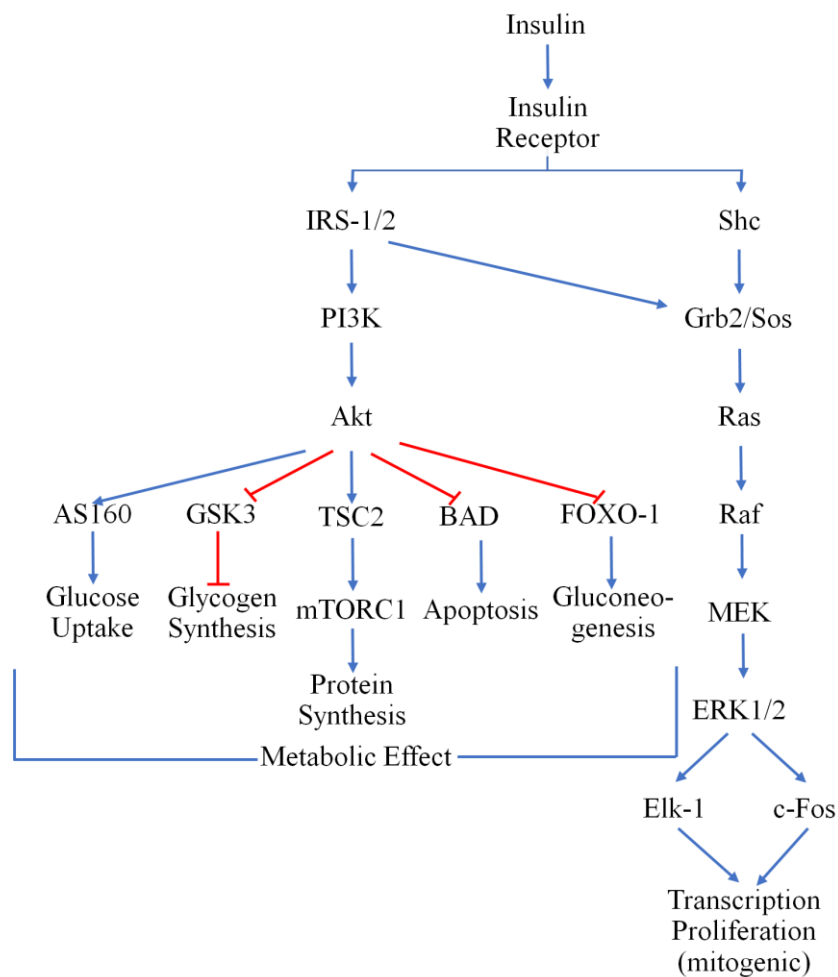
Fig. 1.4: Canonical insulin signaling pathway. GSK3, glycogen synthase kinase 3; TSC2, tuberous sclerosis complex 2; BAD, Bcl-2-associated death promoter; FOXO-1, forkhead box O-1; mTORC1, mammalian target of rapamycin complex 1.

1.5.2 FOXO Transcription Factors

The Forkhead family of transcription factors is comprised of more than 100 members and is characterized by a conserved DNA-binding domain called "Forkhead box" (245). They are named as "Forkhead" because the mutation in the first identified member FOXA resulted in a head structure that resembled a fork in *Drosophila* (245). FOXO transcription factors are part of this family. There are 4 FOXOs namely FOXO-1, FOXO-3, FOXO-4 and FOXO-6, which are regulated similarly by insulin/PI3K/Akt pathway (246).

Active FOXO-1 is localized to the nucleus. Insulin negatively regulates FOXO-1 activity through PI3K-Akt pathway. Insulin-activated Akt phosphorylates FOXO-1 on the conserved residues Threonine 24, Serine 256 and Serine 319 resulting in nuclear exclusion of FOXO-1 and inhibition of FOXO-1-mediated transcription (246). In the cytoplasm, the phosphorylated FOXO-1 is polyubiquitinated and degraded (247).

During low insulin condition, such as fasting, FOXO-1 localizes to the nucleus and induces the expression of FOXO-1-targeted genes involved in activities ranging from gluconeogenesis (248) to inflammation (249, 250) to protection against oxidative stress (251), the stress that could be the result of gluconeogenesis-induced hyperglycemia or inflammation. The majority of the studies highlighting the role of FOXO-1 are from organs which are involved in glucose homeostasis such as liver, adipose tissue and skeletal muscles because of involvement of FOXO-1 in glucose metabolism. The role of FOXO-1 is less studied in epithelial cells and the available literature suggests that FOXO-1 could regulate innate immune functions in epithelial cells from different systems. In keratinocytes, FOXO-1 promoted wound healing in cells cultured in normal glucose media (252, 253), while impaired wound healing in cells cultured in high glucose media (253). This observation may indicate contradicting roles of FOXO-1 in healing of normal wound vs. diabetic wound. In colonic epithelial cells, FOXO-1 is shown to play a role in TNF-mediated impaired barrier function (254). Finally, in AECs, FOXO-1 is activated upon bacterial infections to decrease internalization of bacteria and induce the release of inflammatory mediators and defensins, anti-microbial peptides (255).

1.5.3 Insulin resistance

Inflammation can lead to insulin resistance, a condition where insulin is not able to exert its functions because cells do not respond to insulin. Insulin resistance could be a result of decrease in expression of insulin signaling molecules or blockade in insulin signaling. Insulin resistance is best studied in adipose tissue and skeletal muscles, which are the primary sites of insulin action for glucose uptake. Inflammation is a major contributor to insulin resistance by multiple mechanisms. First, inflammatory mediators activate suppressor of cytokine signaling (SOCS) family of proteins that decrease the intrinsic tyrosine phosphorylation activity of insulin receptor (256) and increase the degradation of IRS-1 (257), which is downstream of insulin receptor in the in insulin signaling cascade. Second, inflammatory mediators such as IL-6 (258), IL-1β (259) and TNF (260) decrease the expression of insulin receptor and IRS-1. Finally, inflammation results in activation of serine / threonine kinases such as c-Jun N-terminal kinase (JNK) (261) and protein kinase C (PKC) (262) that phosphorylates IRS-1 on serine residues, which prevents the recruitment of PI3K and results in blockade of insulin signaling. This results in insulin resistance in adipose tissue and skeletal muscle and hence these tissues do not perform insulin-directed functions including glucose uptake.

1.6 Rationale, Hypotheses and Aims

In asthma, PAR-2 expression is increased on airway epithelial cells but the mechanism underlying PAR-2 upregulation as well as the consequences of this upregulation are poorly studied. As we discussed, PAR-2 expression is increased in a variety of inflammatory diseases. It is believed that the inflammatory milieu upregulates PAR-2 expression in these diseases.

(i) Along with metabolic functions, insulin resistance results in blockade of anti-inflammatory functions of insulin. Insulin acts anti-inflammatory by decreasing expression of pro-inflammatory receptors such as TLRs, which are activated by a particular molecular pattern. Similar to TLRs, PAR-2 is a pro-inflammatory receptor, which is activated by serine proteinases. PAR-2 expression is increased in adipose tissue during insulin resistance (263-265) and improving insulin sensitivity decreases PAR-2 expression (263) indicating insulin may regulate PAR-2 expression. It is also

possible that insulin regulates PAR-2 expression in AECs and upregulated PAR-2 expression in airway epithelium in asthma (185) is due to altered insulin signaling in AECs. This possibility is plausible since asthmatic airways are chronically inflamed and the mediators that induce insulin resistance in adipose tissue, such as IL-6, IL-1β and TNF (266), are also increased in the airways in asthma (267-270). Interestingly, recent evidence has shown decreased expression of insulin receptor in AECs obtained from asthma patients compared to healthy individuals (271) raising the possibility that insulin regulates PAR-2 expression in AECs and the increased PAR-2 expression in AECs in asthma (185) could be the result of decreased insulin signaling in AECs. Finally, an indirect evidence demonstrating potential role of insulin in regulating PAR-2 expression showed that mice lacking insulin had increased PAR-2-mediated inflammation (272). However, PAR-2 expression was not studied in this mouse model it is possible that the increased in PAR-2-mediated inflammation was because of increased PAR-2 expression in the absence of insulin. Based on this evidence, **we hypothesized that insulin regulates PAR-2 expression in AECs**.

(ii) In asthma, airways are infiltrated by inflammatory cells such as eosinophils (273) and neutrophils (274). In addition of these inflammatory cells, other cells such as monocytes, macrophages, epithelial cells and smooth muscle cells, release chemokines, cytokines and reactive oxygen species (ROS) resulting in the presence of high levels of inflammatory mediators (275) and oxidative stress (276) in the airways of asthma patients. It is also shown that airway remodeling of the subepithelium, which is also observed in asthma, may limit the delivery of cell growth nutrients and oxygen (hypoxia) to the airway epithelium (277-279). There is evidence that inflammatory mediators (196, 198, 203), hypoxia (144, 202) and growth factor deprivation (280) regulate PAR-2 expression on cells other than AECs. **We hypothesized that these cellular stressors of the inflammatory microenvironment regulate PAR-2 expression in AECs.**

Aim 1: To study the effect of insulin on PAR-2 expression in mouse lungs *in vivo* and in primary cultures of human AECs *in vitro* (Chapter 2, Chapter 3 and Chapter 4 of the thesis).

Aim 2: To study the effect of oxidative stress, hypoxia and decreased levels of cell growth nutrients on PAR-2 expression in primary cultures of human AECs (Chapter 5, Chapter 6 and Chapter 7 of the thesis).

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Chapter 2: Insulin decreases expression of the pro-inflammatory receptor proteinase-activated receptor-2 on human airway epithelial cells

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A version of this chapter has been accepted for publication.

The chapter here has been expanded and supplemental data and methods have been included in the main manuscript for clarity.

2.1 Abstract

Background: Proteinase-Activated Receptor-2 (PAR-2) is implicated in the pathogenesis of allergic asthma. PAR-2 expression is increased on the airway epithelium of asthmatic individuals, but the factors responsible and the consequences of increased PAR-2 expression are poorly studied. Recently, decreased insulin signaling has been linked with airway epithelial changes in asthma.

Objective: To understand the role of insulin in PAR-2 regulation in the lungs *in vivo* and *in vitro*.

Methods: PAR-2 expression was studied in the lungs of mice with obesity following high fat diet (HFD). Submerged and air-liquid interface cultures of primary human airway epithelial cells (AECs) were used to elucidate the mechanisms underlying regulation of PAR-2 expression on AECs by insulin.

Results: HFD-fed mice that are characterized by increased levels of circulating insulin expressed lower PAR-2 levels in the lungs. Insulin deficiency in human AECs resulted in increased expression of PAR-2 mRNA and protein. PAR-2 upregulation increased PAR-2-dependent pro-inflammatory potential of AECs. PI3K inhibition prevented insulin-mediated regulation of PAR-

2 expression. Insulin, through PI3K, inhibits FOXO-1 transcription factor. Expression of constitutively active FOXO-1 resulted in increased PAR-2 expression in the presence of insulin and inhibition of FOXO-1 prevented insulin deprivation-induced PAR-2 upregulation.

Conclusion: Insulin may have an anti-inflammatory role in the airways by controlling PAR-2 expression on AECs through regulation of FOXO-1 activity. Alteration of insulin signaling through decreased insulin receptor expression or insulin resistance may be responsible for PAR-2 upregulation in asthmatic airway epithelium, an effect that may exacerbate proteinase-mediated airway inflammation.

2.2 Introduction

Proteinase-Activated Receptor-2 (PAR-2) is a G-protein coupled receptor activated by serine proteinases (1). PAR-2 expression is widespread on cells in the gut, skin, liver and other tissues and its activation induces inflammation (2-5). In lungs, PAR-2 is expressed on bronchial smooth muscle cells (6, 7), fibroblasts (8) and airway epithelial cells (AECs) (7), in addition to various immune cells. PAR-2 has been shown to be involved in the pathogenesis of a number of lung diseases such as allergic asthma (9, 10), idiopathic pulmonary fibrosis (IPF) (11) and pulmonary arterial hypertension (12).

PAR-2 activation on AECs induces the release of mediators that play important roles in the development of allergic sensitization (13, 14), recruitment of inflammatory cells (15, 16), eosinophil survival (16) and tissue remodeling (17). PAR-2 activation also affects epithelial barrier properties (18) resulting in increased interactions of inhaled allergens and pollutants with immune cells. These observations indicate that PAR-2 plays an important role in the pathogenesis of asthma.

PAR-2 expression increases in various disease states (3, 11, 19-21) including asthma (22-24), and PAR-2 over-expression can enhance inflammation (9). It is believed that the inflammatory milieu upregulates PAR-2 expression in these disease states, but this has not been proven. Inflammatory stimuli (25-27) and hypoxia (12, 28) can regulate PAR-2 expression on different cells but there have been no systematic studies addressing the detailed mechanisms of PAR-2 regulation. Our main interest has been to understand the role and regulation of PAR-2 in asthma, and especially on AECs, the first line of defense against inhaled inflammatory triggers and an important participant in the pathogenesis of inflammatory lung diseases.

Insulin has anti-inflammatory properties (29) but the role of these mechanisms in regulating airway inflammation in patients with asthma is not known. Insulin resistance, due to decreased expression of insulin receptor and/or blockade in insulin signaling, limits insulin-mediated anti-inflammatory activities. Insulin resistance is associated with allergic sensitization and allergic asthma in humans (30) and also in animal models (31, 32). Recent evidence also shows decreased expression of insulin receptor in the airway epithelium in asthmatic individuals (33),

which may render asthmatic epithelium insulin resistant. These results suggest that airway inflammation in asthma could be, at least in part, the result of decreased anti-inflammatory effects of insulin. Expression of the pro-inflammatory receptor PAR-2 increases in adipose tissue during insulin resistance, an increase that results in exaggerated PAR-2-mediated inflammation (34-36), while improving insulin sensitivity decreases PAR-2 expression and PAR-2-mediated inflammation (34).

We hypothesize that insulin-mediated regulation of PAR-2 expression explains the observed increase in PAR-2 expression on AECs of asthmatic individuals in a background of decreased insulin signaling. Here we studied the effects of insulin on PAR-2 expression in whole lungs and AECs using a mouse model and primary cultures of human bronchial epithelial cells, respectively. We show that PAR-2 expression increases with decreasing insulin concentrations. PAR-2 upregulation in the absence of insulin leads to increased calcium release upon PAR-2 activation, indicating that the pro-inflammatory potential of AECs may increase with decreasing insulin signaling. We also show that insulin-mediated regulation of PAR-2 expression in AECs is phosphoinositide 3-kinase (PI3K)-Akt- Forkhead box protein O1 (FOXO-1) pathway dependent.

2.3 Materials and Methods

Animal study

C57BL6 male mice (6-7 weeks old) were maintained on high fat diet (HFD) (60% kcal fat, Research Diets, New Brunswick, NJ) or standard chow diet (4% kcal fat) *ad libitum* for 30 weeks. Mice were euthanized with an overdose of sodium pentobarbital and blood was collected by cardiac puncture. Lungs were snap frozen in liquid nitrogen. Plasma and lung tissue were stored at -80ºC until further processing. Experiments were approved by the University of Alberta Health Sciences Laboratory Animal Ethics committee.

Insulin measurement

Insulin levels were measured in mouse plasma using the Rodent Insulin Chemiluminescence ELISA kit (ALPCO, Salem, NH).

Cell culture

Primary AECs (normal human bronchial epithelial cells), from five donors, were purchased from Lonza (Walkersville, MD); experiments were done on cells at passages 3-5 after receiving. The cells were cultured on tissue culture flasks or plates (Falcon®, Corning, NY) coated with 30 µg/mL bovine collagen type I (Life Technologies, Burlington, ON) dissolved in bronchial epithelial basal media (BEBM, Lonza). Cells were cultured in bronchial epithelial growth media (BEGM, Lonza) i.e. BEBM supplemented with bovine pituitary extract, hydrocortisone, epidermal growth factor, epinephrine, insulin, transferrin, triiodothyronine, retinoic acid, gentamycin-amphotericin-B and Penicillin-Streptomycin (Life Technologies).

Air-liquid interface (ALI) cultures of AECs were done as described (37). In summary, 90,000 cells /insert were seeded on 0.4 µm inserts (Corning Inc., Corning, NY). The differentiation media contained 50% DMEM (high glucose, Life Technologies) and 50% BEGM with all the mentioned growth supplements except gentamicin-amphotericin B, triiodothyronine and retinoic acid. Gentamycin at final concentration of 50 µg/mL (Life Technologies) was added separately. All-trans retinoic acid (Sigma) was added fresh every time at final concentration of 50 nM. For the first 7 days, the differentiation media was added to apical and basolateral sides. After 7 days,

apical media was removed to establish ALI and new media was added to only basolateral side. Cells were further cultured for 2 weeks to allow differentiation.

BEAS-2B cells (virus transformed AECs) were purchased from ATCC (Manassas, VA). The cells were cultured on tissue culture flasks or plates coated with a mixture of 2 μg/mL fibronectin (Life Technologies), 30 μg/mL bovine collagen type I and 10 μg/mL BSA (Sigma-Aldrich, St. Louis, MO) dissolved in BEBM. BEGM media without gentamycin - amphotericin and retinoic acid was used to culture cells.

For all the cell culture procedure, cells were cultured at 37°C in 5% CO_2 and 90% humidity and media was changed 3 times /week. All cell cultures maintained in our lab were tested monthly for mycoplasma using the MycoAlert kit (Lonza) and only negative cultures were used.

To understand the role of insulin signaling pathway in PAR-2 regulation, AECs were treated with PI3K inhibitors LY294002 (20 μM, (38, 39)) (Tocris, Minneapolis, MN) and wortmannin (5 μM, (40)) (Cayman, Ann-Arbor, MI) and Forkhead box protein O1 (FOXO-1) inhibitor AS1842856 (1 μM, (41)) (Calbiochem, San Diego, CA). All the inhibitors were dissolved in DMSO (Sigma-Aldrich) so that the final concentration of DMSO added to cells was less than 0.1%. DMSO at 0.1% concentration had no effect on PAR-2 expression (appendix-1).

RNA extraction, reverse transcription and quantitative RT-PCR

RNA extraction was performed using RNeasy kit (Qiagen, Germantown, MD); 0.5 μg RNA was reverse transcribed using, 0.5 μg Oligo (dt) 12-18 (Life Technologies) and 200U M-MLV reverse transcriptase (Life Technologies) in 20 μl final volume.

Human *PAR-2* mRNA was quantified using a custom 6FAM-labeled DTAM probe (5'-TAA GGT TGA TGG CAC ATC CCA CGT CAC TGG -3') (ThermoFisher Scientific) and the following primers *PAR-2*-F (5'-TGC TAG CAG CCT CTC TCT CC -3') and *PAR-2*-R (5'-CCA GTG AGG ACA GAT GCA GA-3'). *GAPDH* mRNA was used as an internal control and was quantified using a custom 6FAM-labeled TAMRA probe (5'-AAA TCC CAT CAC CAT CTT CCA GGA GCG A-3') (ThermoFisher Scientific) and the following primers *GAPDH*-F (5'-CTG AGA ACG GGA AGC TTG TCA -3') and *GAPDH*-R (5'-GCA AAT GAG CCC CAG CCT T-

3'). The PCR protocol consisted of 10 minutes at 94˚C followed by 40 cycles of 15 seconds at 94˚C, 25 seconds at 62˚C and 15 seconds at 72˚C. All samples were run in triplicate. The *PAR-2* and *GAPDH* PCR products were cloned into the pCR2.1-TOPO vector (Life Technologies), quantified and serial dilutions of linearized plasmid were used to generate a standard curve of known copy numbers for each gene. Data were calculated and represented *PAR-2* copies /1000 *GAPDH* copies. PAR-2 expression was also normalized to a second housekeeping gene, *Cyclophilin A (PPIA)*. For this Taqman gene expression assays were used for *PAR-2* (Hs00608346_m1, ThermoFisher Scientific) and housekeeping gene *Cyclophilin A* (Hs04194521_s1, ThermoFisher Scientific).

Mouse *PAR-2* expression was measured using Taqman gene expression assay (Mm0043360_m1, ThermoFisher Scientific, Waltham, MA) as per manufacturer's instructions; *HPRT* (Mm03024075_m1, ThermoFisher Scientific) was used as a house keeping gene.

Confocal microscopy

AECs were cultured on collagen coated glass coverslips and subjected to various treatments. Cells were then rinsed with PBS and fixed with 4% paraformaldehyde-PBS for 15 minutes (min) at room temperature, rinsed (with PBS 3X 5 min), permeabilized using 0.5% triton X100-PBS for 10 minutes and then rinsed to remove triton X100. Cells were then blocked using 1% BSA-PBS for 30 min at room temperature to decrease nonspecific binding of antibodies; excess BSA was removed by rinsing cells. Cells were then incubated overnight at 4˚C with 2 μg anti-PAR-2 monoclonal primary antibody (SAM-11, Santa Cruz Biotechnology, Dallas, TX) in 1% BSA-PBS. Cells were then rinsed to remove unbound antibody and incubated with 4 μg secondary antibody (Alexa Fluor 488 goat anti-mouse IgG2a, Molecular Probes) in 1% BSA-PBS for 90 min at room temperature. Cells stained with only secondary antibody were used as negative control. Cells were again rinsed to remove unbound antibody and incubated with 5 μM DAPI (Sigma-Aldrich) for 10 min in dark at room temperature. Cells were rinsed with water and mounted in prolong gold without DAPI (Molecular Probes) and allowed to cure overnight at room temperature in dark. Images were obtained using a 40X objective on a Leica SP5 confocal microscope. Images were analysed using ImageJ software (NIH) to calculate Corrected Total Cell Fluorescence (CTCF).

CTCF = Integrated density – (Area of selected cell X Mean fluorescence of background reading)

In the case of cell transfection with FOXO-1-GFP, live imaging was performed for up to 40 min (picture captured at every 10 min interval) using 20X objective on WaveFX spinning disk confocal microscope (Quorum Technologies).

Ca^{++} release assay

PAR-2-mediated Ca^{++} release from intracellular stores, endoplasmic reticulum (ER), was measured using Fluo-4 NW Calcium Assay Kit (Life Technologies) as per manufacturer's instructions. In summary, AECs were cultured in 96 well plates (Corning® Clear Bottom Black plates) and, when 85% confluent, were treated as required for each experiment. At the end of the treatment, cells were incubated with 100 μl of dye loading solution containing probenecid for 30 min each at 37° C and at room temperature. Florescence measurements representing cytoplasmic Ca^{++} levels were recorded by setting the instrument at 485 nm wavelength for excitation and 516 nm wavelength for emission. The baseline fluorescence was first recorded for 100 seconds. After this 10 μl of PAR-2 activating peptide (PAR-2 AP) or control peptide (PAR-2 CP) (50 μM final concentration) were added and fluorescence was recorded for up to 180 seconds. To study the PAR-2-mediated Ca^{++} release from intracellular store, endoplasmic reticulum (ER), we performed the assay in calcium, magnesium free HBSS buffer (Fisher Scientific). This ensured that the increase in cytoplasmic Ca^{++} is due to the release of Ca^{++} from intracellular stores and not due to entry of Ca^{++} from the assay buffer. After PAR-2 AP or CP, 1 μM Thapsigargin (Sigma-Aldrich) was added and fluorescence was measured up to 300 seconds. Thapsigargin induces Ca^{++} release from ER (42). PAR-2-mediated Ca^{++} release from ER was calculated as % of thapsigargin-induced release from ER.

Preparation of PAR-2 reporter plasmid to study PAR-2 promoter activity

The upstream region of transcription start site is considered as a putative promoter region. We used human genomic DNA as a template to amplify a 670 base pair (bp) fragment covering exon-1 (288 bp) and part of the upstream region of Exon-1 of PAR-2 gene using forward primer 5'-CTCCTCCTGCAGGGTCCACC-3', and reverse primer 5'-AGAGCCCTCCTTGGCCAGGT-3'. A kpn 1 restriction digestion site was added to the forward

primer and a Xho 1 restriction digestion site was added to the reverse primer. The PCR product was gel purified with a gel purification kit (Qiagen) and then ligated into the TOPO vector (Life Technologies) using TA cloning. The vector was then sequenced and confirmed for the orientation of the 670 bp DNA fragment. The TOPO vectors were then digested with kpn I and Xho I restriction enzymes to recover the 670 bp fragment DNA. The pGL3-Basic vector (Promega, Madison, WI) was also digested with kpn I and Xho I restriction enzymes. The recovered 670 bp DNA fragment was ligated into the digested pGL3-Basic vector upstream of the firefly luciferase gene to prepare a PAR-2 reporter plasmid. The plasmid DNA was purified using the plasmid maxi kit (Qiagen). The plasmid concentration and purity were measured by Nanodrop 2000c (Thermo Scientific). The size of 670 bp construct provided us with a small size reporter plasmid to increase transfection efficiency and also enabled us to design specific primers to amplify the fragment of interest. However, further different sizes of promoter constructs need to be studied to fully understand the PAR-2 promoter region.

Transfection studies

A pBluescript containing the coding sequence for a constitutively active form of transcription factor FOXO-1 (FOXO-1 CA) was used (43). AECs were nucleofected with 2-4 µg of FOXO-1 CA or GFP-encoding plasmid (pmax-GFP) or empty pBluescript according to the manufacturer's instruction using Amaxa Basic Nucleofector kit for Primary Mammalian Epithelial cells (Lonza), or Ingenio Electroporation kit (Mirus Bio, Madison, WI) and Amaxa Nucleofector 2s (W-005 program, Lonza).

AECs were transfected with a previously described FOXO-1-GFP plasmid (43) using Mirus TransIT-X2 reagent as per manufacturer's instructions (Please see Appendix-2 for transfection optimization procedure with 3 different transfection reagents from Mirus). In summary, AECs were cultured in 35mm tissue culture dishes (Falcon®, Fisher Scientific) until 85% confluent. Cells were then rinsed with PBS and 2 mL of BEGM media without antibiotics was added. Transfection complexes were formed by mixing plasmid DNA (2.5 µg) and transfection reagent (5 µL) in 250 µl BEBM media for 30 min at room temperature and added drop wise to the culture dishes. The next day, transfected cells were rinsed and incubated with 0.5 µg/mL Hoechst stain at 37˚C for 10 min to stain nuclei. Cells were then rinsed and cultured in insulin-deficient

media for 2-4h to allow nuclear localization of FOXO-1-GFP. Insulin (5 µg/mL) was then added and the movement of FOXO-1-GFP was studied by live imaging for up to 40 min using confocal microscopy.

For promoter studies, BEAS-2B cells were seeded in 12-well plates (Falcon) and cultured until 85% confluent. On the day of transfection, cells containing media was replaced with BEBM media (400 µL) after rinsing the cells with PBS. The transfection complexes were formed by mixing 1 µg PAR-2 reporter plasmid DNA or empty pGL3-Basic vector (negative control), 5 ng Renilla plasmid DNA and 2 µg of Lipofectamine 2000 transfection reagent (Life Technologies) in 100 µL BEBM. They were incubated for 30 min at room temperature, after which 100 µL of each transfection complex was added to the cells drop wise and cells were incubated for 5h. The transfection complexes were then removed and growth media was added for 24h. The next day, to study the effect of insulin on PAR-2 promoter, cells were cultured with or without insulin for 24h and promoter activity was assessed by performing luciferase reporter assay.

Luciferase Reporter Assay

At the end of the incubation period, cells were harvested using a cell scraper, pelleted and lysed with 60 µL passive lysis buffer (Promega) per well. From the 60 µL lysate, a 50 µL aliquot was assayed for luciferase activity using Dual –Luciferase Reporter Assay Kit (Promega) and the signal was measured using Turner BioSystems $20/20^n$ Luminometer. The measured luciferase activity is proportional to the amount of produced firefly luciferase enzyme and thus proportional to the promoter activity of the cloned region. To control for any effect of the plasmid itself, one set of cells were transfected with empty pGL3-Basic vector. Renilla is a constitutive active luciferase, thus Renilla luciferase value is used as a control for transfection efficiency. The firefly luciferase value of each sample was normalized by Renilla luciferase of the same sample (firefly luciferase / Renilla luciferase), which gave us the promoter activity of the PAR-2 promoter construct. Finally the results were expressed as the fold change in promoter activity over empty plasmid vector (promoter activity in cells transfected with PAR-2 reporter construct / promoter activity in cells transfected with empty pGL3-Basic vector). To study the effect of insulin deprivation on PAR-2 promoter activity, results were expressed as fold change in promoter activity over cells cultured with insulin.

Western blot

To study the presence of phospho-Akt in mouse lungs, we used rabbit anti-Akt and rabbit anti-phospho-Akt (Ser473) (Cell Signaling, Danvers, MA) primary antibodies, followed by HRP labelled secondary antibody (goat anti-rabbit IgG, Santa Cruz) and the membranes were developed with ECL reagents (ThermoFisher Scientific). To study insulin-mediated FOXO-1 phosphorylation in AECs, we used rabbit anti-FOXO-1 and rabbit anti-Phospho-FOXO-1 (Ser256) (Cell Signaling) primary antibodies, followed by IRdye® conjugated secondary antibody (goat anti-rabbit IgG, LI-COR, Lincoln, NE) and membranes were imaged using Odyssey Infrared Imager (LI-COR). Images were analyzed using Image Studio Lite software (LI-COR) and results were expressed as a ratio of Phospho-Akt/total Akt and Phospho-FOXO-1/total FOXO-1.

Statistics

The results are expressed as Mean ± SEM. In case of comparisons between two groups, two-tailed paired *t*-test was performed, except for the mouse experiments where two-tailed Mann Whitney test was used. In case of three or more groups, repeated measures ANOVA was used with Tukey's multiple comparison test. The *p*-value of 0.05 or less was considered statistically significant.

2.4 Results

PAR-2 expression in lungs of HFD-fed mice

HFD induces insulin resistance (44) in skeletal muscle, liver and adipose tissue. Insulin resistance modulates PAR-2 expression in adipose tissue (34-36) indicating that insulin regulates PAR-2 expression. We hypothesized that insulin also regulates PAR-2 expression in the lungs of HFD-fed mice. As expected, plasma insulin levels were higher in mice fed with HFD compared to mice fed a control diet (Fig. 2.1A) but there was no significant difference in downstream insulin signaling as measured by phospho-Akt levels in whole lungs (Fig. 2.1B and 2.1C), indicating absence of altered insulin signaling in the lungs of HFD-fed mice. Finally, PAR-2 mRNA expression was decreased in lungs of HFD-fed mice (Fig. 2.1D). From these results we hypothesized that increased circulating insulin levels suppressed PAR-2 expression in lungs of HFD-fed mice. This hypothesis may explain the observed PAR-2 upregulation on AECs of asthmatic individuals (24) in a background of decreased insulin signaling (33).

(A)

(B)

(C)

(D)
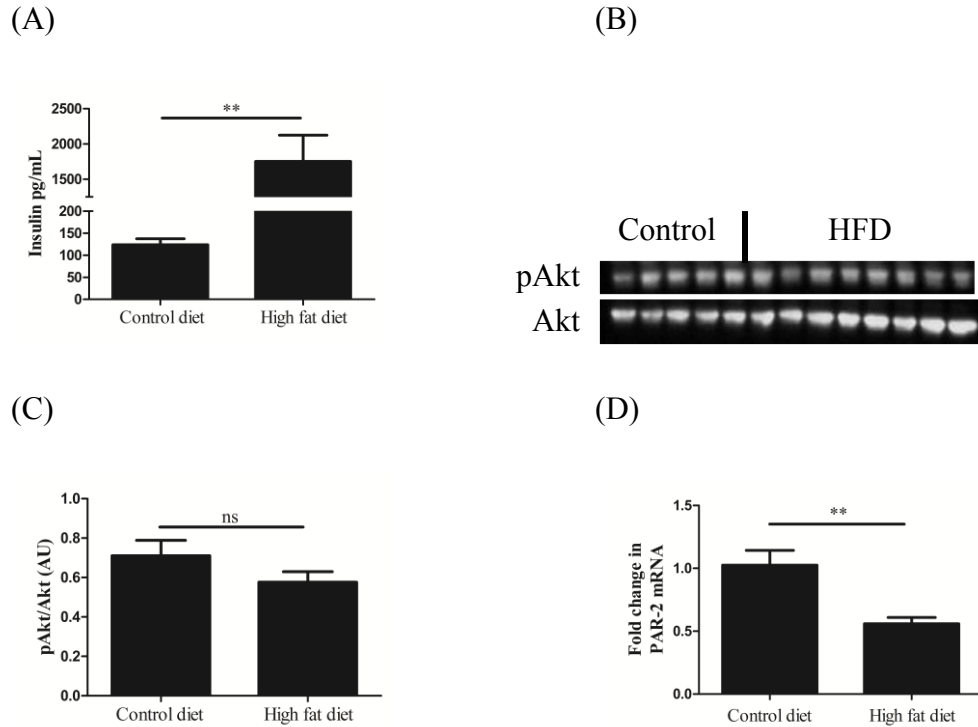
Fig. 2.1 Plasma insulin levels, Akt phosphorylation in lungs and PAR-2 expression in lungs of HFD- and control diet-fed mice (A) Plasma insulin levels in HFD- and control diet-fed mice, (B) a western blot and (C) densitometric analysis of Akt phosphorylation in lungs of HFD- and control diet-fed mice and (D) PAR-2 expression in lungs of HFD- and control diet-fed mice. Data are presented as Mean ± SEM from n=9 for HFD-fed and n=5 for control diet-fed mice; ns (not significant), **$P<0.01$.

Insulin regulates PAR-2 mRNA expression in primary human AECs

To explore the role of insulin in regulating PAR-2 expression in primary human AECs, we cultured cells in media with all the growth supplements (media with insulin) or media with all the growth supplements except insulin (media without insulin) for up to 48h and PAR-2 expression was studied by qRT-PCR. Insulin deficiency resulted in PAR-2 mRNA upregulation at all time points compared to cells cultured in media with insulin (Supplementary Fig. 2.E1A and 2.E1B), while PAR-2 expression remained constant at all time points in cells cultured in media with insulin. PAR-2 mRNA upregulation in the absence of insulin was confirmed using

another housekeeping gene, cyclophilin A (Supplementary Fig. 2.E1C). PAR-2 mRNA upregulation in the absence of insulin plateaued at 24h (Fig. 2.2A). Subsequent studies to define the mechanism of insulin-mediated PAR-2 regulation were performed with cells cultured without insulin for 24h. Addition of insulin reversed insulin deficiency-induced PAR-2 upregulation (Fig. 2.2B), indicating that this is a dynamic change in PAR-2 expression and not the effect of permanent alterations in cellular biology due to the absence of insulin. To study the cause and effect relationship between the levels of insulin and PAR-2 expression, we cultured AECs with various concentration of insulin, in the absence of all other growth supplements. Insulin concentrations between 0.2 μg/mL and 125 μg/mL dose dependently regulated PAR-2 expression (Fig. 2.2C).

We then studied whether insulin deficiency-induced PAR-2 mRNA upregulation is the result of increased PAR-2 mRNA stability or increased PAR-2 gene transcription. The rate of PAR-2 mRNA degradation in the presence of actinomycin D, was the same whether cells were previously incubated for 24h in the presence or absence of insulin, indicated that the absence of insulin did not alter PAR-2 mRNA stability (Fig. 2.2D). To study whether insulin directly affects PAR-2 mRNA transcription we used a 670 bp PAR-2 reporter plasmid that we have generated. Since primary AECs are difficult to transfect we used the BEAS-2B cell line (45) for these experiments. Insulin deficiency resulted in PAR-2 mRNA upregulation in BEAS-2B cells, similar to primary AECs (Fig. 2.2E). Insulin deficiency in BEAS-2B cells transfected with the PAR-2 reporter construct increased PAR-2 promoter activity (Fig. 2.2F) indicating that insulin regulates PAR-2 gene transcription.

(A)

(B)

n=3 for 2h,6h,48h
n=6 for 24h

Fold change in PAR-2 mRNA over "cells with Insulin"

Time (h) 2 6 24 48

PAR-2 copies / 1000 copies of GAPDH

n=4

Time (h) 0 24 24
Insulin   + + -

After 24h w/o insulin

24 24 Time (h)
+   -   Insulin

(C)

(D)

PAR-2 copies / 1000 copies of GAPDH

n=3

Growth media 0.2 1 5 25 125

Insulin µg/mL

24h

% PAR-2 copies remaining

n=4

— Without Insulin
— With Insulin

-6.693 ± 0.9957

-6.469 ± 0.7353

Act D time (h)

(E)

(F)

PAR-2 copies / 1000 copies of GAPDH

n=3

Time (h) 0 24 24
Insulin   + + -

Fold change over "cells with insulin"

n=8

Insulin   +   -

24h

Fig. 2.2 Insulin transcriptionally regulates PAR-2 mRNA expression in AECs. (A) Fold change in PAR-2 mRNA expression in cells cultured in the absence of insulin compared to cells cultured in the presence of insulin for up to 48h (n=3-6). (B) Reversibility of insulin deficiency-induced upregulation of PAR-2 mRNA expression by insulin (n=4). After the first 24h of culture in the presence or absence of insulin (left part of the graph), cells cultured in the absence of insulin were further cultured with or without insulin for next 24h (right part of the graph). (C) PAR-2 mRNA expression in AECs cultured in the presence of various concentrations of insulin for 24h (n=3). (D) The rate of PAR-2 mRNA degradation for 8h after the addition of actinomycin D in cells cultured in the presence (Black) or absence (Red) of insulin for 24h (n=4). PAR-2 levels are

expressed as % PAR-2 expression compared to time "0". (E) PAR-2 mRNA expression in BEAS-2B cells cultured in the presence or absence of insulin for 24h (n=3). (F) PAR-2 promoter activity in BEAS-2B cells transfected with the PAR-2 reporter plasmid and cultured in the presence or absence of insulin for 24h (n=8). Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

Insulin regulates functional PAR-2 expression in AECs

Insulin deficiency also increased PAR-2 protein expression in AECs after 24h, as shown by confocal microscopy (Fig. 2.3A-C). It appears that during insulin deficient condition number of cells expressing PAR-2 is also increased (Fig. 2.3B). However, further studies are required where PAR-2-positive cells from multiple field of view are calculated to reach this conclusion.

As a Gq-coupled receptor, PAR-2 activation leads to release of Ca^{++} from intracellular stores ER, which activates calcium release-activated calcium channels and induces release of inflammatory mediators from AECs (46). To study if PAR-2 upregulation increases PAR-2-mediated calcium signaling, we measured PAR-2-mediated Ca^{++} release from ER using PAR-2 activation peptide (PAR-2 AP) or control peptide (PAR-2 CP). PAR-2 AP induced Ca^{++} release in AECs while PAR-2 CP did not (Supplementary Fig. 2.E2A). PAR-2 AP did not induce further Ca^{++} release in cells activated with thapsigargin indicating PAR-2-mediated Ca^{++} release is from ER (Supplementary Fig. 2.E2B). PAR-2 AP-mediated activation of cells cultured in the absence of insulin led to increased Ca^{++} release compared to activation of cells cultured in the presence of insulin (Fig. 2.4A and 2.4B) indicating that insulin-mediated regulation of PAR-2 expression may affect PAR-2-mediated activation of AECs.
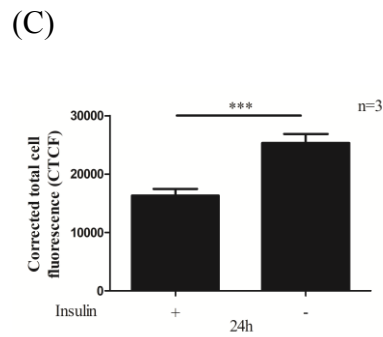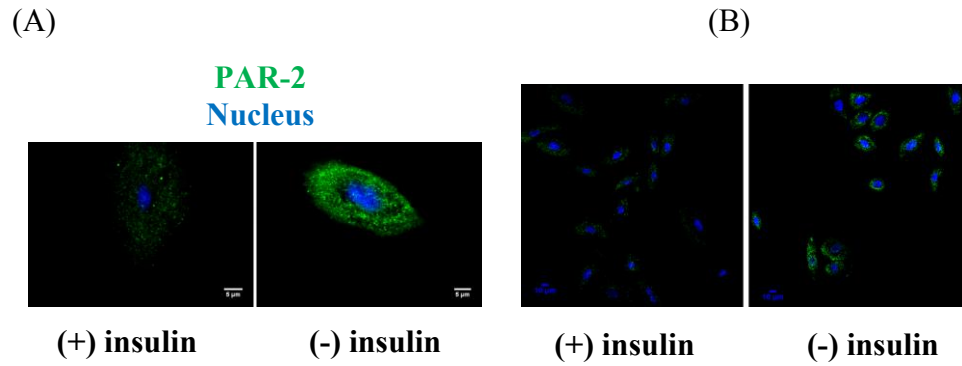
Fig. 2.3 Insulin regulates PAR-2 protein expression in AECs. (A-B) Images of PAR-2 expression (Green) in (A) a representative individual cell and (B) a representative field of view displaying multiple cells cultured in the presence (left image) or absence (right image) of insulin for 24h. Nuclei are stained with DAPI (Blue). (C) Corrected Total Cell Fluorescence intensity of PAR-2 expression in AECs cultured in the presence or absence of insulin (Fluorescence was calculated for 30 cells / experiment, n=3). Data are presented as Mean ± SEM; ***P<0.001.
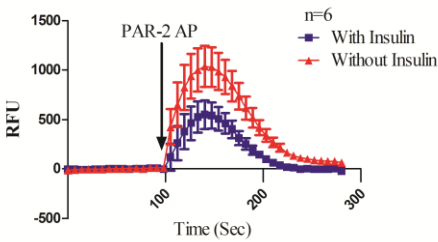
(A)   (B)
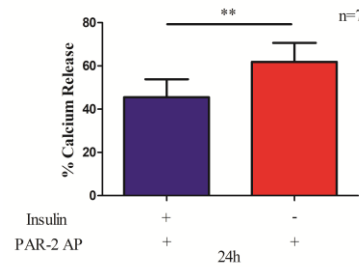
Fig. 2.4 Insulin regulates PAR-2-mediated activation of AECs. (A) PAR-2-mediated Ca^{++} release from ER in cells cultured in the presence (Blue line) and absence of insulin (Red line) for 24h. (B) Quantification of the intracellular Ca^{++} release by PAR-2 as a percentage of the release induced by thapsigargin (n=7). Data are presented as Mean ± SEM; **P<0.01.

Insulin regulates PAR-2 expression through PI3K-Akt pathway

Upon binding to insulin receptor, insulin activates the PI3K-Akt signaling pathway which mediates many of the insulin effects (47). PI3K inhibition using LY294002 in cells cultured in the presence of insulin showed increase in PAR-2 expression compared to cells cultured in the presence of insulin without the inhibitor (Fig. 2.5A). Since insulin in the absence of all other growth supplements regulates PAR-2 expression (Fig. 2.2C), we then cultured cells in the presence of insulin or in media without insulin with or without an irreversible PI3K inhibitor wortmannin. Again, PI3K inhibition in the presence of insulin resulted in increased PAR-2 expression (Fig. 2.5B). These results indicate that insulin regulates PAR-2 through PI3K-Akt pathway. To investigate the role of PI3K-Akt pathway in the observed insulin-mediated reversibility of upregulated PAR-2 expression (Fig. 2.2B), we cultured cells in the absence of insulin to upregulate PAR-2 and then added insulin with or without PI3K inhibitors. PI3K inhibition with LY294002 or wortmannin prevented insulin from downregulating PAR-2 expression (Fig. 2.5C and Fig. 2.5D).

(A)

(B)

(C)

(D)
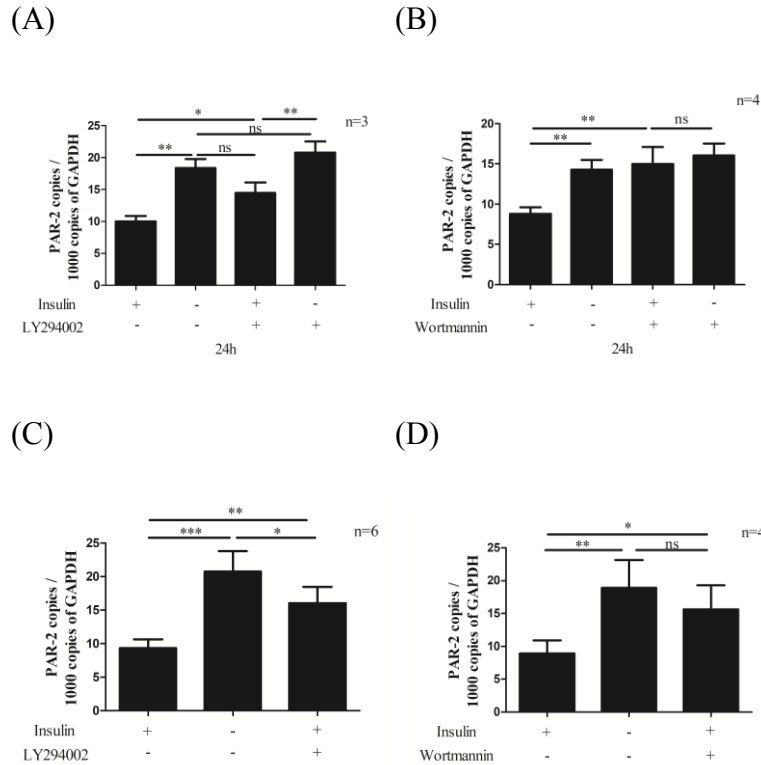
Fig. 2.5 Insulin regulates PAR-2 expression through PI3K activation. PAR-2 mRNA expression in cells cultured in the presence or absence of insulin with or without the PI3K inhibitor (A) LY294002 (n=3) or (B) Wortmannin (n=4) for 24h. PAR-2 mRNA expression in cells that were first cultured in the absence of insulin for 24h and then further cultured in the absence of insulin or in the presence of insulin with PI3K inhibitor (C) LY294002 (n=6) and (D) Wortmannin (n=4) for another 24h. Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

Insulin regulates PAR-2 expression through the transcription factor FOXO-1

FOXO-1 transcription factor is a direct Akt target (48). Insulin, through Akt, phosphorylates FOXO-1 on 3 sites (T24, S253, S316) leading to nuclear exclusion and decreased transcription factor activity (43, 49). Our in silico analysis of the 670 bp PAR-2 promoter indicated that the promoter contains FOXO-1 binding sites (Appendix-3). We therefore hypothesized that FOXO-1 becomes active during insulin deficiency and induces transcription of PAR-2 in AECs.

We first studied if insulin phosphorylates FOXO-1 and induces its nuclear exclusion in primary AECs. Addition of insulin to AECs cultured in the absence of insulin resulted in phosphorylation of endogenous FOXO-1 (Fig. 2.6A and 2.6B). To study if insulin induces nuclear exclusion of FOXO-1, cells were transfected with a FOXO-1-GFP plasmid. FOXO-1-GFP localized to AEC nucleus in the absence of insulin (Fig. 2.6C, left image) and addition of insulin induced nuclear exclusion of FOXO-1-GFP (Fig. 2.6C, right image and Supplementary Video 1). These results indicate that insulin inhibits FOXO-1 activity in primary AECs.

Since insulin was able to inhibit FOXO-1 in AECs, we next studied if FOXO-1 can induce PAR-2 expression in AECs. Cells were transfected with a plasmid coding for constitutively active FOXO-1, where 3 phosphorylation sites are mutated (T24A, S253D, S316A) rendering FOXO-1 insensitive to insulin-Akt-mediated phosphorylation and inhibition. Transfection of AECs with the constitutively active FOXO-1 CA upregulated PAR-2 expression in cells cultured in the presence of insulin (Fig. 2.6D) indicating that FOXO-1 can mediate PAR-2 expression. Finally, to study the role of FOXO-1 in insulin deficiency-induced PAR-2 upregulation, we inhibited FOXO-1 using an inhibitor AS1842856 in cells cultured in the media without insulin. FOXO-1 inhibition prevented insulin deficiency-induced upregulation of PAR-2 (Fig. 2.6E).
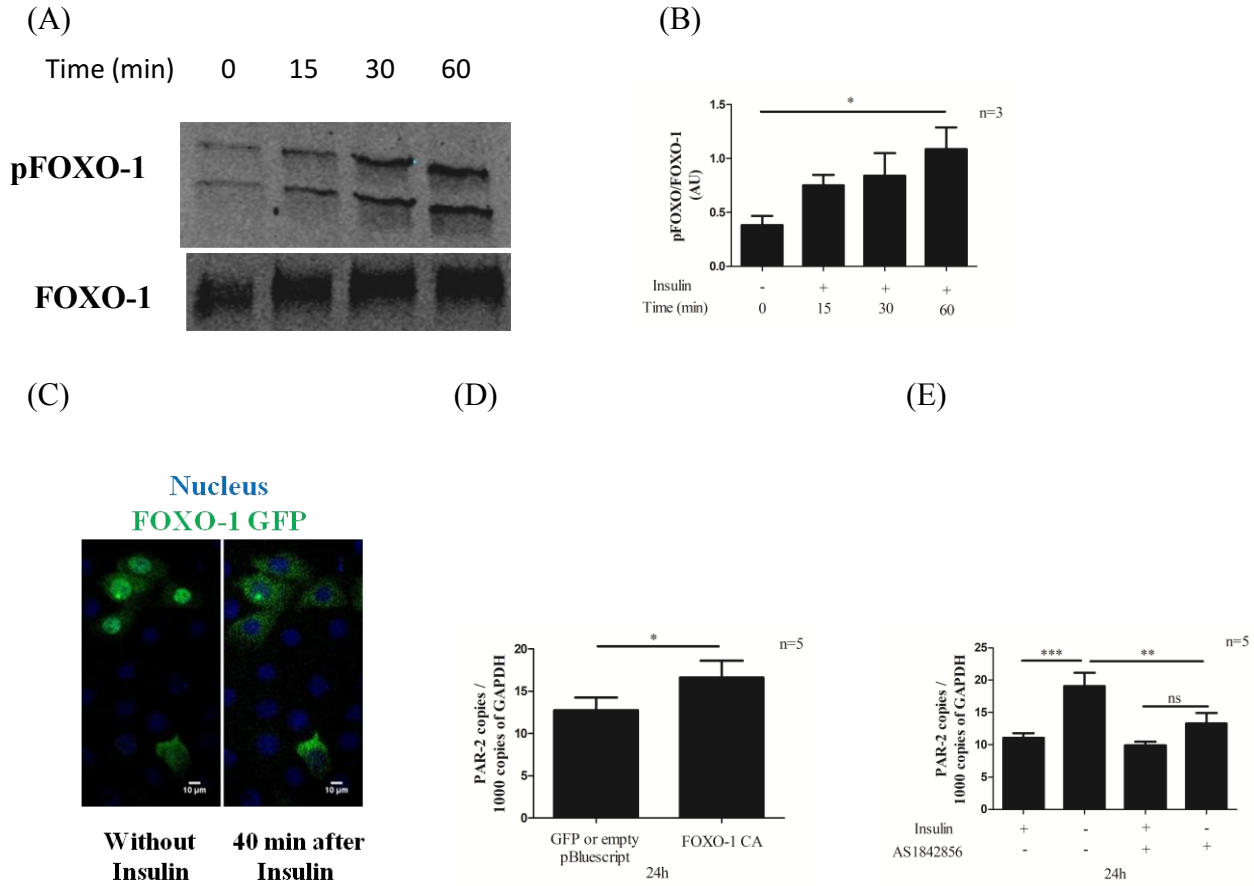
Fig. 2.6 Insulin regulates PAR-2 expression by controlling FOXO-1 transcription factor in AECs. AECs were incubated in media without insulin for 4-6h and then insulin (5 µg/ml) was added for the indicated time. (A) A representative blot and (B) densitometric analysis of FOXO-1 phosphorylation by insulin at indicated time points (n=3). (C) Representative images of insulin-induced nuclear exclusion of FOXO-1 (Green). FOXO-1-GFP transfected cells cultured without insulin (left image) and after addition of insulin (right image) (D). PAR-2 mRNA expression in AECs nucleofected with FOXO-1 CA or pmaxGFP or empty pBluescript (n=5) and cultured in the presence of insulin for 24 h. (E) PAR-2 mRNA expression in AECs cultured in the presence or absence of insulin with or without FOXO-1 inhibitor AS1842856 for 24h. Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

ALI cultures of AECs show similar insulin-mediated PAR-2 regulation

All studies reported above were performed with submerged cultures of AECs. However, submerged cultures are not differentiated and polarized as *in vivo*. We therefore, studied the effect of insulin on ALI cultures. ALI cultures showed the presence of ciliated cells demonstrating differentiation of AECs (Fig. 2.7A). Insulin deficiency in ALI cultures resulted in PAR-2 upregulation (Fig. 2.7B) similar to that observed in submerged cell cultures. In addition, as in submerged cultures, FOXO-1 inhibition prevented insulin deficiency-induced PAR-2 upregulation in ALI cultures (Fig. 2.7C).

(A)   (B)   (C)
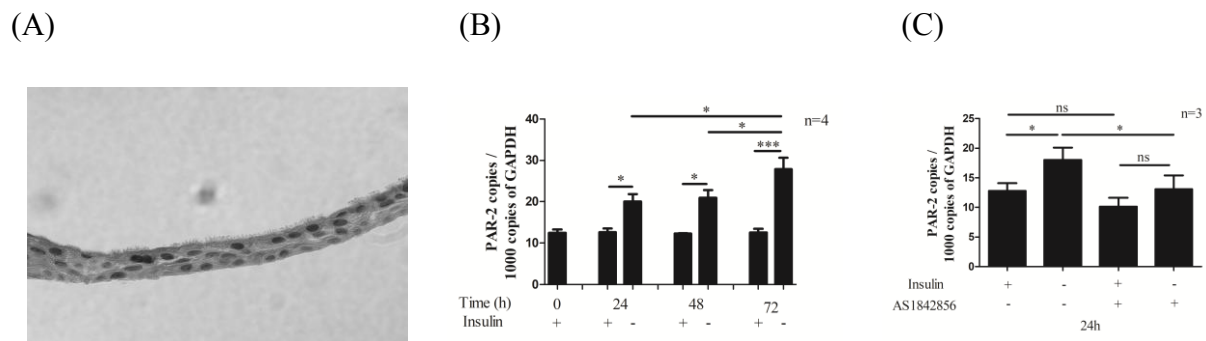
Fig. 2.7 Differentiated cultures of AECs show similar insulin-mediated PAR-2 regulation. (A) Haematoxylin and Eosin (H&E) staining of AECs cultured at Air-Liquid Interface (ALI) to allow cellular differentiation. (B) PAR-2 mRNA expression in differentiated AECs in the presence or absence of insulin for up to 72h (n=4). (C) PAR-2 mRNA expression in differentiated AECs in the presence or absence of insulin with or without FOXO-1 inhibitor AS1842856 for 24h (n=3). Data are presented as Mean ± SEM; ns (not significant), *P<0.05, ***P<0.001 between the groups indicated by horizontal lines.

2.5 Discussion

The serine proteinase receptor PAR-2 is involved in the pathogenesis of allergic asthma (9, 10). In addition, PAR-2 expression is increased in AECs of patients with asthma (24), but the regulation of PAR-2 expression in AECs is poorly studied. We show that insulin regulates expression of PAR-2 on primary AECs through a PI3K-Akt-FOXO-1 pathway. Decreasing concentrations of insulin lead to upregulation of PAR-2 expression and increased potential for PAR-2-mediated activation of epithelial cells. To our knowledge, this is the first report providing a detailed mechanism of PAR-2 regulation in primary human bronchial epithelial cells. In addition, this is the first evidence showing regulation of PAR-2 by a hormone with anti-inflammatory activities.

Recently Loffredo *et al.* (33) analyzed published microarray data and found decreased expression of insulin receptor in AECs obtained from asthmatic individuals. The authors also noted that decreased insulin receptor expression was associated with downregulation of genes involved in epithelial differentiation and validated this observation ex vivo. For their experiments they excluded insulin from ALI cultures of normal AECs throughout the differentiation period and showed that by decreasing insulin signaling they were able to mimic the decreased differentiation of AECs observed in asthma *in vivo*. In our experiments, we cultured AECs with insulin during the differentiation phase of the ALI cultures, and removed insulin after the cultures were fully differentiated. We found that insulin deficiency resulted in PAR-2 upregulation indicating that disruption in insulin signaling could result in increased PAR-2 expression in fully differentiated normal human AECs. We did not validate our confocal microscopy results with another protein detection method for a number of reasons as discussed below. AECs are strongly adherent cells and trypsin is required to detach cells from the cell culture vessels. Trypsin is a PAR-2 agonist and may result in the loss of cell surface PAR-2 expression and recognition of the receptor by the antibody (50) we have been using for detection in flow cytometry in other studies (51). Hence flow cytometry will not be helpful at answering if cell surface PAR-2 expression is increased in the absence of insulin. We also did not use western blot for analysing total PAR-2 expression in the presence or absence of insulin. The molecular weight of PAR-2, based on its amino acid sequence, is 44 kilodalton (kDA) (52). Based on the

post-translational modifications, PAR-2 molecular weight ranges from 44-100 kDA, which results in multiple bands, on a western blot, representing different post-translationally modified forms of PAR-2 (53); multiple bands make difficult to compare and conclude if PAR-2 expression is increased in the absence of insulin.

PAR-2 activation in the airways plays a role in the development of allergic sensitization (54), allergic airway inflammation (10) and airway remodeling (55), while the degree of airway inflammation in these cases may depend on the levels of PAR-2 expression (9). These effects are mediated by the plethora of inflammatory mediators released by PAR-2-activated AECs (13-17); mediator release that in most cases depends on PAR-2-mediated calcium signaling (46). We show here that AECs grown in the absence of insulin exhibit increased calcium signaling following PAR-2-mediated activation, which may result in higher inflammatory mediator release. Thus insulin, by regulating PAR-2 expression, could regulate PAR-2-mediated inflammation.

Insulin has indirect anti-inflammatory properties by preventing hyperglycemia; however, there is increasing evidence that insulin has also direct anti-inflammatory properties by regulating the activity of transcription factors and controlling expression of chemokine receptors, endothelial adhesion molecules and Toll-like receptors (29). There is limited information on the effects of insulin on AECs. Insulin regulates expression of surfactants (56), glucose uptake and barrier function (57) in AECs through the PI3K-Akt pathway. Older studies also suggested that insulin plays a role in wound healing by being a direct chemotactic factor for AECs (58, 59) and/or by regulating expression of fibronectin in AECs (60). Our results demonstrate another anti-inflammatory effect of insulin on AECs through inhibition of the expression of a pro-inflammatory receptor, PAR-2. Thus, alteration in insulin signaling may increase the possibilities for the development of PAR-2-mediated inflammation.

Insulin has also anti-inflammatory effects by regulating the activity of transcription factors and thus inhibiting release of inflammatory mediators. Insulin can inhibit NF-κB transcription factor indirectly by increasing the expression of IκB (61). The FOXO family of transcription factors is under the direct negative regulation of insulin-mediated PI3K-Akt signaling (48). There is limited information on the role of FOXO transcription factors in AEC biology. FOXO-1 is

activated upon bacterial infection of AECs and induces inflammatory mediators and defensins, an effect that is negatively regulated by insulin (62). Our results indicate that with decreasing insulin signaling, FOXO-1 becomes activated and induces PAR-2 transcription leading to increased PAR-2 expression and increased pro-inflammatory potential of the epithelial cells.

An important question is whether decreased *in vivo* insulin signaling in asthma is only the result of decreased receptor expression (33) or there is also an element of insulin resistance. There are two basic animal models to study the effect of insulin *in vivo*; insulin deficient mice (by destruction of pancreatic β cells) and insulin resistant mice. PAR-2-mediated acute paw inflammation is higher in insulin deficient mice (63). Although alterations in PAR-2 expression were not studied in these mice, the effect described could be the result of increased PAR-2 expression in the absence of insulin. HFD-induced obesity induces insulin resistance in mouse adipose tissue, and has been associated with increased PAR-2 expression in adipose tissue and increased PAR-2-mediated adipose tissue inflammation (34-36). However, the authors did not study whether there is a direct pathway between insulin resistance and PAR-2 expression or increased PAR-2 expression is the result of alterations in other inflammatory pathways in HFD-fed mice. Using a similar model of HFD-induced obesity, we did not find alteration in insulin signaling in lung tissue but we found that PAR-2 expression was decreased. We hypothesized that hyperinsulinemia in these mice suppresses PAR-2 expression on AECs. Our *in vitro* data, which show that the higher the concentration of insulin in the media, the lower the PAR-2 expression in AECs, support this observation.

Our results predict that PAR-2 expression should increase in the presence of insulin resistance in airway epithelium. Whether this phenomenon underlies the increased PAR-2 expression seen on the airway epithelium in asthma (24), IPF (11) or inflammatory conditions in other tissues (3, 19-21), requires further study. Inflammatory mediators such as TNF, IL-6 and IL-1β induce insulin resistance in adipose tissue (64). These mediators are also increased in the airway of asthmatics (65-68). Thus, it is possible that increased PAR-2 expression in asthma is the result of localized insulin resistance in the airway epithelium induced by the inflammatory milieu rather than an effect of an individual inflammatory mediator as shown in various *in vitro* studies (25-27). To answer this question, mucosal biopsies from asthmatic patients could be studied for the presence

of insulin resistance markers such as decreased insulin receptor expression (69) or serine phosphorylation of IRS-1 component of insulin receptor signaling cascade (70-73).

In conclusion, our data suggest a potential mechanism for the increased expression of PAR-2 on epithelial cells in asthma and possibly other inflammatory conditions, where PAR-2 plays a role in the disease pathogenesis. Understanding insulin-mediated regulation of PAR-2 expression in a variety of inflammatory diseases may uncover common dysregulatory mechanisms underlying increased PAR-2 expression and lead to the development of novel therapeutic strategies to regulate PAR-2 expression and PAR-2-mediated inflammation.

2.6 References

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2.9 Supplementary Figures

(A)   (B)   (C)
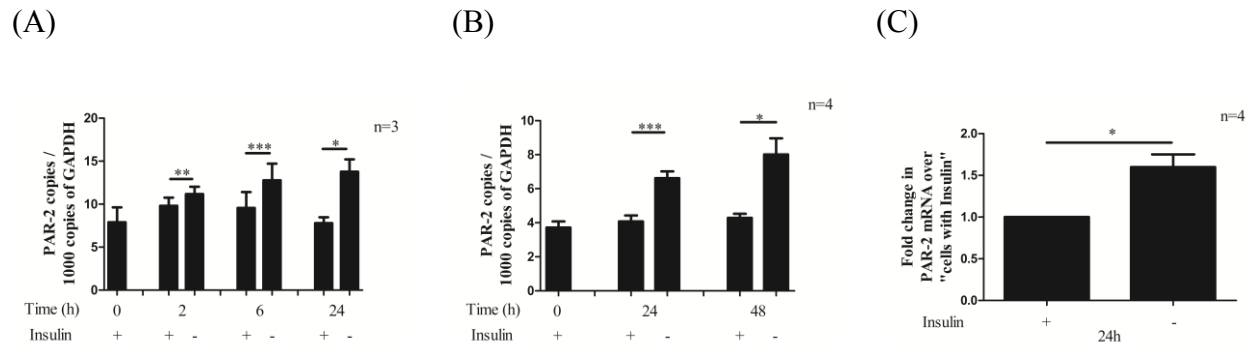
Supplementary Fig. 2.E1 Insulin deficiency upregulates PAR-2 expression in AECs. (A-B) Cells were cultured in media with or without insulin and PAR-2 expression was studied, at indicated time points, by qRT-PCR assay developed by our laboratory (see methods). PAR-2 mRNA expression was increased at all the studied time points (n=3-4). (C) Cells were cultured in media with or without insulin for 24h and PAR-2 expression was studied by qRT-PCR. Taqman gene expression assay was used for *PAR-2* (Hs00608346_m1, ThermoFisher Scientific) and housekeeping gene *Cyclophilin A (PPIA)* (Hs04194521_s1, ThermoFisher Scientific). The normalization of PAR-2 expression to PPIA, a second housekeeping gene, confirmed that insulin deficiency results in PAR-2 upregulation (n=4). Data are presented as Mean ± SEM; *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.
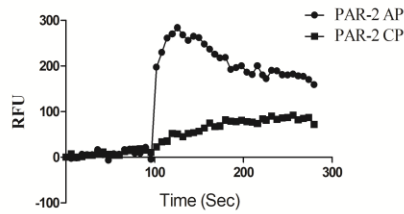
(A) (B)
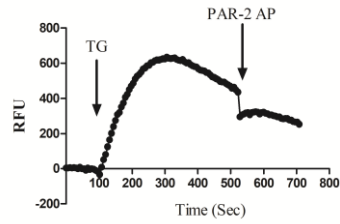
Supplementary Fig. 2.E2 PAR-2 activation induces intracellular Ca^{++} release from endoplasmic reticulum (ER) in AECs. AECs were cultured in 96 well plates (Corning Clear Bottom Black plates) and Ca^{++} release assay was performed (see methods). (A) PAR-2 specific activation peptide (PAR-2 AP) induced Ca^{++} release which was absent in PAR-2 control peptide (PAR-2 CP) treated cells. (B) Treatment of cells with Thapsigargin (TG) resulted in Ca^{++} release from intracellular stores ER. Subsequent PAR-2 activation failed to induce Ca^{++} release indicating PAR-2 activation results in Ca^{++} release from ER.

Chapter 3 Effect of insulin on proteinase-activated receptor-2 expression in the lungs *in vivo*

3.1 Aim: To study PAR-2 expression in the lungs of mice with lower levels of circulating insulin

3.2 Introduction

We showed that insulin dose-dependently downregulates PAR-2 expression in AECs cultured *in vitro* (Chapter 2). We also showed that mice with increased levels of circulating insulin *in vivo* express lower levels of PAR-2 in lungs further supporting the *in vitro* observation (Chapter 2). We now hypothesized that decreased levels of circulating insulin *in vivo* will lead to increased expression of PAR-2 in lungs. Usually the animal model used to study the effect of decreased insulin levels involves destruction of β-cells, using streptozotocin chemical, in order to prevent synthesis of any insulin. However, the effect of streptozotocin is not specific to β-cells (1). Moreover, destruction of β-cells results in hyperglycemia (2). To avoid these confounding factors, we decided to subject mice to overnight fasting as fasting decreases the levels of insulin.

3.3 Methods

C57BL6 male mice (12-14 weeks old) were used. One group of mice were subjected to 14h overnight fasting, while the other group of mice were maintained on regular diet; water was made available to both the groups. Mice were euthanized with an overdose of sodium pentobarbital and blood was collected, in regular 1.5 mL Eppendorf tubes, by cardiac puncture. Blood was left at room temp for 20-30 min to clot and then spun at 1500g for 10 minutes at 4˚C to separate serum; serum was stored at -80ºC. Lungs were stored in RNAlater (ThermoFisher) at 4˚C until used for qRT-PCR. Experiments were approved by the University of Alberta Health Sciences Laboratory Animal Ethics committee.

ELISA, to measure serum insulin levels, and qRT-PCR, to measure PAR-2 expression in lungs, were performed as described before (Chapter 2).

3.4 Results and Discussion

Overnight fasting resulted in decreased levels of circulating insulin (Fig. 3.1A); the levels of insulin were similar to those reported in the literature for the same age group of mice (3). PAR-2 expression was not increased in lungs of these animals with lower levels of insulin (Fig. 3.1B).

(A)   (B)
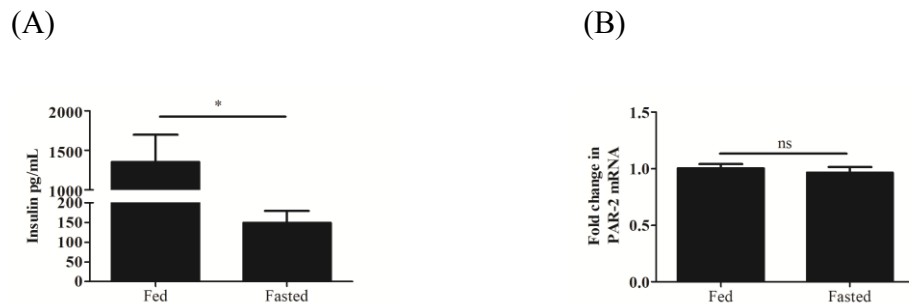
Fig. 3.1 Effect of overnight fasting on insulin levels in serum and PAR-2 expression in lungs. (A) Serum insulin levels and (B) PAR-2 mRNA expression levels in mice subjected to regular diet (Fed, n=4) or overnight fasting (Fasted, n=5). Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, 2-tailed Mann-Whitney t Test.

Our *in vitro* data with primary human AECs show that absence of insulin for 24h results in around 1.8 fold upregulation in PAR-2 mRNA expression. Kinetics of PAR-2 regulation *in vivo*, which is more complex system than pure culture of AECs *in vitro*, could be different and thus 14h of fasting that represents gradual decrease in the levels of insulin may not provide enough time to modulate PAR-2 expression. Other possibility could be that the expression of receptor was modulated on AECs but the modulation of PAR-2 expression was not limited to only AECs. Thus assessing PAR-2 expression in the entire lung may have diluted the signal. To take care of this issue, PAR-2 expression can be studied in lung epithelial cells isolated using laser capture microdissection.

Overnight fasting in mice activates catabolic state and results in loss of body mass by 15% (4). Also overnight fasting may result in improved insulin sensitivity in mice in tissue-specific

manner (5). Due to this reason, the experiments performed with overnight fasting are mainly to understand glucose utilization by different tissues (4). There are very limited studies looking at the expression of insulin-regulated genes after overnight fasting. In these experiments, human subjects (6, 7) or rodents (8) are first subjected to overnight fasting. After this, they are subjected to *in vivo* (in case of human subjects) or *ex vivo* (in case of rodents) insulin treatment and gene expression was compared in the same subject before and after insulin treatment.

A very limited research has focused on the effect of fasting on lungs. These studies have focused on the effect of prolong fasting (4 days) on the expression of glutathione, which provides protection against hyperoxic damage (9, 10) and have not focused on the insulin signaling. Fasting up to 48h is shown to have no effect on lung weight (11, 12). However, fasting resulted in increased expression of genes involved in proteolysis and autophagy pathways indicating the presence of stressors in the lungs (12). Hence it will not be advisable to use animal models subjected to prolong fasting to study the effect of lower levels of circulating insulin on PAR-2 expression in the lungs.

We also noticed a difference in the basal insulin levels in mice used for two different experiments. We used C57BL6 male mice for the following experiments:

1. High fat diet-induced obesity: One group of mice was maintained on high fat diet, while the other group was maintained on control diet (chow diet) (referenced as Exp-1)

2. Overnight fasting to decrease the levels of circulating insulin: One group of mice was subjected to overnight fasting, while the other group was maintained on control diet (chow diet) (referenced as Exp-2)

The average circulating levels of insulin in control diet-fed mice from Exp-1 had 124 pg/mL, while control diet-fed mice from Exp-2 had 1352 pg/mL. The Exp-1 was performed in collaboration with Dr. Jason Dyck hence the experiment was performed in their lab with their mice, while Exp-2 was performed in our lab.

It is known that different batches of mice harbour some differences. Also the same strain of animal obtained from different suppliers is shown to have differences in microbiome (13) and

hematologic variables (14). We compared the mice used in the above mentioned two experiments:

1. Age of the mice: At the end of the experiment, the age of the mice used in Exp-1 was around 9 months, while the age of the mice used in Exp-2 was around 3.5 months. However, in C57BL6 mice, insulin levels do not fluctuate as much between 3 to 9 months of age (15).

2. Composition of control diet: Different control diets could have different ingredients. The control diet used in Exp-1 was PicoLab Laboratory Rodent Diet Catalog#5L0D, while the control diet used in Exp-2 was PicoLab Rodent Diet 20 Catalog#5053. The calorie composition of these two control diets was very similar. However, the control diet used for Exp-1 contained more cholesterol and cholesterol negatively regulates insulin secretion (16). This could be one of the reasons that mice used in Exp-1 had lower insulin levels.

3. Mouse strain: The mouse strain used in the Exp-1 was C57BL6J (from Jackson lab), while the mouse strain used in Exp-2 was C57BL6N (from KnockOut Mouse Project KOMP, UC Davis). It is shown that C57BL6J mice (17) and C57BL6NJ mice (mixed genetic background) (3) have impaired insulin secretion compared to C57BL6N mice. This could be the primary reason that mice used in Exp-1 had lower insulin levels.

   The mice used in Exp-2 were obtained as heterozygous PAR-2 knockout mice. The homozygous PAR-2 mice (wild-type) were obtained by mating heterozygous PAR-2 knockout male mice with heterozygous PAR-2 knockout female mice. The wild-type colony of mice was maintained for 7-9 generations before using it for Exp-2. PAR-2 is shown to inhibit insulin secretion (18). Thus it may be possible that due to lower expression of PAR-2, in PAR-2 heterozygous knockout mice, insulin secretion and thus insulin levels were increased. However, it is not clear if this increased insulin secretion was maintained after the generation of wild-type mice. Measuring insulin levels in PAR-2 knockout, PAR-2 heterozygous and wild-type mice may provide some clarification.

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Chapter 4 Effect of insulin on proteinase-activated receptor-2 expression in primary human airway smooth muscle cells

4.1 Aim: To study the effect of insulin on PAR-2 expression in human airway smooth muscle cells

These experiments were done in collaboration with Dr. Andrew Halayko at the University of Manitoba. I designed the experiments with them. Dr. Halayko's lab performed the experiments and sent us samples and I evaluated PAR-2 expression.

4.2 Introduction

Airway SMCs play an important role in the development of airway remodeling and airway hyperresponsiveness (AHR), which are the characteristics of asthma. One of the factors that contribute to airway remodeling in asthma is increase in airway smooth muscle thickness (1), which could be due to increase in SMC proliferation (2) that may result in narrowing of the airways. Also, airway smooth muscle in asthma has increased contractile property (1), which increases AHR meaning that increased sensitivity of the airways to agonists inducing contraction. PAR-2 is expressed on airway smooth muscle cells (SMCs) (3). PAR-2 activation on airway SMCs is shown to increase smooth muscle proliferation (4, 5) and contraction (6). In asthma, PAR-2 expression is increased in airway SMCs (5) and is associated with airway smooth muscle thickness (7). Thus, in asthma, PAR-2 airway SMCs may contribute to airway remodeling, by inducing SMC proliferation, and to AHR, by making smooth muscle hyperresponsiveness to PAR-2 agonists. Insulin is shown to induce profibrotic and procontractile phenotype in airway SMCs by upregulating collagen release and proteins involved in contraction (8, 9). Since insulin develops procontractile phenotype it could be possible that insulin upregulates PAR-2 expression in airway SMCs, which further increased the smooth muscle sensitivity to contraction by PAR-2 agonists. Also we showed that insulin negatively regulates PAR-2 expression in AECs (Chapter 2) and we wanted to assess if insulin has similar or opposite effect in ASM for the regulation of PAR-2 expression.

4.3 Methods

Cell culture

Airway SMCs, from 3 donors, were isolated as described before (10). Briefly, airway SMCs were isolated from 2nd to 4th generation macroscopically healthy bronchial main stem surgically removed from patients undergoing lung resection surgery for adenocarcinoma. Cells were cultured in DMEM (Lonza) media containing 10% FBS (PAA Laboratories) and penicillin-streptomycin (Lonza) until they reached 80% confluency. Cells were then cultured in FBS-free DMEM media containing penicillin-streptomycin with or without 1X ITS (Insulin 10 µg/mL, Transferrin 10 µg/mL and Selenium 0.01 µg/mL, Lonza) for up to 48h. PAR-2 mRNA expression was studied as described before (Chapter 2). *GAPDH* mRNA was used as an internal control. Data were calculated and represented as *PAR-2* copies /10000 *GAPDH* copies.

Statistics

Results are expressed as Mean ± SEM. Results are analyzed using repeated measures ANOVA with Tukey's multiple comparison test. The *p*-value of less than 0.05 was considered statistically significant.

4.4 Results and Discussion

The number of PCR cycles required to detect PAR-2 mRNA in airway SMCs was higher compared to the number of cycles required in AECs (36 cycles in airway SMCs vs. 29 cycles in AECS) suggesting that PAR-2 expression in airway SMCs was low compared to AECs. However, there were differences in the protocols used to study PAR-2 expression in airway SMCs and AECs, which may explain the observed difference in PAR-2 expression between the two cell types. First, Trizol was used to extract RNA from airway SMCs, while RNeasy mini kit was used for AECs. Even though there is no literature comparing these two techniques for the quality of RNA obtained from airway SMCs or AECs, it is shown that these two methods could provide different qualities of RNA in other cell types (11, 12). The quality of RNA is important as it could affect the cDNA synthesis (reverse transcription) and ultimately gene expression results obtained using qPCR (13). We will not be able to compare the quality of RNA as we do not have that data available to us. Also, for airway SMCs 150 ng RNA was used for reverse transcription using a mixture of random hexamer and oligo dT as primers, while 500 ng RNA was used for reverse transcription. The concentration of RNA used for reverse transcription plays a role in the quality of cDNA synthesized (14), which could also affect the analysis of gene expression. Thus further experiments are required where airway SMCs and AECs are processed by similar protocol to compare the PAR-2 expression between the two cell types.

Following 24h and 48h of culture in the presence of ITS, there was a trend of decreased PAR-2 expression compared to cells cultured without ITS (Fig. 4.1).
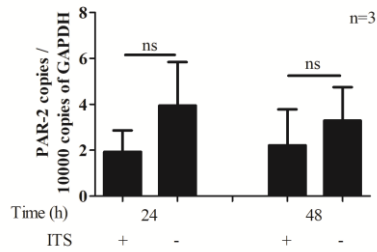
Fig. 4.1 Effect of insulin on PAR-2 expression in airway SMCs. Airway SMCs were cultured in FBS-free DMEM media containing 1X ITS for up to 48h and qRT-PCR for PAR-2 expression was performed. Data are presented as Mean ± SEM; ns (not significant) between the groups indicated by horizontal lines.

ITS mixture contains insulin, transferrin and selenium. If insulin is responsible for the observed trend of decreased PAR-2 expression, then similar to AECs, insulin negatively regulates PAR-2 expression in airway SMCs. Thus insulin-dependent development of procontractile phenotype in airway SMCs may be because of increased expression of proteins involved in smooth muscle contraction (8, 9) and not due to PAR-2 upregulation. Transferrin is used in cell culture media as it provides iron to cells and also prevents toxic effect of excess oxygen radicals. Transferrin is also present in the media we use to culture AECs, where exclusion of transferrin does not affect PAR-2 expression (Chapter 5). Selenium acts as an anti-oxidant in cell culture system. Serum deprivation and growth factor deprivation induce oxidative stress in different cells (15-17) to upregulate PAR-2 expression (18). Hence it is possible that FBS deprivation in our experiments with SMCs may results in oxidative stress and PAR-2 upregulation and the presence of transferrin and selenium prevented PAR-2 upregulation by preventing oxidative stress. More experiments are required to determine if the observed trend of decreased PAR-2 expression in the presence of ITS is due to the presence of insulin or the other components of ITS.

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Chapter 5: Growth media supplements regulate proteinase-activated receptor-2 expression in human bronchial airway epithelial cells

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This is a version of a manuscript that will be submitted after performing a few more experiments.

5.1 Abstract

Background: Proteinase-Activated Receptor-2 (PAR-2) is involved in the pathogenesis of a variety of inflammatory diseases including allergic asthma. Also PAR-2 expression is increased under inflammatory conditions. In asthma, PAR-2 expression is increased on airway epithelium but mechanism of PAR-2 regulation is poorly studied. Since PAR-2 expression is increased under inflammatory conditions, we hypothesize that inflammatory microenvironment, such as decreased availability of cell growth nutrients, regulates PAR-2 expression.

Objectives: To study the effect of decreased cell growth nutrients on PAR-2 expression and functions in primary bronchial epithelial cells (BECs).

Methods: PAR-2 expression and functions were studied in submerged and air-liquid interface cultures of primary BECs from healthy and asthmatic individuals subjected to media supplement deprivation, as a model of decreased cell growth nutrients. Cells were also treated with different inhibitors to elucidate the mechanism underlying PAR-2 expression.

Results: Media supplement deprivation upregulated PAR-2 mRNA and protein expression and also PAR-2-dependent pro-inflammatory potential of BECs from healthy individuals. The upregulation in PAR-2 mRNA was due to increased PAR-2 gene transcription. Also this upregulation was reversible upon re-addition of media supplements. The media supplements involved in PAR-2 regulation were insulin, hydrocortisone, bovine pituitary extract and retinoic acid. Treatment of cells with different inhibitors of signaling molecules showed that media supplement deprivation-induced PAR-2 upregulation is independent of PI3K-Akt-FOXO-1 and ERK1/2 pathways. Finally, BECs from asthmatic individuals expressed increased levels of PAR-

2 compared to BECs from healthy individuals and showed similar media supplement deprivation-mediated regulation of PAR-2 expression and functions, as observed in BECs from healthy individuals.

Conclusions: Our results indicate that decrease in cell growth nutrients in the inflammatory microenvironment may result in increased PAR-2 expression in the airways. Since activation of upregulated PAR-2 by proteinases from inhaled aeroallergens or immune cells may augment PAR-2-mediated airway inflammation, targeting PAR-2 regulation could be an effective therapeutic strategy.

5.2 Introduction

Proteinase-Activated Receptor-2 (PAR-2), a G-coupled pro-inflammatory receptor, is expressed on cells of different tissues and activated by serine proteinases (1). PAR-2 activation is shown to play a role in the pathogenesis of inflammatory diseases in different organs (2-5). In lungs, PAR-2 plays a role in the pathogenesis of allergic asthma (6, 7), idiopathic pulmonary fibrosis (IPF) (8) and pulmonary arterial hypertension (9).

In lungs, PAR-2 is expressed on bronchial smooth muscle cells (10, 11), fibroblasts (12) and bronchial epithelial cells (BECs) (11). BECs are the first line of defense against the inhaled air constituents including allergens, pathogens and pollutants. PAR-2 activation on BECs results in impaired epithelial barrier function (13) and also in the release of mediators (14-18) which are involved in the development of the cardinal features of asthma such as allergic sensitization, allergic inflammation and airway remodeling.

In addition to PAR-2 involvement in the pathogenesis of inflammatory diseases, PAR-2 expression is also increased in these diseases (3, 8, 19-21). In asthma, PAR-2 expression is increased on airway epithelial cells (AECs) (22). By using transgenic mice overexpressing PAR-2, it was shown that increased levels of PAR-2 augments allergic airway inflammation (6). Thus, understanding regulation of PAR-2 expression may provide the knowledge required to inhibit PAR-2 upregulation and control PAR-2-mediated inflammation.

The regulation of PAR-2 expression is poorly understood. Since PAR-2 expression is increased in inflammatory diseases, it is deduced that inflammatory environment plays a role in the regulation of PAR-2 expression. Due to increased number of active inflammatory cells that release mediators and reactive oxygen species, the site of inflammation is characterized by the presence of increased levels of inflammatory mediators and oxidative stress (23). Also higher metabolic demands, consumption of oxygen and cell growth nutrients by increased number of cells may result in decreased availability of metabolic substrates (24), hypoxia (25, 26) and decreased levels of nutrients supporting cell growth at the site of inflammation. In asthma, airways are infiltrated by inflammatory cells such as eosinophils (27) and neutrophils (28). In addition of these inflammatory cells, other cells such as monocytes, macrophages, epithelial cells and smooth muscle cells, release chemokines, cytokines and reactive oxygen species (ROS)

resulting in the presence of high levels of inflammatory mediators (29) and oxidative stress (30) in the airways of asthma patients. It is also shown that airway remodeling of the subepithelium, which is also observed in asthma, may limit the delivery of cell growth nutrients and oxygen (hypoxia) to the airway epithelium (31-33). There is evidence that inflammatory mediators (34-36), hypoxia (9, 37) and growth factor deprivation (38) regulate PAR-2 expression on cells other than AECs.

Asthmatic airways are chronically inflamed and may be under the influence of above mentioned stress stimuli. We hypothesised that decreased availability of nutrients supporting cell growth, in the background of inflammation, regulates PAR-2 expression on BECs. The cell culture media for BECs contains supplements that support cell growth. To study the effect of decreased availability of factors supporting cell growth on PAR-2 expression, we cultured BECs in media deprived of supplements (media supplement deprivation). Media supplement deprivation upregulated PAR-2 expression in primary cultures of normal BECs (NBECs) and also increased PAR-2-dependent pro-inflammatory potential of BECs. The media supplement deprivation-induced increase in PAR-2 expression was independent of PI3K-Akt-FOXO-1 and ERK1/2 signaling pathways. Finally, primary BECs from asthmatics (ABECs) also showed similar media supplement-mediated PAR-2 regulation.

5.3 Materials and Methods

Cell culture

Primary normal BECs (NBECs) from five donors were purchased (normal human bronchial epithelial cells, Lonza, Walkersville, MD); all the experiments were performed on cells at passage 3-5 after receiving. Cells were cultured as described in Chapter 2 of this thesis.

Primary asthmatic BECs (ABECs) were cultured from material obtained from bronchial brushings, from five asthmatic individuals undergoing bronchoscopy for clinical or research indications at the University of Alberta Hospital. All patients gave informed consent before bronchoscopy. The study was approved by the Ethics Committee, University of Alberta. ABECs were cultured under the same conditions with NBECs.

In addition to the ABECs obtained at the University of Alberta hospital, mRNA from airway epithelial cells obtained from five healthy and four asthmatic individuals were received from our collaborator Dr. Jamila Chakir, Laval University to compare PAR-2 mRNA expression in primary healthy vs. asthmatic BECs. These airway epithelial cells were isolated from bronchial biopsy specimens as described before (39).

Air-liquid interphase (ALI) cultures of NBECs and ABECs were performed as described in this reference (40) and in Chapter 2 of this thesis.

The bronchial epithelial cell line BEAS-2B was purchased from ATCC (Manassas, VA) and cultured as described in Chapter 2 of this thesis.

The airway epithelial cell lines A549 and Calu-3 cells were purchased from ATCC and cultured using DMEM media (low glucose, Life Technologies) supplemented with 10% heat inactivated fetal bovine serum (FBS, Life Technologies) and penicillin-streptomycin.

NBECs were treated with phosphoinositide 3-kinase (PI3K) inhibitors LY294002 (20 μM, (41, 42), Tocris, Minneapolis, MN), wortmannin (5 μM, (43), Cayman, Ann-Arbor, MI), Forkhead box protein O1 (FOXO-1) inhibitor AS1842856 (1 μM, (44), Calbiochem, San Diego, CA) and MEK 1/2 inhibitor U0126 (10 μM, (45, 46), Tocris). All the inhibitors were dissolved in DMSO

(Sigma-Aldrich). Final concentration of DMSO in the treatment was 0.1% or less. DMSO at 0.1% concentration had no effect on PAR-2 expression (appendix-1).

For all the cell culture procedure, cells were cultured at 37˚C in 5% CO_2 and 90% humidity and media was changed 3 times /week. All cell cultures maintained in our lab were tested monthly for mycoplasma using the MycoAlert kit (Lonza) and only negative cultures were used.

RNA extraction, reverse transcription and quantitative RT-PCR (qRT-PCR)

RNA extraction and reverse transcription were performed as described in Chapter 2 of this thesis.

qRT-PCR for *PAR-2* was performed using a probe-based assay developed in our lab (47) and as described in Chapter 2 of this thesis. *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH)* was used as a house keeping gene. The results were expressed as *PAR-2* copies/1000 *GAPDH* copies. PAR-2 expression was also normalized to a second housekeeping gene, *Cyclophilin A*. For this Taqman gene expression assays were used for *PAR-2* (Hs00608346_m1, ThermoFisher Scientific) and housekeeping gene *Cyclophilin A* (Hs04194521_s1, ThermoFisher Scientific).

Intracellular calcium release assay

PAR-2-mediated increase in intracellular calcium was measured as described in Chapter 2 of this thesis. PAR-2-mediated calcium release from ER was expressed as % of thapsigargin (TG)-induced calcium release from ER.

Preparations of PAR-2 reporter plasmid

The protocol on the development of this construct is described in Chapter 2 of this thesis.

Transfection studies

For promoter studies, BEAS-2B and Calu-3 cells were seeded in 12-well plates (Falcon) and cultured until 85% confluent. On the day of transfection, BEAS-2B and Calu-3 cells were rinsed with PBS and 400 µL of respective media without supplements (BEBM or DMEM without FBS) was added to the wells. The transfection complexes were formed by mixing 1 µg PAR-2 reporter plasmid DNA (preparation of reporter plasmid is described in the supplementary methods for

Chapter-2) or empty pGL3-Basic vector, 5 ng Renilla plasmid DNA and 2 µg of Lipofectamine® 2000 Transfection Reagent (Life Technologies) in 100 µL respective media lacking supplements and incubating the mixture for 30 min at room temperature. Transfection complexes (100 µL) were then added to the cells dropwise and cells were incubated for 5h. After 5h of incubation, the transfection complexes were removed and appropriate growth media was added for 24h.

Next day, to study the effect of media supplements on PAR-2 promoter activity, cells were cultured in respective media with or without supplements for 24h. The promoter activity was assessed using the Dual-Luciferase Reporter Assay Kit (Promega, Madison, WI).

Luciferase reporter assay

At the end of 24h of culturing cells in media with or without supplements, cells were rinsed with PBS and harvested using a cell scraper. Cells were then pelleted and lysed with 60 µL of passive lysis buffer (Promega, Madison, WI). From this, 50 µL lysate was assayed for luciferase activity using Dual –Luciferase Reporter Assay Kit (Promega) as described in Chapter 2 of this thesis.

To study the effect of growth supplement deprivation on PAR-2 promoter activity, results were expressed as fold change in promoter activity over cells cultured with growth supplements.

Lactate dehydrogenase (LDH) assay

Cells, plated in multi-well plate, were cultured in media with or without supplements for up to 48h. Cell supernatants were collected and assayed for LDH activity using LDH Cytotoxicity Assay kit (Cayman). One well, of the multi-well plate, was treated with 1% Triton X-100 for 30 min to lyse the cells and obtain maximum LDH activity. To measure LDH activity, 100 µL of LDH standards or samples were mixed with 100 µL of Reaction Solution and incubated for 30 min at room temperature; the absorbance was read at 490 nm. Since Triton X-100 will lyse all the cells, the LDH activity in Triton X-100 treated cells was considered as 100% and "% LDH activity" in cells cultured with or without media supplements was calculated relative to the LDH activity obtained in Triton X-100 treated cells. The LDH activity is proportional to the amount of LDH present in cell supernatant.

Apoptosis and cell cycle evaluation using flow cytometry

Apoptosis assay was performed using the Dead Cell Apoptosis Kit (Life Technologies). Cells, plated in multi-well plate, were cultured in media with or without supplements for 24h. Cells were then trypsinized and pelleted down by centrifugation at 4º C at 200 X g for 5min. The pellet was washed by resuspending it in cold PBS and again centrifuged at 4º C at 200 X g for 5min. The pellet was resuspended in 1X annexin-binding buffer at the ratio of 100 µl buffer per 100,000 cells. Annexin V (component-A, 5 µl/100 µl cell suspension) and propidium iodide (component-B, 1 µl of 100 µg/mL per 100 µl cell suspension) were added and cells were incubated for 15 min at room temperature in the dark. At the end of incubation, 400 µl of annexin-binding buffer was added to the samples, mixed gently and kept on ice. Stained cells were examined on BD FACSCanto II flow cytometer and the data were analyzed using FlowJo (Tree Star, Ashland, OR).

Cell cycle analysis was performed using the following protocol. Cells, plated in multi-well plate, were cultured in media with or without supplements for 24h. Cells were then trypsinized and pelleted down by centrifugation at 4º C at 200 X g for 5min. The pellet was washed by resuspending it in cold PBS and again centrifuged at 4º C at 200 X g for 5min. Cell pellet was then resuspended in 500 µl of cold PBS. Cold 3.5 mL of 70% ethanol was added to cells drop wise, mixed gently and kept on ice for 2h. Cells were then centrifuged at 4º C at 200 X g for 5min and resuspended in 5 mL cold PBS. Cells were kept on ice for 2-3 min before spinning them down again at 4º C at 200 X g for 5min. The pellet was resuspended in 500 µl of staining solution comprising of 200 µg/mL ribonuclease A (Sigma-Aldrich) and 20 µg/mL propidium iodide (Sigma-Aldrich) in 0.1% Triton X-100-PBS and incubated for 30 min at room temperature in dark. Stained cells were examined on BD FACSCanto II flow cytometer.

ELISA

CXCL8 levels in cell supernatants were measured using the Human IL-8/CXCL8 DuoSet ELISA kit (R&D Systems, Minneapolis, MN) as per manufacturer's instructions.

Trans epithelial electrical resistance (TEER) measurements

TEER was measured on differentiated ALI cultures of ABECs and NBECs using Millicell$^{®}$ ERS-2 Electrical Resistance System (Sigma-Aldrich). TEER measurements were first taken before wound was created (Pre-cut). Mechanical wound was then created by scratching the insert membrane using the tip of a Pasteur pipette. Post-cut TEER was measured immediately after wounding and also at 24h and 48h after wounding. Pre-cut TEER values were considered as 100% and Post-cut TEER values were expressed as % of Pre-cut TEER value.

Statistics

Results are expressed as Mean ± SEM. In case of comparisons between two groups, two-tailed paired *t*-test was used, except for the comparison between asthmatics and healthy individuals where two-tailed Mann Whitney test was used. In case of three or more groups, repeated measures ANOVA was used with Tukey's multiple comparison test or 2-way repeated measures ANOVA was used with Bonferroni post-test, depending on the experimental set-up. The *p*-value of less than 0.05 was considered statistically significant.

5.4 Results

Media supplement deprivation regulates PAR-2 mRNA expression in NBECs

In submerged NBEC cultures, media supplement deprivation induced an over 2-fold upregulation of PAR-2 mRNA expression at 24h (Fig. 5.1A and 5.1B) and 48h (Fig. 5.1B) without affecting cell viability as measured by LDH assay (Fig. 5.1C and 5.1D). Media supplement deprivation-induced PAR-2 upregulation was confirmed using another housekeeping gene cyclophilin A (Fig. 5.1E). The addition of supplements back to supplement-deprived cells completely reversed PAR-2 upregulation after 6h and 24h (Fig. 5.1F), indicating the dynamic nature of the PAR-2 expression, while continuing the supplement deprivation further increased PAR-2 mRNA expression (Fig. 5.1F). ALI cultures of differentiated NBECs also showed supplement deprivation-induced upregulation in PAR-2 mRNA expression (Fig. 5.1G).
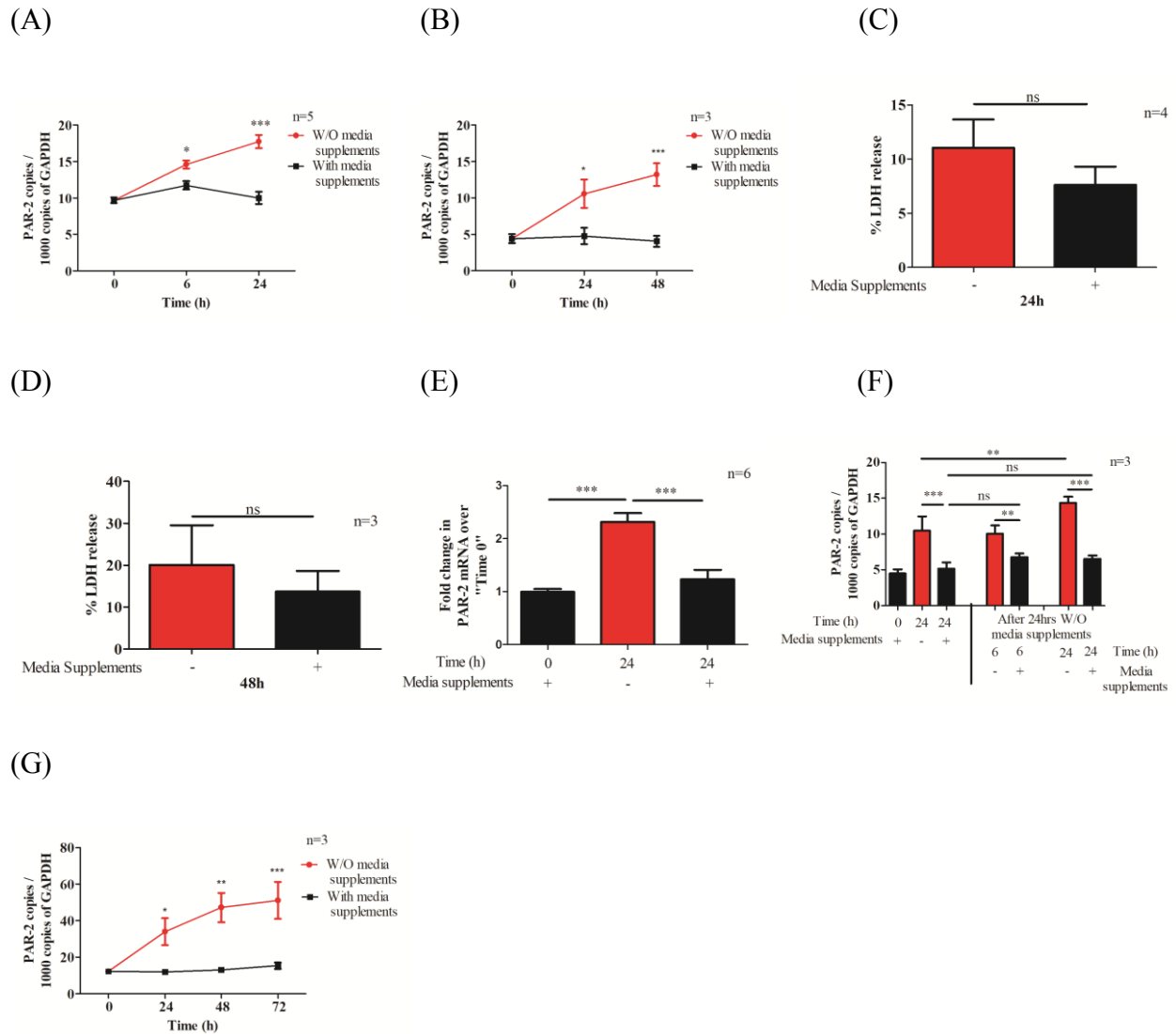
Fig. 5.1 Media supplements regulate PAR-2 expression in submerged and differentiated cultures of NBECs. PAR-2 expression was studied by qRT-PCR. (A-B) Cells were cultured in media with supplements until they reached 85% confluency (Time "0"). Cells were then cultured in media without or with supplements for (A) 6h and 24h (n=5), and (B) 24h and 48h (n=3). (C-D) LDH activity in supernatants of cells cultured in media without or with supplements for (C) 24h (n=4) and (D) 48h (n=3). LDH activity in Triton X-100 treated cells was considered as 100% and "% LDH activity" in cells cultured without or with media supplements was calculated relative to the LDH activity obtained in Triton X-100 treated cells. (E) NBECs were cultured in media with supplements until reached 85% confluency (Time "0"). Cells were then cultured in media without or with supplements for 24h (n=6). Taqman gene expression assays were used for *PAR-2*

and housekeeping gene *Cyclophilin A*. (F) Cells were cultured in media with supplements until reached 85% confluency (Time "0"). Cells were then cultured in media without or with supplements for 24h (left part of the graph). Cells cultured in media without supplements for 24h were further cultured in media without or with supplements for the next 6h and 24h (n=3) (right part of the graph). (G) Differentiated ALI cultures of NBECs were cultured in media without or with supplements for up to 72h (n=3). Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ at an indicated time-point between two groups or between the groups indicated by horizontal lines.

To study the role of individual media supplements on PAR-2 expression, we excluded one supplement at a time. Only insulin exclusion, as we have shown before (Chapter 2), showed increase in PAR-2 expression; hydrocortisone exclusion showed a trend towards increase in PAR-2 expression (p=0.08), while the exclusion of other supplements did not affect PAR-2 expression (Fig. 5.2A). However, insulin deprivation-induced PAR-2 upregulation was significantly lower than the upregulation induced by the absence of all media supplements.

Next, we cultured cells in the absence of media supplements for 24h to upregulate PAR-2 expression and then added selected supplements back individually, mainly hormones, growth factors and ligands for nuclear receptors hydrocortisone and RA. Insulin (Fig. 5.2B), BPE (bovine pituitary extract) (Fig. 5.2C), hydrocortisone (Fig. 5.2D) and RA (retinoic acid) (Fig. 5.2D) decreased the media supplement deprivation-induced upregulation in PAR-2 mRNA expression, while the addition of epinephrine (Fig. 5.2E) and EGF (epidermal growth factor) (Fig. 5.2F) did not induce significant alteration in PAR-2 mRNA expression. These results indicate that the increased upregulation in PAR-2 expression upon exclusion of all the media supplements compared to exclusion of only insulin is a result of combined absence of other media supplements regulating PAR-2 expression.
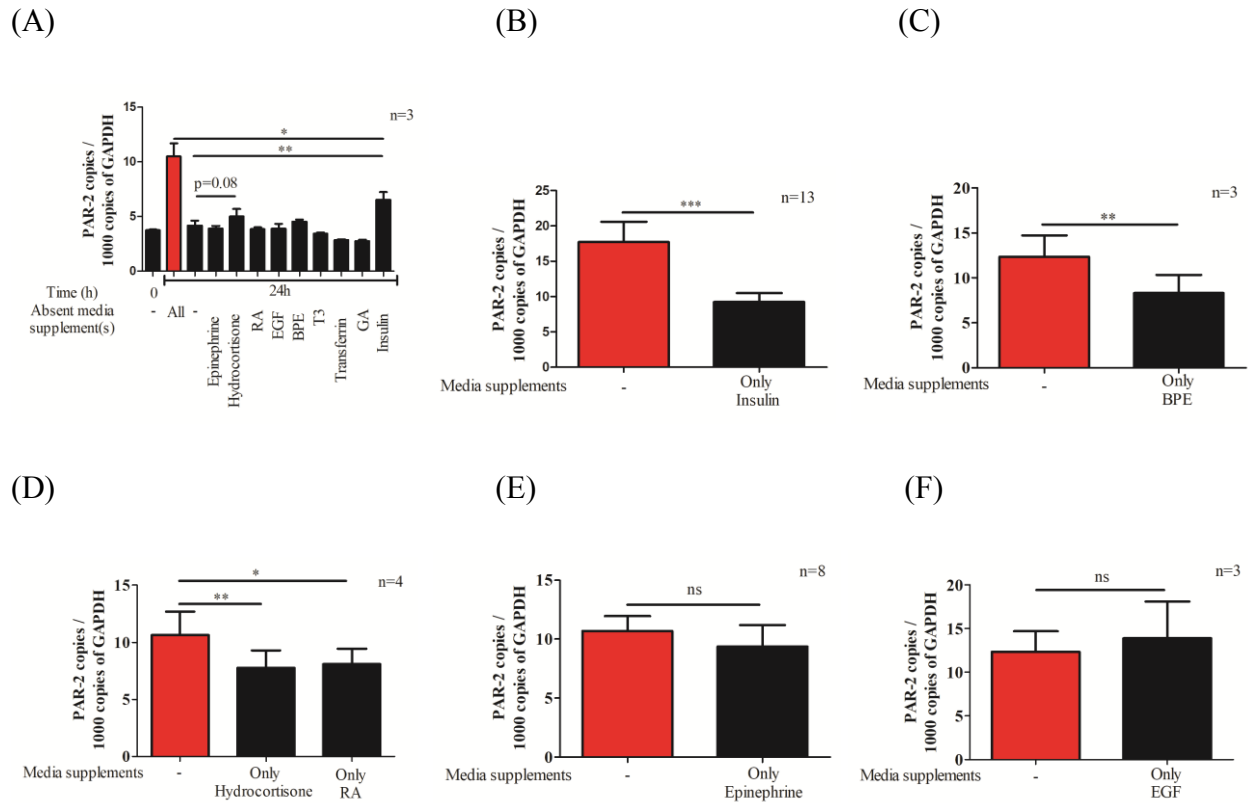
Fig. 5.2 Effect of different media supplements on PAR-2 expression in NBECs. PAR-2 expression was studied by qRT-PCR. (A) Cells were cultured in media with supplements until they reached 85% confluency (Time "0"). Cells were then cultured in media without all supplements or with all supplements or without indicated supplement for 24h (n=3). (B-F) Cells were first cultured in media without supplements for 24h. Cells were then further cultured in media without supplements or with only indicated supplement (B) insulin (n=13) (C) BPE (n=3) (D) hydrocortisone and RA (n=4) (E) epinephrine (n=8) and (F) EGF (n=3) for the next 24h. Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

Role of PI3K-FOXO-1 pathway in media supplement deprivation-induced PAR-2 upregulation

We have shown that insulin, through PI3K pathway, controls FOXO-1 activity to regulate PAR-2 expression. Since, insulin is one of the media supplements that modulates PAR-2 expression,

we evaluated the role of PI3K signaling in PAR-2 expression in supplement-deprived and insulin-deprived cells. PI3K inhibition further increased PAR-2 expression in growth supplement-deprived cells, while PI3K inhibition in insulin-deprived cells had no effect on PAR-2 expression (Fig. 5.3A). We have specifically shown that FOXO-1 inhibition prevents insulin deprivation-induced PAR-2 upregulation (Chapter 2), while FOXO-1 inhibition did not prevent PAR-2 upregulation in supplement-deprived cells (Fig. 5.3B). These results collectively indicate that the mechanism of PAR-2 regulation is different in supplement-deprived cells vs. insulin-deprived cells.
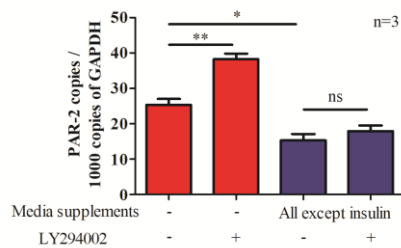
(A)   (B)
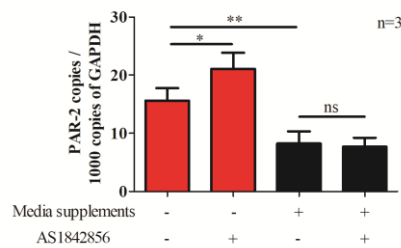
Fig. 5.3 Role of PI3K-FOXO-1 pathway in media supplement deprivation-induced PAR-2 upregulation. PAR-2 expression was studied by qRT-PCR. (A) Cells were cultured in media without all supplements or without only insulin in the presence or absence of PI3K inhibitor LY294002 for 24h (n=3). (B) Cells were cultured in media without supplements or with supplements in the presence or absence of FOXO-1 inhibitor AS1842856 for 24h (n=3). Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, between the groups indicated by horizontal lines.

Role of ERK1/2 in media supplement deprivation-induced PAR-2 upregulation

Media supplement deprivation activates ERK1/2 (48). Insulin and hydrocortisone, that decreased media supplement deprivation-induced PAR-2 upregulation in our BECs, are also shown to inhibit ERK1/2 (49, 50). We hence studied the role of ERK1/2 in media supplement deprivation-induced PAR-2 upregulation in BECs. Inhibition of ERK1/2 decreased media supplement deprivation- and insulin deprivation-induced PAR-2 upregulation (Fig. 5.4A). However,

inhibition of ERK1/2 also decreased PAR-2 expression in cells cultured with media supplements (Fig. 5.4A). The % decrease in PAR-2 expression upon ERK1/2 inhibition was similar in all 3 treatment groups namely; media supplement deprivation, insulin deprivation and cells cultured with media supplements indicating role of ERK1/2 in basal expression of PAR-2 (Fig. 5.4B).
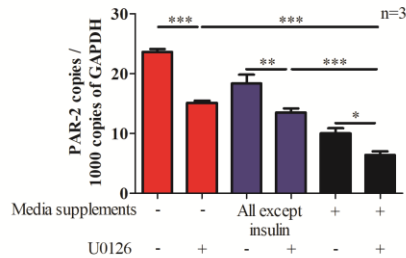
(A)   (B)
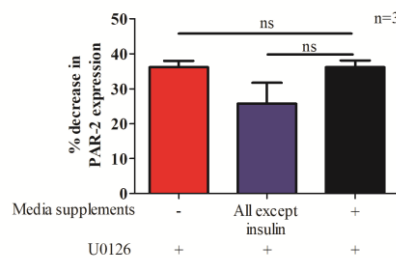
Fig. 5.4 Role of ERK1/2 in media supplement deprivation-induced PAR-2 upregulation. Cells were cultured in media without all supplements, without only insulin or with all supplements in the presence or absence of ERK1/2 inhibitor U0126 for 24h. (A) PAR-2 expression was studied by qRT-PCR (n=3). (B) % decrease in PAR-2 expression was calculated in cells treated with ERK1/2 inhibitor in different media compared to respective untreated cells (n=3). Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

Media supplement deprivation-induced PAR-2 upregulation is due to increased PAR-2 gene transcription

We have shown that insulin deprivation did not alter PAR-2 mRNA stability and resulted in FOXO-1-mediated transcriptional regulation of PAR-2 (Chapter 2). Since media supplement deprivation-induced PAR-2 mRNA upregulation does not involve FOXO-1, it is possible that this upregulation is due to increased PAR-2 mRNA stability. After blocking mRNA transcription using actinomycin D, we found that the rate of PAR-2 mRNA degradation was similar whether cells were cultured without or with supplements indicating media supplement deprivation did not alter PAR-2 mRNA stability (Fig. 5.5A).

To study the effect of media supplement deprivation on PAR-2 gene transcription we used a PAR-2 reporter plasmid. For these experiments, we used immortalized airway epithelial cell lines (AECs) because primary AECs have lower transfection efficiency. We first studied if media supplement deprivation regulates PAR-2 expression in these cells. Media supplement deprivation induced PAR-2 mRNA upregulation in BEAS-2B cells (Fig. 5.5B) as observed in primary NBECs. Other cell lines, Calu-3 and A549, which were cultured with FBS as the source of supplements did not show alteration in PAR-2 expression when cultured in FBS-deprived media (Fig. 5.5C and 5.5D).

Media supplement deprivation in BEAS-2B cells transfected with a PAR-2 reporter plasmid showed increase in promoter activity indicating supplement deprivation-induced increase in PAR-2 mRNA is due to increased transcription of PAR-2 gene (Fig. 5.5E). Serum deprivation in Calu-3 cells transfected with a PAR-2 reporter plasmid did not increase PAR-2 promoter activity (Fig. 5.5F); which was expected as serum deprivation did not increase PAR-2 mRNA expression in these cells (Fig. 5.5C).
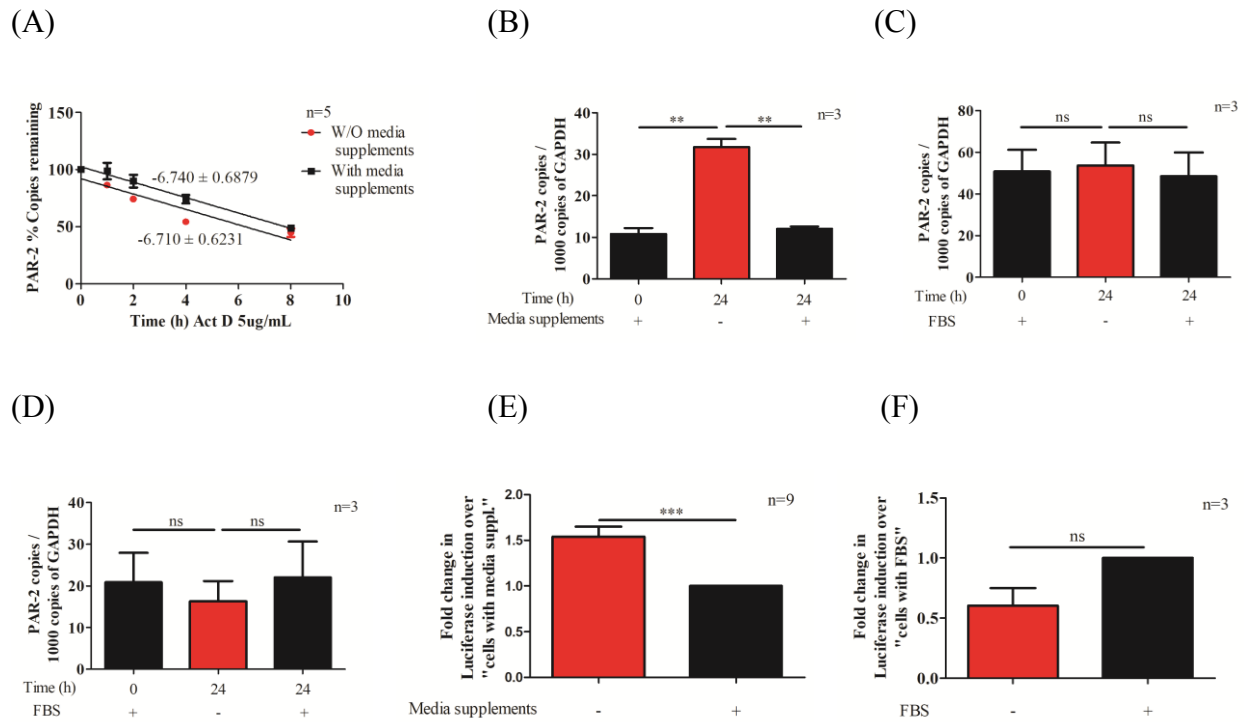
Fig. 5.5 Media supplement deprivation transcriptionally regulates PAR-2 mRNA expression in NBECs. PAR-2 expression was studied by qRT-PCR. (A) Cells were cultured in media with supplements until they reached 85% confluency. Cells were then cultured in media without or with supplements for 24h (Time "0"). Cells were further cultured in media without or with supplements, in the presence of actinomycin D, and PAR-2 mRNA expression was studied at indicated time points (n=5). PAR-2 levels were expressed as % PAR-2 expression compared to the PAR-2 expression at time "0". The numbers on the lines show the rate of PAR-2 mRNA degradation for 8h after the addition of actinomycin D. (B-D) Cells were cultured in media with supplements or FBS until reached 80% confluency (Time "0"). (B) BEAS-2B (n=3), (C) Calu-3 (n=3) and (D) A549 cells (n=3) were then cultured in media without or with respective supplements for 24h. (E-F) PAR-2 promoter activity expressed as fold change in luciferase induction in (E) BEAS-2B cells (n=9) and (F) Calu-3 cells (n=3) transfected with PAR-2 reporter plasmid and cultured in media without or with respective supplements for 24h. Data are presented as Mean ± SEM; ns (not significant), **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

Media supplement deprivation upregulates PAR-2 protein expression and PAR-2-dependent pro-inflammatory potential of BECs

Media supplement deprivation increased PAR-2 protein expression in BECs after 24h, as shown by measuring fluorescence intensity of PAR-2, for 30 cells/ experiment in 6 independent experiments, using confocal microscopy (Fig. 5.6A-C). To study the functional significance of this PAR-2 upregulation, we studied PAR-2-mediated calcium signaling and CXCL8 release in cells cultured in media with or without supplements for 24h.

We showed that PAR-2 activation increases intracellular Ca^{++} by inducing Ca^{++} release from ER (Chapter 2). Also upregulated PAR-2 expression leads to increased PAR-2-mediated intracellular Ca^{++} release (51). We also found that PAR-2-activating peptide (PAR-2 AP)-mediated increase in intracellular Ca^{++} was higher in cells subjected to media supplement deprivation compared to cells cultured in media with supplements (Fig. 5.6D and 5.6E).

Further, PAR-2 AP-mediated calcium signaling is shown to induce the release of inflammatory mediators including CXCL8 (52). The baseline release of CXCL8 was higher in cells cultured with media supplements compared to cells cultured without media supplements. The treatment of cells with PAR-2 control peptide (PAR-2 CP) had no effect on baseline CXCL8 release irrespective of the presence or absence of media supplements. PAR-2 activation induced 1.2 fold higher CXCL8 release over PAR-2 CP in cells cultured with media supplements, while PAR-2 activation induced 1.8 fold higher CXCL8 release over PAR-2 CP in cells subjected to media supplement deprivation, which was higher compared to cells cultured in media with supplements (Fig. 5.6F).
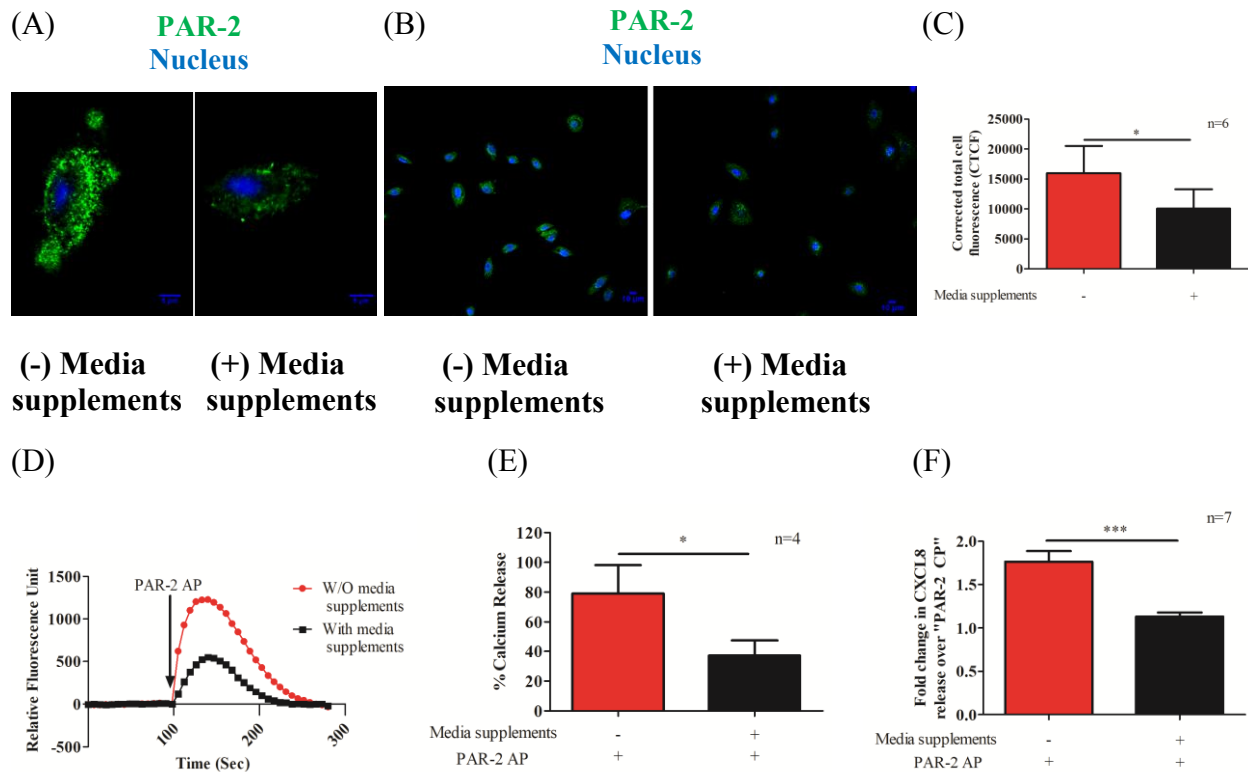
Fig. 5.6 Media supplements deprivation upregulates functional PAR-2 expression. (A-B) Images of PAR-2 expression (Green) in (A) a representative individual cell and (B) a representative field of view displaying multiple cells cultured in media without (left image) or with (right image) supplements for 24h. Nuclei are stained with DAPI (Blue). (C) Corrected Total Cell Fluorescence representing PAR-2 expression in cells cultured in media without or with supplements. CTCF was calculated for 30 cells/experiment in 6 independent experiments (n=6). (D) Representative graph of PAR-2-mediated intracellular Ca^{++} release from ER in cells cultured in media without or with supplements for 24h. Cells were activated using PAR-2 AP (50 µM). (E) PAR-2-mediated Ca^{++} release from ER was quantified as % of thapsigargin-induced Ca^{++} release from ER (n=4). (F) Cells were cultured in media without or with supplements for 24h and then treated with PAR-2 AP or PAR-2 CP (50 µM) for 12h in the same media that they were cultured in. CXCL8 levels (pg/mL) in cell culture supernatant were measured using ELISA (n=7). The graph shows PAR-2 AP-mediated CXCL8 release over PAR-2 CP. Data are presented as Mean ± SEM; *$P<0.05$.

Effect of media supplement deprivation on cell cycle and apoptosis

Media supplement deprivation leads to cell cycle arrest in "G01" phase and activation of apoptosis (53). The cell cycle analysis showed that media supplement deprivation increased cells in G01 phase by only 6% compared to cells with supplements (Fig. 5.7A). We also found that media supplement deprivation marginally increased apoptotic cells (Fig. 5.7B) but not dead cells (Fig. 5.7C). These results indicate that after 24h of media supplement deprivation, the growth of BECs was not significantly altered.

(A)   (B)   (C)
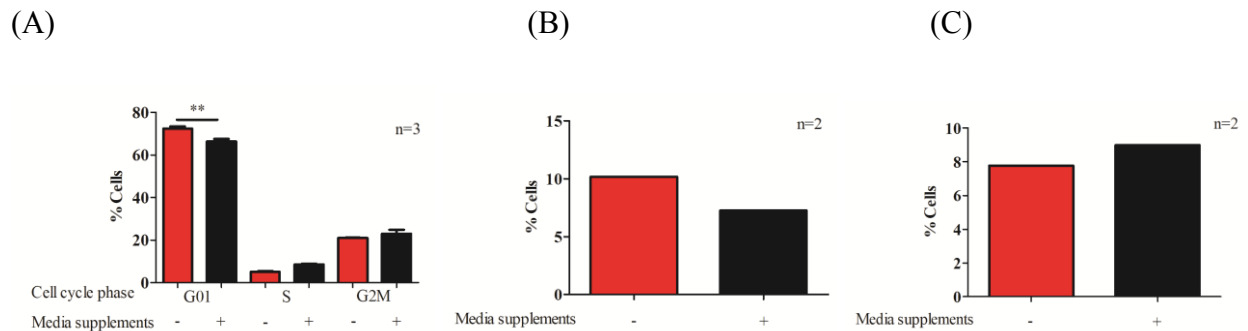
Fig. 5.7 Effect of media supplement deprivation on cell cycle and apoptosis in NBECs. Cells were cultured in media without or with supplements for 24h and subjected to flow cytometry. (A) % cells in different phases of cell cycle (n=3) based on the propidium iodide-stained DNA profile analysis, (B) % apoptotic cells (annexin V-positive and annexin V/propidium iodide double positive cells were considered as apoptotic cells) (n=2) (C) % dead cells (only propidium iodide positive cells were considered as dead cells). Data are presented as Mean ± SEM for (A) and as Mean for (B) and (C); **$P<0.01$

BECs from asthma patients express increased PAR-2 compared to NBECs and show similar media supplement-mediated PAR-2 regulation as NBECs

BECs obtained from asthmatic individuals, when cultured *in vitro*, retain some of their *in vivo* characteristics such as defective barrier property (54), poor differentiation (55) and poor efficiency at wound healing (56, 57). We first studied if our ABECs show the previously demonstrated characteristic of inefficient wound healing. Mechanical wound resulted in decrease in TEER in both NBECs and ABECs as expected. In NBECs, TEER was increased to over 100%

after 24h of injury indicating that the wound was completely healed. Further the TEER was maintained around 100% up to 48h (Fig. 5.8A). Contrary to this, ABECs were not able to reach 100% TEER even after 48h of injury showing defective repair property of ABECs (Fig. 5.8B).

PAR-2 expression is increased in *ex vivo* cultures of ABECs compared to NBECs (Fig. 5.8C). We next studied if ABECs obtained from four donors respond to media supplement deprivation similar to NBECs. Media supplement deprivation induced upregulation in PAR-2 expression in all four cultures of ABECs (Fig. 5.8D), as observed in NBECs. The addition of media supplements, to supplement-deprived cells, reversed PAR-2 upregulation at 6h (Fig. 5.8E) and 24h (Fig. 5.8E). The ALI cultures of ABECs also showed increased PAR-2 expression upon media supplement deprivation (Fig. 5.8F and 5.8G). Finally, similar to NBECs, PAR-2 activation in media supplement deprived ABECs released increased CXCL8 compared to cells cultured with media supplements (Fig. 5.8H).
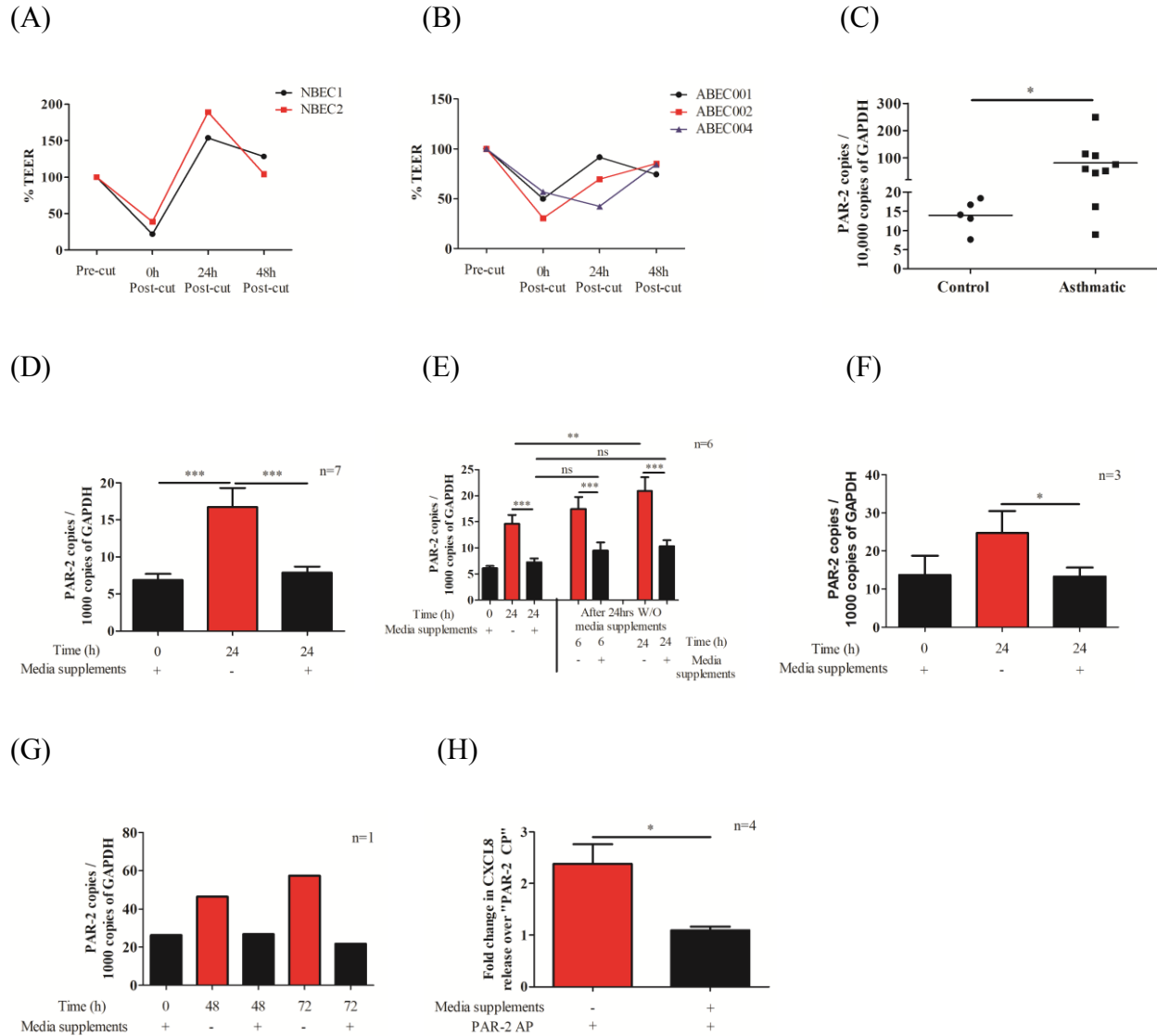
Fig. 5.8 BECs from asthmatic individuals express increased PAR-2 mRNA and show similar media supplement-mediated PAR-2 regulation. (A-C) BECs were obtained from asthmatic and healthy individuals and cultured *in vitro*. (A-B) Differentiated ALI cultures of BECs from (A) healthy (NBEC) and (B) asthmatic (ABEC) individuals were subjected to TEER measurements before (Pre-cut) and after (Post-cut) wound generation. The Pre-cut TEER values were considered as 100% and Post-cut TEER values were expressed as % of Pre-cut TEER value. (C) NBECs and ABECs were cultured in media with supplements PAR-2 expression was studied by qRT-PCR. (D-E) PAR-2 expression was studied by qRT-PCR. Cells were cultured in media with supplements until reached 85% confluency (Time "0"). (D) ABECs were then cultured in media without or with supplements for 24h (n=7). (E) ABECs were then cultured in media without or

with supplements for 24h (left part of the graph). Cells cultured in media without supplements for 24h were further cultured in media without or with supplements for the next 6h and 24h (n=6) (right part of the graph). (F-G) PAR-2 expression was studied by qRT-PCR. Differentiated ALI cultures of ABECs were cultured in media without or with supplements for (F) 24h (n=3) or (G) 48h and 72h (n=1). (H) Cells were cultured in media without or with supplements for 24h and then treated with PAR-2 AP or PAR-2 CP (50 µM) for 12h in the same media that they were cultured in. CXCL8 levels (pg/mL) in cell culture supernatant were measured using ELISA (n=4). The graph shows PAR-2 AP-mediated CXCL8 release over PAR-2 CP. Data are presented as Mean ± SEM; ns (not significant), *$P<0.05$, **$P<0.01$, ***$P<0.001$ between the groups indicated by horizontal lines.

5.5 Discussion

The expression of PAR-2, a G-coupled receptor, is increased in inflammatory conditions in many organ systems (3, 8, 19-21). PAR-2 upregulation can further augment inflammation (6). In asthma, PAR-2 expression is increased on AECs (22), but the mechanisms underlying regulation of PAR-2 expression is poorly studied. The presence of increased number of metabolically active inflammatory cells may result in decreased levels of cell growth nutrients at the site of inflammation (24) and it was shown that growth factor deprivation can regulate PAR-2 expression in cells other than AECs (38). We hypothesized that the localized deprivation of growth nutrients, in the background of inflammation, upregulates PAR-2 expression in AECs. To mimic the decreased availability of factors supporting cell growth *in vivo*, we excluded cell growth supplements from cell culture media *in vitro*. We observed that media supplement deprivation resulted in PAR-2 upregulation in undifferentiated submerged and differentiated ALI cultures of BECs obtained from healthy individuals. PAR-2 upregulation also increased PAR-2-dependent pro-inflammatory potential of the epithelial cells as observed by increased calcium signaling and CXCL8 release upon PAR-2 activation.

To understand the exclusion of which supplement resulted in increased PAR-2 expression, we excluded one supplement at a time. As demonstrated (Chapter 2), exclusion of insulin resulted in increased PAR-2 expression on NBECs, while exclusion of other media supplements individually did not upregulate PAR-2 expression. We also used another approach to identify the supplements involved in the regulation of PAR-2 expression. We first cultured cells in the media lacking supplements to upregulate PAR-2 expression and then added individual supplement. Insulin had the maximum effect in decreasing PAR-2 expression while hydrocortisone, BPE and RA were able to decrease PAR-2 expression partially. Inhaled corticosteroids are the most effective treatment to control airway inflammation in asthma hence, downregulation of PAR-2 expression by hydrocortisone could be an anti-inflammatory mechanism of corticosteroids. BPE is a non-defined source of growth factors and hormones; the observation again supports the notion that decreased levels of cell growth supplement at the site of inflammation may result in increased PAR-2 expression. RA is shown to be important for the differentiation and secretory function of BECs (58). RA is a metabolite of vitamin A; vitamin A is an anti-oxidant vitamin, which is found to be decreased in asthmatic kids (59) and is a suggested dietary supplement to

control oxidative stress (60). Thus the observation suggest that the stress stimuli present in the lung microenvironment such as decreased cell growth nutrients and increased oxidative stress can modulate PAR-2 expression.

Media supplement deprivation upregulated PAR-2 expression in primary BECs and virally transformed bronchial epithelial cell line BEAS-2B but not in cancerous epithelial cell lines Calu-3 (61) and alveolar epithelial cells, A549 (62). The observed difference could be because of the cancer phenotype of Calu-3 and A549 cell line, as cancer cell lines may behave differently than primary cells and transformed cell lines. Also, specialized cell culture media was used for BECs and BEAS-2B, where the media supplements are defined, while FBS, an undefined mixture of supplements, was used to culture Calu-3 and A549 cells. We also tried growing primary BECs in media containing FBS to study if FBS deprivation affects PAR-2 expression in primary BECs but experiments were terminated as cell morphology was altered.

Media supplement deprivation activates ERK1/2 (48). Insulin and hydrocortisone that decreased PAR-2 upregulation in our BECs are shown to inhibit ERK1/2 (49, 50). Hence it is possible that media supplement deprivation in BECs activated ERK1/2 to upregulate PAR-2 expression. Inhibition of ERK1/2 decreased media supplement deprivation- and insulin deprivation-induced PAR-2 upregulation but it also decreased, at the same extent, basal PAR-2 expression in cells cultured with media supplements. These results suggest that ERK1/2 is not involved in media supplement deprivation- and insulin deprivation-induced PAR-2 upregulation.

We also studied the involvement of PI3K in media supplement deprivation-induced PAR-2 regulation as we have shown that insulin regulates PAR-2 expression by inhibiting FOXO-1 transcription factor through PI3K-Akt pathway (Chapter 2). PAR-2 expression was not affected upon PI3K inhibition in insulin deprived cells, while in media supplement deprived cells, PAR-2 expression was increased upon PI3K inhibition. We have shown that insulin deprivation-induced PAR-2 upregulation is FOXO-1 dependent (Chapter 2). In current study inhibition of FOXO-1 did not prevent the supplement deprivation-induced PAR-2 upregulation indicating the involvement of different mechanisms in PAR-2 upregulation induced by supplement deprivation vs. insulin deprivation.

It is shown that during growth factor deprivation / serum deprivation, FOXO-1 gets activated and localizes to nucleus to induce apoptosis (63) but if FOXO-1 is present in cytoplasm (inactive FOXO-1) then autophagy is induced. It is also shown that growth factor deprivation activates stress-activated protein kinase (SAPK) c-Jun N-terminal kinase (JNK), which inhibits FOXO-1-induced apoptosis by decreasing FOXO-1 expression (64) and induces autophagy (65). Autophagy is a cell survival mechanism that usually occurs before apoptosis but prolonged autophagy results in apoptosis (66). Since we did not see increase in apoptosis in our cells, it is possible that FOXO-1 was not activated in our media supplement-deprived cells, hence was not involved in media supplement deprivation-induced PAR-2 upregulation. This can be evaluated by studying (i) if growth factor deprivation results in less nuclear FOXO-1 localization compared to insulin deprivation (ii) if media supplement deprivation-induced JNK activation decreases FOXO-1 expression.

There is other evidence that support the notion that the mechanisms involved in PAR-2 upregulation, induced by media supplement deprivation vs. insulin deprivation, are different. First, supplements other than insulin also had an effect on PAR-2 expression and thus, compared to removal of only insulin, removal of all the supplements may result in activation or repression of different signaling pathways involved in PAR-2 regulation. Second, the time course for PAR-2 upregulation is different between the cells subjected to insulin deprivation vs. media supplement deprivation. In our previous study, we did not find increase in PAR-2 expression after 6h of insulin deprivation (Chapter 2), but in this study PAR-2 expression was increased after 6h of supplement deprivation. Since exclusion of all the supplements could exert more cellular stress than the exclusion of only insulin, this early upregulation in PAR-2 expression in cells subjected to supplement deprivation may be the result of early cellular responses such as activation of stressed-induced signaling pathways involving stress kinases (67).

As discussed, we observed a marginal increase in apoptotic cells under the growth supplement deprivation condition. It is possible that autophagy, which precedes apoptosis, was activated in our culture system against the cellular stress that could result upon supplement deprivation. PAR-2 upregulation at this pre-apoptotic phase could be a protective mechanism for cell survival as PAR-2 activation is shown to inhibit apoptosis in airway (68) and colonic (69) epithelial cells.

Further experiments are required to assess if autophagy is activated upon supplement deprivation and if autophagy plays any role in supplement deprivation-induced PAR-2 upregulation.

We obtained ABECs through bronchial brushes and cultured them *ex vivo*. The baseline expression of PAR-2 was higher in ABECs compared to NBECs. The upregulated PAR-2 expression was maintained in *ex vivo* cultures of ABECs, which suggests an involvement of epigenetic changes in PAR-2 upregulation. Since media supplement deprivation increased PAR-2 expression in NBECs, we then studied the effect of media supplement deprivation on PAR-2 expression in ABECs. We hypothesized that if upregulated PAR-2 expression in ABECs is due to the epigenetic changes induced by decreased cell growth nutrients, then ABECs will not further respond to media supplement deprivation for PAR-2 expression. However, ABECs showed media supplement deprivation-induced PAR-2 upregulation. Further addition of media supplements to supplement-deprived ABECs decreased PAR-2 expression and brought it down close to baseline PAR-2 expression of ABECs, which is higher than baseline PAR-2 expression of NBECs. These results support the notion that increased PAR-2 expression in ABECs is imprinted into the cells due to epigenetic changes, however the factor responsible these epigenetic changes are not known. It is possible that other stress stimuli present in the *in vivo* inflammatory lung microenvironment may play a role in the development of these epigenetic changes.

In conclusion, our results suggest that decrease in cell growth nutrients, which could occur locally in the background of inflammation, may be a possible mechanism involved in PAR-2 upregulation observed in inflammatory conditions. Further studies are required to understand if the pre-apoptotic pathways such as autophagy or stress-activated protein kinases are activated and involved in this limiting cell growth nutrient condition-induced PAR-2 upregulation. Also the results suggest that regulation of PAR-2 expression could be one of the anti-inflammatory mechanisms of hydrocortisone. Understanding the mechanisms of PAR-2 upregulation may provide us new therapeutic strategies to inhibit PAR-2 upregulation and thus PAR-2-mediated inflammation in different inflammatory conditions.

5.6 References

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Chapter 6: Effect of cellular stress on proteinase-activated receptor-2 expression in primary human airway epithelial cells

6.1 Aim: To study the effect of oxidative stress, hypoxia, cyclic stretch and human rhino virus infection on PAR-2 expression in primary human airway epithelial cells (AECs)

6.2 Introduction

The regulation of PAR-2 expression is poorly understood. Since PAR-2 expression is increased in inflammatory diseases (1-5), it is deduced that inflammatory environment plays a role in the regulation of PAR-2 expression. Due to increased number of active inflammatory cells that release reactive oxygen species, sites of inflammation are characterized by the presence of oxidative stress (6); also increased metabolic demands of cells and decreased availability of metabolic substrates at the site of inflammation may result in hypoxia (7).

Asthmatic airways are chronically inflamed. The presence of oxidative stress in the asthmatic airways is well documented (8, 9). Also hypoxia-induced transcription factors are activated in the airways of asthmatic individuals (10), suggesting the presence of hypoxic environment in the airways in asthma. There is evidence that oxidative stress (11) and hypoxia (12, 13) regulate PAR-2 expression on cells other than AECs.

AECs undergo normal mechanical stretch during respiration. Mechanical stretch can influence cell growth, differentiation and also cell activation (14, 15). Human rhino virus (HRV) are major cause of asthma exacerbation (16, 17) and mechanical stretching could be more pronounced during exacerbations.

We studied effect of these cellular stress stimuli on PAR-2 expression in primary human AECs.

6.3 Methods

Studies in this chapter were performed on AECs cultured as described in Chapter 2. After subjecting AECs to the stresses described below PAR-2 expression was studied using qRT-PCR as described in Chapter 2.

Oxidative stress

SIN-1 hydrochloride spontaneously decomposes to yield reactive oxygen species (ROS) such as nitric oxide, superoxide anion and peroxynitrite leading to oxidative stress and is used to study the effects of ROS on cells. AECs were treated with SIN-1 hydrochloride (Calbiochem) for up to 24h.

Hypoxia

AECs cultured in media with or without supplements (as described in chapter 5) were subjected to hypoxia (1% oxygen, 5% CO_2, 94% nitrogen; Baker Ruskinn hypoxia chamber) or normoxia (Thermo Scientific Forma Steri-Cult CO_2 incubator) for 24h. To confirm that cells experienced hypoxia, we performed qRT-PCR for hypoxia-inducible factor-1-alpha (HIF-1A) using Taqman gene expression assay for HIF-1A (ThermoFisher, Hs00153153_m1) and housekeeping gene GAPDH (ThermoFisher, Hs02786624_g1).

Cyclic stretch and human rhino virus (HRV) infection

These experiments were performed in collaboration with the laboratories of Dr. Richard Leigh and Dr. David Proud at the University of Calgary. I designed the experiments with them. The labs of Dr. Leigh and Dr. Proud performed the experiments and sent us samples to evaluate PAR-2 expression.

Primary human bronchial epithelial cells (AECs) were obtained by protease digestion of dissected airways from non-transplanted human lungs as described before (18). Cells were cultured in BEGM media (Lonza) and then subjected to the following treatments in BEBM media (Lonza) containing 1X ITS (Insulin 10 μg/mL, Transferrin 10 μg/mL and Selenium 0.01 μg/mL, Lonza) for 24h:

a. Media

b. Human rhino virus-16 (HRV-16): Cells were infected with $10^{4.5}$ Tissue Culture Infective Dose (TCID50) U/ml of HRV-16.

c. Stretch: Cells were cultured on BioFlex® Culture Plates and subjected to 30 cycles of 18% stretch intensity per minute using FLEXCELL® FX-4000™ Tension Plus™ System and Tissue Train™ System.

d. HRV-16 and Stretch

CXCL8 mRNA expression was studied using Taqman gene expression assay for CXCL8 (ThermoFisher, Hs00174103_m1 and housekeeping gene GAPDH (ThermoFisher, Hs02786624_g1).

Statistics

Results are expressed as Mean ± SEM. Results are analyzed using repeated measures ANOVA with Tukey's multiple comparison test. The *p*-value of less than 0.05 was considered statistically significant.

6.4 Results and Discussion

Oxidative stress

Preliminary results show that treatment of AECs with a compound generating ROS does not upregulate PAR-2 expression (Fig. 6.1). The limitation of the experiments is that whether cells experienced oxidative stress upon treatment with SIN-1 hydrochloride was not assessed, which can be done by measuring the levels of ROS and anti-oxidant enzymes. Hence it would not be appropriate to say that preliminary results show no effect of oxidative stress on PAR-2 expression in AECs.
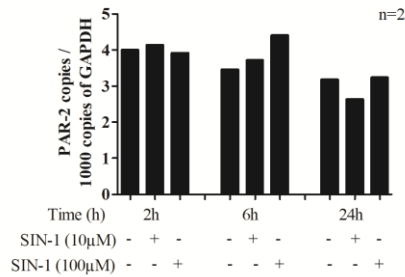
Fig. 6.1 Effect of oxidative stress on PAR-2 expression in AECs. AECs were treated with only growth media or growth media containing different concentration of SIN-1 and qRT-PCR for PAR-2 expression was performed at indicated time points. Data are presented as Mean of the two experiments.

Hypoxia

To validate that cells underwent hypoxia, upregulation in HIF-1A protein levels or HIF-1A-mediated gene expression can be studied. HIF-1A mRNA expression is shown to decrease under prolonged hypoxic condition (12h to 24h) in alveolar epithelial cells (19), human lung microvascular endothelial cells (mentioned but data not shown in the referenced paper) (19) and human umbilical vein endothelial cells (20). We also observed decrease in HIF-1A expression upon 24h hypoxia in bronchial AECs (Fig. 6.2A) indicating that cells in fact underwent hypoxia. We also studied if simultaneous exposure to two stressors (hypoxia and media supplement deprivation) has any synergistic or additive effect on PAR-2 expression in AECs. Under

normoxic condition, cells cultured in media without supplements showed 2.50 fold increase in PAR-2 expression compared to cells culture in media with supplements (Fig. 6.2B, black bars). Similarly, under hypoxic condition also, cells cultured in media without supplements showed 2.44 fold upregulation in PAR-2 expression compared to cells cultured in media with supplements (Fig. 6.2B, red bars), suggesting the absence of any interaction between the two stressors to modulate PAR-2 expression.

Cell subjected to hypoxia showed a tendency of decreased PAR-2 expression compared to cells subjected to normoxia. This hypoxia-induced decreased in PAR-2 expression was similar whether cells were cultured in media with supplements (1.36 fold decrease, right part of the Fig. 6.2B) or in media without supplements (1.39 fold decrease, left part of the Fig. 6.2B).

(A) (B)
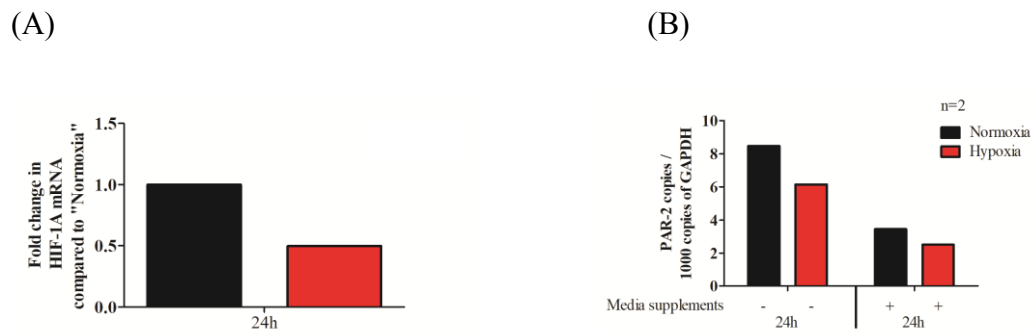
Fig. 6.2 Effect of hypoxia on PAR-2 expression in AECs. (A) AECs were cultured in media with supplements and subjected to normoxia (black bar) or hypoxia (red bar) for 24h and qRT-PCR for HIF-1A expression was done. (B) AECs were cultured in media without or with supplements and subjected to normoxia (black bar) or hypoxia (red bar) for 24h. qRT-PCR for PAR-2 expression was performed. Data are presented as Mean of the two experiments.

Using pulmonary arterial smooth muscle cells, it was shown that HIF-1A is not involved in PAR-2 expression under normoxic condition but was involved in hypoxia-induced PAR-2 upregulation (13). Even though our preliminary data indicates the activation of HIF-1A in AECs under hypoxic condition, more markers should be studied such as upregulation in HIF-1A protein levels and increase in HIF-1A-mediated gene expression to confirm HIF-1A activation in our cell culture system. PAR-2 mRNA expression was repressed under hypoxic condition

compared to normoxic condition in AECs. The mechanism responsible for hypoxia-induced gene repression is not well studied but there is evidence that Reptin, a chromatin-remodelling factor (21) and Repressor Element 1-Silencing Transcription Factor (22) may play a role in hypoxia-induced gene repression. Hence, further studies are required to understand if these mechanisms are involved in hypoxia-induced decrease in PAR-2 expression in AECs. It could also be possible that PAR-2 mRNA stability is decreased under hypoxic condition, which results in decreased expression of PAR-2 mRNA irrespective of the presence or absence of media supplements. This can be studied by comparing the rate of PAR-2 mRNA degradation in cells subjected to normoxia and hypoxia.

Cyclic stretch and HRV

Our collaborators have shown that mechanical stretch and HRV infection independently and synergistically upregulate CXCL8 mRNA expression in AECs (23). We first checked the induction of CXCL8 in our experiments as an experimental control to make sure that cells responded to virus infection and mechanical stretch similarly to what is observed before. Even though we observed similar trend to what our collaborators have shown, there was no significant upregulation in CXCL8 mRNA expression due to variability in the results (Fig. 6.3A). The variability could be due to differences in the susceptibility of AECs obtained from different donors to the studied stressors. We looked at the individual experiments and found that in one of the three experiments AECs did not upregulate CXCL8 mRNA expression in response to HRV infection or mechanical stretch alone or in combination.

Mechanical stretch and HRV infection, independently or in combination, did not modulate PAR-2 mRNA expression in AECs (Fig. 6.3B). There are not many reports demonstrating the effect of mechanical stress or virus infection on PAR-2 expression in primary cultures of AECs.

Mechanical stressors such as centrifugation and repeated pipetting are shown to upregulate PAR-2 expression in peripheral blood monocytes by mobilizing the intracellular stores of PAR-2 to the cell surface; authors did not study the effect of mechanical stressors on PAR-2 mRNA expression (24). In our study, it is possible that mechanical stretch upregulated PAR-2 protein expression on cell surface of AECs. We could not study this due to methodological limitations. For e.g. (i) as explained in Chapter 2, we were not able to study PAR-2 protein expression using

flow cytometry or western blot in AECs. (ii) We also could not perform confocal microscopy as the mechanical stretch treatment requires cells to be grown on a specific plate which cannot be mounted on microscope.

For virus infection, there are no reports of virus infection increasing PAR-2 expression in primary bronchial epithelial cells, the cell culture system that we used in our study. However there is evidence that influenza A upregulates PAR-2 expression in A549 cells (25), which is a cancerous alveolar epithelial cell line (26). Thus it is possible that, for PAR-2 expression, virus infections have different effect on primary cells vs. cell line and also have different effect on epithelial cells from different parts of the airways such as bronchi vs. alveoli. The other difference is that, in addition to the difference in the cell culture system used, influenza A is negative-sense single stranded RNA virus while HRV is positive-sense single stranded RNA virus. Hence, further experiments are required to understand if virus-induced PAR-2 upregulation is cell type specific or virus specific phenomenon.

(A)   (B)
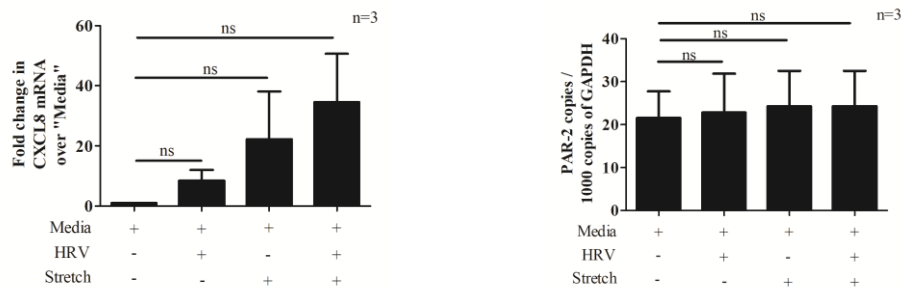

Fig. 6.3 cyclic stretch and HRV infection do not modulate PAR-2 expression in AECs. NHBE cells were treated with only media or HRV or mechanical stretch or combination of HRV and mechanical stretch for 24h. qRT-PCR for (A) CXCL8 expression and (B) PAR-2 expression were performed. Data are presented as Mean ± SEM; ns (not significant) between the groups indicated by horizontal lines.

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Chapter 7: Effect of media supplement deprivation on the expression of G protein-coupled receptors in primary human AECs

7.1 Aim: To study effect of media supplement deprivation on the expression of G-coupled receptors in primary human airway epithelial cells (AECs)

7.2 Introduction and rationale for selecting PAR-1, ADRB2 and P2RY2 for study

We showed that the expression of PAR-2, a G-coupled protein receptor (GPCRs) that belongs to the PAR family, is increased in AECs cultured in media without supplements (media supplement deprivation) compared to cells cultured in media with supplements (Chapter 4). AECs express all four receptors of the PAR family, PAR-1 to PAR-4 (1, 2). To study if media supplement deprivation specifically regulates expression of PAR-2 within PAR family of receptors, we examined the effect of media supplement deprivation on the expression of PAR-1 in AECs. We opted to study the expression PAR-1 because, in contrast to PAR-3 and PAR-4, there are standardized reagents available for PAR-1, mainly antagonists, which will enable us to study the functional significance of media supplement deprivation-mediated regulation of PAR-1 expression, if the expression of PAR-1 is regulated.

> PAR-1: PAR-1 and PAR-2 both are activated by serine proteinases. However, in contrast to PAR-2, which is activated by trypsin, PAR-1 is activated by thrombin and neutrophil elastase. PAR-1 is coupled with multiple G-proteins such as G_q, $G_{12/13}$, G_i. On AECs, PAR-1 activation increases the survival of basal cells through a tissue factor-dependent pathway (3). Further, similar to PAR-2, PAR-1 activation on AECs releases inflammatory mediators CXCL8 (4), CCL2 (5), IL-6 and PGE2 (1) but there are no studies showing involvement of PAR-1 in the pathogenesis of asthma. Finally, PAR-1 activation of fibroblasts induces proliferation (6) and myofibroblast differentiation (7), suggesting a role in the pathogenesis of fibroproliferative lung diseases (8).

In addition to PAR family, primary cultures of AECs are shown to express GPCRs belonging to adrenergic receptor family (9), purinergic receptor family (10), prostanoid receptor family (11, 12) and chemokine receptor family (13, 14). To study if media supplement deprivation affects expression of other families of GPCRs in AECs, we studied the expression of receptors that

belong to adrenergic receptor family and purinergic receptor family as these families of GPCRs are known to play a regulatory role on primary innate defense mechanisms of AECs as described below:

Beta-2 adrenergic receptor (ADRB2, adrenergic receptor family): The agonist for this G_s-coupled receptor is epinephrine. On AECs, activation of ADRB2 regulates innate defense functions such as ciliary beating frequency (15) and airway epithelial barrier properties (16). Beta-2 agonists (bronchodilators) are the primary medication for asthma that act through ADRB2 receptors to induce smooth muscle relaxation and bronchodilation (17). Interestingly, ADRB2 activation on airway epithelium plays a protective role against bronchoconstriction. By removing epithelial layer from tracheal preparations *in vitro* (18, 19) and by using transgenic mice over-expressing ADRB2 in airway epithelium (20), it was shown that ADRB2 activation on airway epithelial cells may regulate airway responsiveness and have bronchodilating effect by inducing smooth muscle relaxation.

P2Y purinoceptor 2 (P2RY2, purinergic receptor family): The agonists for this purinergic receptor are ATP and UTP nucleotides. P2RY2 is a G_q-coupled receptor that on airway epithelium regulates ciliary beating frequency and mucus secretion (10), thus provides protection against invading foreign particles. Interestingly, recent evidence has showed that aeroallergens acts on AECs to release ATP, which works as a "danger signal" and activates P2RY2 in an autocrine manner to induce the release of IL-33 from AECs (21); IL-33 is a mediator that plays a vital role in the pathogenesis of Th2 inflammation (22).

Finally, to evaluate if the media supplement deprivation-mediated regulation of receptor expression in AECs is limited to GPCRs, we also studied the effect of media supplement deprivation on expression of a non-GPCR, toll-like receptor 3 (TLR-3).

TLR-3: This receptor of the innate immune system belongs to the family of pattern recognition receptors (PRRs), a receptor family that gets activated by pathogen-associated molecular patterns (molecules associated with microbial pathogens). TLR-3 is a receptor for double-stranded RNA viruses. In AECs, TLR-3 activation induces the release of cytokines, chemokines and interferons as an immune response (23, 24). Rhinoviruses (RV) are a major cause of asthma exacerbation (25, 26). TLR-3 is shown to

have an anti-viral response against RV by decreasing virus replication in AECs (27). However, in asthma, the anti-viral effects of TLR-3 in AECs are decreased not because of decreased expression of TLR-3 but because of impaired TLR-3 signaling (28).

7.3 Methods

AECs were cultured and subjected to media supplement deprivation as described before (Chapter 5). The following Taqman gene expression assays (ThermoFisher) were used to study the expression of gene of interest and *Cyclophilin A (PPIA)* (housekeeping gene) by qRT-PCR.

Gene of interest	Taqman Gene expression assay
PAR-1	Hs00169258_m1
ADRB2	Hs00240532_s1
P2RY2	Hs01856611_s1
TLR-3	Hs01551079_g1
Cyclophilin A (PPIA)	Hs04194521_s1

7.4 Results and Discussion

(A)   (B)

(C)   (D)
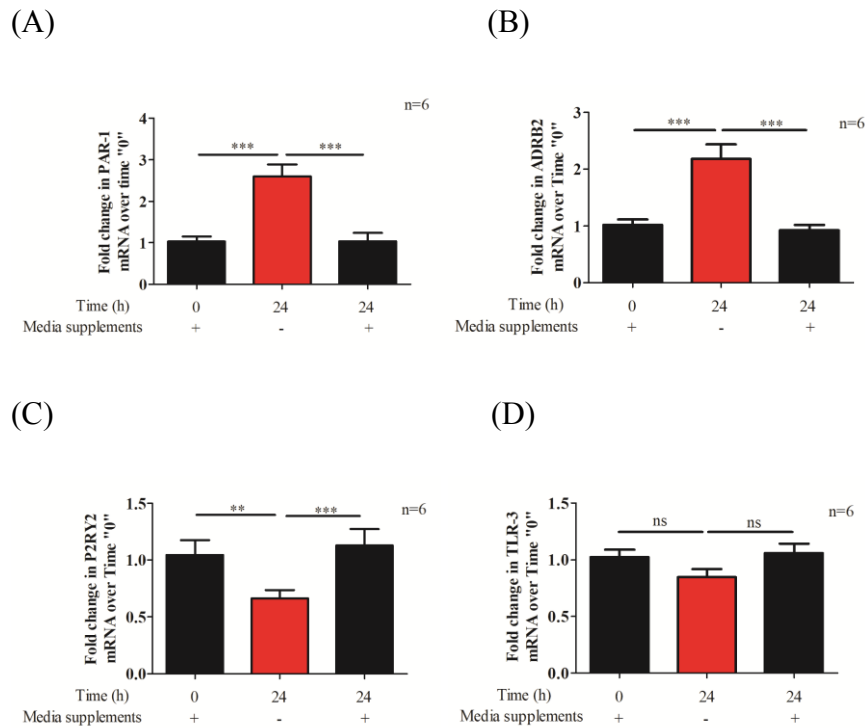
Fig. 7.1 Effect of media supplement deprivation on the expression of PAR-1, ADRB2, P2RY2 and TLR-3 receptors. AECs were cultured in media with supplements until reached 80% confluency (Time "0"). Cells were further cultured in media without supplements (red bars) or media with supplements (black bars) for 24h. The graphs show relative mRNA expression for (A) PAR-1, (B) ADRB2 (C) P2RY2 and (D) TLR-3. Data are presented as Mean ± SEM; ns (not significant), $**P<0.01$, $***P<0.001$ between the groups indicated by horizontal lines.

Six experiments that were used to analyze the effect of media supplement deprivation on the expression of PAR-2 were also evaluated for the expression of other receptors. Media supplement deprivation modulated expression of all 3 G-coupled receptors. The expression of PAR-1 (Fig. 7.1A) and ADRB2 (Fig. 7.1B) was increased, while the expression of P2RY2 (Fig. 7.1C) was decreased. The expression of TLR-3, a non-GPCR was not affected (Fig. 7.1D).

TLR-3 activation is shown to release type I interferons and increase TLR-3 expression in AECs (29), probably through an autocrine action of type I interferons (30). It is shown that

inflammatory mediators such as TLR-4 agonist lipopolysaccharide (LPS), TNF and IL-1β do not modulate TLR-3 expression in AECs (29). On the other hand, the expression of GPCRs is regulated by inflammatory mediators. For example, TGFβ is shown to increase PAR-1 expression in AECs (31). Otherwise there is no information if inflammatory mediators alter expression of ADRB2 and P2RY2 in AECs. For cells other than AECs, TNF upregulates PAR-1 expression in fibroblast (32), while LPS, TNF and IL-1β upregulate P2RY2 expression in vascular smooth muscle cells (33). In summary, the literature suggests that expression of TLR-3 and GPCRs is regulated differently; this is what we observed in our experiments also. It is possible that media supplement deprivation may affect expression of only GPCRs but still expression of other GPCRs and non-GPCRs need to be studied before reaching this conclusion.

Similar to PAR-2 (2.3 fold upregulation, Fig. 5.1E in Chapter 5), the expression of PAR-1 was also increased (2.6 fold) upon media supplement deprivation. For PAR-2, the primary media supplement regulating PAR-2 expression was insulin (Chapter 2). Hence further experiments are required to study if insulin also regulates expression of PAR-1, another member of the same receptor family. There is one study showing that LPS increased PAR-2 expression in AECs without affecting PAR-1 expression (34). This suggests that the regulation of these two receptors may be stimulus specific and hence it is possible that media supplement other than insulin may regulate PAR-1 expression in AECs.

Very limited information is available regarding the mechanisms that regulate expression of the studied GPCRs in general and especially on AECs. So there are a few possibilities for what could regulate expression of GPCRs studied:

(i) The exclusion of media supplements regulate expression of receptors by activating signaling pathways which are otherwise not activated when a single supplement is excluded from the cell culture media. For example, media supplement deprivation can activate stress-activated protein kinase JNK and p38 signaling pathways (35) and ERK1/2 (36), which result in activation of a range of transcription factors including AP-1, ELK-1, ATF-2. The study of these signaling pathways will show whether these GPCRs are regulated by a single signaling pathway.

Cells release ATP as a result of respiration and ATP is an agonist for P2RY2. Cellular stressors such as injury (37), exposure to air pollutants (38) or virus infections (39) modulate the release of ATP or the expression of P2RY2. Some of the media supplements are growth factors for cells and it is possible that exclusion of media supplements may generate stress that could alter ATP release and thus modulate P2RY2 expression in an autocrine manner. This can be studied by measuring ATP release in cell supernatants in cell cultured with and without media supplements. Further, cells cultured in the absence of media supplements can be supplied with ATP and P2RY2 expression can be studied.

Out of GPCR families studied, media supplement deprivation upregulated receptors belonging to PAR and adrenergic receptor families, while downregulated receptor belonging to purinergic receptor family. It may be possible that the cellular stress induced by media supplement deprivation exerts differential effects on the expression of different families of GPCRs. The expression of other members of these receptor families should be studied to understand if all the members of a particular GPCR family are regulated similarly by media supplement deprivation. To do this we require information about what other receptors are expressed in AECs, but this information is limited in case of adrenergic and purinergic receptor families. Microarray-based detection can be performed to identify the GPCRs expressed in AECs. However, it is also noted that microarray-based detection of GPCRs can be challenging as microarrays that study genome-wide RNA expression are not optimized to detect GPCRs (40). This is most probably because GPCRs are expressed at low levels; qRT-PCR based approaches are developed to overcome this limitation (41). Once known, other relevant receptors can be selected for future studies.

(ii) It is also possible that a particular media supplement exerts regulatory role on all or a particular GPCR. For example (a) we showed that exclusion of insulin from cell culture media upregulated PAR-2 expression (Chapter 2). Even though there is no evidence that insulin has a regulatory role for a wide range of GPCRs, further experiments are required to identify if insulin regulates GPCRs belonging to various families in our cell culture system. (b) One of the components of media supplement is epinephrine. Epinephrine is

shown to regulate the expression of a class of adrenergic receptors, Alpha-2A adrenergic receptors (42). Epinephrine is a ligand for ADRB2, so exclusion of epinephrine may modulate the expression of adrenergic receptors. (c) Finally, retinoic acid, another component of media supplements, is shown to upregulate the expression of P2RY2 in dose-dependent manner in keratinocytes (43). If retinoic acid has the similar effect on P2RY2 expression in AECs, then it is possible that the removal of retinoic acid may result in the downregulation of P2RY2 expression. Thus further experiments are required to study all these possibilities.

(iii) Another possibility is that there is an interaction between two or more media supplements for regulating expression of these GPCRs. Thus the observed effect on the expression of GPCRs is may be the result of exclusion of more than one media supplements but not all. For e.g. in human uterine cervical epithelial cells, epinephrine increases the degradation of purinergic receptor P2X7, the effect which is potentiated by EGF. The study to check this possibility will involve very complicated experiments where absence or presence of different media supplement combinations need to be studied to understand their role in regulation of GPCRs expression.

Further experiments are required to understand if the observed changes in mRNA expression of these receptors are the result of alteration in gene transcription or mRNA stability. It is also important to study whether the observed changes in mRNA expression are translated at protein levels. For example, in cells other than AECs, it is shown that increase in PAR-1 mRNA did not result in increase in PAR-1 protein (44, 45). If the observed changes in mRNA expression are translated into protein expression, the upregulation in PAR-1 (3) and ADRB2 (15, 16) could be protective from cell death or airway hyperresponsiveness, respectively based on their described roles in AECs. The decreased expression of P2RY2 may play an important role in controlling inflammation by decreasing the release of IL-33 but on the other hand this may limit the mucociliary clearance function of AECs as P2RY2 regulates ciliary beating frequency and mucus secretion in airway epithelium (10). Differentiated cultures of AECs can be subjected to media supplement deprivation and then treated with agonists of these receptors or allergens and AEC functions, such as barrier property, ciliary beating and mucus secretion, and inflammatory

mediator release can be studied to understand the functional significance of this modulation in receptor expression.

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Chapter 8: General Discussion

8.1 Discussion and Future Directions

In asthma, PAR-2 expression is increased on AECs (1); cells that play an important role in the pathogenesis of lung inflammatory diseases. Upregulated PAR-2 expression leads to increased lung inflammation (2). Thus inhibition of PAR-2 upregulation is a rational strategy to control PAR-2-mediated inflammation, but regulation of PAR-2 expression is poorly studied. Since upregulated PAR-2 expression is reported in a variety of inflammatory conditions, it is believed that the inflammatory milieu regulates PAR-2 expression.

Inflammation leads to insulin resistance, a condition where insulin signaling is decreased or blocked and the function of insulin is impaired. HFD-induced obesity in mice is a standard model to induce insulin resistance in adipose tissue and skeletal muscle tissue. PAR-2 expression increases in insulin resistant adipose tissue in HFD fed obese mice (3-5). Further, PAR-2 expression decreases upon improving insulin sensitivity in insulin resistant adipose tissue (3). Although it was not shown directly, it is possible that insulin may directly regulate PAR-2 expression in these tissues. Due to insulin resistance in adipose tissue and skeletal muscle, these tissues do not perform insulin-directed glucose uptake. Pancreatic β-cells sense the increased levels of circulating glucose and, in response, secrete more insulin, which results in higher levels of insulin in circulation. Even though this state is known as systemic insulin resistance, the status of insulin sensitivity in lungs is not known.

To study if insulin modulates PAR-2 expression in lungs *in vivo*, we used a similar mouse model of HFD-induced obesity. We found that the mice fed with HFD had higher levels of circulating insulin, compared to mice fed normal diet, but the baseline insulin signaling was not altered in the lungs of obese mice. Further, we showed that higher levels of circulating insulin in obese mice were associated with lower levels of lung PAR-2 expression, hence it is likely that insulin regulates PAR-2 expression in lungs and increased levels of insulin decreased PAR-2 expression in lungs of obese mice.

Obesity is associated with allergic sensitization and allergic asthma in humans (6). Using similar model of HFD-induced obesity, obese animals are shown to be prone to develop allergic

sensitization (7) and eosinophilic airway inflammation (7, 8). PAR-2 is shown to play a vital role in the development of allergic sensitization, allergic airway inflammation and airway remodeling (9-13) and thus it may be possible that PAR-2 expression would increase in lungs of obese mice and humans, which makes them more susceptible to develop allergic asthma. Opposite to this rationale, we found decreased PAR-2 expression in lungs of obese mice. Hence it may be possible that the mechanism underlying obesity-induced asthma does not involve PAR-2.

There are several mechanisms proposed for the development of asthma in obesity (14). Low grade systemic inflammation is present in obesity resulting in increased systemic levels of inflammatory cytokines IL-6, TNF and IL-1. These cytokines could affect lung functions by acting on SMCs, fibroblasts and epithelial cells in the airways (15, 16). Also during obesity, the expression of adipokines is altered and this alteration may favor the development of asthma. For example, adipokine leptin, whose level is increased in obesity, is shown to increase AHR (17). On the other hand, adipokine adiponectin prevents allergic airway inflammation, AHR and airway remodeling (18, 19) but the level of adiponectin is decreased in obesity. Also higher levels of circulating insulin, in obesity, is shown to develop AHR (20), alter lung structure (21) and decrease lung function (22) by upregulating collagen deposition and peribronchial fibrosis. Finally, insulin increases expression of contractile phenotypic markers in airway smooth muscle (23-25) indicating insulin may upregulate contractile potential of the airways. These results indicate that the alteration in the levels of cytokines, adipokines and hormones in obesity make animals susceptible to the development of asthma. Hence the decreased expression of PAR-2 in the lungs of these obese animals may indicate that PAR-2 play a very limited role in the development of asthma. Further studies are required to understand the role of PAR-2 in the development of allergic sensitization and allergic airway inflammation in obesity. This can be done by developing animal model of asthma in PAR-2 KO obese mice or by developing animal model of asthma in wild type obese mice with the allergens containing PAR-2 activating property or PAR-2 agonists.

Using primary human AECs, we show that insulin regulates PAR-2 expression through the PI3K-Akt-FOXO-1 pathway and alterations in insulin signaling result in increased PAR-2 expression in AECs. Thus, *in vivo*, alteration in insulin signaling may result in PAR-2

upregulation. One of the mechanisms that alters insulin signaling *in vivo* is insulin resistance. In adipose tissue, where PAR-2 upregulation is correlated with insulin resistance, increased levels of inflammatory mediators such as IL-6, IL-1β and TNF are shown to induce insulin resistance (26). Since asthmatic airways are chronically inflamed and the mediators that induce insulin resistance in adipose tissue are also increased in the airways in asthma (27-30), it would be an interesting hypothesis to study whether the observed upregulation in expression of epithelial PAR-2 in asthma is due to localized insulin resistance in the airway epithelium. Our hypothesis can be studied by obtaining bronchial biopsies, from healthy and asthmatic individuals, and correlating the presence of markers of insulin resistance with expression of PAR-2 in the airway epithelium. A positive correlation between presence of insulin resistance and PAR-2 expression will further support our hypothesis. Finally, to strengthen the idea that insulin resistance upregulates PAR-2 expression in asthmatic AECs, it is important to show that PAR-2 expression is increased upon inducing insulin resistance in AECs *in vitro*. Currently, there is no established *in vitro* cellular model of insulin resistance in AECs. Also it is not known whether AECs develop insulin resistance. A recent paper has shown decreased expression of insulin receptors in AECs obtained from asthma patients compared to healthy individuals (31). However, it is not clear whether decrease in insulin receptor expression, in these AECs, alters insulin signaling to induce insulin resistance. Other *in vitro* cultures of hepatoma cells and adipocytes have shown that phorbol myristate acetate (PMA), anisomycin (c-Jun N-terminal kinase-1 (JNK-1) activator), calyculin A (inhibitor of serine/threonine phosphatase) (32) and TNF (33) can induce insulin resistance by serine phosphorylation of IRS-1. On the other hand IL-1β (34), IL-6 (33) and dexamethasone (35) decrease IRS-1 expression to induce insulin resistance. Similar strategies can be used to develop *in vitro* cellular model of insulin resistance in AECs.

In addition to evaluating the effect of insulin resistance on PAR-2 expression, *in vitro* cellular models of insulin resistance in AECs will be a valuable tool to understand the unstudied area of role of insulin in innate immune functions and homeostatic functions of AECs. A couple of studies published in 1990s suggested chemotactic activity of insulin for AECs (36, 37). This function of insulin can play an important role in epithelial repair after airway injury and inefficient insulin function could result in delayed wound healing. Recent evidence indicates an important role of insulin in AEC differentiation (31) and regulation of their innate immune

functions, such as barrier function (38) and inflammatory mediator release (39). Further, it will be worth evaluating if insulin has any role in other defense mechanisms of AECs, such as ciliary beating and mucus secretion. Altered insulin signaling may limit these immune functions of AECs and result in decreased protection against inhaled particles. Finally, in AECs, insulin regulates glucose uptake and thus maintains the levels of glucose in the airway surface liquid (ASL) (38). Altered insulin signaling in the airway epithelium could result in dysregulated glucose homeostasis and increased levels of glucose in ASL. Using an animal model, it was shown that higher levels of glucose in ASL could increase the likelihood of bacterial respiratory infections (40). The discussed literature describing the effect of insulin on AECs is derived from proof of principle experiments where insulin was either added to or removed from cell culture media. However, the currently used approaches such as addition or exclusion of insulin or the use of inhibitors of insulin signaling do not mimic the actual *in vivo* condition of altered insulin signaling.

We have used *in vitro* differentiated cultures of AECs that mimic the *in vivo* airway epithelium. Thus, developing an *in vitro* model of insulin resistance in these differentiated AECs will not provide insight into the role of insulin in the functions of AECs, but will also show if AECs have any alternate strategies to cope up with altered insulin signaling. For example, insulin regulates glucose uptake in cells by regulating glucose transporters. There are two types of glucose transporters; one being dependent on insulin actions, while the other is independent of insulin actions for glucose uptake. AECs express both insulin-dependent and -independent glucose transporters (38, 41, 42). Hence it is possible that under conditions where insulin signaling is altered, AECs increase their efficiency of glucose uptake through insulin independent glucose transporters to maintain their glucose uptake.

After understanding the mechanism of insulin-mediated regulation of PAR-2 expression, we focused on the other potential mechanisms involved in the regulation of PAR-2 expression in AECs. There is evidence that characteristics of inflammatory microenvironment such as inflammatory mediators (43-45), hypoxia (46, 47) and growth factor deprivation (48) regulate PAR-2 expression on cells other than AECs. We hypothesized that these cellular stressors of inflammatory microenvironment regulates PAR-2 expression in AECs.

We have not yet studied the effect of inflammatory mediators on PAR-2 expression in AECs. The literature suggests that mainly TNF and IL-1β upregulate PAR-2 expression (Table- 1.2). Thus future studies are required to identify if TNF and IL-1β upregulates PAR-2 expression in AECs.

Our preliminary results indicate that hypoxia may decrease PAR-2 expression on AECs. Hypoxia is shown to upregulate PAR-2 expression in smooth muscle cells (47), where the upregulation was HIF-1A and platelet-derived growth factor-BB dependent. We found that hypoxia decreased HIF-1A mRNA expression in AECs indicating possible HIF-1A activation, but PAR-2 expression was decreased. Due to limited literature available on the effect of hypoxia in AECs and airway SMCs, it is not clear why hypoxia exerts opposite effects on PAR-2 expression in these two cell types. It has been shown that hypoxia decreases proliferation of airway SMCs (49), while increases proliferation of goblet cells, a type of AECs (50). Hence it may be possible that hypoxia has different effects on these cells. In our experiments we assessed HIF-1A mRNA levels, which is an indirect approach to evaluate if AECs underwent hypoxia and activated HIF-1A. HIF-1A activation should be confirmed by showing increase in HIF-1A protein expression or increase in nuclear localization of HIF-1A, since it is a transcription factor, or by studying expression of HIF-1A-regulated genes. For example, in AECs, HIF-1A increases mucin gene expression upon hypoxia (50). So, further analysis is required to confirm that cells underwent hypoxia in our experimental settings.

To mimic decreased availability of nutrients supporting cell growth *in vivo*, we excluded culture media supplements from the growth media in our AEC cultures *in vitro*. These supplements include growth factors and hormones among other factors. We found that exclusion of media supplements resulted in higher upregulation in PAR-2 expression compared to PAR-2 upregulation induced by the exclusion of only insulin. The media supplements, other than insulin, that had a regulatory effect on PAR-2 expression were hydrocortisone, BPE and RA.

The exclusion of hydrocortisone showed a trend of increased PAR-2 expression in AECs, while the addition of hydrocortisone to cells subjected to media supplement deprivation, decreased the media supplement deprivation-induced upregulation in PAR-2 expression. Since hydrocortisone has anti-inflammatory properties, PAR-2 downregulation could be one of its anti-inflammatory

mechanisms. However, it is shown that asthmatics that were on inhaled steroids had similar upregulation in PAR-2 expression as asthmatics which were not using inhaled steroids (1). There are a few possibilities that could explain this difference in effect of corticosteroid on PAR-2 expression *in vivo* vs. *in vitro*. First, the regulation of PAR-2 expression by hydrocortisone *in vitro* could be an artifact of cell culture system and corticosteroids do not regulate PAR-2 expression *in vivo*. Second, the study that showed increased PAR-2 expression in airway epithelium of asthmatics that were on inhaled steroid included only 10 patients. Airway epithelial PAR-2 expression in 4 out of 10 asthma patients was very similar to healthy controls. Within these 10 patients, the dose of steroid ranged 400-1600 µg/day. The authors did not show any correlation between the dose of steroid and PAR-2 expression, but it may be possible that steroids decrease PAR-2 expression and the patients who were on higher dose of steroids had lower expression of airway epithelial PAR-2. Thus a study with larger sample size, where patients are divided into groups based on their dose of steroids, might explain if patients taking higher dose of steroids have lower PAR-2 expression. Third, it may be possible that the upregulation of PAR-2 expression in asthma is the result of epigenetic changes in the airway epithelium. Hence even though steroids act anti-inflammatory, PAR-2 expression stayed upregulated. Finally, corticosteroids are shown to induce insulin resistance (51, 52); this observation is true about systemic delivery of steroid that shows the development of insulin resistance in liver, skeletal muscle and adipose tissues. Moreover, direct *in vitro* exposure of dexamethasone is shown to induce insulin resistance in cultures of adipocytes (35). AECs are directly exposed to inhaled steroids. It may be possible that the dose of inhaled steroids and duration of exposure to inhaled steroids play a role in the development of insulin resistance in AECs. This may disrupt the insulin-mediated regulation of PAR-2 expression resulting in PAR-2 upregulation. Further studies can be done to understand the time- and dose-dependent effects of hydrocortisone on insulin sensitivity and PAR-2 expression in AECs.

The addition of BPE and RA to cells subjected to media supplement deprivation decreased the media supplement deprivation-induced upregulation in PAR-2 expression. Growth factor deprivation induces oxidative stress in different cells (53-55) to upregulate PAR-2 expression (56). Media supplements for AECs also contained growth factors hence it is possible that deprivation of media supplements in AECs may induce oxidative stress to upregulate PAR-2

expression. BPE and RA have anti-oxidant property (57, 58). Thus when we add BPE and RA to media supplement-deprived cells, BPE and RA may decrease PAR-2 expression by decreasing oxidative stress induced by media supplement deprivation.

Contrary to the hypothesis, that oxidative stress upregulates PAR-2 expression in AECs, our preliminary experiments did not show upregulation in PAR-2 expression when AECs were subjected to oxidative stress. To induce oxidative stress in AECs, we treated cells with SIN-1 hydrochloride compound, which spontaneously decomposes to yield reactive oxygen species (ROS) such as nitric oxide, superoxide anion and peroxynitrite. However the limitation of our experiments is that we did not evaluate if oxidative stress was induced, in AECs, at the used concentration of SIN-1 hydrochloride. Further experiments are required where AECs will be treated with different compounds, such as SIN-1 hydrochloride and hydrogen peroxide, to induce oxidative stress. First the induction of oxidative stress will be confirmed by measuring the levels of ROS and anti-oxidant enzymes and then PAR-2 expression will be studied.

In addition to oxidative stress, growth factor deprivation or media supplement deprivation can lead to activation of cellular stress responses such as stress-activated protein kinase (SAPK) JNK or p38 kinase (59, 60) or autophagy (59). Whether SAPKs are activated upon media supplement deprivation in AECs should be studied first. SAPK signaling leads to activation of AP-1, ELK-1, ATF-2 and other transcription factors, hence further experiments are required to understand the signaling pathways involved in media supplement deprivation-induced PAR-2 upregulation. Further, these SAPKs are known to activate autophagy (59, 61) as a survival mechanism. Autophagy occurs before apoptosis but prolonged autophagy results in apoptosis (62); activated SAPK maintain balance between autophagy and apoptosis (63). Since we did not see significant upregulation in the number of apoptotic cells upon media supplement deprivation, it is possible that autophagy was activated to upregulate PAR-2 expression. Further experiments are required to assess if autophagy is activated upon supplement deprivation and if autophagy plays any role in supplement deprivation-induced PAR-2 upregulation. PAR-2 upregulation in AECs during autophagy (pre-apoptotic stage) could be a protective mechanism because PAR-2 activation is shown to inhibit apoptosis in colonic (64) and airway (65) epithelial cells.

We showed that exclusion of insulin, one of the media supplements, activates FOXO-1 transcription factor to upregulate PAR-2 expression. We then showed that exclusion of all the media supplements resulted in higher upregulation in PAR-2 expression compared to PAR-2 upregulation induced by the exclusion of only insulin. Based on these observations, we hypothesize that PAR-2 upregulation upon exclusion of all the media supplements will partially be dependent on FOXO-1. However, FOXO-1 inhibition further increased media supplement deprivation-induced PAR-2 upregulation indicating the involvement of different mechanisms in media supplement deprivation- vs. insulin deprivation-induced PAR-2 upregulation. The question is why does FOXO-1 involve in insulin deprivation-induced PAR-2 upregulation and not involved in media supplement deprivation-induced PAR-2 upregulation? Media supplements contained multiple growth factors. It is shown that under growth factor deprived condition, FOXO-1 activation (nuclear localization of FOXO-1) induces apoptosis (66, 67), while cytoplasmic localization of FOXO-1 (inactive FOXO-1 due to acetylation) during growth factor deprivation prevents apoptosis (66). It is also shown that growth factor deprivation activates JNK, which inhibits FOXO-1-induced apoptosis by decreasing FOXO-1 expression (68). In our hands, media supplement deprivation led to marginal increase in apoptotic cells. Thus, this evidence suggests that FOXO-1 was not activated during media supplement deprivation in our cells and hence played no role in PAR-2 upregulation. This possibility can be studied by (i) evaluating whether growth factor deprivation results in less nuclear localization of FOXO-1, compared to insulin deprivation and (ii) if FOXO-1 expression is decreased during media supplement deprivation, in AECs.

Studies have shown that asthmatic AECs cultured *ex vivo* maintain the intrinsic phenotypic differences (69-73). We have an observation that PAR-2 expression is increased in AECs from asthmatic individuals compared to healthy individuals and this upregulated PAR-2 expression is maintained when cells were cultured *ex vivo* for multiple passages. This property of asthmatic AECs will be useful to understand differences in the functions of epithelial PAR-2 between healthy and asthmatics individuals in order to understand the consequences of upregulated PAR-2 expression in asthma. Cell culture supernatants of PAR-2-activated healthy and asthmatics AECs can be analyzed for an array of cytokine and chemokines to identify the inflammatory mediators that are released in increased amount from asthmatic AECs cells. Further to confirm

that the increased release of mediators from asthmatic AECs is because of the higher expression of PAR-2, AECs from healthy individuals can be transfected with PAR-2 overexpression vector to upregulate PAR-2 expression to a level similar to that of asthmatic AECs. PAR-2 activation in these cells expressing higher levels of PAR-2 mimics increased PAR-2 functions of asthmatic cells (74). Thus higher release of inflammatory mediators by PAR-2 in these transfected cells will confirm that increased release of mediators from asthmatic AECs is because of the upregulated expression of PAR-2. This analysis of PAR-2-mediated release of inflammatory mediators from healthy vs. asthmatic AECs may guide us for the differential release of mediators between the two different phenotypes of cells. Finally, comparison of other outcomes of PAR-2 activation, such as mucus secretion (75) and epithelial barrier disruption (76, 77), between healthy and asthmatic AECs may show significant differences. Over all these comparative studies of PAR-2 functions between healthy and asthmatic AECs will help us understand the role of epithelial PAR-2 in the pathogenesis of asthma.

It is also reported that the phenotypic differences in asthmatic AECs are associated with the severity of asthma (78). In our study, two of the asthmatic individuals showed PAR-2 expression similar to healthy controls. Due to limited available information about the asthmatic individuals, we have not correlated PAR-2 expression with asthma severity and other characteristics of patients that the epithelial cells came from to identify if any clinical characteristic is associated with PAR-2 expression. We have shown that in patients having severe asthma compared to mild/moderate asthma (i) PAR-2 expression on peripheral pro-inflammatory monocytes is higher and (ii) higher number of pro-inflammatory monocytes expresses PAR-2 (79). In the same manuscript, we also showed that the patients that had higher number of PAR-2-expressing pro-inflammatory monocytes also experienced asthma exacerbation over the last year. Further studies are required to understand if PAR-2 expression in AECs varies based on the disease severity, as upregulated PAR-2 could exacerbate proteinase-mediated airway inflammation.

We are not sure at what point PAR-2 upregulation in AECs in asthma becomes irreversible as upregulated PAR-2 expression is maintained in asthmatic AECs cultured *ex vivo*. It could be possible that *in vivo* inflammatory microenvironment induces epigenetic changes that render asthmatic airway epithelium with higher PAR-2 expression. Epigenetic changes in the gene for

PAR-2, F2RL1, can be compared between asthmatic and healthy individuals to understand the epigenetic changes present in asthma. To identify if the observed epigenetic changes are involved in upregulated PAR-2 expression in asthma, *in vitro* epigenome editing assays can be performed where site-directed epigenetic changes can be introduced on DNA to determine the function of the epigenetic modifications. Using *in vitro* epigenome editing assays, we can introduce the same epigenetic changes that we observed in asthma in F2RL1 gene and study if these epigenetic changes increase PAR-2 expression. Even after this point, the questions still remain that what induces these epigenetic changes and at what time these epigenetic changes are incorporated into the PAR-2 gene? The recent literature suggests that chronic inflammation may induce epigenetic changes (80). Asthmatic airways are chronically inflamed. Chronic localized inflammation may lead to insulin resistance, decrease in nutrients supporting cell growth, oxidative stress and hypoxia in the airways. Insulin resistance (81), oxidative stress (82) and hypoxia (83) may induce epigenetic changes. We showed that insulin and cell growth nutrients regulate PAR-2 expression in AECs. Hence, it may be possible that localized insulin resistance in the airway epithelium and decrease in cell growth nutrients in the airways, in the background of inflammation, upregulate PAR-2 expression in AECs in asthma (Fig. 8.1 Conceptual model).

Our findings will encourage studies to evaluate if similar mechanisms are involved in the regulation of PAR-2 expression in other cells of lungs or cells of other organs such as colon and skin because in inflammatory diseases of these organs, localized inflammation is present and PAR-2 expression is increased. These studies may identify common dysregulatory mechanisms involved in increased PAR-2 expression in different inflammatory conditions and may lead to develop novel therapeutic strategies to inhibit PAR-2 upregulation and control PAR-2-mediated inflammation.

8.2 Conceptual Model

In AECs, binding of insulin to insulin receptor activates the PI3K-Akt signaling cascade, which inactivates FOXO-1transcription factor. Also the presence of cell growth nutrients prevents activation of any SAPK signaling pathways. In this condition, PAR-2 expression is maintained at lower levels on AECs and PAR-2 activation results in controlled inflammation. Chronic airway inflammation leads to localized insulin resistance in the airway epithelium, as characterized by decrease in insulin receptor expression or serine phosphorylation of IRS-1, which blocks insulin signaling. The blockade in insulin signaling results in FOXO-1 activation and FOXO-1-mediated PAR-2 expression. Chronic airway inflammation also leads to decrease in the levels of cell growth nutrients, resulting in activation of the SAPK signaling cascade leading to upregulated PAR-2 expression through unknown transcription factors. The activation of upregulated PAR-2 on AECs augments airway inflammation.
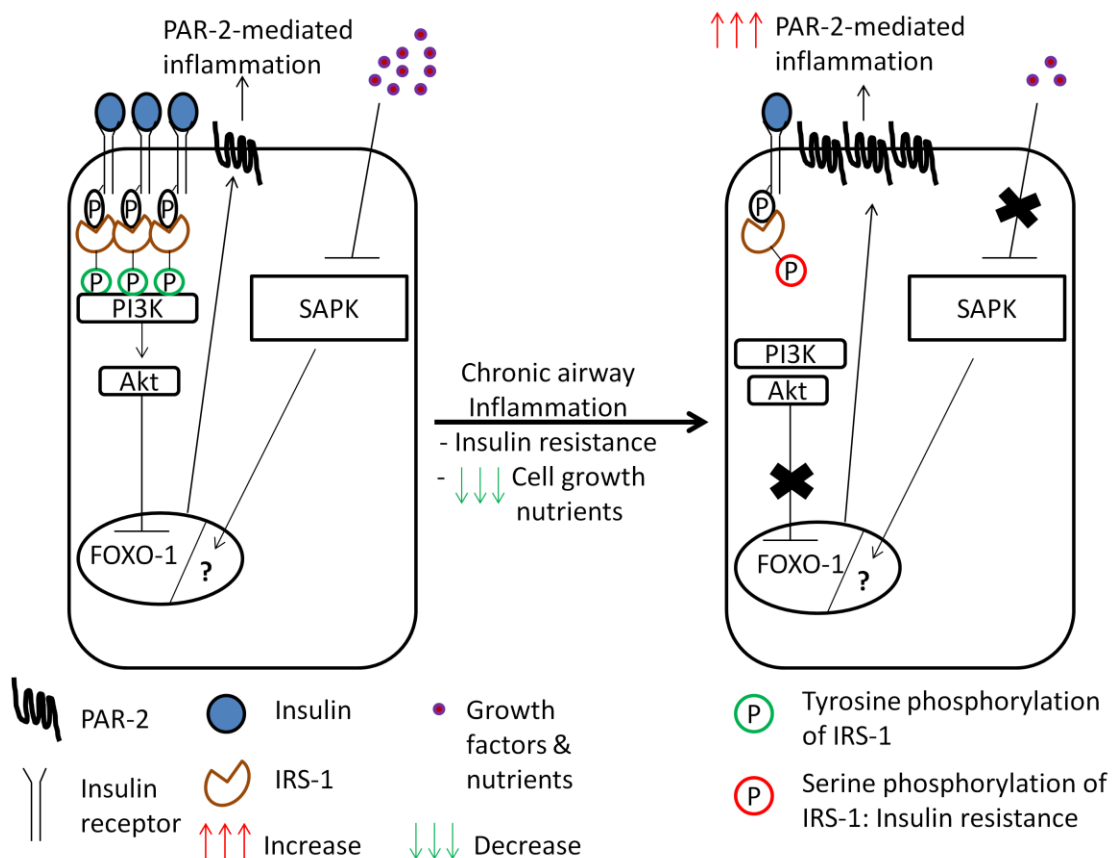

Fig. 8.1 Conceptual Model.

8.3 References

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Appendix-1

To understand the mechanism of PAR-2 regulation, AECs were treated with inhibitors of ERK1/2, PI3K and FOXO-1 (data was shown in chapter 2 and 3 of the thesis). All the inhibitors were dissolved in DMSO (Sigma-Aldrich) so that the final concentration of DMSO added to cells was equal to or less than 0.1%. It is important to evaluate if DMSO, at the concentration used, has any effect on PAR-2 expression (vehicle control treatment).

Methods

To study if this concentration of DMSO has any effect on PAR-2 expression, we cultured AECs in media without supplements or with all supplements or with all supplements except insulin in the absence or presence of 0.1% DMSO. PAR-2 expression was studied using qRT-PCR as described before.
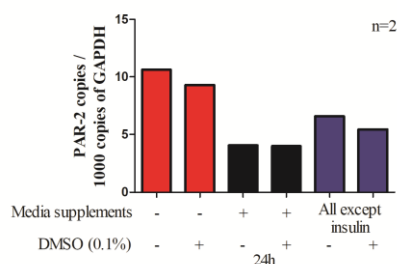

Fig. AECs were cultured in media without supplements (Red bars) or media with all supplements (Black bars) or media with all supplements except insulin (Blue bars) for 24h in the absence or presence of 0.1% DMSO and qRT-PCR for PAR-2 expression was performed. Data are presented as Mean of the two experiments.

Results

Preliminary results suggest that DMSO at 0.1% concentration had no effect on PAR-2 expression in AECs.

Appendix-2

Transfection optimization

BEAS-2B and NHBE cells were transfected with pmaxGFP (Lonza) using 3 different transfection reagents from Mirus; TransIT-X2, TransIT-2020 and TransIT-LI1.

The common transfection protocol for all 3 transfection reagents: BEAS-2B and NHBE cells were cultured in multi-well plate until 85% confluent. Cells were then rinsed with PBS and 1 mL of BEGM media without antibiotics was added. Transfection complexes were formed by mixing pmaxGFP (1 µg) and transfection reagent in 100 µl BEBM media for 30 min at room temperature and added drop wise to the culture dishes. Cells were trypsinized after 24h and assessed for transfection efficiency using flow cytometry.

First, BEAS-2B cells were transfected with the transfection complexes prepared using 1 µg pmaxGFP and 3ul of the transfection reagents.

Table 1. Transfection efficiency of 3 different transfection reagents based on % pmaxGFP positive BEAS-2B cells

Transfection reagent	pmaxGFP positive cells (%)	pmaxGFP positive cells (%)	Average
TransIT-2020	13.3	15.2	14.25
TransIT-LT1	9.88	10.3	10.09
TransIT-X2	**30.4**	**28.2**	**29.30**

Results showed that TransIT-X2 was the most effective at transfecting BEAS-2B cells followed by TransIT-2020 and TransIT-LT1.

Next, BEAS-2B cells were transfected with transfection complexes prepared using 1 µg pmaxGFP and different amount of TransIT-X2 transfection reagent (2ul - 6ul).

Table 2. Transfection efficiency of different volumes of TransIT-X2 based on % pmax GFP positive BEAS-2B cells

Volume of TransIT-X2	pmaxGFP positive cells (%)	pmaxGFP positive cells (%)	Average
2ul	**30.3**	**29.4**	**29.85**
3ul	18.3	16.2	17.25
4ul	11.1	12.1	11.60
5ul	8.11	7.29	7.70
6ul	8.09	5.78	6.94

Results showed that transfection was most efficient with the transfection complexes prepared using 2ul of TransIT-X2.

We then transfected NHBE cells with 1 µg pmaxGFP using 2ul - 4ul TransIT-X2 transfection reagent.

Table 3. Transfection efficiency of different volumes of TransIT-X2 based on % pmax GFP positive NHBE cells

Volume of TransIT-X2	pmaxGFP positive cells (%)	pmaxGFP positive cells (%)	Average
2ul	**39.8**	**41.5**	**40.65**
3ul	20.7	21.6	21.15
4ul	15.2	11.3	13.25

Similar to BEAS-2B cells, TransIT-X2 reagent was most effective at transfecting NHBE cells when used at 2ul transfection reagent to 1 µg pmaxGFP ratio. This transfection protocol was further used for FOXO-1-GFP experiments.

Appendix-3

Sequence of 670 bp promoter region (putative FOXO-1 binding site highlighted)

CTCCTCCTGCAGGGTCCACCGCACCGGGAAGCCCTGTCTGTATCAGTTACCAACCAC
AATTGCAGTGAGTACGAATCGTGGCTTTCCCACAGTCAGGAAAGGCAAGGGAGACC
GACGACCCGCTTCTCTAGGAGTAAGTAAAGATTAAAGGTAGTTCGCGGTATAGCCTA
GGCAGGGATTAACCCGTGGTCCCAGCGCTCCTGC<mark>TATTTG</mark>CATTCCAAAGCAGACAC
CTCATGCGCTCAACCCCGCCCGCAGGCGGCTCCCGCAGTCTAAGGGACCTGGCGCG
AGTCCGGGAAGCGGAGGGCGCAGCTGCGCAGGGAAGGGGGCCGGGGGCGGGACCA
GGGCGCGCGTTCCGGTCCCGGGGCGTGGCCTCCCGCAGGTGAGTACGCTGCTCCTTC
GGTTTCCCTGAAACCTAACCCGCCCTGGGGAGGCGCGCAGCAGAGGCTCCGATTCG
GGGCAGGTGAGAGGCTGACTTTCTCTCGGTGCGTCCAGTGGAGCTCTGAGTTTCGAA
TCGGTGGCGGCGGATTCCCCGCGCGCCCGGCGTCGGGGCTTCCAGGAGGATGCGGA
GCCCCAGCGCGGCGTGGCTGCTGGGGGCCGCCATCCTGCTAGCAGCCTCTCTCTCCT
GCAGTGGCACCATCCAAGGTGAGAAACCTGGCCAAGGAGGGCTCT

FOXO-1 core binding site: T(G/A)TTTG (1-3)

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