Characterization of β-Adrenergic Mechanisms and their Interaction with Corticosteroids in Human Pulmonary Fibroblasts

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To my Parents,

and those who supported me in any respect during the completion of this project.

Contents

Abbreviations

A	
AC	Adenylyl Cyclase
Ach	Acetylcholine
AHR	Airway Hyperresponsiveness
AKAP	A Kinase-Anchoring Protein
ASMC	Airway Smooth Muscle Culture
ATP	Adenosine Triphosphate
A.T.	Annealing Temperature
ATRAP	Angiotensin II Type I Receptor-associated Protein
C	
cAMP	Cyclic Adenosine Monophosphate
Ca2+	Calcium
CBD	cAMP-Binding Domain
C/EBP-α	CCAAT/enhancer binding protein alpha
CDC25HD	CDC25-Homology Domain
cDNA	Complementary Deoxyribonucleic Acid
CNB	Cyclic Nucleotide Binding
COPD	Chronic Obstructive Pulmonary Disease
D	
DEP	Disheveled, Egl-10, and Pleckstrin
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E	
EDTA	Ethylenediaminetetraacetate
Epac	Exchange Proteins Directly Activated by cAMP
ECM	Extracellular Matrix

F	
FCS	Fetal Calf Serum
FEV1	Forced Expiratory Volume/Second
G	
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
gDNA	Genomic DNA
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCRs	G-Protein-Coupled Receptors
GR	Glucocorticoid Receptor
GRKs	G protein-coupled receptor kinases
GTP	Guanosine triphosphate
G protin	Guanine Nucleotide-Binding Proteins
H	
hr	Hour
hrs	Hours
I	
ICSs	Inhaled Corticosteroids
Ig E	Immunoglobulin E
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
L	
LABA	Long-Acting β_2-Agonists
M	
MEM	Minimal Essential Medium Eagle
MAPK	Mitogen-Activated Protein Kinase
mRNA	Messenger Ribonucleic Acid
N	
NEAA	Non-Essential Amino Acids
NF-kB	Nuclear factor-kappa B

O

OD	Optical Density

P

P38MAPK	p38 Mitogen-Activated Protein Kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PhLFb	Primary Human Lung Fibroblasts
PI	Proteases Inhibitors
PKA	Protein Kinase A
PMSF	Protease Inhibitors Phenyl Methansulfonylfluorid
PTX	Pertussis Toxin
PVDF	Polyvinylidene Difluoride

Q

Q-PCR	Quantitative Real Time Polymerase Chain Reaction

R

Rap	Ras-Related Protein
RIPA	Radio Immuno Precipitation Assay
Rpm	Revolutions (or Rotations) Per Minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction

S

SABA	Short-Acting β_2-Adrenoceptor Agonists
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfatepolyacrylamide Gelelectrophoresis
α-SMA	Alpha Smooth Muscle Actin
SPS	Sodium Pyruvate Solution

T

TGF-β	Transforming growth factor-β
Th	T helper
TNF	Tumor Necrosis Factor

1. Introduction

1.1 Obstructive Airways Diseases

Bronchial asthma and chronic obstructive pulmonary disease (COPD) are two lung diseases that are continuously increasing worldwide and represent major world causes of disability and death (Welte and Groneberg 2006).

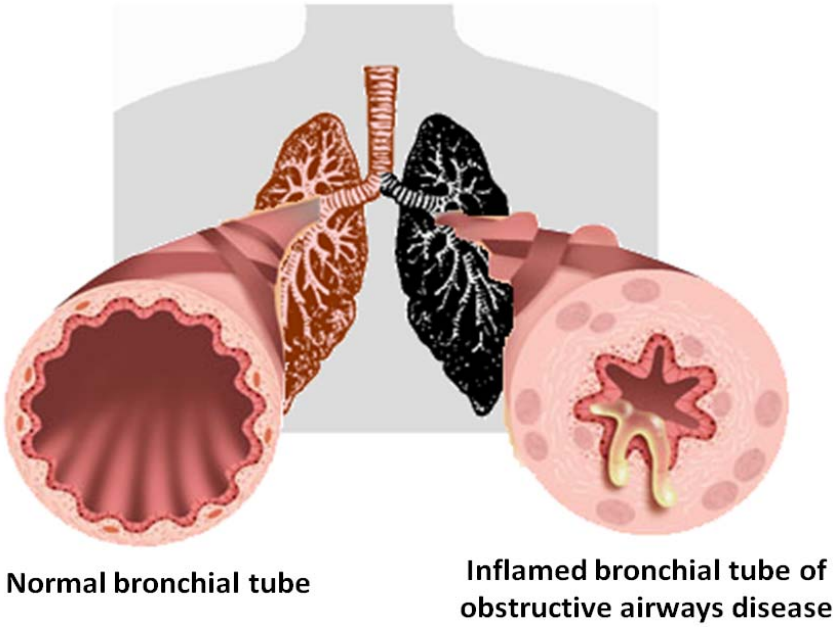


Figure 1-1: Representation of airways in the normal lung (left) and in obstructive airways disease (right) (Jeffery 2001).

Both are chronic inflammatory disorders associated with irreversible structural changes of lung composition "remodelling" (Fig. 1-1) in which several cells and components play a crucial role (Descalzi et al. 2008; Jeffery 2001).

In the following, asthma and COPD, their pathophysiology and airway remodelling will be briefly described.

1.1.1 Bronchial Asthma

Asthma is a common disease that afflicts all ages and can differ greatly in severity. It is an inflammatory disorder condition characterized by reversible airflow obstruction, accompanied with lower airway inflammation and remodelling (Holgate and Davies 2009; Wilson and Bamford 2001). The chronic inflammation is associated with increased airway hyper-responsiveness causing recurrent episodes of wheezing, breathlessness, and chest tightness.

Clinically, asthmatic subjects have difficulty exhaling air because of an increase in airway resistance that is a consequence of smooth muscle contraction, inflammation and remodelling (Fig. 1-2) (Weiss et al. 2006).

The severity of asthma is dependent on a combination of symptoms and use of medications. Currently, asthma cannot be cured but can be controlled.

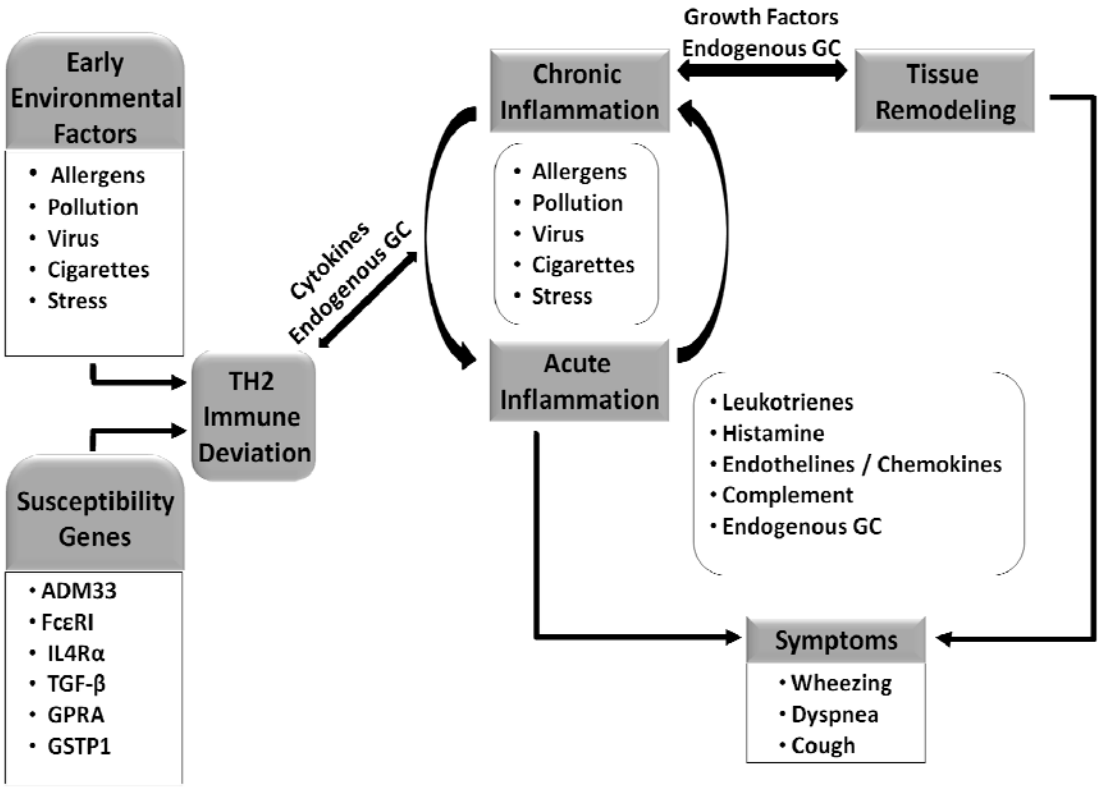


Figure 1-2: Schematic representation of asthma pathobiology (Weiss et al. 2006).

Asthma is caused by environmental and genetic factors (Martinez 2007).
In asthmatic subjects, the airway inflammation is characterized by an increase in eosinophils numbers and $CD4^{+}$T lymphocytes (Busse and Lemanske 2001). Allergen-IgE directed processes are predominant features of airway pathology with mast cells, Th2 lymphocytes, and eosinophils the predominant histological features in acute inflammatory aspects of asthma (Bousquet et al. 2000).

The Th2 cytokine profile involved in these processes often includes IL-3, IL-4, IL-5, IL-9, and IL-13 (Barnes 2008). Transforming growth factor-β (TGF-β) also may play an important role as a fibrogenic and immunomodulatory factor in leading to structural changes in asthmatic airways (Duvernelle et al. 2003).

In asthma, large and small airways are structurally changed but, in the asthmatic non-smoker, there is no parenchymal destruction. However, the large walls of the airways in asthma are thickened by between 50 and 300%, leading to luminal narrowing compromised by excessive mucus (Jeffery 2001).

It has been elucidated in previous reports that lower airway fibroblasts may play a critical role in the remodelling process of airway mucosa, as they are involved in continuous cycles of collagen synthesis, proliferation, activation and cross-talk with inflammatory cells (Descalzi et al. 2008).

1.1.2 Chronic Obstructive Pulmonary Disease (COPD)

According to WHO, chronic obstructive pulmonary disease (COPD) is a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is not fully reversible. It is also defined as a multicomponent disease characterized by progressive airflow limitation and an inflammatory response of the lung (Hanania and Donohue 2007). Principally, COPD is caused by prolonged exposure to tobacco smoke (80 to 90% of cases of COPD are due to smoking), fine airborne particles, air pollution, genetics (severe hereditary deficiency of alpha1-antitrypsin) and autoimmune disease (Halbert et al. 2006; Young et al. 2009).

In contrast to asthma, the airway limitations are poorly reversible and usually get progressively worse over time. COPD is ranked as the sixth leading cause of death in 1990. It is also expected to be the fourth leading cause of death worldwide by 2030 due to an increase in smoking rates and demographic changes in many countries.

The most upsetting symptoms for COPD patients are; cough, mucous hyper-production, dyspnea ('Shortness of Breath'), physical disability and limitation of daily activities (Hanania and Donohue 2007; Hnizdo et al. 2004; Mathers and Loncar 2006). COPD develops slowly over many years and is usually diagnosed when the disease is already advanced.

Histologically, biopsy samples of COPD subjects show clear structural changes. For example, an increase of smooth muscle mass, sub-epithelial fibrosis, and thickening of small airway walls associated with predominant infiltration and accumulation of inflammatory cells in airways and lungs, such as $CD8^+$ T lymphocytes, macrophages, and neutrophils (Hamid et al. 2004; Hogg 2004; Jeffery 2001).

1.1.3 Airway Remodelling

In chronic inflammatory and obstructive airway diseases, such as bronchial asthma and COPD, airway structural changes are a pathological feature that appear to be an essential determinant of the long-term outcome of these diseases (Chiappara, et al. 2001; Hogg 2004; Holgate 2002; Jeffery 2001; Ward et al. 2002; Wilson and Bamford 2001). Fibrotic processes appear to be of significance in airway remodelling in both asthma and COPD (Hogg 2004; Jeffery 2004; Molfino and Jeffery 2007).

1.1.3.1 Remodelling and Pathophysiology in Asthma

The term "airway remodelling" is used to refer to the development of specific structural changes in the asthmatic airway wall that occur as secondary phenomena arising late in the disease process as a result of chronic inflammation of the airway. Airway remodelling and structural changes (Fig. 1-3) mainly occur in epithelium, lamina propria and the submucosa which include abnormal deposition of collagen,

increase in cell number (hyperplasia) and increase of cell size (hypertrophy) of goblet cells, submucosal gland cells, smooth muscle cells and blood vessel cells (Fahy et al. 2000).

In bronchial asthma, thickening of the reticular basement membrane is observed essentially in the large airways (Beasley et al. 1989; Brewster et al. 1990).

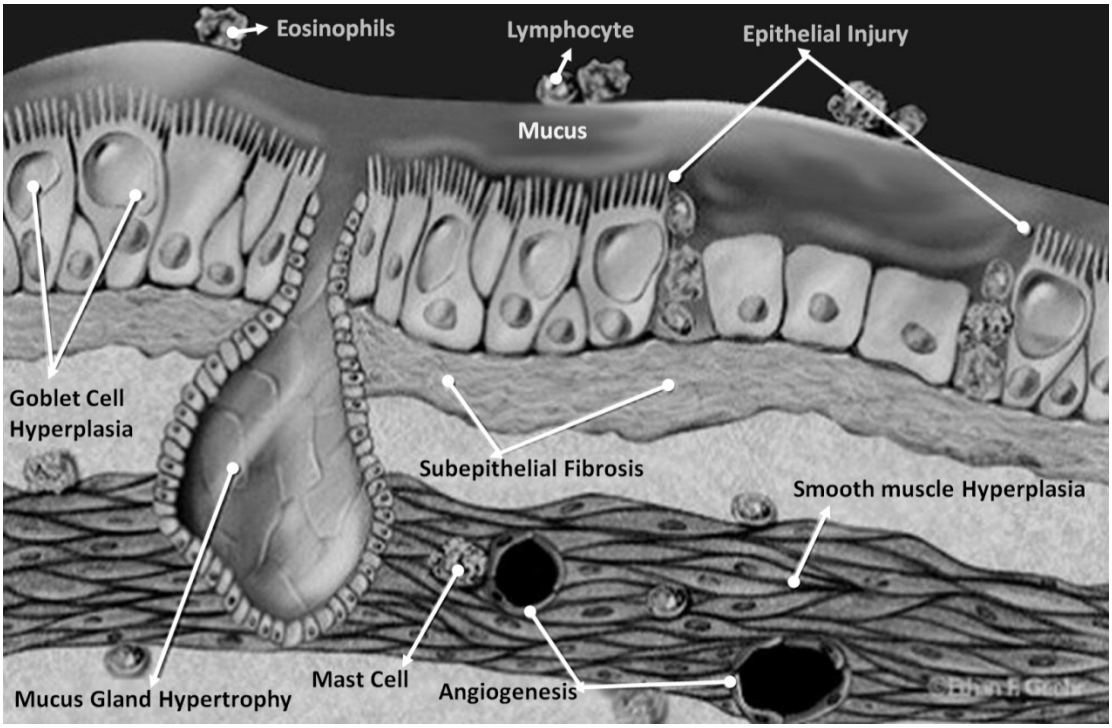


Figure 1-3: Model of the airway remodelling with inflammatory cells, epithelial damage, goblet cell hyperplasia, sub epithelial fibrosis, mucus hyper secretion and smooth muscle cell hypertrophy and hyperplasia (Panettieri et al. 2008).

Most inflammation that occurs during asthma is a result of interaction between epithelial cells and mesenchymal cells, such as fibroblasts and myofibroblasts (Olman 2003).

A variety of mediators, cytokines, chemokines and growth factors are released from inflammatory cells such as eosinophils, activated T cells, mast cells and macrophages (Chung and Barnes 1999). These cytokines and chemokines can results in an acute inflammatory response, which is characterized by vascular leakage and mucus hyper-secretion. At the same time, by release of mediators, cytokines, chemokines and

growth factors, these cells cause chronic inflammatory infiltrate and induce structural changes in the airway wall (Vignola et al. 2001).

Several inflammatory mediators increase airway smooth muscle proliferation, which strongly induces the deposition of extracellular matrix (ECM) proteins and growth factors in fibroblasts and epithelial cells (Meerschaert et al. 1999). The epithelial cells are normally injured in asthmatic airways leading to activation of epithelial-mesenchymal trophic unit inducing their proliferation and differentiation. Fibroblasts and myofibroblasts are the major mesenchymal cells representing the main target of the cytokines and growth factors produced by epithelial cells during the damage-repair process, which results in production of collagen, elastic fibers, and proteoglycans by fibroblasts. Whereas myofibroblasts are implicated in airway remodelling by production of extracellular matrix (ECM) components such as elastin, fibronectin and laminin (Leslie et al. 1992; Vignola et al. 2001; Yamauchi and Inoue 2007).

Furthermore, a variety of cells such as macrophages, lymphocytes, fibroblasts, airway epithelial cells, eosinophils, mast cells and T cells release TGF-β, a cytokine that increases the synthesis of extracellular matrix (ECM) (Hoshino et al. 1998; Ohno et al. 1996; Vignola et al. 1996; Vignola et al. 1997). It is known that only the type2 pattern of Th2 cells, such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, are associated with asthma, while the type1 cytokines, such as interferon-γ, tumor necrosis factor-α (TNF-α) and lymphotoxin oppose type2 cytokines, leading to attenuation of allergic inflammation. Many pathological features in the airway occur during injury-repair cycles, including epithelial changes, eosinophilic inflammation, subepithelial changes and mucus abnormality (Fahy et al. 2000).

1.1.3.2 Remodelling and Pathophysiology in COPD

COPD is characterized by physiological abnormalities including airflow and gas exchange limitations, and many clinical symptoms such as increased sputum production, expectoration cough, wheezing and dyspnea. COPD include two main phenotypes: chronic bronchitis and emphysema.

Bronchitis is characterized by inflammation of the "bronchial tree", the airways that carry airflow from the trachea into the lungs. This results in tissue swelling and excessive secretions of mucus into the bronchi, with progressive airflow limitation.

1. Introduction

Chronic bronchitis is caused by recurring injury or irritation to the respiratory epithelium of the bronchi, resulting in chronic inflammation, edema (swelling), and increased production of mucus by goblet cells. The inflammatory response associated with chronic bronchitis is usually located in the epithelium of the central airways, which, larger than 4 mm in internal diameter, extends along the gland ducts into the mucus-producing glands (Mullen et al. 1985; Saetta et al. 1997).
Chronic bronchitis is characterized by two main characteristics, excessive production of sputum and chronic inflammatory cell infiltration of the bronchial wall (Yoshida and Tuder 2007). Clinically, chronic bronchitis is characterized by wheezing and a persistent expectoration cough which is usually associated with airflow limitation.

From a pathological point of view, emphysema has been defined as an irreversible destruction of alveolar structures with airspace enlargement, not associated with significant amount of fibrosis. In other words, emphysema is characterized by destruction of the tissues, loss of lung parenchyma, and over-inflation of the alveoli. Much attention is focused on the pathophysiological processes interacting in the periphery of the lung in COPD including oxidative stress, alveolar cell apoptosis, and extracellular matrix proteolysis (Yoshida and Tuder 2007).
The bronchial mucosa of large and small airways in patients with chronic obstructive pulmonary disease is characterized by squamous-cell metaplasia, goblet-cell hyperplasia, mucus gland enlargement, smooth-muscle hypertrophy and infiltration of inflammatory cells (Bergeron and Boulet 2006). A small airway (< 2 mm) is believed to be the main site of airways obstruction occurring during COPD. Processes contributing to small airways obstruction involve; destruction of the epithelial barrier, interference with mucociliary clearance leading to accumulation of inflammatory mucous, and deposition of connective tissue in the airway wall resulting in lumen diameter reduction (Hogg 2004; Skold 2010). Recent insights into the pathophysiological processes that contribute to development of emphysema provide at least two main mechanisms including protease-antiprotease imbalance and apoptosis of structural cells (Skold 2010). Interestingly, the fibrosis and stenosis of airway wall was observed in small airways, along with loss of the activity of antiprotease enzymes such as alpha 1-antitrypsin, allowing protease enzymes to damage the lung and development of emphysema (Saetta et al. 1985). In other words, in emphysema, collagen

and elastin per weight unit respectively is increased compared with patients without emphysema. This suggests that along with destruction of the lung tissue, fibrotic mechanisms may occur in parallel (Skold 2010).

The COPD inflammatory pattern is characterized by an increase in neutrophils, macrophages, CD8+, T-cells, eosinophils and inflammatory mediators such as IL-8, TNF-α, leukotriene B4 and TGF-β (Crooks et al. 2000; Keatings et al. 1996; Lane et al. 2010; Saetta et al. 1998; Turato et al. 2001; Yamamoto et al. 1997). Figure (1-4).

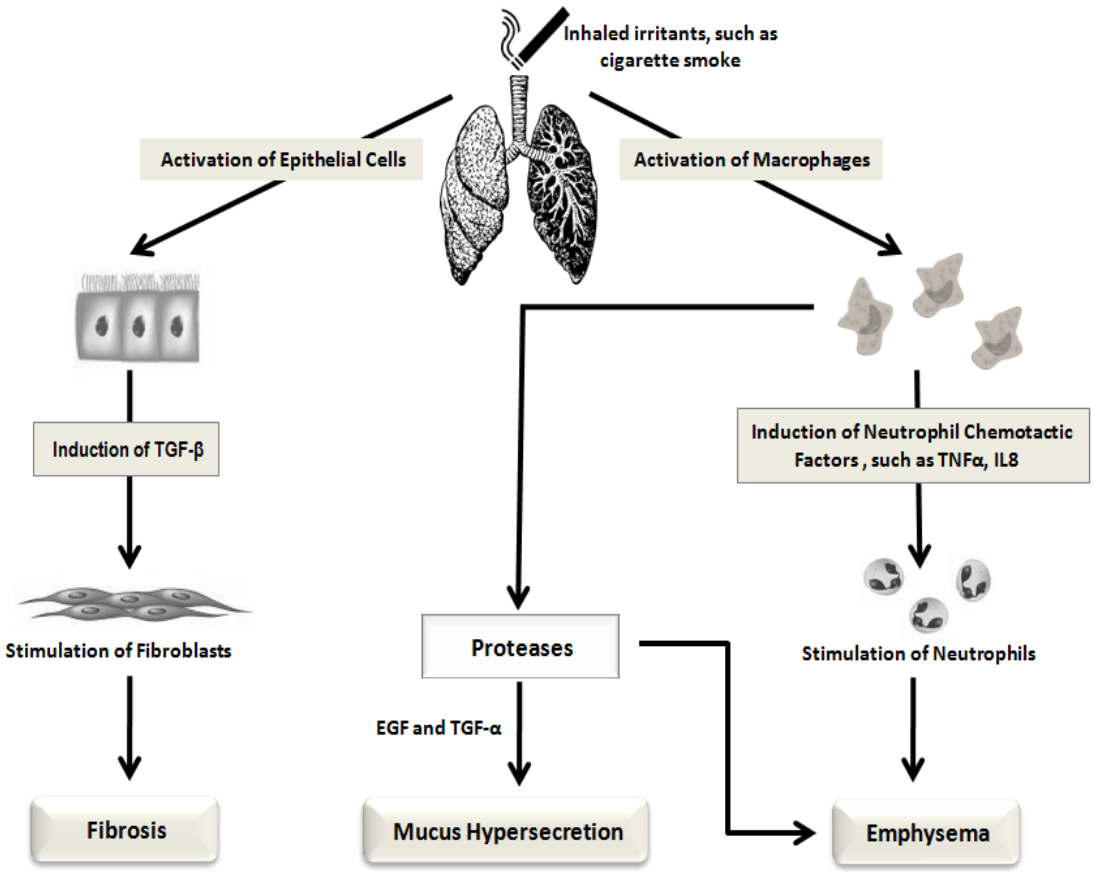


Figure 1-4: Inflammatory cells involved and stages of pathogenesis of COPD (Lane et al. 2010).

1.1.3.3 Role of Mesenchymal Cells in Airways Remodelling

The main structural components of mesenchyme are fibroblasts and myofibroblasts. After stimulation these cells have the ability to produce immune-modulatory cytokines and chemokines. These products can, in turn activate or attract inflammatory cells in the lungs, and to mediate the majority of the events contributing to the subepithelial fibrosis and remodelling in airways. It is also known that airways remodelling is the main result of altered fibroblasts behavior (Baouz et al. 2005).

A number of previous studies have shown that there is an increase in fibroblasts and myofibroblasts in biopsy samples obtained from pulmonary obstructed disease subjects (Benayoun et al. 2003; Brewster et al. 1990; Descalzi et al. 2007; Roche et al. 1989). Fibroblasts and myofibroblasts play a pivotal role in the exaggerated deposition of collagen and structural changes in airways. Bone marrow-derived fibroblasts may also play a role in fibrotic remodelling in airways (Kotton et al. 2001). It has been demonstrated that bone marrow-derived fibroblasts play a critical role in pulmonary fibrosis in rats treated with bleomycin (Hashimoto et al. 2004).

The circulating fibrocytes can migrate to injury-repair site to serve as a source for fibroblasts and myofibroblasts which usually participate in repair process (Abe et al. 2001). Allergen exposure induces the accumulation of fibrocyte-like cells in the bronchial mucosa, where $CD34^+$, expressed collagen I and α-smooth muscle actin (α-SMA) are present (Schmidt et al. 2003).

In simple words, normal homeostasis in human tissues requires complicated balanced interactions between cells and the network of secreted proteins known as the extracellular matrix. In these cooperative interactions numerous cytokines are involved and act via specific receptors located on the cell surface. Any disturbance or alterations in the balance between the cells and the extracellular matrix can result in human disease. One of these interactions is mediated by TGF-β (Blobe et al. 2000).

TGF-β is a member of the family of secreted polypeptide and has various functions in significant cell cycle processes including; biological activity, regulating cell function, cellular proliferation, differentiation, development and other cell functions. TGF-β has been implicated as a master switch in many chronic fibrotic conditions such as pulmonary and hepatic fibrosis. (Hassoba et al. 2005; Willis and Borok 2007).

There are three isoforms of TGF-β (β_1, β_2, and β_3) which are important for normal embryonic and fetal development and play a role in normal organ homeostasis and function (Bartram and Speer 2004). All isoforms are expressed at high levels during normal lung development. In addition, TGF-β has a role in modulating inflammation, wound repair, and immune homeostasis and tolerance (Taylor 2009). It plays a crucial role in normal pulmonary morphogenesis activity and also in lung pathogenesis.

Originally, small amounts of TGF-β are present in adult lung and are involved in tissue healing after lung injury. It was demonstrated that, in various forms of pulmonary diseases, the expression levels of TGF-β are increased. In other words, damage of lung tissue by chemicals, bacteriologic, or immunologic noxious effects leads to an induction of TGF-β that limits some of the inflammatory reactions and plays an important role in mediating tissue remodelling and repair. In addition, TGF-β induces expression of α-SMA, which is a well-accepted marker of myofibroblast differentiation, and has been suggested to play a role in the production of contractile force during wound healing and fibrocontractive diseases (Baouz et al. 2005; Bartram and Speer 2004; Hinz et al. 2001).

The communication between the epithelium and the underlying mesenchyme (the epithelial mesenchymal trophic unit) plays an important role in the structure alterations (Fig. 1-5). TGF-β is released from damaged epithelial cells, fibroblasts, smooth muscle cells and inflammatory cells, and can mediate its effects by interacting with TGF-β receptors on fibroblasts. This interaction can promote the transformation of fibroblasts into myofibroblasts (Boxall et al. 2006). The biological action of TGF-β is regulated by a selective pathway of TGF-β synthesis and signaling that involves activation of latent TGF-β, specific TGF-β receptors, and intracellular signaling by Smad molecules (Bartram and Speer 2004).

Transformed myofibroblasts are more biosynthetic in nature than fibroblasts and can synthesise and release an array of growth factors and cytokines, leading to remodelling of airways and persistence of the chronic inflammatory events. The remodelling process is increased by release of various factors such as: endothelin-1, a mitogen for airway smooth muscle cells (Glassberg et al. 1994) and fibroblasts (Takuwa et al. 1989) and also stimulates collagen synthesis; eotaxin, a chemoattra-

ctant for eosinophils; and vascular endothelial growth factor, an angiogenic factor that promotes the growth of new blood vessels (Richter et al. 2001; Vignola et al. 2002).

Moreover, human lung fibroblasts are thought to be directly activated by Th2 cytokines such as IL-4 and IL-13, which later induce signal pathways resulting in production of several pro-inflammatory molecules (Doucet et al. 1998; Richter et al. 2001).

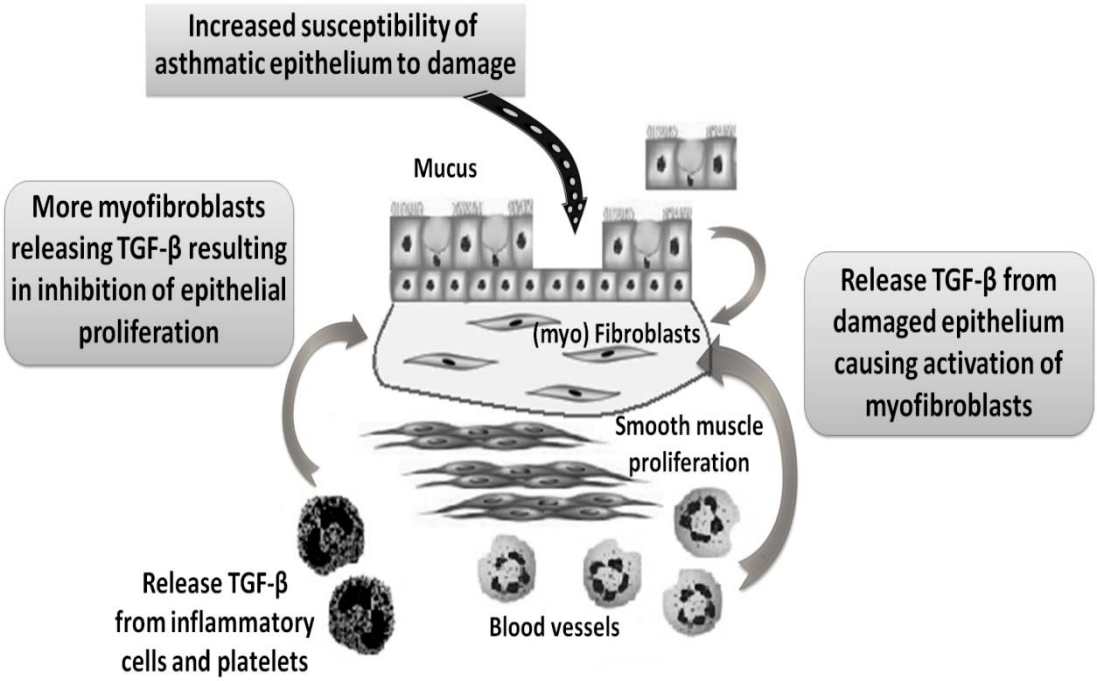


Figure 1-5: Expression and release of TGF-β in asthmatic airways, from the epithelium, fibroblasts, smooth muscle cells and inflammatory cells, and its role in the fibrosis associated with airway remodelling (Boxall et al. 2006).

Airway remodelling has been documented to be an irreversible process, although there is evidence suggesting that early control and treatment of airway obstructive disease can delay or even reverse the structural changes (Selroos et al. 1995).

1.1.4 Overlapping, Similarities and Differentiation of Asthma and COPD

As summarized in figure 1-6 recent studies have focused on the overlap between asthma and COPD. From a clinical point of view, both diseases are characterized by airflow obstruction and chronic persistent airway inflammation. In addition, a number of clinical studies have shown that many patients with asthma have characteristics of COPD, and approximately 10% of patients with COPD also have asthma and therefore share pathologic features, namely "wheezy bronchitis". In other word, COPD and asthma are two different forms of chronic pulmonary diseases with considerable overlap in presentation and management.

Both disorders have different disease mechanisms, however there are structural and inflammatory similarities. Most overlap is between severe asthmatics and COPD individuals, where Th1 and neutrophilia are common features. The management with pharmacologic and non-pharmacologic strategies for the treatment of these diseases is similar, focusing on treating bronchoconstriction and airway inflammation (Barnes 2000; Guerra 2005).

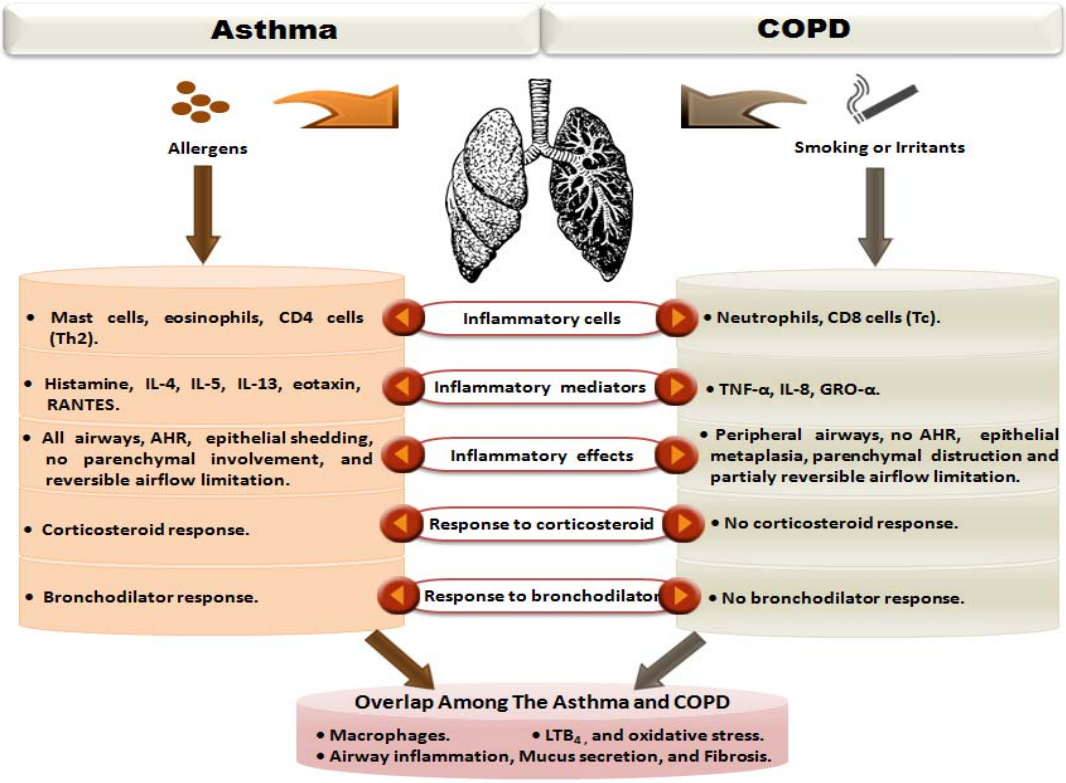


Figure 1-6: Overlapping, similarities and differentiation between COPD and asthma. (Modified from Barnes 2000).

Airway inflammation in asthma, characterized by an eosinophilic inflammation affecting all the airways but not lung parenchyma, is linked to airway hyper-responsiveness (Barnes 2000). In COPD, there is a predominantly neutrophilic inflammation in the airways. Parenchymal destruction is an important irreversible feature and leads to airflow obstruction through dynamic compression.
The eosinophilic inflammation in asthma is markedly suppressed by corticosteroids, but they have no appreciable effect on the inflammation in COPD, consistent with failure of long-term corticosteroids to alter the progression of COPD (Barnes 2000).

Briefly, airway inflammation in asthma is characterized by mast cells, eosinophils and $CD4^+$ T lymphocytes; whereas in COPD, macrophages, neutrophils and $CD8^+$ T lymphocytes are predominantly involved. Regarding the structural changes, epithelial shedding and reticular basement membrane thickening occur only in asthma, increased smooth muscle occurs in large airways in asthma and in small airways in COPD. Additionally, emphysema is a feature only of COPD.

1.2 Obstructive Airways Diseases Therapy

The purpose of therapy in asthma is to be symptom-free with as near to normal lung function as possible; while the purpose of COPD therapy is to stop the ultimate progression of damage to the lung, reduce exacerbations and help improve quality of life. Generally, treatment options depend on general medical condition and severity of disease. Bronchodilators are widely used in the treatment of patients with these conditions. The main bronchodilators are β_2-adrenoceptors agonists, muscarinic antagonists, theophylline and their combinations (Gupta and O'Mahony 2008). In addition, inhaled corticosteroids (ICSs) are the most effective anti-inflammatory agents and controllers currently available for the long-term treatment of asthma. By contrast, ICSs have established to be less effective in patients with severe asthma, smoking asthmatics and in patients with COPD; but at high-doses they have been shown to decrease the frequency and severity of exacerbations of the disease (Adcock et al. 2010; Barnes 2010; Paggiaro et al. 1998; Vestbo, et al. 1999).

As known, the lungs are innervated by both the sympathetic and parasympathetic nervous systems, which lead to activation of β-adrenoceptors and muscarinic receptors respectively. Both β-adrenoceptors and muscarinic receptors are widely expressed and distributed in the lungs and the specific receptor profile expression can vary significantly between the species. The location and the subtype of receptor expressed are important in the regulation of normal lung function. Furthermore, lung function and airway tone relies on the communication between parasympathetic and sympathetic nerves which are mediated by negative cross-talk between muscarinic receptors, especially M_2 and β_2-adrenergic receptors. (Proskocil and Fryer 2005).
A combination of inhaled β_2-adrenoceptors agonists and inhaled corticosteroids represent the cornerstone in treatment of obstructive airways diseases. In combination with corticosteroids, β_2-adrenoceptors agonists can reduce exacerbation and disease progression and improve the airway limitations (Cazzola et al. 2003; Hancox and Taylor 2001; Pauwels et al. 1997). In general, there is no curative treatment for asthma and COPD, and no medications have been found to cure the disease or reverse the loss of lung function. The treatment of asthma is characterized by suppression and prevention of inflammations, while treatment of COPD is characterized by relief of symptoms (Donohue 2004).

In other words, the purposes of asthma treatment are to maintain pulmonary function as close as possible to normal, prevent the development of irreversible airflow limitations, and prevent exacerbation. While the purposes of COPD treatment are relief of symptoms, prevention and treatment of exacerbations, and prevention of the progression of lung tissue damage, which helps in slowing the accelerated decline in lung function (Buist 2003; Scanlon et al. 2000; Spina and Page 2002).

Most medications used in the treatment of asthma such as bronchodilators and corticosteroids are used in the treatment of COPD. Whereas some medications, like PDE4 inhibitors, may be effective in the treatment of asthma and are especially useful in COPD (Donohue 2004), some others are used specifically in the treatment of bronchial asthma such as, leukotriene antagonists, immunomodulator, cromolyn and nedocromil.

1.2.1 Muscarinic Receptors Antagonists

There are five subtypes of muscarinic receptors (M_1-M_5) which have been identified by pharmacological experiments and molecular cloning technique. Each is the product of a distinct gene and member of the superfamily of G-protein coupled receptors that have seven transmembrane domains and are coupled to a guanosine tri-phosphate (GTP) binding protein (Caulfield and Birdsall 1998). Of these five sub-types, human lung airways contain a mixed population of M_1, M_2 and M_3 muscarinic receptors. The M_2 muscarinic receptors preferentially couples to the G-protein belonging to ($G_{i/o}$) family and functions to counteract the β-adrenoceptors mediated relaxant pathway by inhibiting the production and accumulation of cyclic adenosine mono-phosphate (cAMP) (Jones et al. 1987; Kume and Kotlikoff 1991). The M_3 receptor couples preferentially to stimulatory G-protein (G_q) resulting in stimulation of phospholipase C and an increase in intracellular calcium which mediates contraction of airway smooth muscle (Caulfield and Birdsall 1998).

As described in previous studies, two types of anticholinergic agents are presently used in clinical therapy: short-acting compounds such as ipratropium bromide and long-acting drugs such as tiotropium bromide (Matthiesen et al. 2006). At the present, numerous new long-acting anticholinergic compounds are under development, such as NVA237 (glycopyrronium bromide) and LAS 34273 (aclidinium bromide) (Barnes 2011; Matthiesen et al. 2006; Zhang et al. 2012).

1.2.2 β-Adrenergic Receptors Agonists

The human β-adrenoceptors is a member of the metabotropic G-protein-coupled receptors (GPCRs) that act as a target of the catecholamines, especially adrenaline and noradrenaline. GPCRs represent the largest family of membrane proteins in the human genome and are transmembrane proteins that include seven helical transmembrane segments (TM helices, TMs).

Structurally, GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane (7-TM) α-helices connected by three intracellular and three extracellular loops and finally an intracellular C-terminus. GPCRs are known and po-

pular as drug targets since it has been estimated that more than 400 GPCRs are of potential therapeutic interest and more than 800 GPCRs sequences in the human genome have been identified (Fredriksson et al. 2003). GPCRs indicates that these receptors communicate with G-proteins, but the main common characteristic of GPCRs are seven stretches of about 20-35 sequential amino acid residues, which show a high degree of hydrophobicity and represent α-helixes that span the plasma membrane (Lagerstrom et al. 2006).

The natural ligands which react with GPCRs are particularly different such as ions, amines, peptides, proteins, lipids, and nucleotides. In addition to signaling transduction mediated via heterotrimeric G proteins, it is now known that GPCRs act via many other alternative G protein-independent signaling pathways. An increasing number of proteins have been identified that bind GPCRs and either couple GPCRs to G protein-independent signal transduction pathways or alter G protein specificity and agonist selectivity, such as G protein-coupled receptor kinases (GRKs), arrestins, calmodulin, calcyon, A kinase-anchoring protein (AKAP) and Angiotensin II Type I Receptor-associated Protein (ATRAP) (Ferguson 2001).

β-Adrenoceptors is coded by an intronless gene product of approximately 1200 base pairs situated on the long arm of chromosome 5 (Johnson 2006) and has been subdivided into three subtypes: β_1, β_2 and β_3. β_1-Adrenoceptors are located mainly in the heart and in the kidneys. β_2-Adrenoceptors are located mainly in the lungs, gastrointestinal tract, liver, uterus, vascular smooth muscle, and skeletal muscle. Whereas β_3-Adrenoceptors located in adipose tissue (Frielle et al. 1988). All three β-adrenoceptors subtypes couple to the stimulatory GTP-binding protein (Gs) to activate adenylyl cyclase which increases the intracellular 3',5' cyclic adenosine-monophosphate (cAMP) level resulting in an increase of protein kinase A (PKA) activity, which phosphorylates downstream protein modulators (Proskocil and Fryer, 2005; Tang et al. 1999).

This thesis focuses on β_2-adrenoceptors subtype and their role in the treatment of airway obstructive diseases and modulation of long term remodelling processes.

As described, β_2-adrenoceptors agonists are considered as first-line medications in the management of asthma and COPD. These medications are used both as disease symptoms relievers, and in combination with inhaled corticosteroids as disease controllers. However, β_2-adrenoceptors agonists are classified depending on their duration of action, selectivity, potency and efficacy.

In addition to their bronchodilator effect, it was reported that the long acting β_2-adrenoceptor agonist salmeterol also inhibited inflammatory cells. This effect appears to be a β_2-adrenoceptors-independent mechanism, and mostly appears to be as a result from the stabilization of cell membranes of inflammatory cells, such as mast cells and their inhibition of mediators' release from eosinophils, macrophages, T lymphocytes, and neutrophils (Anderson et al. 1996; Hanania and Moore 2004).

Furthermore, β_2-adrenoceptors agonists were elucidated to exert anti-inflammatory and inhibitory effects on airway smooth muscle cells and human lung fibroblasts (Korn, et al. 2001; Loven et al. 2007; Pang and Knox 2000; Spoelstra et al. 2002). It is an emerging question whether the action of these drugs is confined to bronchodilation or whether they can exert additional action on inflammatory and/or structural cells, which may either contribute to the therapeutic benefit or result in undesired effects. However, although β_2-adrenoceptors agonists showed anti-inflammatory properties in vitro, depending on the changes in the number of activated inflammatory cells, they have failed to demonstrate any anti-inflammatory effects during clinical trials (Hanania and Moore 2004).

It was reported in previous studies that chronic use of β_2-adrenoceptors agonists alone for treatment of obstructive airways diseases treatment could induce receptor tolerance and desensitization that limits the efficacy of drugs, leading to disease exacerbation and poor disease control (Cheung et al. 1992; Cooper and Panettieri 2008; Haney and Hancox 2005).

As known already, the desensitization phenomenon of receptors is characterized by a down-regulation of β_2-adrenoceptors in the inflammatory cells with the progression of the disease in patients with COPD and the destruction of β-adrenoceptors-GS-adenylyl cyclase system, or a tolerance to stimulant effects. The mechanisms which are applied in β_2-adrenoceptors desensitization consist of firstly, uncoupling of the

receptors from the adenylyl cyclase which occurs after phosphorylation of the receptor by cAMP-independent β-adrenoceptors kinase; and secondly, internalization or sequestration of the uncoupled receptors from the cell surface, which is observed after more prolonged exposure. Finally, after some hours of exposure a breakdown of receptors can occur and they can be replaced by synthesis of new receptors (Zhang et al. 2012).

Inhaled short-acting β_2-adrenoceptors agonists (SABA) are the most effective therapy for rapid reversal of airflow obstruction and immediate relief of symptoms. They are used for symptomatic treatment of bronchospasm, providing quick relief of acute bronchoconstriction in patients with mild asthma. Salbutamol and terbutaline are the most commonly used and their action occurs maximally in 5 min and lasts 4 to 6 hrs (Nelson 1995; Weiss et al. 2006).

Inhaled selective long-acting β_2-adrenoceptors agonists (LABA) including salmeterol and formoterol are approved for use in both asthma and COPD. They given by inhalation, and the extensive clinical trials data indicated that they both provide significant bronchodilation effects lasting for at least 12 hrs (Anderson et al. 1994). Both of these drugs have become important therapy in the regular management of asthmatic patients, as well as regular use of these compounds in COPD patients can improve health status than inhaled short-acting β_2-adrenoceptors agonists. They can be used in combination with compounds from other bronchodilator classes and ICSs for better symptom control, (Donohue 2004). They improve lung function, reduce symptoms, and protect against exercise-induced dyspnea in patients with COPD (Johnson and Rennard 2001).

However, in looking at the ability of these medications to effect prompt reversal of airflow limitations, some reports have demonstrated that daily regular use of inhaled β_2-adrenoceptors agonists has resulted in loss of asthma control (Sears et al. 1990; Taylor et al. 2000). Most studies commonly explained this adverse effect of chronic use of β_2-adrenoceptors agonists to desensitization of β_2-adrenoceptors (Hanania et al. 2002; Hancox et al. 1998). The principal action of β_2-adrenoceptors agonists is to relax airway smooth muscle by stimulating β_2-adrenergic receptors, see (1.2.3) (Johnson and Rennard 2001; Tashkin and Fabbri 2010).

1.2.3 β_2-Adrenoceptors Signaling Pathways

According to classical signaling, agonism of β_2-adrenoceptors and activation of the signal transduction pathway is mediated by elevation of intracellular cAMP levels after stimulation of adenylate cyclase, which catalyzes the conversion of adenosine triphosphate (ATP) into cAMP.
cAMP levels are then regulated through the activity of phosphodiesterase isozymes /isoforms, which degrade it to 5'-AMP.
The airway smooth muscle cell relaxation induced by cAMP is not fully understood, but it is accepted that cAMP activates the protein kinase A (PKA) resulting in inhibition of phosphoinositol hydrolysis, decreasing intracellular Ca^{2+} levels; or via cAMP-independent mechanisms involving direct activation of large conductance potassium channels which are present in the cell membrane, resulting in hyperpolarization and thereby airway smooth muscle relaxation and bronchodilation response (Johnson 2006; Jones et al. 1990).

Although most of the β_2-adrenergic receptor actions are usually mediated via Gs proteins and the cAMP-dependent PKA system, the receptor can also couple to Gi proteins leading to stimulation of the extracellular signal-regulated kinase and p38-mitogen-activated protein kinase (p38MAPK) pathways (Daaka et al. 1997).
β_2-Adrenergic receptor agonists may also reduce cholinergic neurotransmission due to stimulation of β_2-adrenergic receptors on parasympathetic ganglia (Johnson and Rennard 2001; Tashkin and Fabbri 2010). It was also reported that activation of β-adrenergic receptors localized in the mucosa mediates inhibition of [^{3}H]-acetylcholine release from the isolated rat and guinea-pig trachea probably via liberation of inhibitory prostaglandins from the airway mucosa (Wessler et al. 1994).

1.2.3.1 cAMP

The discovery of cAMP in 1958 by Earl Wilbur Sutherland marked the birth of second messenger theory and the age of signal transduction.
cAMP is utilized by a large number of hormones and neurotransmitters as an intracellular second messenger.

It plays a pivotal role in the various processes of cellular regulation, such as cell growth and differentiation, ion channel conductivity, release of neurotransmitters, metabolism, and gene transcription (Skalhegg and Tasken 2000).

Upon binding of ligand, the G-protein couple receptors on the cell surface transduce the extracellular signal across the cell membrane and mediate stimulatory or inhibitory G-protein that then interacts with adenylyl cyclase (AC). Elevation of cAMP via adenylyl cyclase regulates different cell processes that are mediated by two downstream signaling pathways effectors: the classic cAMP-dependent protein kinase A (PKA) and the recently discovered alternative cAMP effector, exchange protein directly activated by cAMP (Epac).

Depending upon their relative abundance, distributions and localization, as well as the specific cellular environments, PKA and Epac may act independently, converge synergistically, or oppose each other in regulating a specific cellular function (Cheng et al. 2008).

1.2.3.2 Protein Kinase A (PKA)

PKA refers to a family of enzymes whose activity is dependent on the level of cAMP in the cell. PKA was one of the first protein kinases to be discovered and purified from rabbit skeletal muscle (Walsh et al. 1968).

As described in Figure 1-7 (Skalhegg and Tasken 1997), PKA composed of two separate subunits, the catalytic (C) subunit that is serine/threonine protein kinase and regulatory (R) subunit. In the absence of cAMP the catalytic subunit combines with the regulatory subunit to form an inactive form of PKA (Holoenzyme) (Uhler et al. 1986). Upon phosphorylation of the catalytic subunit of PKA at threonine 197 (T197) by phosphoinositid dependent protein kinase it becomes fully active (Cauthron et al. 1998; Cheng et al. 1998).
As the concentration of cAMP increases it binds to the regulatory subunits leading to conformational changes that induce the Holoenzyme dissociation into its constituent (C) and (R) subunits (Taylor et al. 1990). When the active (C) subunits become free

they can phosphorylate their targets of different cellular processes of cytoplasmic and nuclear protein substrates including enzymes and transcriptional factors (Zetterqvist et al. 1990).

PKA was classified into two general classes or two isoforms; PKA type (I) and type (II), depending on differences in (R) subunits, RI and RII that interact with an identical (C) subunit (Taylor et al. 1990). At present four different (R) subunit genes (RIα, RIβ, RIIα and RIIβ) and three different (C) subunits (Cα, Cβ, and Cγ) have been identified in humans (Scott 1991). However, preferential expression of any of the catalytic subunits with either (RI or RII) has not been found (Beebe et al. 1990).

Both (RI and RII) contain two in-tandem and highly conserved cAMP binding domains (CBD) at carboxy terminus (Weber et al. 1987). Both (RI and RII) are signifycantly different at their amino terminus, especially at a proteolytically sensitive hinge region that binds to the peptide recognition site of the (C) subunits. The hinge region of (RII) subunits contains a serine at the (P) site that can be auto phosphorylated by the (C) subunits (Rosen and Erlichman 1975). But (RI) contains a pseudophosphorylation site.

Furthermore, existence of a family of A-kinase anchoring proteins (AKAPs) which is structurally diverse but functionally related proteins that share the capacity to bind protein kinase A (PKA) (Sarkar et al. 1984; Theurkauf and Vallee 1982). AKAPs contain a targeting sequence that serves to tether the (RII) subunits to specific subcellular compartment (Colledge and Scott 1999; Scott and McCartney 1994). The majority of AKAPs preferentially bind (RII) subunits, however AKAPs specific for both (RI and RII) have also been identified (Huang et al. 1997).

It was demonstrated that AKAPs interact with the (R) subunits dimerization domain and only the first 50 N-terminal amino acid residues of the (R) subunits are required for binding of AKAPs (Newlon et al. 1999). It is worth noting that large numbers of AKAPs have been identified.

It was found that the ratio of the total (R) subunits/(C) subunits in normal tissue remains constant at around 1:1 while the relative amount of (RI) and (RII) varies and depends highly on physiological conditions and hormonal status of the tissue (Doskeland et al. 1993; Hofmann et al. 1977; Lohmann and Walter 1984).

The switching of PKA isoforms from (PKA IIβ) to (PKA Iα) demonstrates clearly that the (RIα and RIIβ) are functionally different. Although, many of the physiological

actions of cAMP are mediated by one or more of the PKA isoforms, some of the cAMP-dependent effects cannot be explained based on the functions of PKA. For example the induction of insulin secretion from pancreatic beta cells mediated via cAMP is not affected after using specific PKA inhibitors (Renstrom et al. 1997).

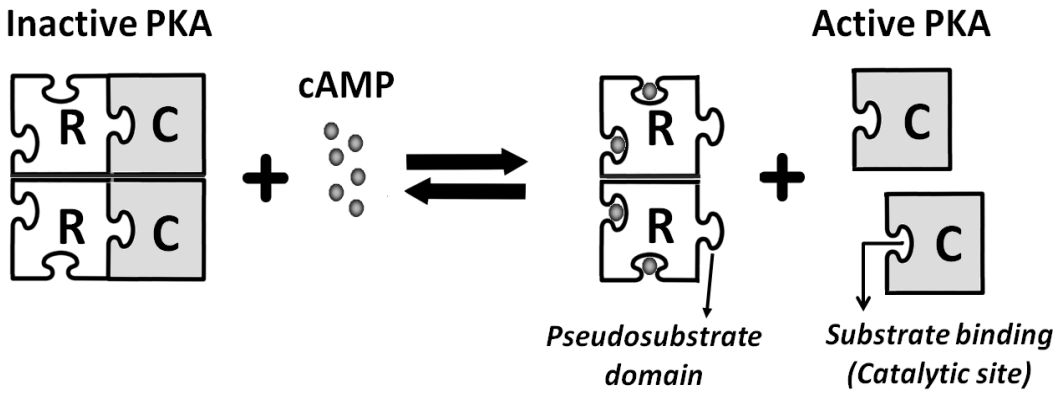


Figure 1-7: Regulation (activation and inactivation) mechanisms of PKA: (C): catalytic subunits, (R): a regulatory subunit dimer. (Modified from Skalhegg and Tasken 1997).

1.2.3.3 Epac

Epac are exchange proteins directly activated by cAMP, named also cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs) that mediate PKA-independent signal transduction properties of the second messenger adenosine-3',5'-cAMP. $Epac_s$ are also known as Rap1 guanine-nucleotide-exchange factors directly activated by cAMP and represent a family of novel cAMP-binding effector proteins. Rap1 and Rap2 proteins are a small Ras-like GTPase activated by specific extracellular stimuli to regulate several cellular processes such as cell proliferation, cell differentiation, signaling and survival. Rap1 was previously described as a protein that could suppress the oncogenic transformation by Ras (de Rooij et al. 2000; de Rooij et al. 1998; Fu et al. 2007; Kawasaki et al. 1998; Kraemer et al. 2001).

In the past, the description of $Epac_s$ and the recent development of pharmacological compounds that segregate between cAMP-mediated pathways have been explained as unrecognized roles for cAMP that are independent of it is classical target cAMP-dependent protein kinase A (Purves et al. 2009).

$Epac_s$ contain a cAMP binding domain (CBD) that is similar to that of PKA (R) sub-units. Epac proteins bind to cAMP with high affinity resulting in stimulation of the exchange of GTP for GDP on small GTPases of the Rap family (Rap1 and Rap2). This nucleotide exchange activates the GTPases and allows Rap proteins to interact with and stimulate effector signaling molecules, as shown in figure 1-8, B (Holz et al. 2008).

As described in figure 1-8, A and B (Bos 2006), Epac proteins are expressed in mammals' genes in two different variants named as Epac1, Epac2 officially known as (RAPGEF3, Rap guanine nucleotide exchange factor 3) and (RAPGEF4, Rap guanine nucleotide exchange factor 4) alternatively, both of which are activated in living cells by physiological relative concentrations of cAMP, as previously described.

It was reported that Epac proteins are differentially distributed in the body tissue, where Epac1 mRNA is ubiquitously expressed, Epac2 mRNA is predominantly expressed in specific tissue (Kawasaki et al. 1998). Both Epac1 and Epac2 share common homo-logy domain structures, where both contain a regulatory region (N-terminal) and a catalytic region (C-terminal).

The regulatory region (N-terminal) carries one (Epac1) or two (Epac2) CNB (Cyclic Nucleotide Binding) domains which contain a (PRAA) motif that binds cAMP with high affinity and a DEP (Disheveled, Eg l-10, and Pleckstrin) domain. The CNB do-mains of Epac2 are designated as "A" or "B". The "A" CNB domain carries a PRHA motif, and it binds cAMP with low affinity. The "B" CNB domain carries a PRAA motif also found in Epac1, and it confers high affinity binding of cAMP.

The catalytic region (C-terminal) of Epac 1 and Epac 2 consists of classic CDC25-homology domain (CDC25HD), RAS association (RA) domain and a RAS exchange motif (REM) domain. Additionally, CNBD of Epac2 contains PRHA motif and binds cAMP with low affinity whose role is unknown and does not seem to be essential for Epac2 activation by cAMP (de Rooij et al. 2000; Holz et al. 2008). Upon binding of cAMP Epac undergoes conformational changes where the regulatory region moves to expose the catalytic region. The DEP domain is involved in membrane targeting of the active molecule and the REM domain participates in Ras activation when the two molecules are closely approximated.

The opinion surrounding cAMP and protein kinase A, which suggests that the cAMP-mediated signaling mechanism is much more complex than previously believed, was broken after identification of Epac proteins as novel sensors for cAMP (Cheng et al. 2008; Metrich et al. 2008).

Many cAMP-mediated physiological and pathological process that were previously thought to be induced via PKA alone may also be transduced by Epac, such as effects on cell adhesion (Enserink et al. 2004; Rangarajan et al. 2003) and cell differentiation (Kiermayer et al. 2005), proliferation, gene expression and apoptosis.

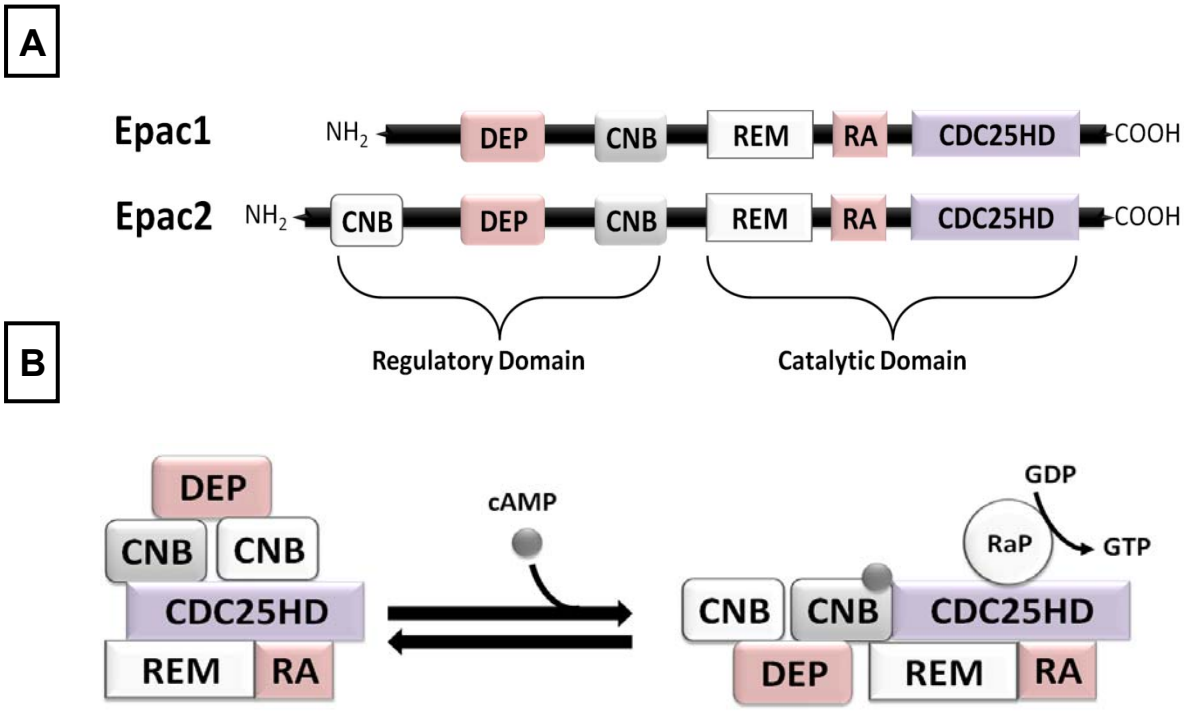


Figure 1-8: The multi domain structure of Epac. Domain structure of Epac1 and Epac2 **(A)**.The activation of Epac (indicated for Epac2) by cAMP **(B)**. (CDC25HD): Is CDC25-homology domain; (CNB): Is cyclic nucleotide-binding domain(s) ;(DEP): Is Desheveled-Egl-10-Pleckstrin domain ;(RA): Is Ras-association domain; (REM): Ras Exchange Motif (Bos 2006).

1.2.4 Inhaled Corticosteroids (ICSs)

Corticosteroids (also named glucocorticosteroids or glucocorticoids) are the most commonly used effective drugs in many inflammatory and immune diseases.

In 1948 hydrocortisone was the first clinically used glucocorticoid and since that time progress has been made in improving glucocorticoid treatment (Ito et al. 2006). Inhaled corticosteroids (ICSs) such as budesonide and fluticasone represent the most efficacious therapy currently available for asthma treatment and the only controllers that can effectively suppress the characteristic inflammation in asthmatic airways, even in very low doses at all levels of persistent asthma. This group of drug can control symptoms, reduce exacerbation and improve health status in most patients irrespective of disease severity (Barnes 2004; Barnes 2006).

ICSs act primarily as anti-inflammatory agents in asthma and partially reduce the associated airway hyper-responsiveness (AHR) in this disease (Giembycz et al. 2008; Hirst and Lee 1998).

In COPD prevention of exacerbation represents one of the main aims in disease management (Wedzicha and Seemungal 2007). However, there is evidence that ICSs reduce exacerbation in patients with COPD when compared with a placebo, their actions on lower airway inflammation in COPD is still doubtful (Adcock and Ito 2005). Although administration of high-dose inhaled corticosteroids have been demonstrated to decrease the frequency and severity of exacerbation during severe COPD, they were ineffective in reducing the progression of the disease and lung function decline (Paggiaro et al. 1998; Pauwels et al. 1999; Vestbo et al. 1999).

ICSs are supposed to exert their major effects in pulmonary airways after transfer into the respiratory epithelial cells and other cells in the airways through the glucocorticoid receptor (GR) (Ito et al. 2006; Leung and Bloom 2003). Simply, after passage the cell membrane corticosteroids bind to glucocorticoids receptor proteins (GR) in cytoplasm resulting in formation of steroid-receptor complex. The anti-inflammatory effects are mediated by direct moves of this complex into the nucleus where it binds to DNA, resulting in production of a glycoprotein called lipocortin. The formed lipo-

cortin inhibits the activity of phospholipase A2, which releases arachidonic acid, the precursor of prostanoids and leukotrienes, from phospholipids, (Kragballe 1989; van der Velden 1998). The steroid-receptor complex can regulate gene products in other mechanisms. First, by inducing the expression of the nuclear factor-kappa B (NF-kB) inhibitor (IKB-α) in certain cell types (Auphan et al. 1995). Second, by direct or indirect binding with other transcription factors, in particular activating protein-1 (AP-1) and NF-kB, resulting in inhibition of the pro-inflammatory effects of cytokines. Third, by binding to a pro-inflammatory transcription factor which causes blocking of gene expression (Adcock 2003). Or by increasing the level of cell ribonucleases and mRNA-destabilizing proteins, causing reduction in mRNA levels (Shim and Karin 2002).

Inhibition of the inflammatory cells repress release of cytokines from stimulated inflammatory and airway epithelial cells and also repress expression of their receptors. The ability of epithelial and smooth muscle cells to release lipids, cytokines, chemokines and pro-fibrotic mediators makes these tissues primary and critical targets for the anti-inflammatory actions of ICSs (Barnes 1996; Panettieri 2004; Schwiebert et al. 1996).

However, ICSs may have several other actions and cellular targets in the airways that contribute to their therapeutic action in asthma management (Hirst and Lee 1998).

Dexamethasone and ICSs like budesonide have been demonstrated to inhibit ICAM-1 and VCAM-1 expression on stimulated epithelial, endothelial cells (Paolieri et al. 1997; Wheller and Perretti 1997) and on fibroblasts (Spoelstra et al. 2000). Furthermore, they also inhibit GM-CSF and IL-6 production by epithelial cells and fibroblasts (Marini et al. 1992; Tobler et al. 1992).

1.2.5 Combination Therapy for Airway Obstructive Disease

As reported in many previous studies, it has been suggested and believed for a long time that β_2-adrenoceptors agonists used in obstructive airway diseases management exert their actions mostly on airway smooth muscle cells, leading to relaxation,

whereas glucocorticoids improve the airway function mostly by their effect as anti-inflammatory drugs. These effects could be explained as a synergistic action occuring at the lung rather than the cellular level. Recently it is becoming clear that both drugs can interact at different levels, including an integrated effect on several cell types (Schmidt and Michel 2011).

Combination of inhaled β_2-adrenoceptors agonists and inhaled corticosteroids represent the cornerstone in treatment of obstructive airways diseases. In combination with corticosteroids, β_2-adrenoceptors agonists can reduce the exacerbation and disease progression and improve the airway limitations (Cazzola et al. 2003; Hancox and Taylor 2001; Pauwels et al. 1997).

There is a large and growing body of experimental and clinical evidence supporting the use of ICSs and LABA as a combination therapy for long-term treatment of subjects with moderate-to-severe asthma and COPD. Although both ICSs and LABA are effective when used separately in improving lung function and reducing exacerbation (Sin and Man 2006), the combination therapy results in better outcomes than higher doses of corticosteroids in asthma (Pauwels et al. 1997; van Noord, et al. 1999) and COPD (Cazzola et al. 2003) with better symptom control and an additive or synergistic reduction of airway inflammation.

The combination of steroids and a LABA in one inhaler was effective in improving symptoms compared with a placebo or with one of the individual components alone (Nannini et al. 2004). It was observed that adrenalectomy in experimental animals caused general loss of responsiveness to catecholamines (Davies et al. 1981; Davies and Lefkowitz 1984). Conversely, in presence of glucocorticoids the physiological processes mediated by β_2-adrenoceptors such as myocardial contractility, glucose metabolism, and bronchial smooth muscle relaxation were enhanced.

It has been reported that ICSs may promote the action of β_2-adrenoceptors agonists by increasing the concentrations of β_2-adrenoceptors on smooth muscle cells (Mak et al. 1995a) and decreasing airway smooth muscle cell hyperresponsiveness to β_2-adrenoceptors agonists (Moore et al. 1999).

The interactions between glucocorticoids and β_2-adrenoceptors agonists are different and range from synergistic or additional to opposite effects (Adcock et al. 1996 Adcock et al. 2002).

In the context of rescue therapy with budesonide/formoterol, this could prevent the evolution of an acute exacerbation by suppressing the increase in inflammation, thus resulting in marked reduction in the number of mild and severe exacerbations. Thus, both budesonide and formoterol have inhibitory effects on the inflammatory process during an exacerbation (Barnes 2007).

Furthermore, there are positive interactions between β_2-adrenoceptors agonists and corticosteroids. These interactions may contribute to the efficacy of combination therapy as a rescue therapy as well as a maintenance treatment (Barnes 2007).
Treatment with ICSs plus LABA improves lung function, increases the number of days and nights without symptoms, decreases the frequency of use of rescue medication, and decreases exacerbation at a lower daily dose of ICSs (Sobande and Kercsmar 2008). Furthermore, systemic administration of corticosteroids can modulate lymphocyte-β_2-adrenoceptors function, both preventing and reversing tolerance (Grove and Lipworth 1995).

Despite little available evidence about the treatment of obstructive airways diseases with a combination of long-acting anticholinergic agent and inhaled corticosteroids, recent trials have suggested that this combination could be the most effective treatment for reducing exacerbation in patients with low FEV_1 (Aaron et al. 2007; Um et al. 2007).

The combination of an anticholinergic and β_2-adrenoceptors agonists used in COPD treatment has been shown to produce superior bronchodilation compared to its individual components without additional side effects and may be helpful in patients for whom a single inhaled bronchodilator has failed to provide an adequate response (Friedman et al. 1999).

1.2.6 Effect of Corticosteroids and β_2-Adrenoceptors Agonists on Airway Remodelling

Despite an impressive body of evidence on the effect of inhaled corticosteroids on airway inflammation in asthma, data on remodelling are more limited and not clear. Yet there is no doubt that early treatment of airway inflammation could modify the outcome of asthma by preventing a permanent loss of pulmonary function and prevent or delay airway remodelling (Boulet et al. 2000; Selroos et al. 1995).

It has been demonstrated that short-term ICSs treatment showed no significant change on the thickness of the reticular basement membrane (Chakir et al. 2003; Jeffery et al. 1992), whereas long-term therapy mostly resulted in a significant reduction in thickness of the reticular basement membrane, mediated via inhibition of ECM deposition, growth factor and collagen deposition (Goulet et al. 2007; Ward et al. 2002). Furthermore ICSs have also been shown to reduce the vascularity (vascular component) in airway wall in subjects with mild to moderate asthma (Chetta et al. 2003; Orsida et al. 1999).

Although glucocorticoids exert an inhibitory effect on cell proliferation of airway smooth muscle culture (ASMC) and primary fibroblast cultures derived from healthy subjects (Descalzi et al. 2008; Stewart et al. 1995), it has been shown that the absence of CCAAT/enhancer binding protein alpha (C/EBP-α) is responsible for the enhanced proliferation of ASMC, lymphocytes and mesenchymal, and that might explain the failure of corticosteroids to inhibit their proliferation in vitro (Roth et al. 2004; Rudiger et al. 2002). All these structural changes result in an overall airway wall thickness that seems to be reduced by treatment of airway obstructive disease with inhaled corticosteroids.

Little is known about the effect of β_2-adrenoceptors agonists on airway remodelling. Briefly, LABA have been shown to reduce airway vascularity in asthma in vivo (Orsida et al. 2001), cell proliferation of ASMC derived from healthy subjects (Stewart et al. 1997), and on total ECM, collagen deposition, gene expression, cell proliferation, and IL-6, IL-8, and TGF-β levels by primary human lung fibroblasts (Goulet et al. 2007).

Finally, it has been shown that combination therapy significantly inhibits ASMC and primary human lung fibroblasts proliferation and exerts an additive anti-inflammatory effect, rather than each drug alone through synchronized activation of GR and C/EBP-α (Descalzi et al. 2008; Roth et al. 2002; Spoelstra et al. 2002).

1.3 Aim of The Study

Fibrotic alterations are part of the airway remodelling which is closely associated with the severity of airways inflammatory diseases, including asthma and COPD. Airways remodelling is a process in response to long-term, unresolved airway inflammation that results in permanent structural changes in the airways walls. Airways structural alterations mostly cause irreversible airflow obstruction, and make treatment of COPD and asthma more difficult.

Sub-epithelial fibrosis as a characteristic of airways remodelling, and the role of pulmonary fibroblasts in airways remodelling has grown in the last few years and represent one of the most prolific cell population considered to play a pivotal role in airways inflammation and structural changes due to their capacity to produce inflammatory mediators, cytokines and extracellular matrix (ECM) perpetuating the inflammation in obstructive airways diseases. This suggests that they might represent one important target for the major drugs used in treatment and control of these diseases.

Little is known about the effects of β-adrenergic agonists and corticosteroids on pulmonary fibroblasts. Therefore, this study was designed to elucidate the role of β-adrenoceptors in mediating inhibitory effects on proliferation and collagen synthesis. However, a detailed pharmacological characterization of the receptors involved is missing and there is only limited knowledge about possible interactions with corticosteroids and the underlying molecular mechanisms.

Furthermore, there is increasing evidence that in addition to acute bronchodilatory effects, classical anti-obstructive drugs such β-adrenoceptors agonists may also modu-

late long-term remodelling processes. Therefore, this study was aimed to explore and analyze β-adrenoceptors subtypes expression, illuminate the transduction pathways mediating inhibitory effects of cAMP-elevating agents on α-smooth muscle actin expression (a marker of myofibroblast differentiation), and to characterize in detail putative β-adrenergic mechanisms and their interaction with corticosteroids in human pulmonary fibroblasts used in this study as in vitro model.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

A	
Agarose NEEO	Roth, Karlruhe
L-Ascorbic acid	Merck, Darmstadt
B	
Boric acid	Roth, Karlruhe
Bromophenol blue	Sigma-Aldrich, München
BSA (bovines Serum albumin)	Sigma-Aldrich, München
D	
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Developing - Stock solution	Sigma-Aldrich, München
E	
Ethanol, absolute	Merck, Darmstadt
Ethidium bromide	Sigma-Aldrich, München
Ethylenediaminetetraacetate (EDTA), disodium Salt	Sigma-Aldrich, München
F	
Fetal Calf Serum (FCS)	Biochrom, Berlin
Ficoll 400	Sigma-Aldrich, München
Fixing- Stock solution	Sigma-Aldrich, München
G	
Glycine	Roth, Karlruhe
H	
Hydrochloric acid 32% (conc. HCl)	Merck, Darmstadt
L	
Lowry Kit	Bio-Rad, München
Leupeptin	Sigma-Aldrich, München
Lumasafe plus Scintillation Cocktail	Lumac LSC, Groningen

M	
β-Mercaptoethanol	Sigma-Aldrich, München
Methanol	Roth, Karlruhe
MOPS SDS Running-Buffer (20X)	Invitrogen, Karlsruhe
N	
Non essential amino acids (100X)	Sgima-Aldrich, Munich
Nonidet P-40	Roche, Mannheim
P	
Penicillin/Streptomycine solution (10000 U/ml, 10 mg/ml)	Sigma-Aldrich, Munich
Pepstatin A	Sigma-Aldrich, Munich
Phenylmethylsulfonylfluoride (PMSF)	Sigma-Aldrich, Munich
Ponceau S	Sigma-Aldrich, Munich
Potassium chloride (KCl)	Merck, Darmstadt
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt
Powdered Milk, Fat free	Heirler, Radolfzell
R	
Roti-Load 1	Optical Density
S	
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium deoxycholate	Sigma-Aldrich, Munich
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich
Di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt
Sodium hydroxide pellets (NaOH)	Merck, Darmstadt
Sodium Pyruvate Solution (SPS) 100 mM	PAA, Cölbe
T	
Trichloro-acetic acid (TCA)	Merck, Darmstadt
Tris (hydroxymethyl) aminomethane	Roth, Karlruhe
Tris-HCL (hydroxymethyl) aminomethane hydrochloride	Boehringer ingelheim,
Triton X-100	Pharmacia, Biotech
Trypan Blue Stain (0.4%)	Sigma-Aldrich, Munich
10X Trypsine-EDTA solution	Sigma-Aldrich, Munich
Tween 20	Sigma-Aldrich, Munich

2.1.2 Culture Medium

Substance	Supplier
Basis medium EARLE`S MEM	PAA, Cölbe
Opti-MEM® I Reduced-Serum Medium (1 x)	Invitrogen, Karlsruhe

2.1.3 Enzymes

Substance	Supplier
DNase (RNase free DNase Set)	Qiagen, Hilden & PeqLab, Erlangen
Omniscript Reverse Transcriptase	Qiagen, Hilden
RNase Inhibitor RNasin Plus (40 U/µl)	Promega, Madison, USA & Fermentas, St. Leon-Rot
Taq DNA polymerase	Invitrogen, Karlsruhe

2.1.4 Markers and Nucleic Acids

Substance	Supplier
BLOCK-iTTM Fluorescent Oligo	Invitrogen, Karlsruhe
DNA Ladder Ready-Load 100bp	Invitrogen, Karlsruhe
PageRuler™ Prestained Protein Ladder	Fermentas, St. Leon-Rot
dNTP-Mix (je 10 mM pro Nucleotid)	Fermentas, St. Leon-Rot
Oligonucleotide (dT)18	MWG, Ebersberg

2.1.5 Kits for Molecular Biology

Substance	Supplier
BM Chemiluminescence Blotting Substrate (POD)	Roche, Mannheim
Omniscript RT Kit	Qiagen, Hilden
QuantiTect® SYBR® Green PCR	Qiagen, Hilden
RNeasy Mini Kit	Qiagen, Hilden
TaqDNA Polymerase	Invitrogen, Karlsruhe

2.1.6 Test Drugs

Test Drug	Supplier	Stock Solution Solvent
A		
Actinonomycin-D	Sigma	Methanol
B		
N6- Benzoyladenosine- 3', 5'- cyclic monophosphate, acetoxymethyl ester (6-Bnz-cAMP)	Biolog	H_2O
8-Bromoadenosine 3':5'-cyclic monophosphate (8-Bromo-cAMP)	Sigma	10 mM NaOH
Budesonide	Astra - Zeneca	Ethanol
Butaprost	Sigma	DMSO
C		
1-[2-((3-Carbamoyl-4-hydroxy) phenoxy) ethylamino]-3-[4-(1-methyl-4-trifluoro-methyl-2-imidazolyl) phenoxy]-2-propanol dihydrochloride (CGP 20712-dihydrochloride)	Tocris	H_2O/ Ethanol
Cycloheximide	Sigma	Ethanol
D		
Dexamethasone	Sigma	Ethanol
F		
Formoterol fumarate dihydrate	Astra-Zeneca	Ethanol
Forskolin	Hoechst	DMSO

I		
(±)-1-[2,3-(Dihydro-7-methyl-1H-inden-4-yl) -oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI 118.551 HCl)	Sigma	Ethanol
Indomethacin	Sigma	Ethanol
Interleukin-4	Sigma	0.1% BSA in1X PBS
Interleukin-13	Sigma	0.1% BSA in1X PBS
Isoprenaline	Sigma	H_2O (0.01 mg/mL Ascorbic acid)
P		
2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD 98059)	Sigma	DMSO
Protein kinase inhibitor-(14-22)-amide, myristoylated (PKI 14-22)	Sigma	H_2O
S		
8-(4-Chlorophenylthio)- 2'- O-methyladenosine- 3', 5'- cyclic monophosphorothioate, Sp- isomer (Sp-8-pCPT-2'-O-Me-cAMPS)	Biolog	H_2O
T		
Transforming growth factor (TGF-β Human Recombinant)	Sigma	0.1% BSA in 4 mM HCl
6,7-Dimethoxy-2-phenylquinoxaline (Tyrphostin AG 1296)	Sigma	DMSO

2.2 Radio Chemicals

Reagents	Supplier
[^{3}H]-Proline (specific activity = 1.813 TBq/mmol)	Perkin Elmer, Boston
[^{3}H]-Thymidine (specific activity = 370 GBq/mmol)	Perkin Elmer, Boston

2.3 Antibody

2.3.1 Primary Antibody

Substances	Species	Dilution	Supplier
α-smooth-muscle-actin	Mouse	1:1000	Sigma
β_2-AR (H-73)	Rabbit	1:200	Santa Cruz
β_2-AR (H-20)	Rabbit	1:200	Santa Cruz
Epac2 (A7)	Mouse	1:200	Santa Cruz
α-Tubulin	Mouse	1:1000	Cedarlane

Adrenoceptor: AR

2.3.2 Secondary Antibody

Reagents	Species	Dilution	Supplier
Anti-Mouse	Goat	1:8000	Santa Cruz
Anti-Rabbit	Goat	1:2667	Santa Cruz

2.4 Primer

2.4.1 Primer for Semi-Quantitative PCR

Primer	Sequence (sense and anti-sense)	Length (bp)	A.T (°C)	Cycles
α-SMA	5′-GAC GAAGCA CAG AGC AAA AGAG-3′ 5′-TGG TGATGA TGC CAT GTT CTA TCG-3′	141	58	30
β-actin	5′-CAC TCT TCC AGC CTT CCT TC-3′ 5′-CTC GTC ATA CTC CTG CTT GC-3′	813	56	23
β_1-AR	5′-TCG TGT GCA CCG TGT GG G CC-3′ 5′-AGG AAA CGG CGC TCG CAG CTG TCG-3′	240	63	35
β_2-AR	5′-GCC TGC TGA CCA AGA ATA AGG CC-3′ 5′-CCC ATC CTG CTC CAC CT-3′	260	59	35
β_3-AR	5′-GCT CCG TGG CCT CAC GAG AA-3′ 5′-CCC AAC GGC CAG TGG CCA GTC AGC G-3′	300	62	35

Annealing temperature: A.T

2.4.2 Primer for Real-Time Quantitative PCR

Primer	Sequence (sense and anti-sense)	Length (bp)	A.T (°C)	Cycles
α-SMA	5′-GACAGCTACGTGGGTGACGAAG -3′ 5′-CAGATCTTTTCCATGTCGTCCC -3′	107	58	35
β_2-AR	5′-GAT TTC AGG ATT GCC TTC CAG -3′ 5′-GTG ATA TCC ACT CTG CTC CCC -3′	110	59	40
GAPDH	5′-CTG CAC CAC CAA CTG CTT AGC -3′ 5′-GGC ATG GAC TGT GGT CAT GAG -3′	87	55	50

Annealing temperature: A.T

2.5 Buffers

2.5.1 Buffers for Cell Culture

Buffer	Components
♦ 10x PBS, pH 7.4-7.5	27 mM KCl 15 mM KH_2PO_4 1.38 M NaCl 81 mM Na_2HPO_4 x H_2O Distilled Water
♦ 1x PBS, pH 7.4-7.5	10x PBS, 1:10 dilution with Distilled Water

2.5.2 Buffers for Proliferation Assay

Buffer	Components
♦ 1 M Tris-HCl pH 7.4	Tris-HCl 1 M Distilled Water Adjust to pH 7.4 with HCl
♦ 1x PBS, pH 7.4-7.5	10x PBS, 1:10 dilution with Distilled Water

2.5.3 Buffers for Protein Determination Using The Lowry Method

Buffer	Components
♦ RIPA Lysis Buffer	50 mM Tris-HCl pH 7.5 150 mM NaCl 0.5% Sodium deoxycholate 1% Nonidet P-40 0.1% SDS
♦ RIPA Lysis Buffer with Protease Inhibitor	RIPA Lysis Buffer 2 mM of 0.1 M EDTA, pH 8 0.7 µg/mL (1 µM) of 125 µg/mL Pepstatin A 170 µg/mL (1 mM) of 100 mM PMSF 0.5 µg/mL (1 µM) of 10 µg/mL Leupeptin
♦ NP-40 Lysis Buffer	100 mM Tris-HCl, pH 7.5 100 mM NaCl 10 mM $MgCl_2$ 100% (v/v) Nonidet P-40 Distilled water
♦ NP-40 Lysis Buffer with Protease Inhibitor	NP-40 Buffer 2 mM of 0.1 M EDTA, pH 8 0.7 µg/mL (1 µM) of 125 µg/mL Pepstatin A 170 µg/mL (1 mM) of 100 mM PMSF 0.5 µg/mL (1 µM) of 10 µg/mL Leupeptin
♦ PBS + Protease Inhibitor (PBS-PI)	BPS Buffer 2 mM of 0.1 M EDTA, pH 8 0.7 µg/mL (1 µM) of 125 µg/mL Pepstatin A 170 µg/mL (1 mM) of 100 mM PMSF 0.5 µg/mL (1 µM) of 10 µg/mL Leupeptin

2.5.4 Buffers for RT/PCR "Gel Electrophoresis"

Buffer	Components
♦ 5x TBE-Buffer	0.45 M Tris 0.44 M Boric acid 0.012 M EDTA Distilled Water
♦ 0.5x TBE-Buffer	5x TBE-Buffer 1:10 diluted in Distilled Water
♦ PCR loading Buffer	15% (w/v) Ficoll 400 0.25% (w/v) 1% Bromophenol blue solution 0.5x TBE-Buffer Distilled Water

2.5.5 Buffers for Protein Gel Electrophoresis and Immunoblot

Buffer	Components
♦ 1x NuPAGE MOPS SDS Running Buffer	5% (v/v) 20x NuPAGE MOPS SDS Running Buffer Distilled Water
♦ Transfer Buffer	25 mM Tris 192 mM Glycine 20% (v/v) Methanol Distilled Water
♦ Tris Buffered Saline (TBS) Buffer, pH 7.5	50 mM Tris 150 mM NaCl HCl Distilled Water
♦ TBST 0.1% (v/v)	1 mL Tween 20 1000 mL TBS
♦ TBST 0.05% (v/v)	500 mL TBS 500 mL TBST 0.1% (v/v)

2.6 Solutions

2.6.1 Solutions for Cell Culture

Solutions	Components
♦ 0.15% (w/v) Trypan blue staining dye	0.4% Trypan Blue Stain, 3:8 dilution with 1x PBS-Buffer
♦ 1x Trypsin-EDTA-solution	10x Trypsin-EDTA, 1:10 dilution with 1x Sterile PBS-Buffer

2.6.2 Solutions for Proliferation Assay

Solutions	Components
♦ 5% Trichloro-acetic acid	5% (w/v) Trichloro-acetic acid crystals in Distilled Water
♦ 0.1 N Sodium hydroxide	0.1 N Sodium hydroxide pellets Distilled Water

2.6.3 Solutions for Proline Assay

Solutions	Components
♦ 20% Trichloro-acetic acid	20% (w/v) Trichloro-acetic acid crystals in Distilled Water
♦ 10% Trichloro-acetic acid	10% (w/v) Trichloro-acetic acid crystals in Distilled Water
♦ 0.2 N HCl	0.2 N HCl in Distilled Water
♦ 0.2 N Sodium hydroxide	0.1N Sodium hydroxide pellets Distilled Water

2.6.4 Solutions for Protein Determination Using The Lowry Method

Solutions	Components
♦ BSA-Standard solution	4 mg/mL BSA 0.1% Tris/Triton X-100 solution
♦ 1% Tris/Triton X-100 solution	100 M of 1M Tris-HCL-solution, pH 7.4 1% (v/v) Triton X-100 Distilled Water
♦ Reagent A	1% (v/v) Reagent S 99% (v/v) Starting Reagent A

2.6.5 Solutions for RT/PCR

Solutions	Components
♦ Agarose Gel 1.2%	1.2% Agarose 0.5x TBE-Buffer 5 µL Ethidium bromide solution (10 mg/mL)
♦ Ethidium bromide solution 1%	1 mL Ethidium bromide 100 mL Distilled Water

2.6.6 Solutions for Protein Gel Electrophoresis and Immunoblot

Solutions	Components
♦ Ponceau S solution	0.2% (w/v) Ponceau S 3% (w/v) Tris-HCl Distilled Water
♦ 5% Blocking solution	5 g Dried milk powder 100 mL Distilled Water
♦ 3% Blocking solution	3 g Dried milk powder 100 mL Distilled Water
♦ Chemiluminescence detection solution 1% (v/v)	30 µL Starting solution 2970 µL Luminescence solution
♦ Developing solution 20% (v/v)	60 mL Developing stock solution 240 mL Distilled Water
♦ Fixing solution 20% (v/v)	60 mL Fixing stock solution 240 mL Distilled Water

2.7 Culture Medium

2.7.1 Standard Fibroblast Medium

Culture Medium	Components
♦ Basis medium EARLE`S MEM	0-15 % FCS 100 U/mL Penicillin, 100µg/mL Streptomycin 1 mM SPS 1x NEAA

2.7.2 Culture Medium for Human Fibroblast without FCS

Culture Medium	Components
♦ Basis medium EARLE`S MEM	100 U/mL Penicillin, 100 µg/mL Streptomycin 1 mM SPS 1x NEAA

2.7.3 Culture Medium for Human Fibroblast With 10% FCS

Culture Medium	Components
♦ Basis medium EARLE`S MEM	10% (v/v) FCS 100 U/mL Penicillin, 100 µg/mL Streptomycin 1 mM SPS 1x NEAA

2.8 Equipment

A

Analytical Balance BP221 D, 2258	Sartorius, Goettingen
Autoclave 80230	Webeco, Bad Schwartau

C

Centrifuges:	
Biofuge Primo	Heathrow Scientific; United Kingdom
C 54 115, 5804 R refrigerated centrifuge	Eppendorf, Hamburg
Labofuge GL, Biofuge pico	Heraeus, Hanau
Mini-Centrifuge MCF 2360	LMS, Tokyo, Japan
Sprout® Mini-Centrifuge	Thermo Electron, Dreieich

E

Electrophoresis and Blotting chamber	Invitrogen, Karlsruhe

F

Forma* 1800 Series Class 100 Clean Benches	Thermo Scientific; Braunschweig

H	
Heating blocks and thermal cycler:	
▪ My Cycler	Bio-Rad, Munich
▪ PCR Thermal Reactor	MWG, Ebersberg
I	
Incubators:	
▪ HERAcell150 CO2-Incubator	ThermoElectron, Dreieich
▪ Infrared CO2 Incubator	Forma Scientific, Marietta,USA
▪ Steri-Cycle CO2 Incubator	Thermo Electron, Dreieich
N	
NuPAGE Microscope:	
▪ Leica DMIL	leica-microsystems; Dreieich
▪ IMT2-RFL	Olympus, Hamburg
P	
PVDF Blotting Membrane Immobilion P	Millipore, Eschborn
pH electrode SenTix 81	WTW GmbH, Weilheim
pH meter inoLab 1	WTW GmbH, Weilheim
Photo Documentation System Power Shot G5	Canon, Krefeld
Photometer:	
▪ DU-64	Beckman, Munich
▪ Gene Quant II	Pharmacia, Freiburg
▪ SmartSpecTM Plus	BioRad, Munich
R	
Real-Time PCR, MX3000P	Stratagene, La Jolla,USA
Rocking Platform:	
▪ WT.15	Biometra; Goettingen
▪ VWR	VWR; Langenfeld
S	
Safety Cabinets LaminAir HB HBB 2436	Holten, Alleroed, Denmark
Scintillation Counter Tri-Carb 2100TR	Packard, Dreieich
T	
Thermo mixer Compact	Eppendorf, Hamburg
V	
Voltage devices:	
▪ 2297 Macro drive 5	LKB Bromme, Sweden
▪ Power Pac 300	BioRad, , Munich

2.9 Culture of Human Lung Fibroblast Cell Line

Most of this work was carried out on MRC-5 cell line human lung fibroblasts, but also some experiments were carried out on HEL-299 cell line & primary human lung fibroblasts (PhLFb). For all cells the minimum essential medium Eagle (MEM with Earle's salts) was supplemented with antibiotic solution, non-essential amino acids, sodium Pyruvate, and different concentrations of fetal calf serum (FCS).

► MRC-5 Cell Line

The MRC-5 cell line (CCL-171, ATCC, Manassas, VA, USA) is human fibroblasts that were isolated from normal lung tissue of a 14-week-old human male, Caucasian fetus by J.P. Jacobs in September of 1966.

► HEL- 299 Cell Line

The HEL-299 cell line (CCL-137, ATCC, Manassas, VA, USA) is human fibroblasts that were derived from normal lung tissue of a human male, black fetus.

2.10 Primary Human Lung Fibroblast (PhLFb)

Cell cultures of primary human lung fibroblasts (PhLFb) were established from lung tissue obtained from patients undergoing thoracotomy due to lung cancer. Lung tissue was taken from histologically normal areas of surgically resected lung, either central or peripheral region of the lung, then the tissue was cut into small pieces, treated with pronase (1 mg/mL Calbiochem Novabiochem, San Diego, CA, USA) at 37°C for 30 minutes, and placed into cell culture plates, incubated in human fibroblast medium supplemented with 15% FCS. After fibroblasts had grown out from the tissues (2 weeks), the slices were removed and the cells were allowed to reach confluence. Confluent fibroblasts were then passaged by trypsinization and used for the experiments between passages 3-11. The procedure for generating primary human lung cell cultures from tissue obtained during surgery had been approved by the ethical committee of the Faculty of Medicine, University of Bonn (Bonn, Germany).

2.11 Culture of Human Lung Fibroblast

The cryogenically preserved human lung fibroblasts (MRC-5, HEL 299, and PhLFb) were thawed at 37°C on a water bath. The cells suspension was then transferred to 175 cm^2 culture flask containing 10% FCS human Fibroblast medium which shortly pre-incubated in a humidified incubator at 37°C and 5% CO_2.

After about 24 hrs the medium was changed to a fresh one in order to remove remnants of DMSO. The cells were then allowed to grow with medium changed twice per week. After reaching confluence cells could be seeded and passaged further.

For cultivation the cells were washed with 10 mL 37°C warm sterile 1x PBS, trypsinized with 5-10 mL warm sterile 1x Trypsin/EDTA solution for 10 seconds after which the Trypsin was aspirated out. Then the cells were washed with 10% FCS medium and centrifuged at 1000 rpm for 5 minutes. The medium was discarded and the cells' pellet again resuspended in a defined amount of 10% FCS medium.

Cells were stained with 0.15% Trypan blue (20 µL of cell suspension plus 80 µL of the stain), counted in a Neubauer-counting chamber under the microscope and cultivated as required.

Required number of cells was cultured further in a 175 cm^2 culture flask containing 10% FCS human lung fibroblast medium. For refreezing, the cells were resuspended in freezing medium consisting of 9 parts of 10% FCS medium and 1 part of 5% DMSO and aliquots were frozen in Cryovial at -80°C for slow cooling and then stored in a liquid nitrogen tank.

2.12 RNA Extraction

Total RNA isolation was carried out by using silica-gel-based membrane, either by using an RNeasy tissue kit provided by Qiagen, or peqGOLD total RNA Kit provided by peqlab, according to the manufacturer's instructions.

1.5-3x10^5 Cells were seeded in 35 mm dishes and/or 6 well plats containing 2 mL of 10% FCS human fibroblast medium. After 24 hrs of starvation time and depending on the protocol, cells were allowed to grow in presence or absence of tested substances. At the end the medium was sucked out and the cells were lysed by addition of 350 µL the lysis buffer containing β-mercaptoethanol. The cells lysate was transferred to a shredder column placed in a 2 mL collection tube and homogenized by centrifugation for 2 minutes at maximum speed (13000-14000 rpm) at room temperature, followed by addition of equal volume of ethanol (70%) to the flow-through lysate and mixed thoroughly by pipette to provide appropriate binding conditions. The lysate was then transferred to an RNeasy spin column placed in a 2 mL collection tube and centrifuged at 10000 rpm for 15 seconds. The flow-through was poured-off. Afterward 700 µL of the wash buffer RW1 (RNeasy tissue kit) or 600 µL of the wash buffer I (peqGOLD total RNA Kit) was added on the spin column and centrifuged for 15 seconds at 10000 rpm. The flow-through and collection tube were discarded and the spin column was placed on a new collection tube. The β-adrenoceptor genes are intronless and contamination of RNA preparation by genomic DNA would cause robust false-positive PCR results, hence the remaining traces of DNA were digested by addition of 75-80 µL RNase-free DNase according to the manufacturer's instructions on the spin column and incubated at room temperature for 15 minutes. The first washing step was repeated and the flow-through was discarded.

500 µL of the RPE (RNeasy tissue kit) or of wash buffer II (peqGOLD total RNA Kit) was added on the spin column and centrifuged for 15 seconds at 10000 rpm. The buffer wash step was repeated with an extended centrifugation time for 2 minutes. The filtrate was discarded and the spin column was dried by centrifugation at 10000 rpm for 15 seconds. The spin column was placed on fresh 1.5 mL tube and the elution was done using 30-40 µL of RNase free water at 10000 rpm for 1 minute. Sometimes a second elution was necessary to increase the total RNA yield.

2.13 DNA Isolation

Genomic DNA was isolated from human lung fibroblasts cell line, either MRC-5 or HEL-299 cell line and used as a reference control for the effectiveness of primer pairs by using a semi-quantitative PCR.

Fibroblasts were cultured in a 175 cm^2 tissue culture flask to confluence, then washed with 1x PBS and trypsinized with 1x Trypsin/EDTA solution for 8-10 seconds. Subsequently the cells were washed out by using culture medium and centrifuged at 1000-2000 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 200 µL 1x PBS and 40 µL RNase A [10 mg/mL] and incubated for 2 minutes at room temperature.

Afterward 20 µL proteinase K and 200 µL buffer AL was added to the reaction tube, mixed by using vortex mixer and incubated at 70 ^oC for 10 minutes. The sample was homogenized by addition of 200 µL (96%) ethanol and mixed again. The probe was transferred into the DNeasy spin column fixed in a 2 mL collection tube and centrifuged at 8000 rpm for one minute, the collection tube was discarded and spin column fixed on a new 2 mL collection tube. 500 µL buffer AW1 was added and the tube centrifuged at 8000 rpm for 1 minute. The collection tube was discarded and the spin column fixed on another new 2 mL collection tube and washed by addition of 500 µL of buffer AW2 and centrifuged at maximum speed (14000 rpm) for 3 minutes to dry the DNeasy membrane. Thereafter the spin column was placed on a clean fresh 1.5 mL Eppendorf tube followed by addition of 100 µL water and incubation of the probe was carried out for 1 minute at room temperature, and then centrifuged at 8000 rpm for 1 minute.

The eluted DNA was then placed in a pipette again on the spine column with 100 µL water and incubated for 1 minute at room temperature, and centrifuged again at 8000 rpm for 1 minute for final DNA elution. The concentration of the product was measured by using a photometer at 260 nm.

2.14 Determination of RNA Concentration by Spectrophotometric Estimation

The concentration of RNA in the collected sample was measured photometrically at a wavelength of 260 nm (OD_{260}) and 280 nm (OD_{280}). The ratio between the absorbance values gives an estimate of the purity of RNA. In a 1:20 or 1:40 dilution with either RNase free water or DEPC-water, the measurement of the concentration was carried out either with the Gene Quant II photometer (Pharmacia) or by Smart-SpecTMPlus (Bio-Rad). Lower ratios indicate the presence of contaminants such as proteins. The sample preparations were stored at -80^{o} C.
The RNA content was calculated as follows: *OD_{260} x dilution x 40 = RNA content (µg/mL)*.

2.15 Reverse Transcription

The transcription reaction was carried out using an Omniscript RT Kit provided by Qiagen, and a PCR Thermal Reactor according to the manufacturer's instructions. To start the reaction, 1 µg of RNA was used as a starting template (x µL), the volume completed to 12.5 µL with RNase free water (12.5 µL - x µL).

The RT-Master mix containing Oligo dT primer in a reverse transcriptase buffer was added to this probe in a 0.5 mL reaction tube making up the volume to 20 µL/probe. The probes were incubated at 37^{o}C for 1 h. Subsequently the reaction was blocked by incubation at 93^{o}C for 5 minutes. Afterward the probes were cooled-down on ice, centrifuged and followed by addition of 80 µL of sterile water per probe. The cDNA probes were stored at -20^{o}C and 5 µL of this cDNA was used as a template for further PCR reactions.

2.15.1 Semi Quantitative Polymerase Chain Reaction (PCR)

All reactions were carried out in a thermal cycler (MyCycler^{R}) provided by BioRad in total volume of 50 µL, where 5 µL of cDNA was used as a template and mixed with 45 µL *PCR-Master mix*. consisting of:

Reagents	Volume per Probe
Water	32.5 µL
10x PCR Reaction Buffer	5 µL
50 mM $MgCl_2$	1.5 µL
10 mM Desoxynucleotide-Mix	1 µL
10 µM Sense-Primer	2.5 µL
10 µM Antisense-Primer	2.5 µL
5 U/µl TaqDNA Polymerase	0.5 µL
Total volume	**45 µL**

Finally, the reaction runs according to the following specifications:

Step	Temperature	Time	
Initial Denaturation	94° C	3 min	
Denaturation	94° C	45 s	23-35 Cycles
Annealing	53° C to 63° C	30 s	
Elongation	72° C	1 min	
Final Elongation	72° C	10 mins	

2.15.2 Quantitative Real Time Polymerase Chain Reaction (qPCR)

qPCR was performed by monitoring the fluorescence of SYBR Green dye on a Stratagene Mx3000P real time PCR system, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard for normalization.
cDNA was diluted 1:3 with RNase free water, where 5 µL of cDNA was used as a template and mixed with 10 µL *PCR-Master mix.* consisting of:

Reagents	Volume per Probe
Master Mix SYBR® Green	7.5 µL
Primer Sense	0.45 µL
Primer Antisense	0.45 µL
Distal Water	1.6 µL
Total volume	**10 µL**

Where the cycling conditions were:

Step	Temperature	Time	
Initial activation of Hot Star-Taq ®DNA Polymerase	95° C	3 mins	
Denaturation	95° C	30 s	35-45 Cycles
Annealing	55° C to 59° C	30 s	
Elongation	72° C	30 s	

Subsequently the results were exported to the Excel program for further analysis. Fluorescence data from each sample were analysed with the $2^{-[\Delta\Delta Ct]}$ method: fold induction = $2^{-[\Delta\Delta Ct]}$, where ΔΔCt = [Ct GI (unknown sample) - Ct GAPDH (unknown sample)] - [Ct GI (calibrator sample) - Ct GAPDH (calibrator sample)], GI is the gene of interest.

2.16 Agarose Gel Electrophoresis

PCR amplification products were separated through agarose gel matrix in response to an electric current.

For agarose gel electrophoresis a 1.2% agarose gel was prepared in 150 mL of 0.5x TBE buffer and 0.5 µg/mL ethidium bromide was added. The prepared gel was transferred to a running chamber containing 0.5x TBE buffer solutions.

5 µL of a PCR buffer (Marker dye) was added to each PCR probe, subsequently 7.5 µL of 100 bp DNA ladder stained with PCR buffer was loaded onto the first gel lane and 25-30 µL of each stained probe were loaded onto the other gel slots. The gel was allowed to run at a constant current of 75 mA for 60-75 minutes. The gel was placed on the UV light and the DNA bands were documented.

Finally, optical density of bands was determined by RFLPscan 2.01 software (MWG, Ebersberg, Germany), and corrected over β-actin bands.

2.17 Western Blot Assay

2.17.1 Protein Extraction

$2*10^5$ - $3.5*10^5$ cells/dish MRC-5 human lung fibroblasts were seeded respectively in 35 or 55 mm culture dishes containing 2 mL of human lung fibroblast medium supplemented with 10% FCS and allowed to grow in humidified conditions with 5% CO2 for 24 hrs. Cells were grown in a serum-deprived medium for a further 24 hrs. Subsequently the cells were incubated with or without tested substance for 24-48 hrs depending on the protocol. The cells were then washed twice with ice cold 1x PBS, and the cellular proteins were extracted in 200-300 µL RIPA buffer or NP-40 buffer containing the protease inhibitors.

2.17.2 Total Protein Extraction

Cells were lysed and cellular proteins were extracted in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodiumdeoxycholat, 1% Nonidet P-40, 0.1% (w/v) SDS, 2 mM EDTA, pH 8.0) containing the protease inhibitors phenylmethansulfonylfluorid (PMSF, 1 mM), pepstatin A (0.7 µg/mL) and leupeptin (0.5 µg/mL). The lysed cells were transferred to a 1.5 mL tube and centrifuged for 15 minutes at

13000 rpm and 4°C where the supernatant used for protein estimation and the rests were stored at -20°C for long-term storage.

2.17.3 Membrane Protein Preparation

This extraction protocol was used for β_2-adrenoceptors protein analysis. Here the cells were lysed and the proteins were extracted in NP-40 lysis buffer (100 mM Tris-HCl, pH 7.5,100 mM NaCl,10 mM $MgCl_2$, 100% (v/v) Nonidet P-40) containing the protease inhibitors phenylmethansulfonylfluorid (PMSF, 1 mM), pepstatin A (0.7 µg/mL) and leupeptin (0.5 µg/mL). The cells lysate was transferred to 1.5 mL Eppendorf tubes and centrifuged for 10 minutes at 6000 rpm and 4°C, the supernatant was transferred to a fresh 1.5 mL tube and centrifuged for 15 minutes at 13000 rpm and 4° C. The supernatants containing cytosolic protein fraction was measured and used as control. The pellet was washed in 100 µL PBS containing the protease inhibitors and centrifuged at 13000 rpm at 4°C for 15 minutes. Supernatant was discarded and the pellet was resuspended in 30 µL of NP-40 + PI where 10 µL used for protein determination and the rest 20 µL proteins were used for β_2-adrenoceptors protein estimation.

2.17.4 Protein Determination

Protein determination was carried out using a Bio-Rad DC protein assay kit which is a colorimetric assay and used according to the manufacturer's instructions.

2.18 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For protein analysis 20-50 µg proteins equivalents for β_2-adrenoceptors expression analysis or 1.5-2.5 µg proteins equivalents to α-SMA regulations analysis were mixed with the reducing loading buffer Roti®-Load (4:1) and denaturated at 70°C for 10 minutes.

Gel running was performed by using XCell II™ Mini-Cells apparatus from Invitrogen. The normalized proteins and 5 µL of kaleidoscope marker were loaded on the gel with clamps in the running chamber containing running buffer (1x MOPS). The gel run was carried out by being attached to a power supply at 200 V constant at 60 mA current for 80-90 minutes.

Analysis of β_2-adrenoceptors expression was carried out by separation of the samples on 10% (NuPAGE®Novex®Bis-Tris Gel) using the 1x MOPS-SDS running buffer system. Whereas, a 4-12% NuPAGE Bis-Tris Gel using the 1x MOPS-SDS running buffer system was utilized for α-SMA analysis.

2.19 Protein Blotting

In order to make the separated proteins susceptible to detection by specific antibody, they were transferred onto a membrane made of polyvinylidene difluoride (PVDF). For this purpose, the membrane was equilibrated by soaking in methanol for 1-3 seconds and thereafter washed in distilled water followed by 20 minutes equilibration in a transfer buffer. In the meantime, filter paper and blotting sponge pads were preincubated in transfer buffer before being used in order to remove any air bubbles.

At the end of the gel running process, the gel was removed from the gel cassette and washed to remove the adhering salts and detergents for 5 minutes in transfer buffer.

At the end of washing and equilibration process a transfer sandwich assembly of the membrane, gel, filter paper and blotting sponge pads were built and transferred to the XCell II™ Mini-Cells apparatus for electro blotting and moving of proteins from gel to PVDF membrane. The chambers were then filled with 4°C cold transfer buffer.
The blotting process was carried out at a constant current of 250 mA and 100V for 90 minutes. At the end of the blotting step the PVDF membrane was transferred onto a tray on a shaker platform followed by staining using Ponceau S (0.2% w/v) in order to ensure and control the blotting quality and efficacy.

The marked lanes were decolorized by washing twice with distilled water followed by washing with TBS solution for 3 minutes. Afterwards, the membrane was blocked by using 8-10 mL of 5% blocking solution overnight at 4^{o}C to saturate and block non-specific protein binding sites.

2.20 Immuno-detection

The blocking solution was discarded and the membrane was incubated in 8 mL of the primary antibody prepared in 3% blocking solution in appropriate dilution for 90 minutes at room temperature on the shaker platform. Thereafter the membrane was washed twice with 0.3% TBST solution for 10 minutes and blocked again twice with 3% blocking solution for 10 minutes each.

This was followed by incubation of the membrane with secondary antibody, linked with a reporter enzyme (horseradish peroxidase), and diluted to appropriate dilution for 45 minutes on a shaker platform. After the end of the incubation period with the secondary antibody the membrane was washed four times with 0.1% TBST solution for 15 minutes each.

Chemiluminescence detection technique was performed by incubation of the membrane with Chemiluminescence solution which was prepared during the last washing step according to the manufacturer's manual and kept at room temperature.

For the detection process the washing buffer was discarded and the membrane transferred to a plastic sleeve and incubated with 2-3 mL of Chemiluminescence detection solution for one minute, then wrapped in a plastic sleeve where the air bubbles were removed from the membrane wrap sandwich. Thereafter the membrane wrap sandwich was fixed in a film cassette and exposed to an X-ray film for 5 seconds to 5 minutes depending on the intensity of the signal. After exposing the film for the appropriate time the signals were developed by exposing the film to the developing solution for 3-5 minutes, washed in water for 3 minutes and finally fixed for 5 minutes in fixing solution. Depending on the intensity of the signal a second film was exposed and developed to optimal exposure time.
After drying, the films were then scanned. The optical density of protein bands was measured densitometrically by using of RFLP scan software.

2.21 Radioactive Assays

2.21.1 [^{3}H]-Thymidine Incorporation Assay

For this assay human lung fibroblasts cells were trypsinized, harvested and seeded into 12-well plates at a density of $4*10^4$ cells per well. Different protocols were tested in order to find conditions under which adrenergic effects might be particularly prominent. Cells were first cultured for 24 hrs in the presence of 10% FCS, followed by 16-18 hrs under FCS-free conditions. Thereafter, test drugs were added and present for 26-30 hrs in most experiments under FCS-free conditions. [^{3}H]-Thymidine (37 kBq) was present during the last 24 hrs in most experiments. At the end of incubation cells were washed in ice-cold PBS and denatured in TCA (5%) for 10 minutes, followed by washing in ice-cold PBS and DNA was extracted during incubation for 1 h in 0.1 mol/l NaOH at 37°C or by incubation with 0.1 mol/l NaOH at 4°C overnight. Samples of the supernatant solution (300 µL portions) were neutralized with 200 µL of Tris HCl (pH 7.4) combined with the scintillation cocktail and the radioactivity was measured by liquid scintillation spectrometry in a Packard 2100 liquid scintillation analyzer. External standardization was used to correct for counting efficiency. [^{3}H]-Thymidine incorporation was expressed either in absolute terms (d.p.m) or as a percentage of the mean of the control group of each cell preparation.

2.21.2 [^{3}H]-Proline Incorporation Assay

Collagen synthesis and deposition into the extracellular matrix were assessed by [^{3}H]-proline incorporation assay, originally developed by Peterkofsky and Diegelmann (1997) and subsequently established also in our laboratory. Cells were trypsinized, harvested and seeded into 12-well dishes at a density of 10^5 cells per well. (Haag et al. 2008a).

Cells were first cultured for 24 hrs in presence of 10% FCS, followed by an additional 18-24 hrs under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for a further 24 hrs. At the end, culture medium was removed and cells were washed twice with 4°C

cold PBS followed by 1-2 hrs incubation in 1 mL 20% trichloroacetic acid (TCA) at 4°C. Denatured cells were scraped off, transferred into a reaction tube and centrifuged at 13000 rpm for 10 minutes. The pellet was washed with 1 mL 10% TCA, and centrifuged again at 13000 rpm for 5 minutes and dissolved in 300 µL, 0.2 M NaOH followed by neutralization with 300 µL 0.2 M HCl. 300 µL portions were combined with scintillation cocktail and radioactivity was determined as described in 2.21.1.
In previous experiments it was confirmed by collagenase digestion that total radioactivity incorporated into proteins largely reflects *de novo* synthesis of collagen.

2.21.3 Calculations and Statistical Analysis

Graph Pad InStat program (GraphPad software, San Diego, CA, USA) was used to calculate statistical significance of differences using ANOVA, followed by Dunnet's or Bonferroni's test. All values are mean ± SEM of n experiments. When normal distribution could not be confirmed (i.e. in all experimental series in which one treatment group had less than eight observations), significance of differences was evaluated by the kruskal-wallis one way test followed by Dunn's test.
P value of < 0.05 was accepted as significant. IC50 values were calculated by the use of computer programs (*GraphPad Prism, GraphPad Software, San Diego, CA, USA*). Antagonism was quantified by calculation of –log KB (apparent pA2) values according to Eq.4 given by Furchgott (1972). All graphical representations were generated using the GraphPad Prism software.

3. Results

3.1 β-Adrenoceptors Expression in Human Lung Fibroblasts

Semi-quantitative RT-PCR's were performed to determine the expression pattern of the mRNA encoding for different β-adrenoceptors subtypes in MRC-5 and HEL-299 human lung fibroblast cell line as well as primary human lung fibroblasts. Total RNA was isolated as described in 2.12 and first strand cDNA was synthesized using reverse transcriptase.

In addition, genomic DNA (gDNA) isolated from MRC-5 was used as a positive control to confirm the effectiveness of all primer pairs. One negative control was regularly performed with each PCR assay by using a lacking template DNA sample. As shown in figure 3-1, A-C, the MRC-5 and HEL-299 human lung fibroblasts cell lines, as well as primary cells show clear expression of mRNA encoding β_2-adrenoceptors, whereas no transcripts for β_1 and β_3-adrenoceptors could be detected in those cells. The expression pattern was found to be constant under different culture conditions, as a similar expression pattern was found in cells of different passages.

MRC-5 cells were then chosen for further studies and the expression of β_2-adrenoceptors in these cells was confirmed at protein level by Western blot analysis using a specific commercially available polyclonal antibody for β_2-adrenoceptors. As expected, most of the receptor protein was expressed in the membrane fraction (Fig. 3-1, E).

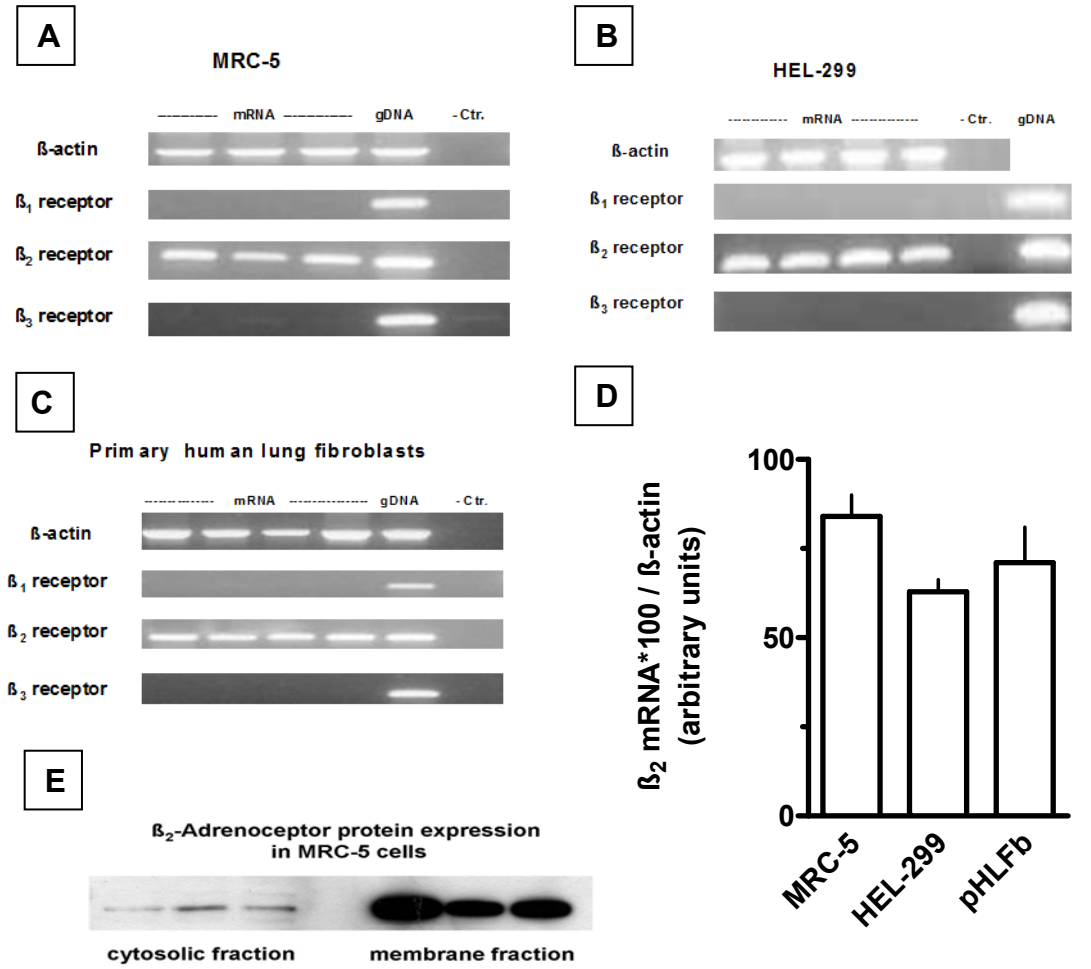


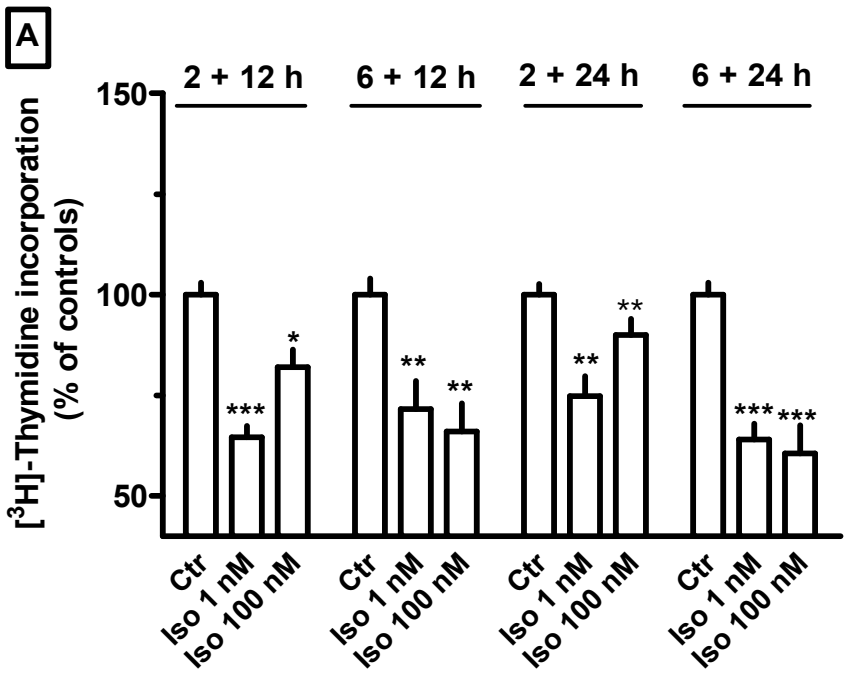
Figure 3-1: β-Adrenoceptors expression in human lung fibroblasts: Samples of RT-PCRs of human β-adrenoceptors (β_1-β_3) on RNA isolated from MRC-5 **(A)**, HEL-299 **(B)**, and primary human lung fibroblasts **(C)**. Cells were grown in presence of 10% FCS in 35 mm culture dishes to confluency, total RNA was extracted, treated with DNase and used for RT-PCR with primers specific for β-actin (23 PCR cycles) or human β-adrenoceptors (35 PCR cycles). Genomic DNA (gDNA) isolated from MRC-5 cells was used as positive control (+Ctr.). One PCR lacking template DNA was regularly performed to exclude any contamination (-Ctr.). PCR products were separated on a 1.2% agarose gel. **(D)**: Densitometrical evaluation of a series of experiments. Given are mean values + SEM of n=4-12. Values were normalized over β-actin to correct for quality of the cDNA preparation. **(E)**: Samples of immunoblots for human β_2-adrenoceptors with protein extracts from MRC-5 human lung fibroblasts, grown in presence of 10% FCS in 55 mm culture dishes to confluency. Cytosolic and membrane proteins were extracted differentially and used for Western blot analysis using a primary antibody directed against human β_2-adrenoceptors.

3.2 Functional Role of β_2-Adrenoceptors in Human Lung Fibroblasts

3.2.1 Effects on Proliferation

Proliferation was measured by determination of [^{3}H]-thymidine incorporation in cell culture.

The first sets of experiments had suggested that β_2-adrenoceptors desensitisation might affect the response levels of the tested β-adrenoceptors agonists. Therefore, in order to examine whether supposed β-adrenoceptors mediated effects on fibroblast proliferation depending on culture conditions and exposure time, the effect of different exposure times was examined. As described in figure 3-2, A and B, tested β-adrenoceptors agonists demonstrated an inhibitory effect on [^{3}H]-thymidine incorporation in MRC-5 cells under all the time schedules tested. Nonetheless, the nethermost variability of the inhibitory effect was detected, when the agonists were added 6 hrs prior to [^{3}H]-thymidine, and lasted together for 24 hrs. Therefore, these culture conditions were used in all following experiments. In several series of experiments the maximum inhibition caused by isoprenaline or formoterol was between about 30% and 40%. Although, the effectiveness of these two agonists was similar, formoterol proved to be markedly more potent (IC_{50} 0.06 nM) than isoprenaline (IC_{50} of 3 nM).



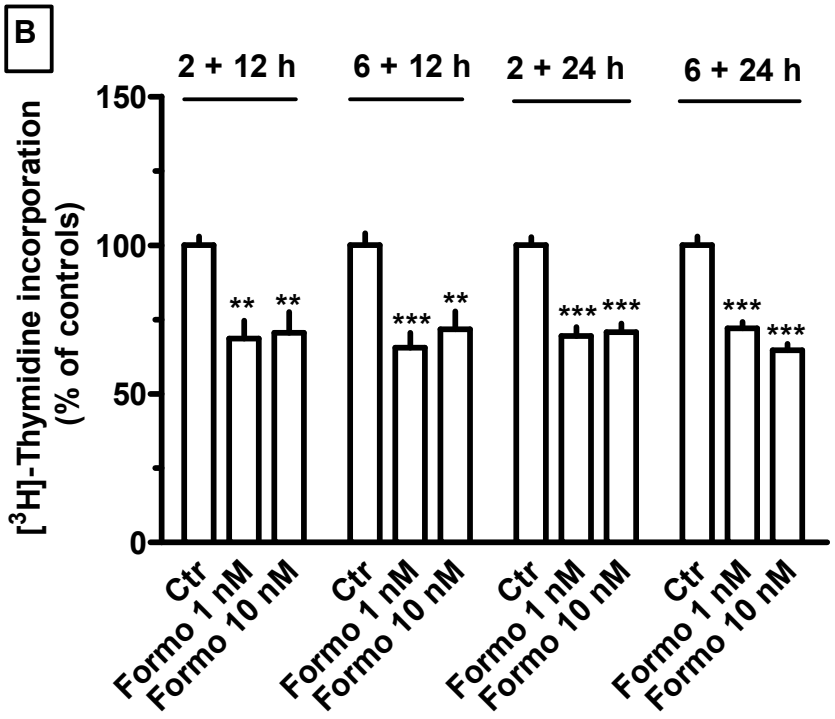


Figure 3-2: Concentration and time dependent effects of isoprenaline (Iso) **(A)**, formoterol (Formo) **(B)**, on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. 4*10^4 cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period test drugs were added as indicated for 14 (2+12 hrs), 18 (6+12 hrs), 26 (2+24 hrs) or 30 (6+24 hrs). [^{3}H]-Thymidine (37 kBq) was present for the last 12 or 24 hrs (i.e. it was added 2 or 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=6-12. Significance of differences:*P<0.05, **P<0.01, ***P<0.001 vs control (Ctr.).

In order to elucidate whether the concentration dependent effect of the β-adrenoceptors agonist formoterol was mediated via β_2-adrenoceptors, interaction experiments with the selective β_2-adrenoceptors antagonist ICI 118,551 and the selective β_1-adrenoceptors antagonist CGP 20712 were performed.

As summarized in figure 3-3, A, the concentration response curve of formoterol was markedly shifted to the right in a concentration-dependent manner by the β_2-adrenoceptors selective antagonist ICI 118,551 (mean apparent pA_2 value: 9.6), but not affected by the β_1-adrenoceptors selective antagonist CGP 2071 (present in the relatively high concentration of 3 μM). Neither ICI 118,551 (0.3 and 3 μM) nor CGP 20712 (3 μM) alone showed any significant effect on [^{3}H]-thymidine incorporation (Fig. 3-3, B).

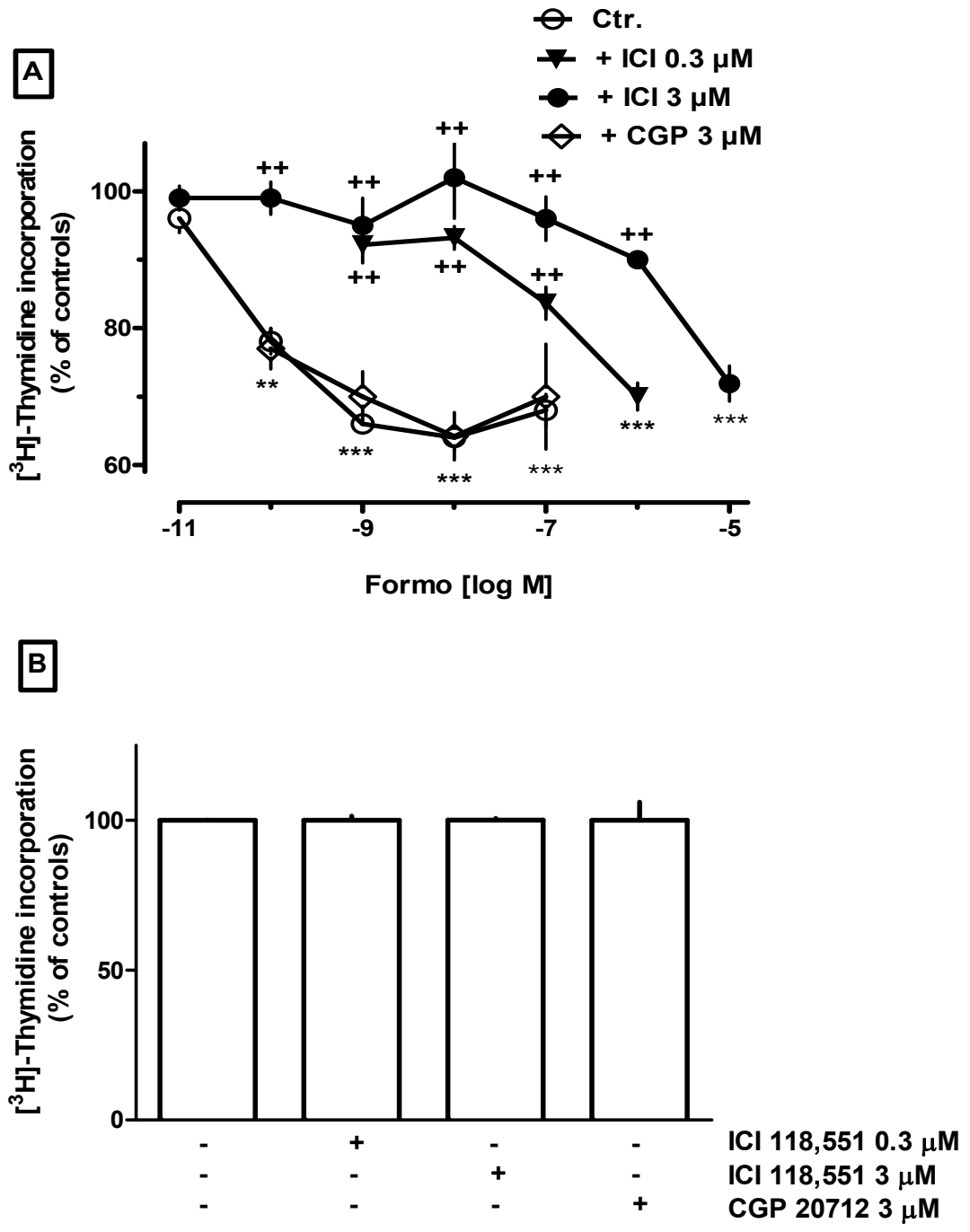


Figure 3-3: Concentration-dependent effects of formoterol in absence or presence of the β_2-adrenoceptors selective antagonist ICI 118,551 (ICI, 0.3 and 3 µM) or the β_1-adrenoceptors selective antagonist CGP 20712 (CGP, 3 µM) **(A)** and the effect of β-adrenoceptors antagonists (β_1 and β_2) **(B)** on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. $4*10^4$ cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period test drugs were added as indicated for 30 hrs where antagonist was added 30 minutes prior agonist. [^{3}H]-Thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means ± SEM of n=6-9. Significance of differences: vs. respective controls (Ctr.); **P<0.01, ***P<0.001; vs. respective value in absence of antagonist, $^{++}$P<0.01.

As shown in figure 3-4, direct stimulation of adenylyl cyclase by 30 µM forskolin caused an inhibition of [^{3}H]-thymidine incorporation by about 70%. It had previously been shown that EP_2 receptors, like β-adrenoceptors, mediate stimulation of adenylyl cyclase and exert a marker anti-proliferative effect in human lung fibroblasts (Haag et al. 2008b), and this was also confirmed in the present study. 100 nM Butaprost caused a reduction of [^{3}H]-thymidine incorporation by about 60%. To elucidate whether prostaglandins released from lung fibroblasts during the experiments interfere with the β-adrenergic mediated inhibition of cells proliferation, an additional set of experiments were performed in which cyclooxygenase was inhibited by addition of 3 µM indomethacin.

As shown in figure 3-5, A and B, indomethacin on its own neither affected the proliferation rate of the cells nor significantly affected the inhibitory effect of isoprenaline. Thus, at least under the present standard culture conditions, any interference with endogenously released prostaglandins can be excluded.

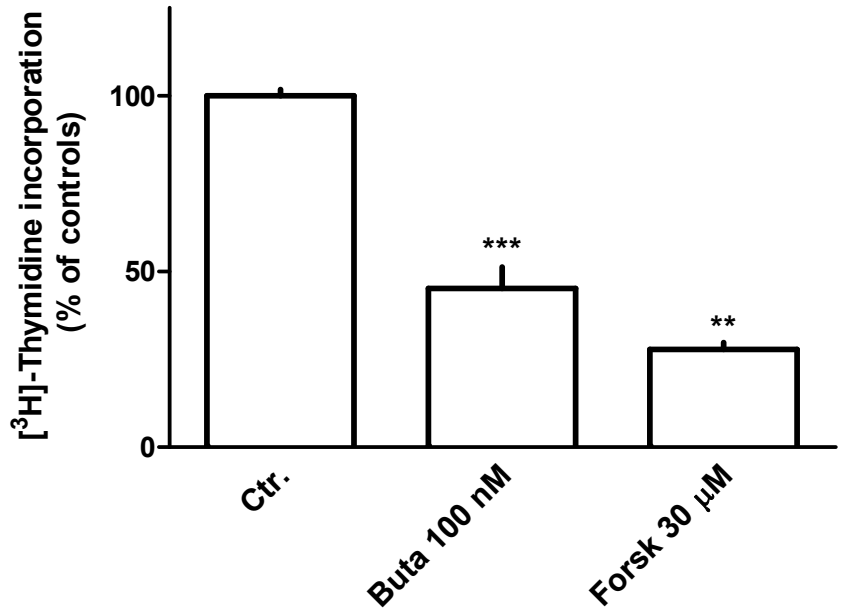


Figure 3-4: Effects of butaprost and forskolin, on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. $4*10^4$ cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period test drugs were added as indicated for 24 hrs [^{3}H]-thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=17-20. Significance of differences: **P<0.01, ***P<0.001 vs control (Ctr.).

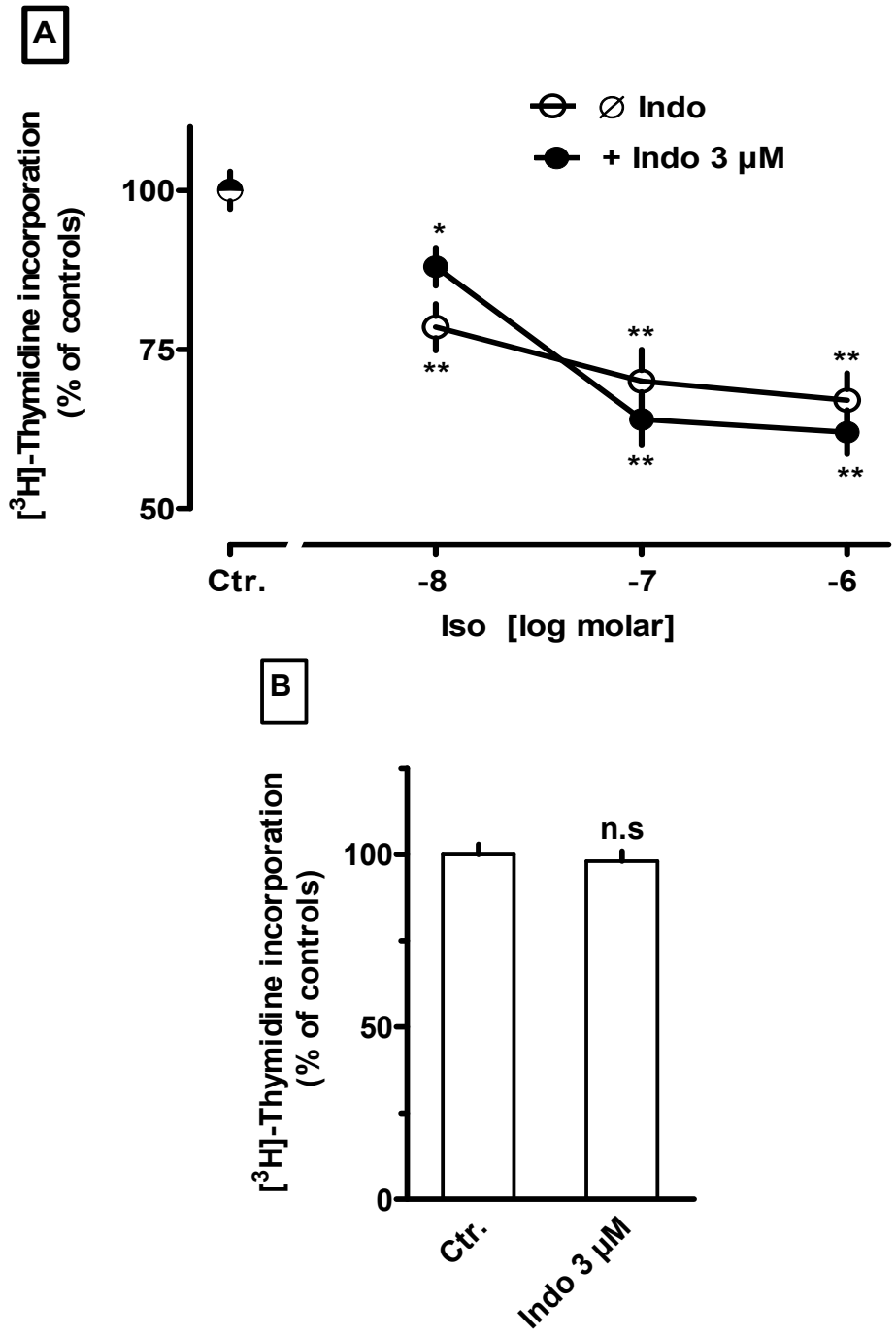


Figure 3-5: Effects of isoprenaline (Iso) **(A)** in absence or presence of indomethacin (Indo) and indomethacin alone **(B)** on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. 4*10^4 cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period test drugs were added as indicated for 30 hrs. [^{3}H]-Thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means ± SEM of n=6-17. Significance of differences: *P<0.05, **P<0.01 vs control (Ctr.); ns = not significant.

Since transforming growth factor (TGF-β) has been widely identified as a key pro-fibrogenic cytokine (see 1.1.3.3) in inflammatory conditions, the effects of TGF-β on cell proliferation and potential interaction with corticosteroids were studied. [^{3}H]-Thymidine incorporation was not significantly affected by 5 ng/mL of TGF-β or 100 nM of budesonide alone or by combined presence of these test substances (Fig. 3-6).

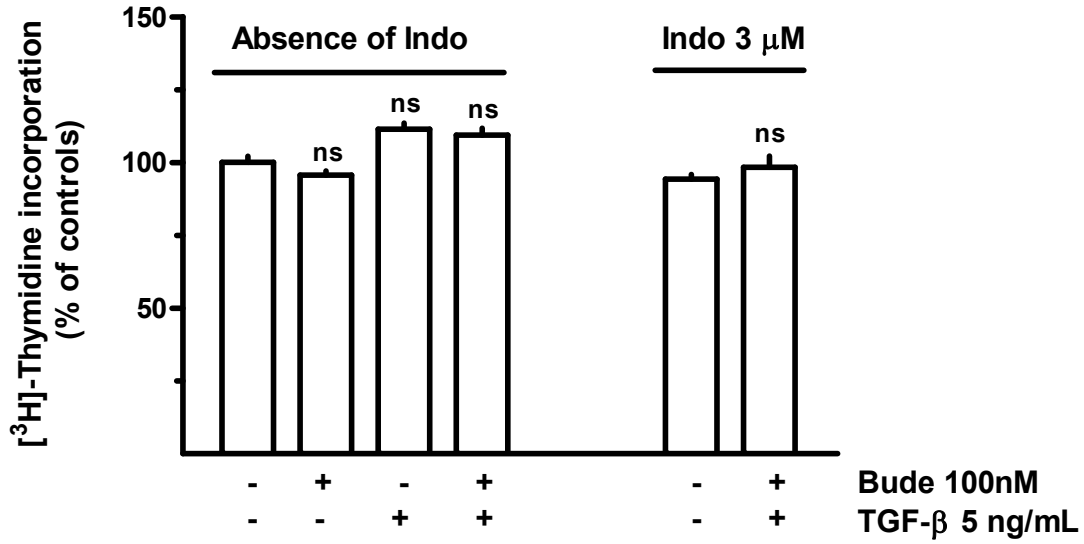


Figure 3-6: Effects of TGF-β (5 ng/mL) and/or budesonide (Bude 100 nM) in absence or presence of indomethacin (Indo 3 μM) on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. $4*10^4$ cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period test drugs were added as indicated for 30 hrs. [^{3}H]-Thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=18-36. Significance of differences: vs control (Ctr.); ns = not significant.

The effect of TGF-β on [^{3}H]-thymidine incorporation inhibitory effect mediated by β_2-adrenoceptors was also investigated in MRC-5 human lung fibroblasts. Cells were incubated with 10 nM formoterol and for comparison reasons with 100 nM of EP_2 receptor agonist butaprost in absence or presence of 5 ng/ml TGF-β for 6 hrs followed by 24 hrs in additional presence of [^{3}H]-thymidine. As summarized in figure 3-7, A and B, incubation of cells with TGF-β induced a loss of the inhibitory effect of formoterol, and the effect was not opposed by additional presence of 100 nM budesonide. Furthermore, the strong inhibitory effect caused by butaprost was also largely

reduced but not completely prevented. These effects of TGF-β were not affected by additional presence of budesonide (Fig. 3-7 B).

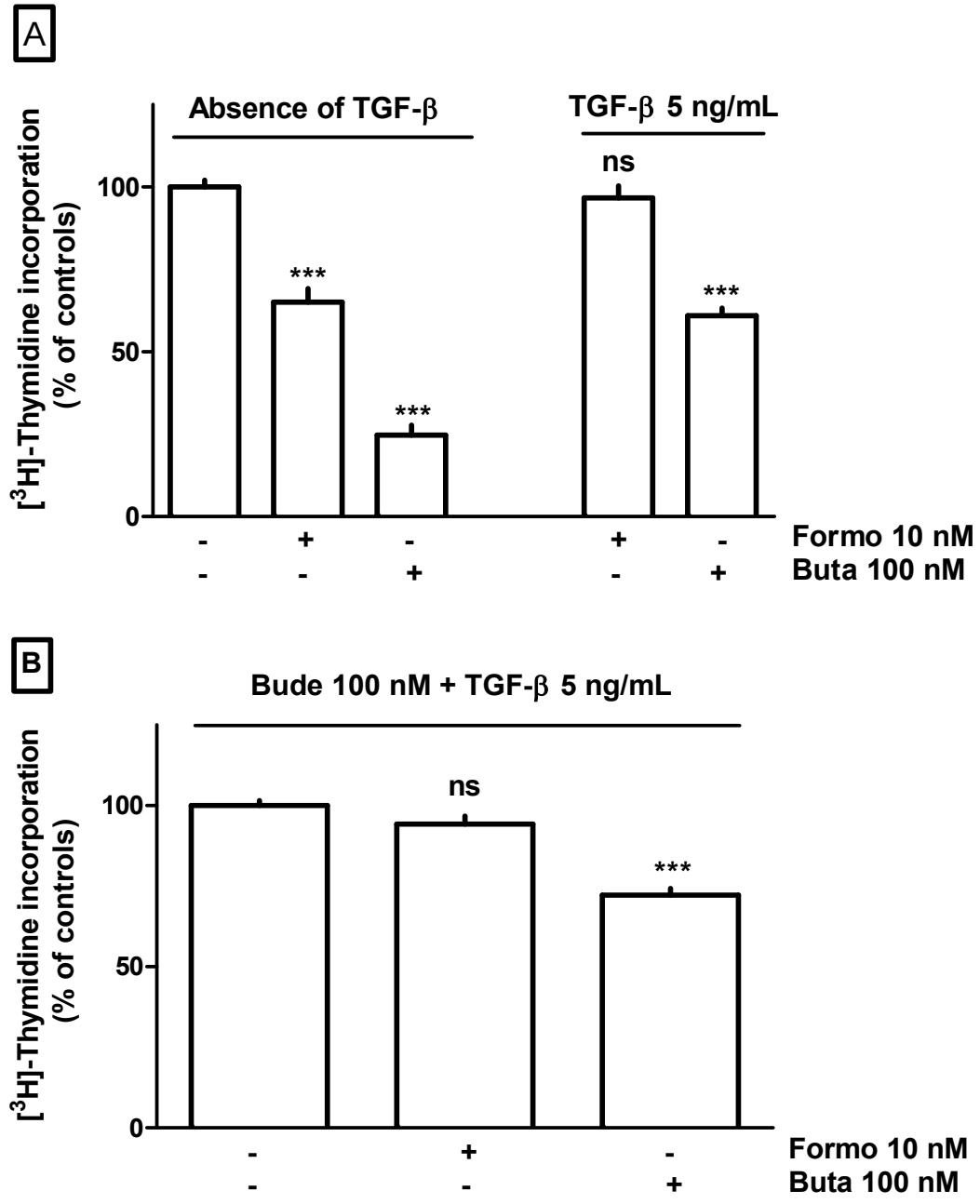


Figure 3-7: Effects of formoterol (Formo 10 nM) and butaprost (Buta 100 nM) in absence or presence of TGF-β 5 ng/mL alone **(A)** or in combination with 100 nM of budesonide **(B)** on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. 4*10^4 cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs FCS-free period formoterol or butaprost were added in absence or in the presence of TGF-β (5 ng/mL) alone or in combination with budesonide (100 nM) as indicated for 30 hrs. [^{3}H]-Thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of the number of experiments indicated. Significance of differences: vs control (Ctr.); ***P<0.001; ns = not significant.

As summarized in figure 3-8, since Th2 cytokines play a crucial role in the pathogenesis of bronchial asthma, it was tested whether IL-4 or IL-13 might affect proliferation of MRC-5 cells or β-adrenoceptors mediated modulation. Neither IL-4 nor IL-13 significantly affects the basal proliferation of MRC-5 cells and the inhibitory effect of formoterol.

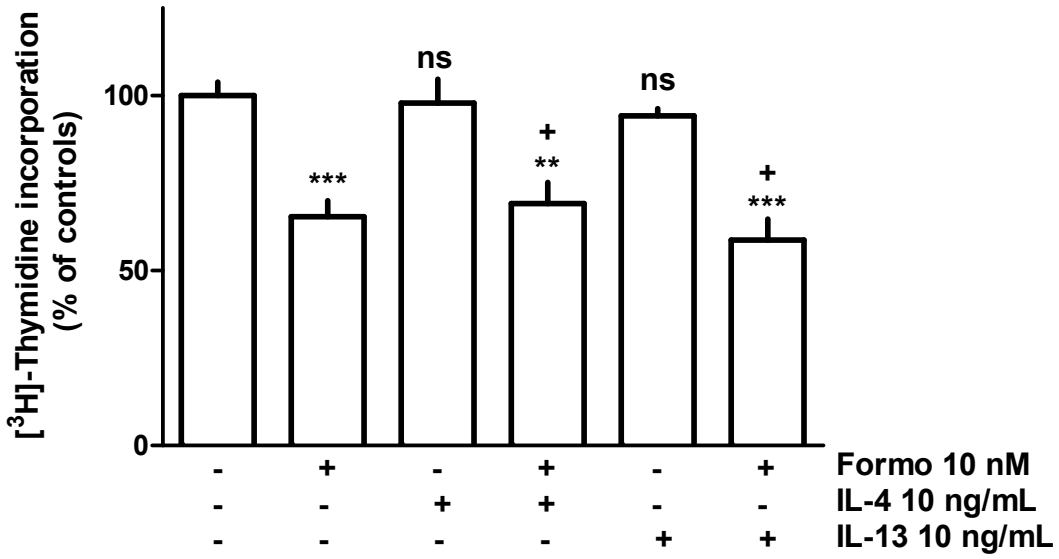
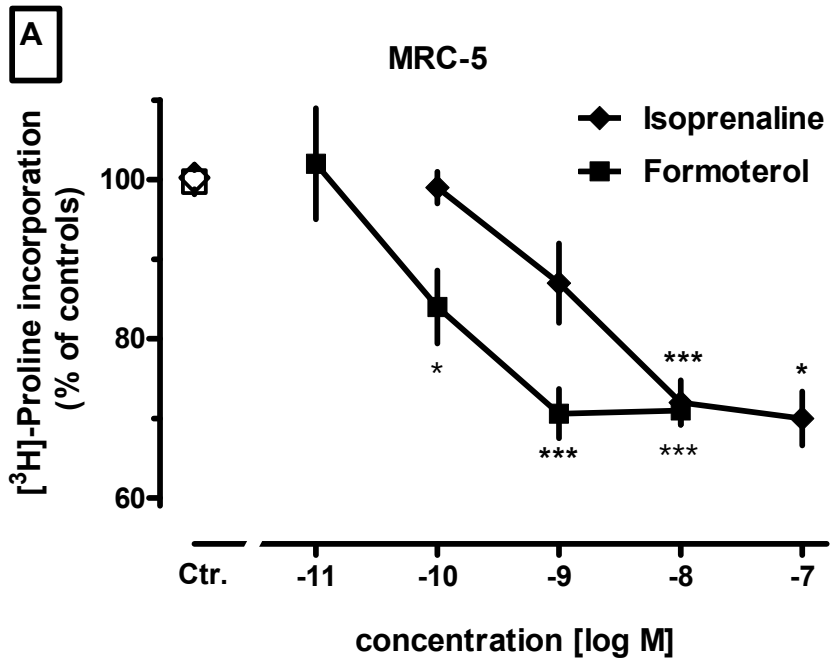


Figure 3-8: Effects of formoterol (Formo 10 nM), and/or IL-4 (10 ng/mL) or IL-13 (10 ng/mL) on [^{3}H]-thymidine incorporation in MRC-5 human lung fibroblasts. $4*10^4$ cells were seeded in 12-well dishes in presence of 10% FCS. After a 24 hrs serum deprived period test drugs were added for 30 hrs. [^{3}H]-Thymidine (37 kBq) was present for the last 24 hrs (i.e. it was added 6 hrs after addition of test drugs). Cellular radioactivity is expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=6-17. Significance of differences: **P<0.01, ***P<0.001 vs control (Ctr.); +P<0.05 vs IL-4; ++P<0.01 vs IL-13; ns = not significant

3.2.2 Effects on Collagen Synthesis

Collagen synthesis was measured by determination of [^{3}H]-proline incorporated in protein during cell culture. Cells were trypsinized, harvested and seeded into 12-well dishes at a density of 10^5 cells per well, cultured for 24 hrs in presence of 10% FCS and allowed to attach, followed by an additional 18-20 hrs under serum deprived conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with different concentration of β-adrenoceptors agonists (isoprenaline and formoterol) as indicated, and incubated for additional 24 hrs. At the end of the experiment the radioactivity incorporated was determined as described previously. As shown in figure 3-9, A and B, [^{3}H]-proline incorporation in MRC-5 and PhLFb was inhibited by both the non-selective β-adrenoceptors agonist isoprenaline and the β_2-adrenoceptors selective agonist formoterol in a concentration-dependent manner. Similar to the observations on [^{3}H]-thymidine incorporation described above, both agonists showed similar effectiveness, but formoterol was substantially more potent (IC_{50} 0.09 nM) than isoprenaline (IC_{50} of 1.3 nM). Additionally, the inhibitory effect of 10 nM formoterol was blocked by the β_2-selective antagonist ICI 118,551(0.3 μM) but was not affected by the β_1-adrenoceptors selective antagonist CGP 20712 (3 μM). In line with the result seen on [^{3}H]-thymidine incorporation neither 0.3 μM ICI 118,551 nor 3 μM CGP 20712 showed significant effects on basal [^{3}H]-proline incorporation (data not shown, each n=6) (Fig. 3-9, C).



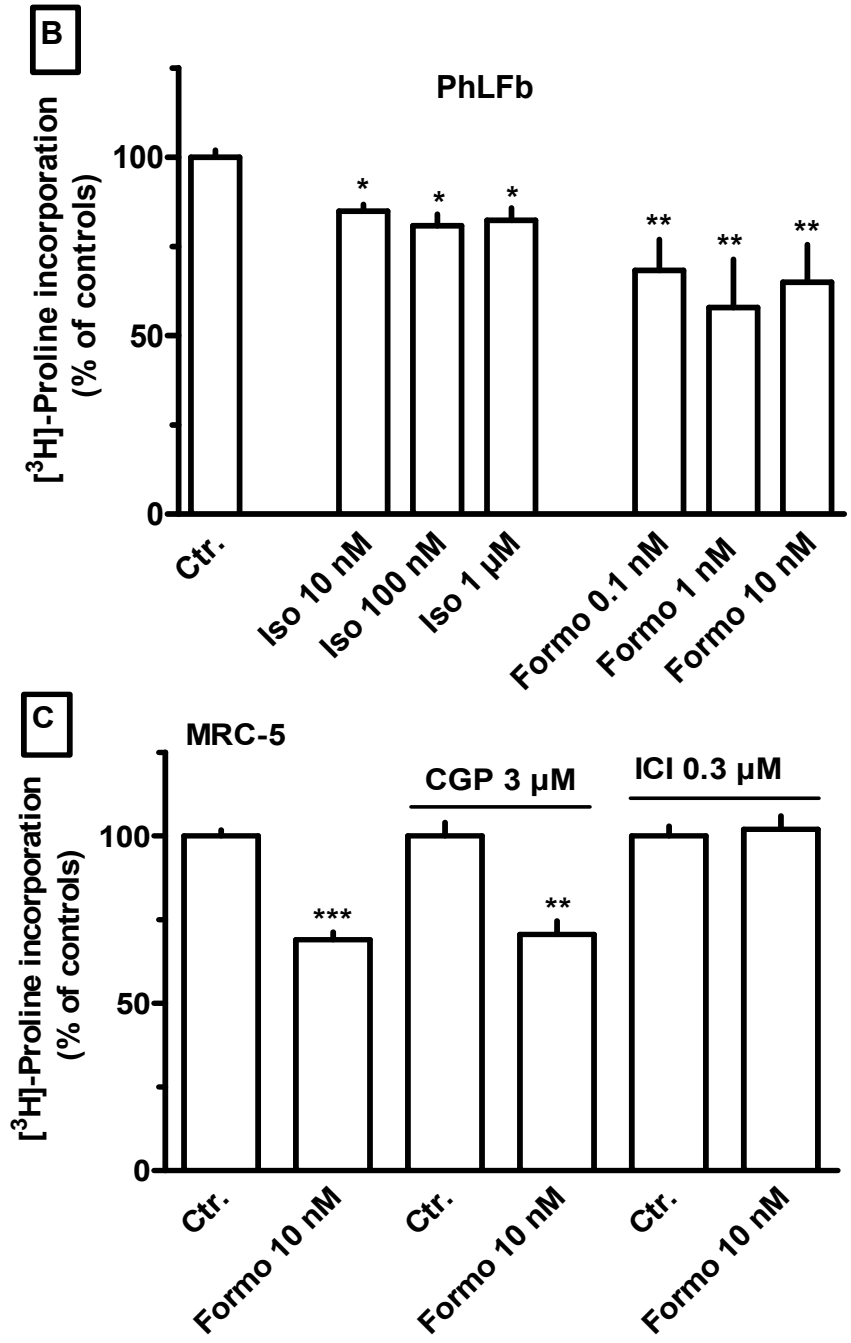


Figure 3-9: Concentration-dependent effects of isoprenaline (Iso 10-100 nM &1 µM) and formoterol (0.1-10 nM) on [^{3}H]-proline incorporation in MRC-5 cell line **(A)** and PhLFb **(B)**. And effect of formoterol (Formo 10 nM) in absence and presence of β_2-adrenoceptors selective antagonist, ICI 118,551 (0.3 µM) or β_1-adrenoceptors selective antagonist, CGP 20712 (3 µM) on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts **(C)**. 10^5 cells were seeded and cultured in 12-well dishes for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived period [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hrs. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=9-18. Significance of differences: vs. control (Ctr.), *P<0.05; **P<0.01; ***P<0.001.

Again, in line with previous observations on proliferation the maximum inhibition of collagen synthesis caused by β-adrenoceptors agonist was significant but smaller than that caused by 30 μM of forskolin, or 100 nM of butaprost (Fig. 3-10).

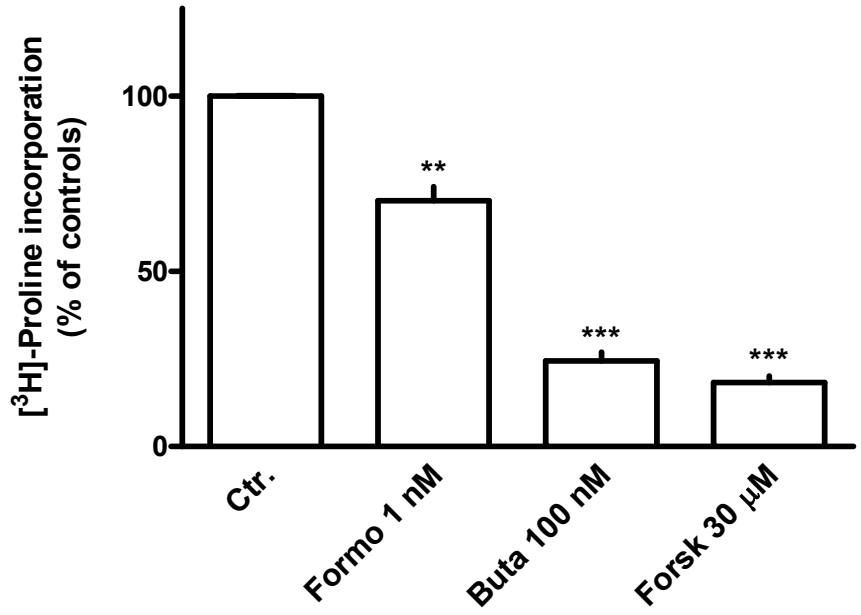
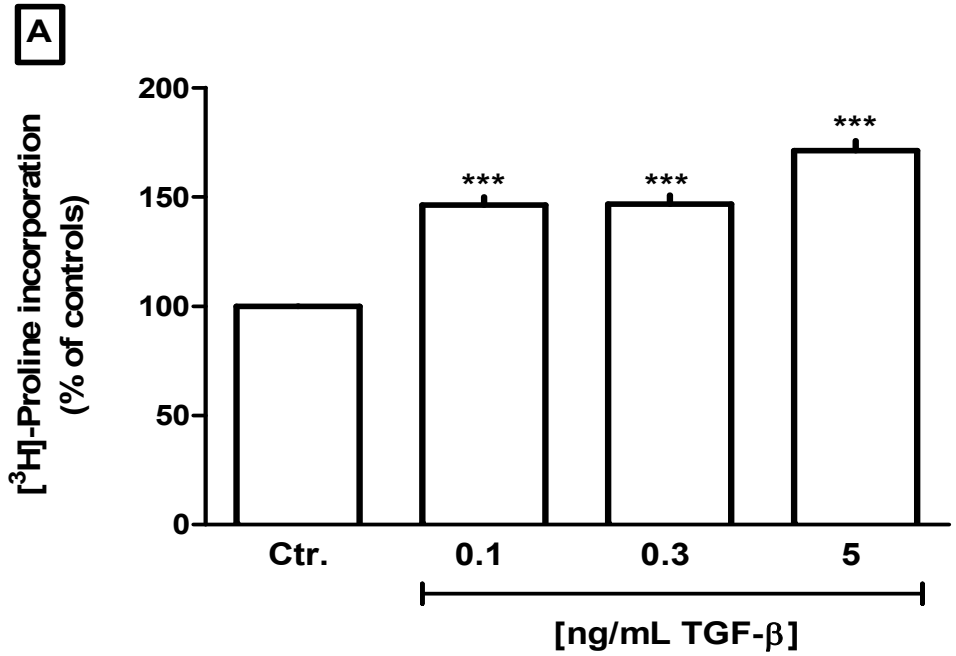


Figure 3-10: Comparison between the effects of formoterol (Formo 1 nM), butaprost (Buta 100 nM), and forskolin (Forsk 30 μM) on [^{3}H]-proline incorporation in MRC-5 cell line. 10^5 Cells were seeded and cultured in 12-well dishes for 24 hrs in presence of 10% FCS, after a 24 hrs serum deprived period [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hrs. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of the number of experiments indicated. Significance of differences: vs. control (Ctr.), **P<0.01; ***P<0.001.

Finally, possible interactions of TGF-β with corticosteroids were studied. As described in figure 3-11, A-D in contrast to the lack of effect of TGF-β on cells proliferation, it caused a marked increase in [^{3}H]-proline incorporation in human lung fibroblasts. Budesonide and dexamethasone, which alone had no significant effect, augment the stimulatory effect of TGF-β on [^{3}H]-proline incorporation (Fig. 3-11, B and C). The strong stimulatory effect of TGF-β in combination with budesonide was preserved in presence of indomethacin (Fig. 3-11, D).


A
[3H]-Proline incorporation
(% of controls)
200
150
100
50
0
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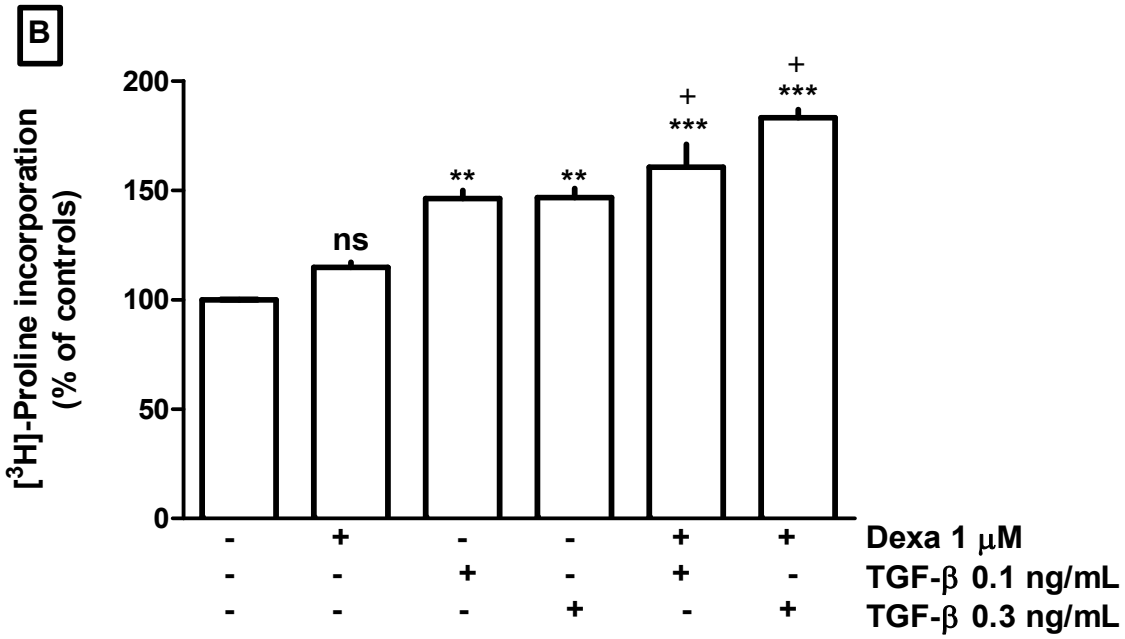
***
***
Ctr.
0.1
0.3
5
[ng/mL TGF-β]


B
[3H]-Proline incorporation
(% of controls)
200
150
100
50
0
ns
**
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Dexa 1 μM
TGF-β 0.1 ng/mL
TGF-β 0.3 ng/mL

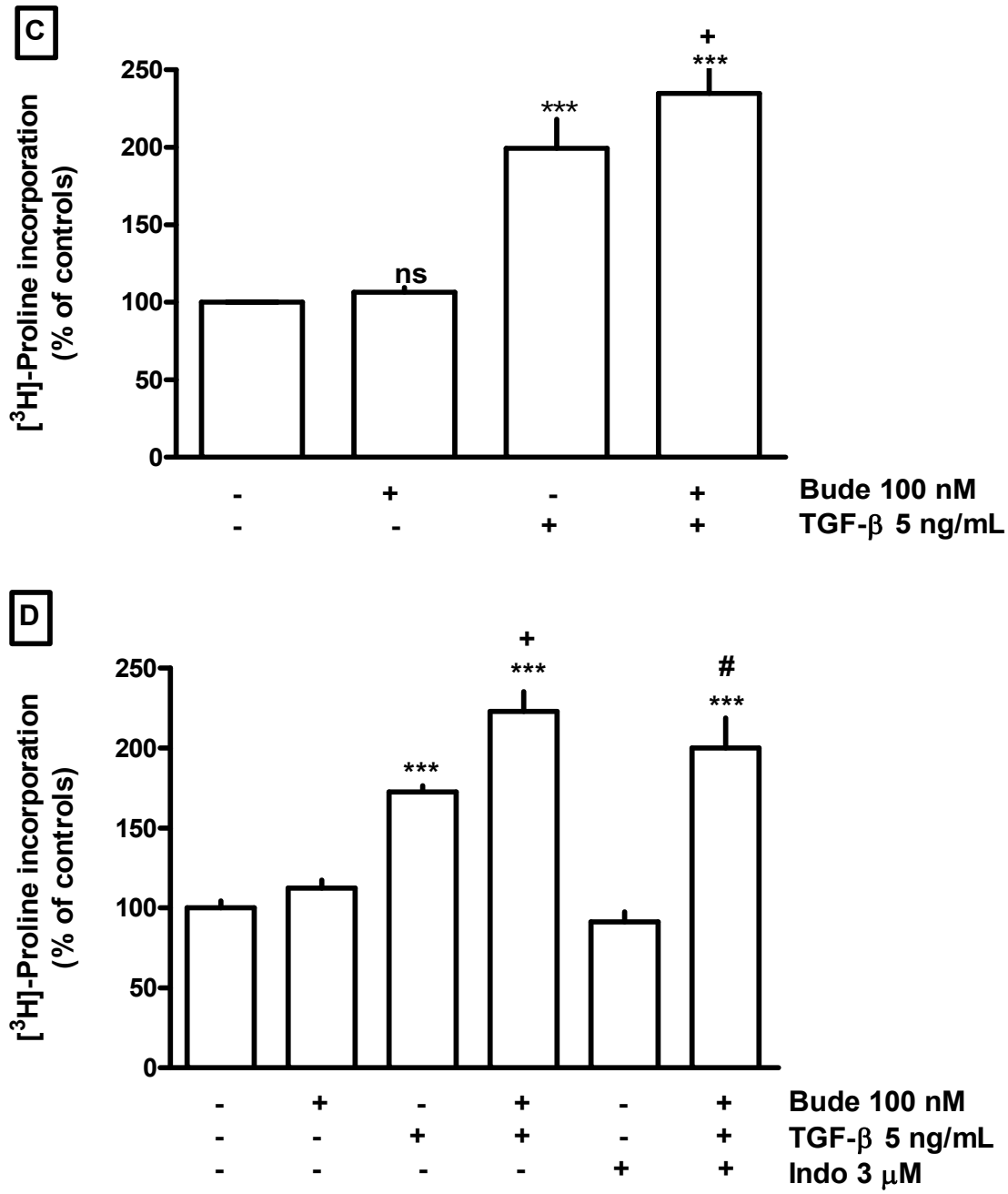
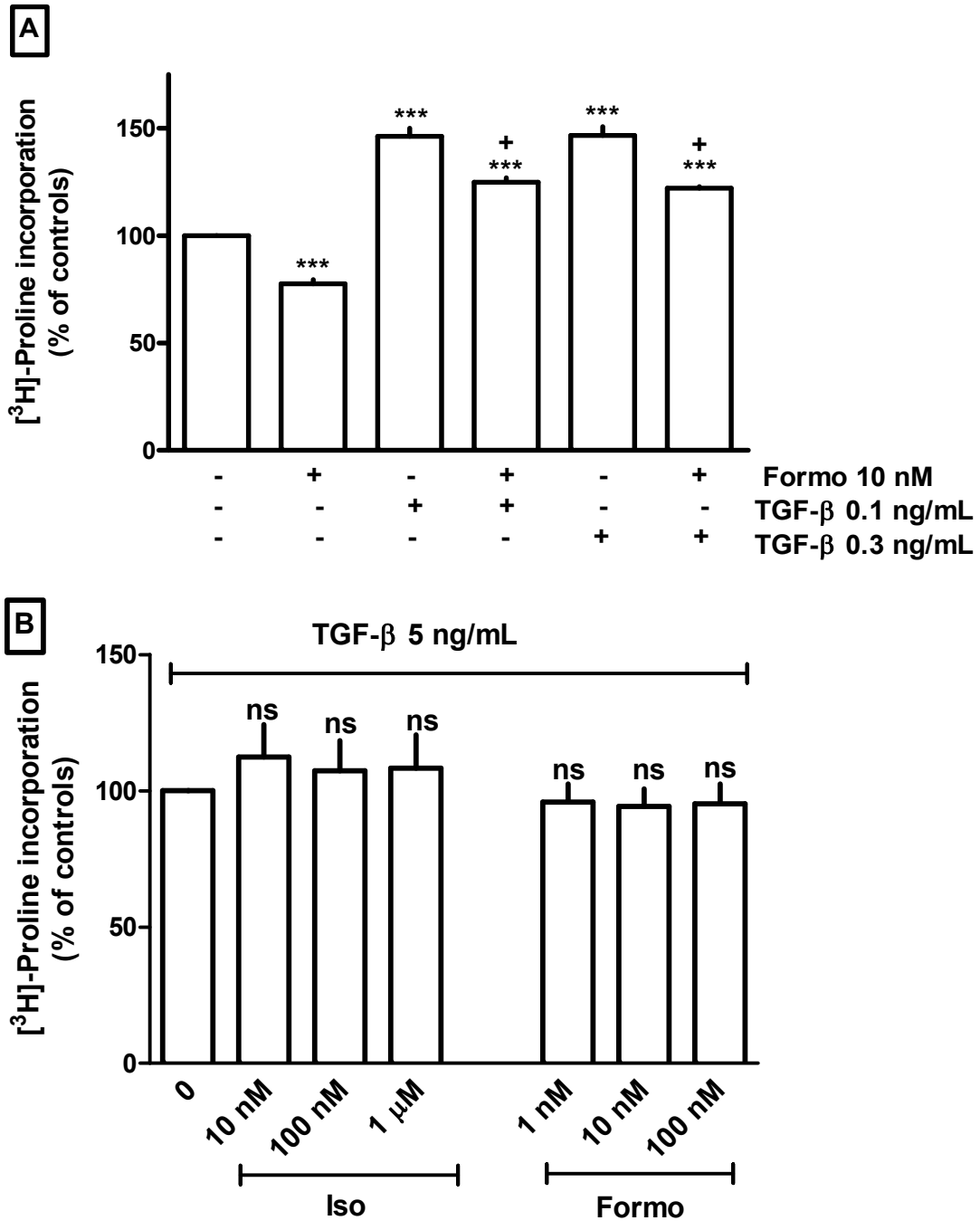


Figure 3-11: Effects of TGF-β (5 ng/mL), dexamethasone (Dexa 1μM), Budesonide (Bude 100 nM) and indomethacin (Indo 3 μM) alone or in combination on [^{3}H] proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded and cultured in 12-well dishes for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived period [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hrs. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of the number of experiments indicated. Significance of differences: **P<0.01; ***P<0.001 vs control (Ctr.); + P< 0.01 vs TGF-β; #P<0.001 vs Indo; ns = not significant vs control (Ctr.).

The inhibitory effect of β-adrenoceptors agonists on proline incorporation in pulmonary fibroblasts was abolished in presence of TGF-β. As shown in figure 3-12, A and B, treatment of cells with TGF-β caused an increase of collagen synthesis which was not significantly affected by addition of different concentrations of β-adrenoceptor agonists. Furthermore, the effect of TGF-β was examined in presence of a formoterol and budesonide combination. Likewise, the stimulatory effect of TGF-β was not affected by additional presence of the combination (Fig. 3-12, C).



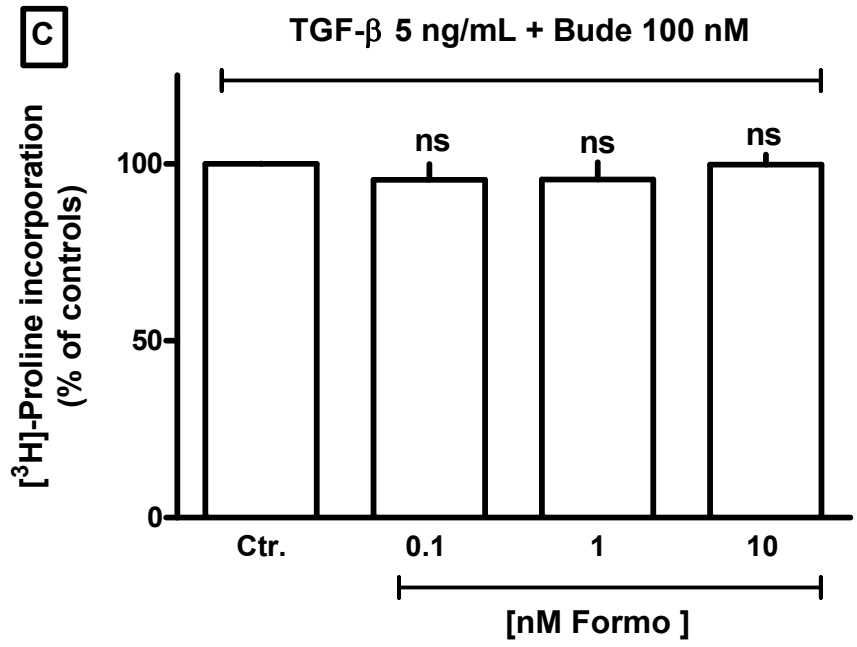


Figure 3-12: Effects of formoterol (Formo 0.1-10 nM), isoprenalin (Iso 10-100 nM &1 μM), TGF-β (5 ng/mL) and budesonide (Bude 100 nM), alone or in combination (as indicated) on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded and cultured in 12-well dishes for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived period [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hrs. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of the number of experiments indicated. Significance of differences: ***P<0.001 vs control (Ctr.); + P< 0.01 vs TGF-β; ns = not significant vs control (Ctr.).

The effect of the Th2 cytokines IL-4 and IL-13 were also tested on proline incorporation and its modulation by β-adrenoceptors agonists. As summarized in figure 3-13, both IL-4 and IL-13 exerted slight inhibitory effects on basal collagen synthesis of MRC-5. Interestingly, additional presence of formoterol with IL-4 caused further reduction in collagen synthesis, and the effect of IL-4 and formoterol appear to be completely additive. Whereas the inhibitory effect of formoterol and IL-13 were not additive, and resulted in an inhibition of proline incorporation by about 25%, an effect similar to that seen in presence of formoterol alone.

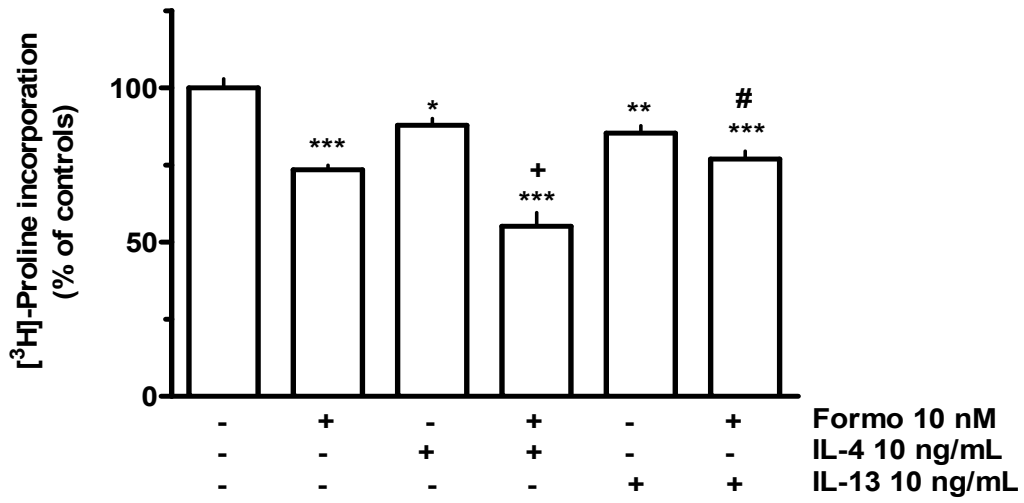


Figure 3-13: Effects of formoterol (Formo 10 nM) and/or IL-4 (10 ng/mL) or IL-13 (10 ng/mL) as indicated on [^{3}H]-proline incorporation in MRC-5 human lung fibroblasts. 10^5 cells were seeded and cultured in 12-well dishes for 24 hrs in presence of 10% FCS, After a 24 hrs serum deprived period [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs and cells were cultured for further 24 hrs. Radioactivity incorporated in cellular and extracellular protein was determined and expressed as % of the mean value of the controls of each cell preparation. Given are means + SEM of n=9-18. Significance of differences: *P<0.05, **P<0.001, ***P<0.001 vs control (Ctr); +P< 0.001 vs IL-4; #P<0.05 vs. IL-13.

3.2.3 Effects on α-Smooth Muscle Actin (α-SMA) Expression

It is already known that α-smooth muscle actin (α-SMA) is a marker of myo-fibroblast differentiation. Therefore the expression of α-SMA in MRC-5 human lung fibroblasts was studied at mRNA level and protein level.

3.2.3.1 Effect on mRNA

As shown in figure 3-14 a clear basal expression of α-SMA at mRNA was detected.

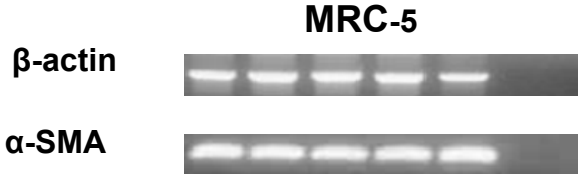
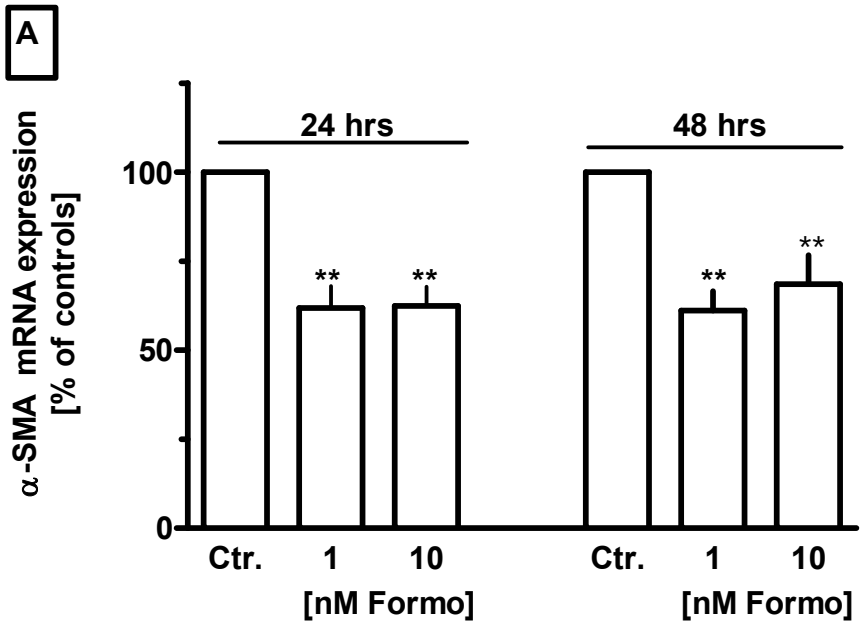


Figure 3-14: Basal expression of α-SMA mRNA in MRC-5 human lung fibroblasts. Cells were grown in presence of 10% FCS in 35 mm culture dishes to confluency, total RNA was extracted, treated with DNase and used for RT-PCR with primers specific for β-actin (23 PCR cycles) or α-SMA (30 PCR cycles). PCR products were separated on a 1.2% agarose gel.

To investigate the effect of formoterol on α-SMA mRNA expression level, MRC-5 were treated with 1 and 10 nM formoterol for 24 and 48 hrs. Real time (qPCR) measurements showed that formoterol induced down-regulation of α-SMA mRNA expression level in a non-concentration or time dependent manner (Fig. 3-15, A).

In order to investigate the effect of TGF-β on α-SMA mRNA expression level, cells were stimulated with various concentrations of TGF-β (0.1, 0.3, and 1 ng/mL) for 48 hrs. TGF-β (1 ng/mL) induced a marked up-regulation of α-SMA mRNA expression level by about 3000-4000% (Fig. 3-15, B). In presence of TGF-β, the inhibitory effect of formoterol (1 nM, a maximum effective concentration) on α-SMA mRNA expression was abolished (Fig. 3-15, C).



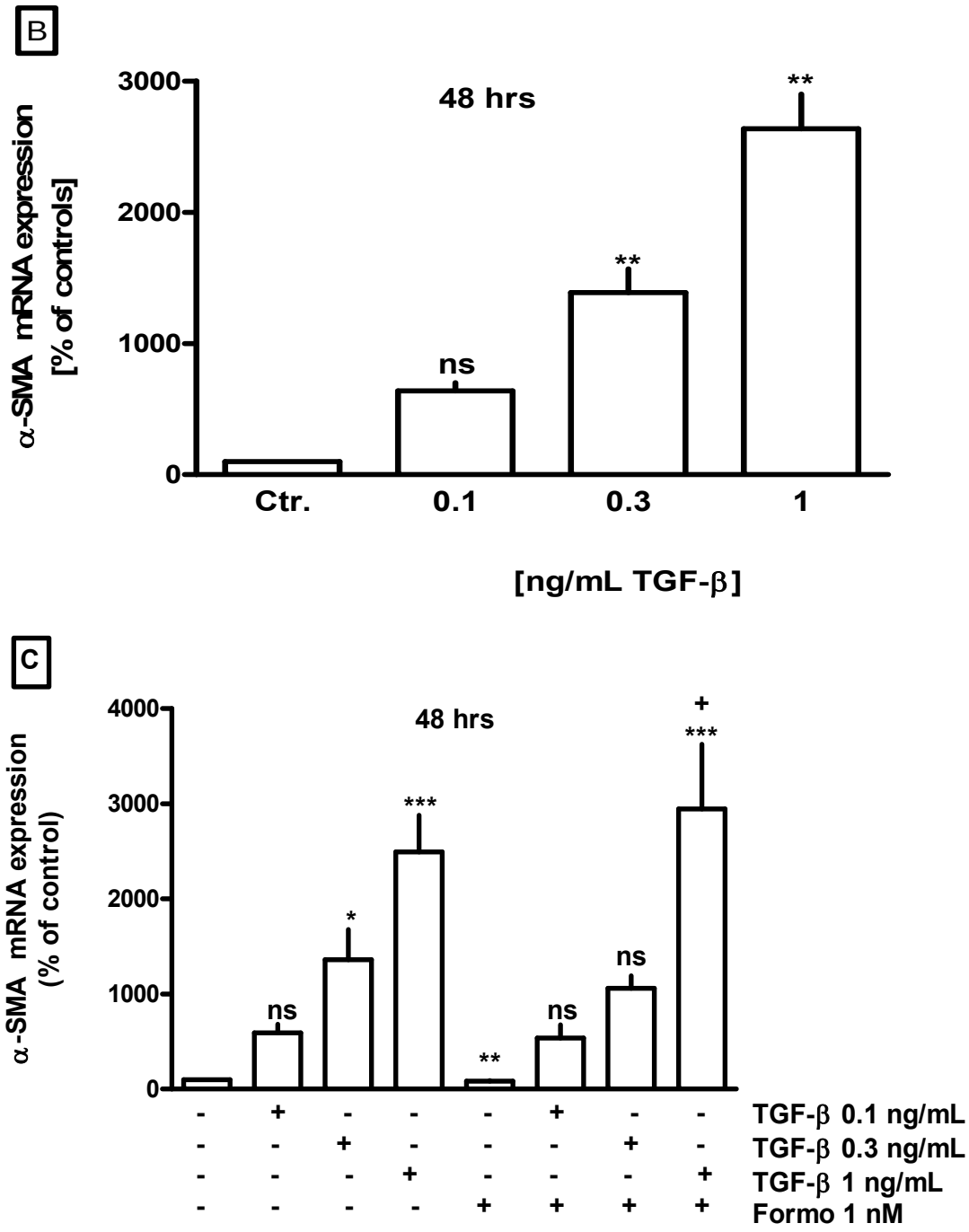
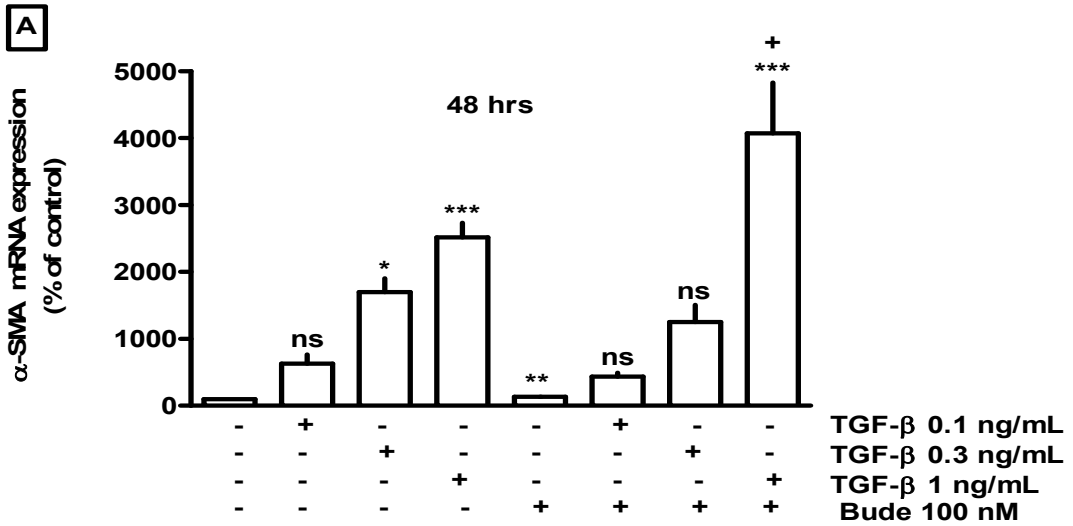
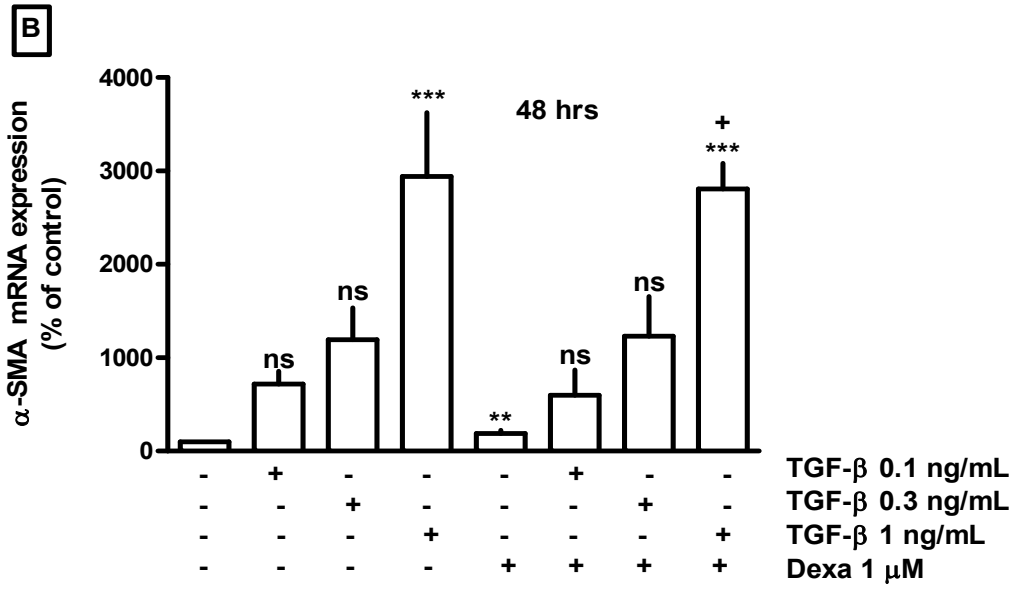


Figure 3-15: Effects of formoterol (Formo 1 nM) (**A**), TGF-β (0.1, 0.3 & 1ng/ml) (**B**) and their combinations (**C**) on α-SMA expression in MRC-5 human lung fibroblasts. $3.5*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived period, cells were cultured for additional 24-48 hrs in serum deprived medium in absence or presence of test substances. RNA was extracted, DNase-treated and used for RT-qPCR. α-SMA mRNA normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-9, significance of differences: *P<0.05, **P<0.001, ***P<0.001 vs control (Ctr); +P< 0.001 vs Formo; ns = not significant.

To elucidate whether corticosteroids affect the marked TGF-β induced up-regulation of α-SMA mRNA, cells were stimulated with various concentrations of TGF-β in absence or presence of 100 nM of budesonide or 1μM of dexamethasone for 48 hrs. Both budesonide and dexamethasone induced up-regulation of α-smooth muscle actin mRNA expression level by about 33% and 86% respectively, whereas the marked up-regulation induced by TGF-β was not significantly affected in presence of budesonide or dexamethasone (Fig. 3-16 A-C).





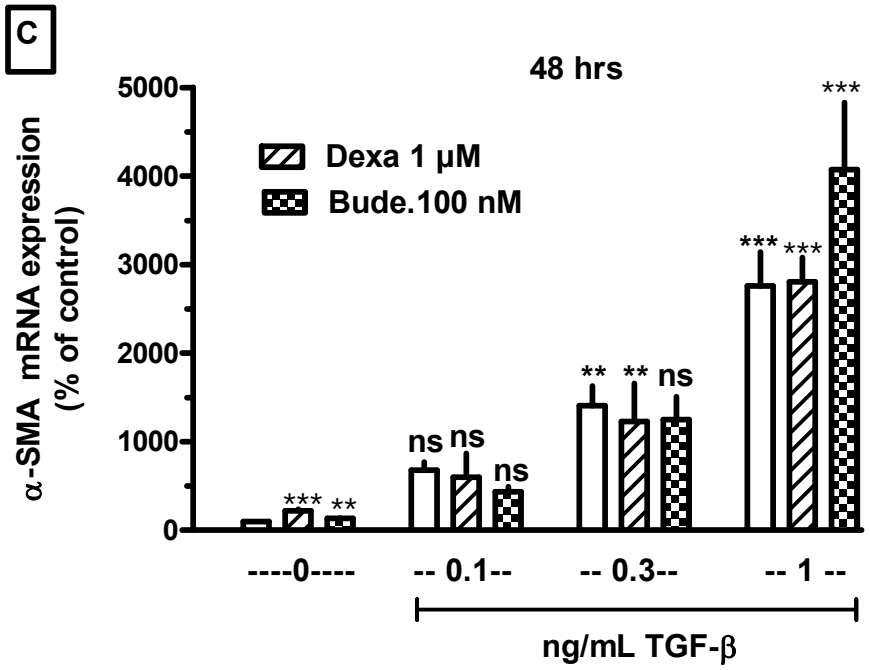
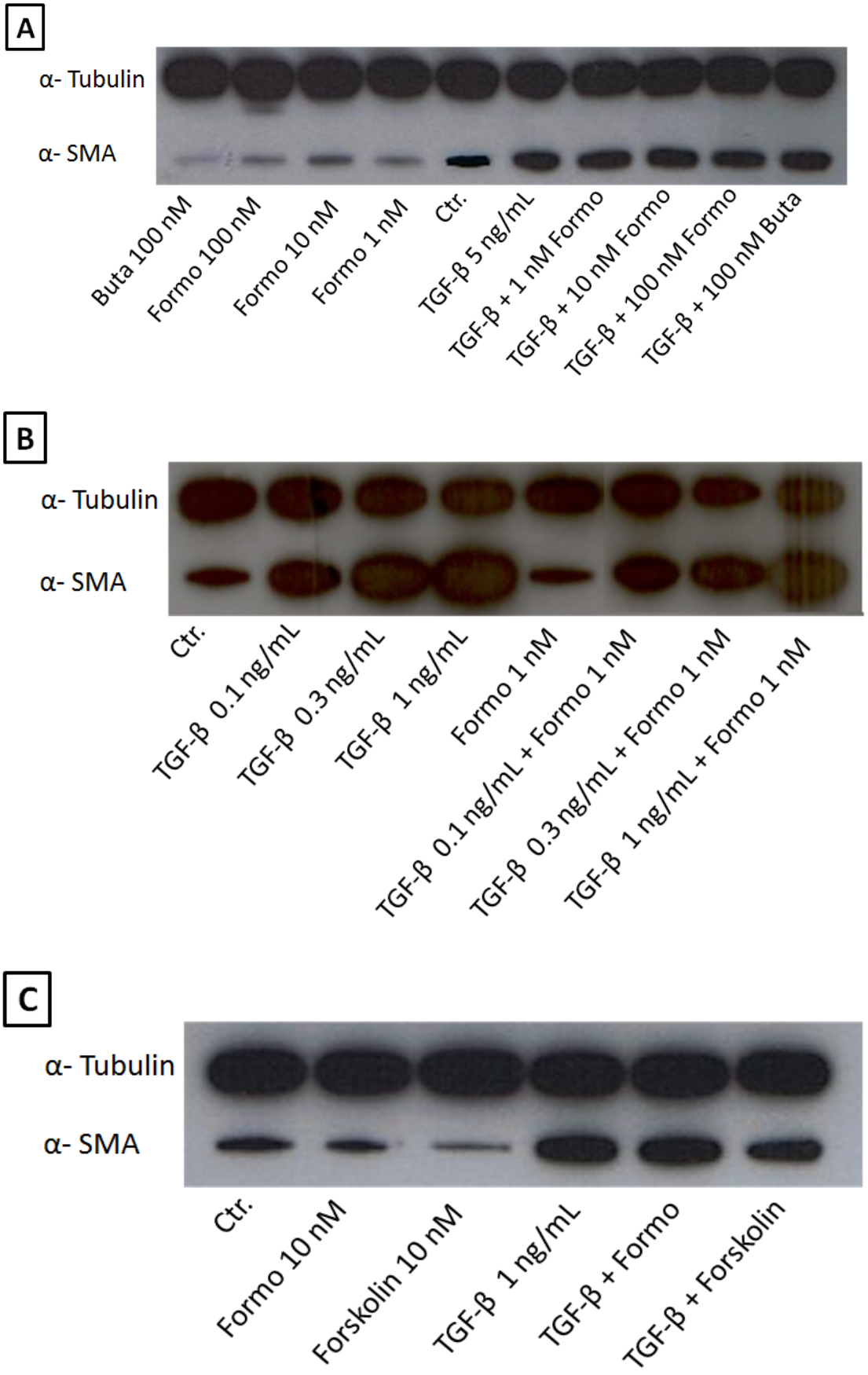


Figure 3-16: Effects of TGF-β (0.1-1 ng/mL) in absence and presence of dexamethasone (Dexa 1µM) and Budesonide (Bude 100 nM) on α-SMA mRNA expression levels in MRC-5 human lung fibroblasts. $3.5*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs FCS-free period, cells were cultured for additional 24-48 hrs in serum-deprived medium in absence or presence of test substances. RNA was extracted, DNase-treated and used for RT-qPCR. α-SMA mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-6, significance of differences:*P<0.05, **P<0.001, ***P<0.001 vs control (Ctr); +P< 0.001 vs Bude and Dexa; ns = not significant.

3.2.3.2 Effects on Protein

Western blot analysis was carried out to investigate and confirm the effect of formoterol on α-SMA protein expression level, either alone or in combination with TGF-β. Incubation of cells with various concentration of formoterol (1-100 nM) induced a reduction in α-SMA protein expression level in non-stimulated cells (under control culture conditions) by about 50% (Fig. 3-17, A-D). For comparison 10 µM of the direct activator of AC forskolin, or 100 nM of the EP_2 receptor agonist butaprost caused a reduction by about 60% and 70% respectively (Fig. 3-17, A and C). However, the TGF-β induced increase of α-SMA protein expression was not prevented or opposed by formoterol (1-10 nM). Similarly, the strong inhibitory effects of forskolin and butaprost were also abolished in cells treated with TGF-β (Fig. 17, A-C).


A
α- Tubulin
α- SMA
Buta 100 nM
Formo 100 nM
Formo 10 nM
Formo 1 nM
Ctr.
TGF-β 5 ng/mL
TGF-β + 1 nM Formo
TGF-β + 10 nM Formo
TGF-β + 100 nM Formo
TGF-β + 100 nM Buta
B
α- Tubulin
α- SMA
Ctr.
TGF-β 0.1 ng/mL
TGF-β 0.3 ng/mL
TGF-β 1 ng/mL
Formo 1 nM
TGF-β 0.1 ng/mL + Formo 1 nM
TGF-β 0.3 ng/mL + Formo 1 nM
TGF-β 1 ng/mL + Formo 1 nM
C
α- Tubulin
α- SMA
Ctr.
Formo 10 nM
Forskolin 10 nM
TGF-β 1 ng/mL
TGF-β + Formo
TGF-β + Forskolin

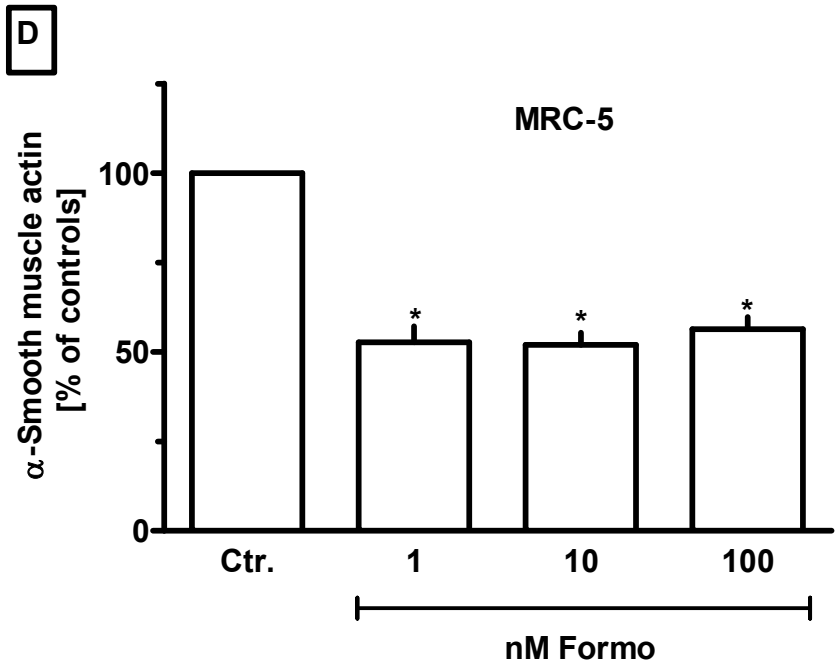


Figure 3-17: Effects of formoterol (Formo 1-100 nM), butaprost (Buta 100 nM) forskolin (Forsk 10 µM) and TGF-β (0.1-5 ng/mL) alone or in the given combinations on α-smooth muscle actin (α-SMA) expression in MRC-5 human lung fibroblasts. $2*10^5$ cells were seeded in 12-well dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived medium period, cells were cultured for additional 24 hrs in serum deprived medium in absence or presence of test drugs (at the concentrations indicated). Cellular proteins were extracted and Western blot analysis for α-SMA and α-tubulin (α-Tub) was performed. **(A-C):** Samples of original Western blots. **(D):** Densitometric analysis of a series of Western blots, ratio α-SMA/α-Tub, expressed as % of controls, means + SEM of n=4. Significance of differences: vs controls (Ctr.), *P<0.05.

Pulmonary fibrosis is a disease characterized by the formation of aggregates of myofibroblasts, which play an important role in tissue remodelling occurring in lung fibrosis. In order to investigate the effect of budesonide as an anti-inflammatory substance on TGF-β induced increase of α-SMA protein expression, western blot analysis was carried out. As showed in figure 3-18, A, incubation of cells with formoterol (10 nM) or/and budesonide (100 nM) shows inhibition of protein expression level, Western blot analysis showed that both budesonide and formoterol, either alone or in combination, induced a decrease in α-SMA protein expression by about 30-50%. However, neither budesonide nor their combinations with formoterol were able to oppose the TGF-β induced increase in α-SMA protein expression (Fig. 3-18, A-B).

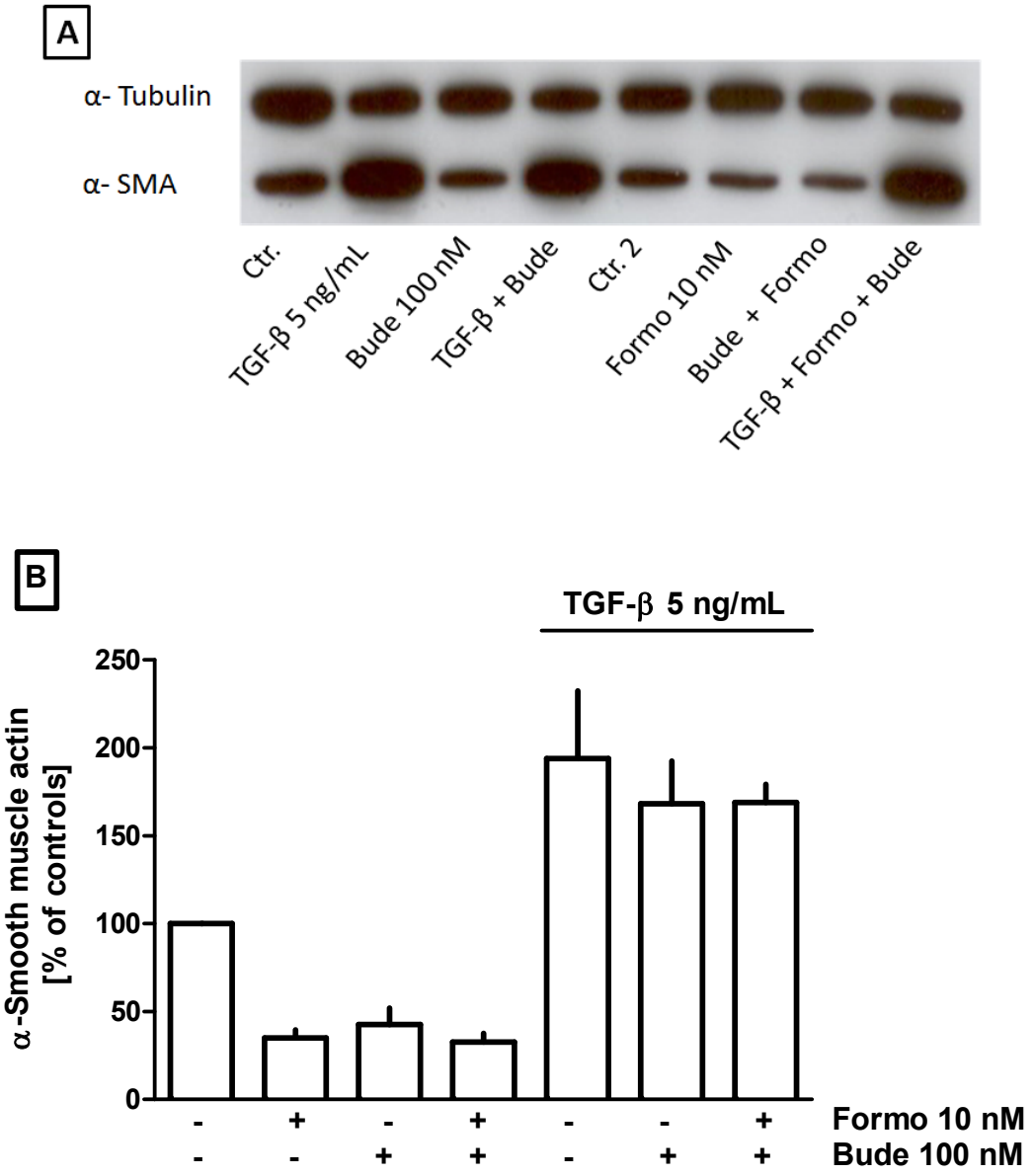


Figure 3-18: Effects of formoterol (Formo 1-10 nM), budesonide (Bude 100 nM) and TGF-β (5 ng/mL) alone or in the given combinations on α-SMA expression in MRC-5 human lung fibroblasts. 2-3.5*10^5 cells were seeded in 6-well dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived medium period, cells were cultured for additional 24 hrs in serum deprived medium in absence or presence of test drugs. Cellular proteins were extracted and Western blot analysis for α-SMA and α-tubulin was performed (**A):** Samples of original Western blots. (**B):** Densitometric analysis of a series of Western blots, ratio α-SMA/α-Tub, expressed as % of controls, means + SEM of n=3.

3.2.4 Effects of Selective Epac Agonist, PKA Agonist and their Combination on α-Smooth Muscle Actin (α-SMA) Expression

3.2.4.1 Effects on mRNA

To investigate the effect of cAMP effectors, Epac and PKA agonists, each alone or in combination, cells in serum-deprived medium were treated with 300 µM of the selective Epac (8-CPT-2'-O-Me-cAMP) and 500 µM selective PKA (6-Bnz-cAMP), either alone or in combination for 24 hrs. RT-qPCR measurements indicated that both Epac and PKA agonists caused significant reduction of α-SMA mRNA expression level in MRC-5 human lung fibroblasts by about 35% and 50% respectively, whereas the combination of both drugs tended to be stronger and caused a reduction of expression by about 59% (Fig. 3-19).

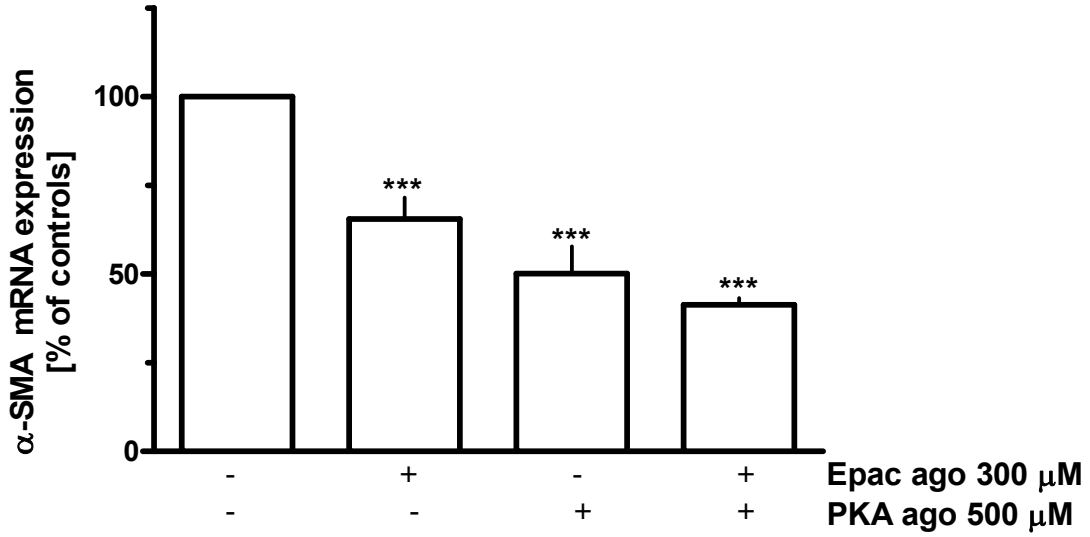


Figure 3-19: Effects of the selective PKA (6-Bnz-cAMP, 500 µM) and/or Epac (8-CPT-2'-O-Me-cAMP, 300 µM) agonist (ago) on α-SMA expression in MRC-5 human lung fibroblasts. $3*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs FCS-free period, cells were cultured for additional 24 hrs in FCS-free medium in absence or presence of test substances. RNA was extracted, DNase-treated and used for RT-qPCR. α-SMA mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-9. Significance of differences vs. control: ***p<0.001.

3.2.4.2 Effects on Protein

Moreover, the effect of selective activators of cAMP effectors, Epac and PKA agonists and their combination on α-SMA protein expression was also studied. Interestingly, similar to the effect observed at the mRNA level, analysis of protein by Western blot showed a reduction in expression of protein level of α-SMA induced by Epac and PKA by about 22% and 40% respectively, while the combination of both leaned towa-rds stronger reduction effect by about 55% (Fig. 3-20, A and B).

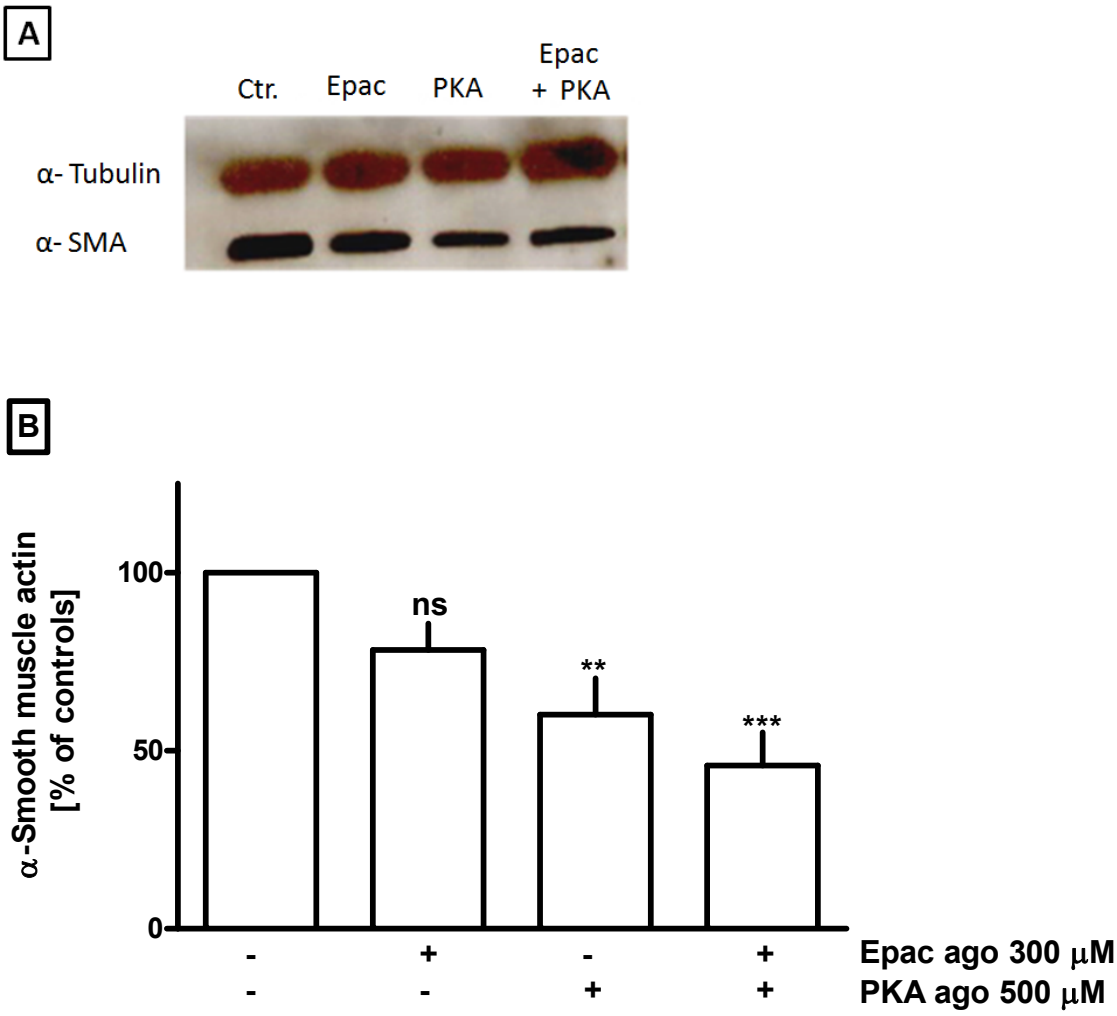


Figure 3-20: Effects of the selective PKA (6-Bnz-cAMP, 500 µM) and/or Epac (8-CPT-2'-O-Me-cAMP, 300 µM) agonist (ago) on α-SMA expression in MRC-5 human lung fibroblasts. $2.5*10^5$ cells were seeded in 6- well plates and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs serum deprived period, cells were cultured for additional 24 hrs in serum deprived medium in absence or presence of test substances. Cellular proteins were extracted and Western blot analysis for α-SMA and α-tubulin was performed (**A**): Samples of original Western blots. (**B**): Densitometric analysis of a series of Western blots, ratio α-SMA/α-tubulin, expressed as % of controls. Given are means + SEM of n=3-7. Significance of differences vs. control: **p<0.01; ***p<0.001; ns = not significant.

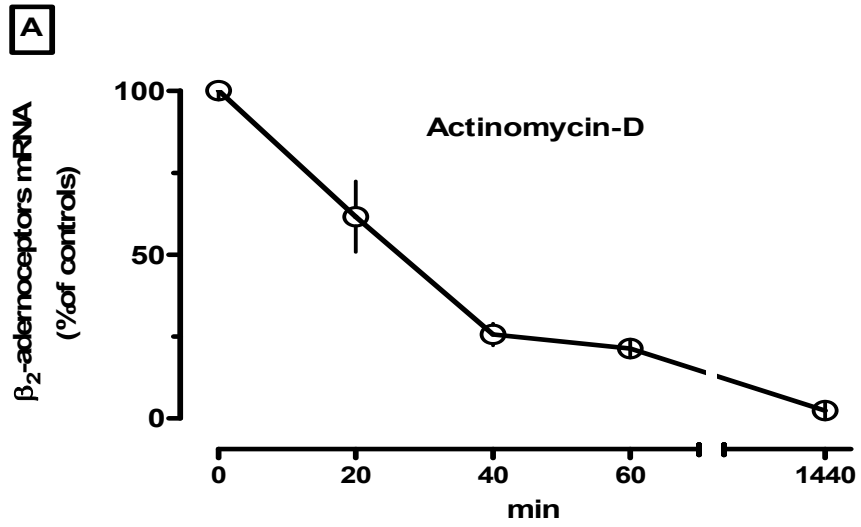
3.3 Regulation of β_2-Adrenoceptors Expression in MRC-5 Human Lung Fibroblast Cells

3.3.1 Stability of β_2-Adrenoceptors mRNA in MRC-5 Human Lung Fibroblasts

To find out the stability (half-life) of transcribed β_2-adrenoceptors mRNA, cells were exposed to actinomycin D (30 µM) for the indicated time. As described in figure 3-21, A, after inhibition of de-novo RNA synthesis by actinomycin D, β_2-adrenoceptors mRNA showed a rapid decline, with a half-life of about 25 minutes.

The effect of inhibition of protein synthesis was also studied to explore whether the up-regulation of β_2-adrenoceptors mRNA involves new protein synthesis. As described in figure 3-21, B, inhibition of protein synthesis by cycloheximide (30 µM) resulted in a rapid, marked increase in β_2-adrenoceptors mRNA level by about 5 fold within 1.5 hrs and only slightly higher after 4 and 6 hrs.

The effect of cycloheximide is largely attenuated after inhibition of de novo RNA synthesis. The reduced β_2-adrenoceptors mRNA levels caused by actinomycin D were only slightly increased (42 ± 21%, n=3) (data not shown) by cycloheximide after 1.5 hrs, indicating that the large increase of β_2-adrenoceptors mRNA caused by cycloheximide is mainly the result of an increased transcription rather than increased stability of mRNA.



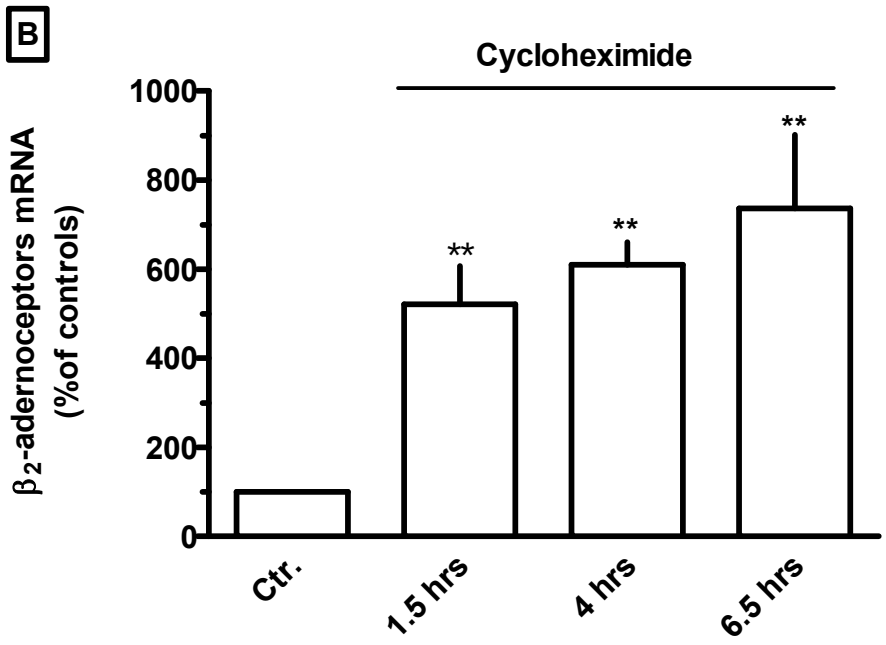


Figure 3-21: Time-dependent effects of actinomycin D (30 µM) **(A)** and cycloheximide (30 µM) **(B)** on β_2-adrenoceptors mRNA expression in MRC-5 human lung fibroblasts. $3*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS. After 24 hrs FCS-free period, cells were cultured for additional 24 hrs in FCS-free medium. Subsequently, the tested substances were added according to the described time. RNA was extracted, DNase-treated and used for RT-qPCR.β_2-adrenoceptors mRNA normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n = 4-6. Significance of differences vs. control: **p<0.01.

3.3.2 Effects of β-Adrenoceptors Agonists and their Interaction with β-Adrenoceptors Antagonists

The effect of β-adrenoceptors agonists on β_2-adrenoceptors mRNA was studied. Incubation of MRC-5 human lung fibroblasts with β-adrenoceptors agonists showed time-dependent dual effects on β_2-adrenoceptors mRNA expression. As summarized in figure 3-22, A, formoterol (1 nM) resulted in a significant rapid sharp increase in β_2-adrenoceptors mRNA already after 20 minutes, and a maximal increase by about 130% was observed within 1 h. This effect disappeared gradually within 2 hrs and an inhibition of about 55% was seen after 4 hrs, but also this effect was lost over time and an increase in expression was again seen after prolongation of exposure time to 48 hrs.

The stimulatory effects of β-adrenoceptors agonists were also mimicked by direct activation of adenylyl cyclase by exposure to forskolin (10 μM) for 1 h (Fig. 3-22, B).

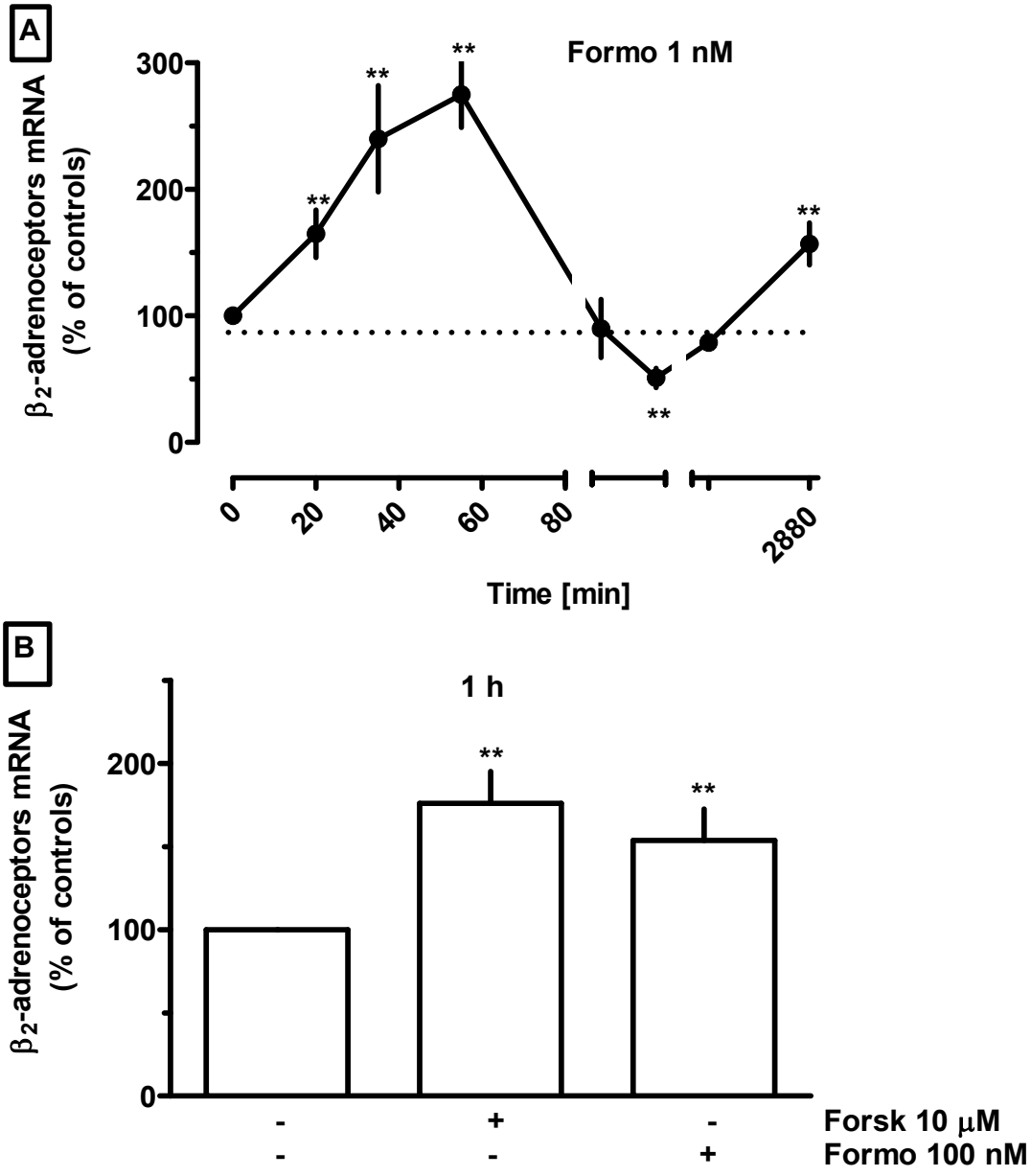


Figure 3-22: Time-dependent effects of formoterol (Formo 100 nM) **(A)** and comparison of the effects of formoterol (Formo 100 nM) and forskolin (Forsk 10 μM) **(B)** on β_2-adrenoceptor mRNA expression in MRC-5 human lung fibroblasts. $3*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS. After a 24 hrs FCS-free period, cells were cultured for additional 24 hrs in FCS-free medium. Subsequently, the tested substances were added according to the described time. RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)*}100$). Given are means + SEM of n=4-6. Significance of differences vs. control: **p<0.01.

In order to investigate whether the rapid up-regulation of β_2-adrenoceptors mRNA levels are mediated through β_2-adrenoceptors, a selective β_2-adrenoceptors antagonist ICI 118,551 (3μM) was added 30 minutes prior to 20 and 60 minutes exposure to 100 nM formoterol. As described in figure 3-23, A and B the rapid up-regulation of β_2-adrenoceptors mRNA was prevented in presence of β_2-adrenoceptors selective antagonist ICI 118, 551.

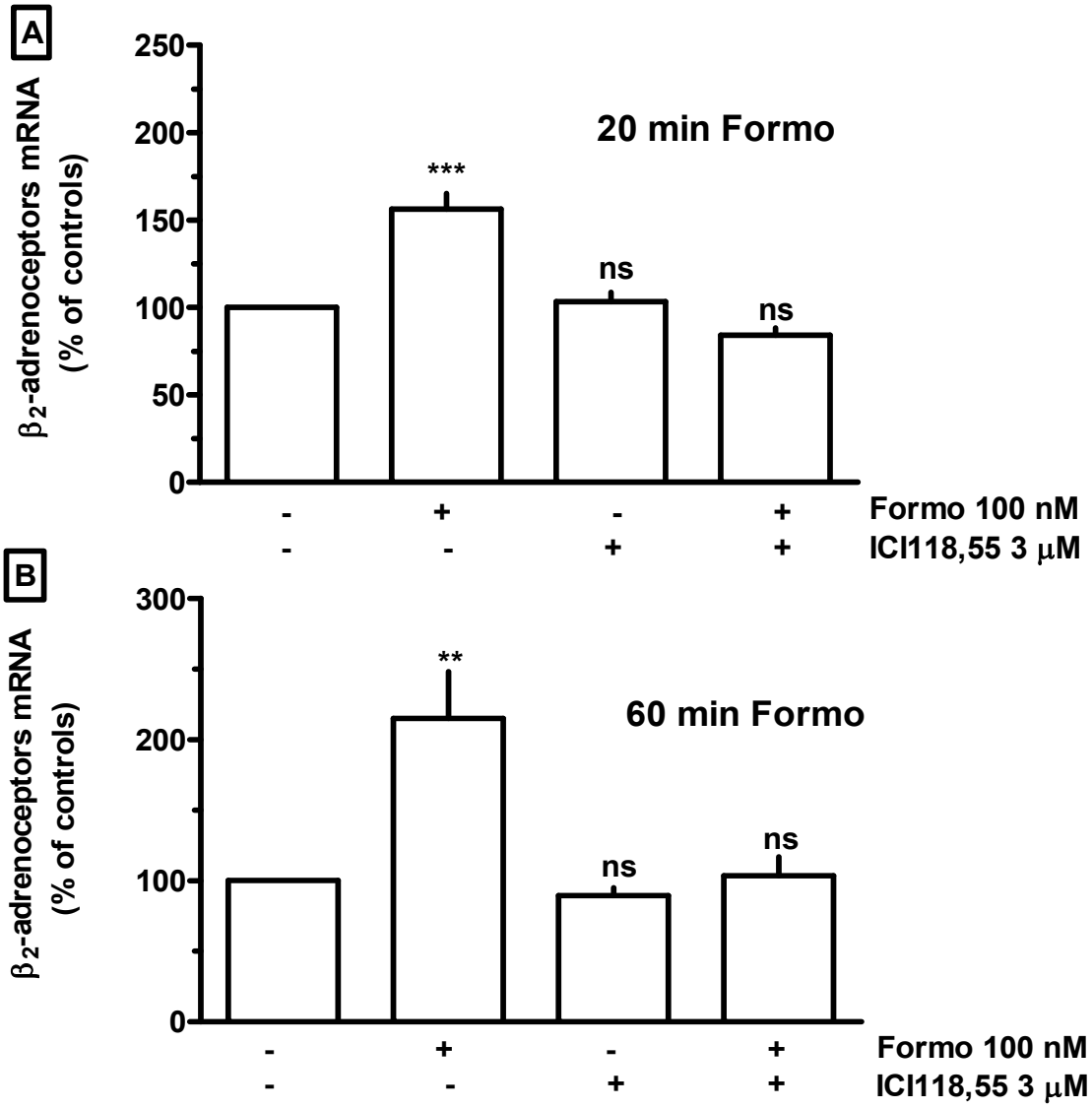
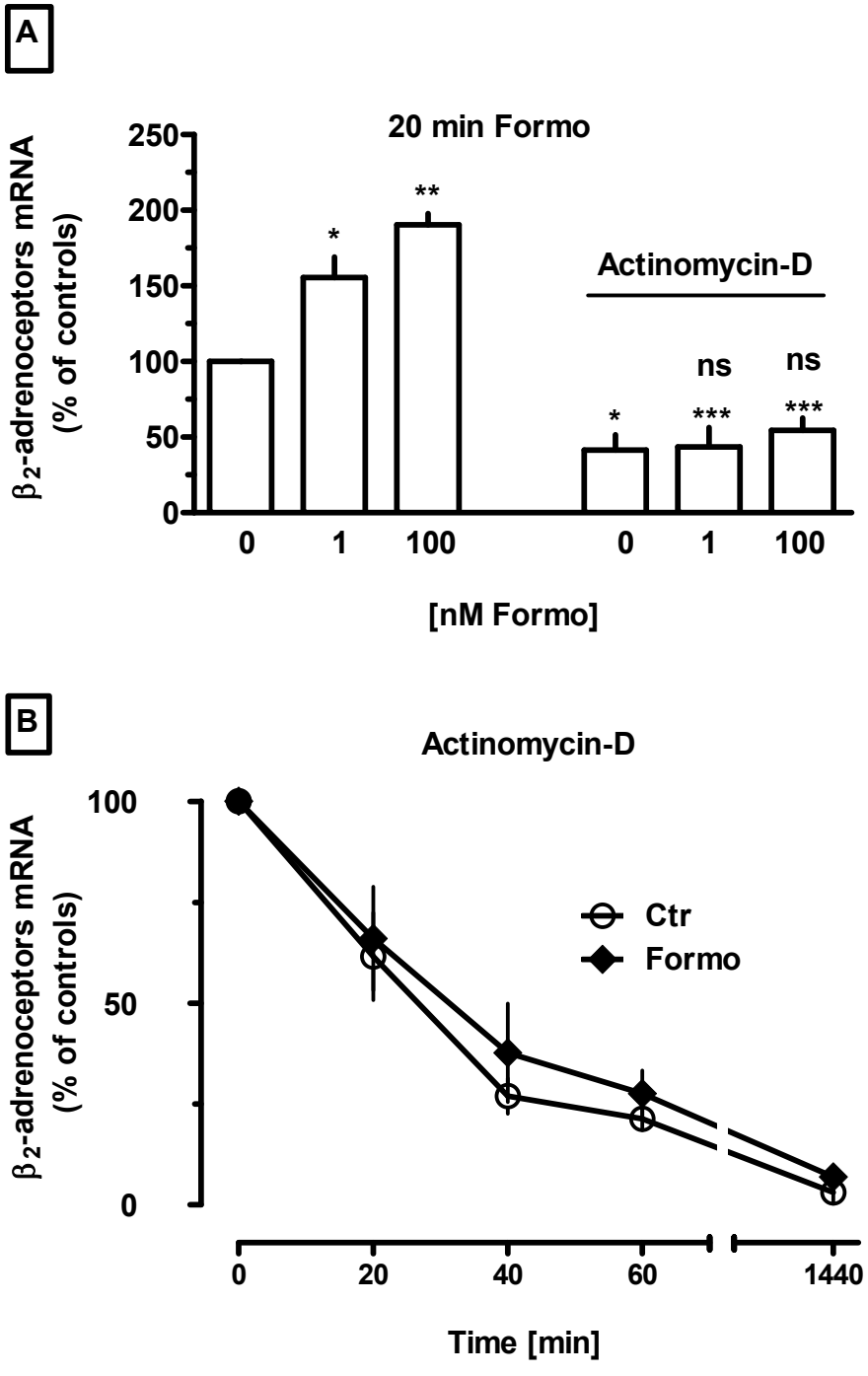


Figure 3-23: Effect of formoterol (100 nM) and/or ICI 118,551 (3 μM) on β_2-adrenoceptors mRNA. $3.5*10^5$ cells were cultured and grown in presence of 10% FCS for 24 hrs (to nearly confluence) followed by 24 hrs under serum deprived conditions. Cells were pre-incubated for 30 minutes with 3 μM of ICI 118,551 before treating with 100 nM of formoterol for 20 minutes **(A)**, and 60 minutes **(B)**. Thereafter, RNA was isolated, DNase-treated and analysed by real time PCR. β_2-Adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-6. Significance of differences vs. control: **p<0.001, ***p<0.0001; ns = not significant

Furthermore, the effect of inhibition of de novo RNA and protein synthesis on the stimulatory effect of β_2-adrenoceptors agonists was also studied. The stimulatory effect of formoterol was abolished after inhibition of de novo RNA synthesis (Fig. 3-24, A-B). However, the stimulatory effect of formoterol on β_2-adrenoceptors mRNA was synergistically increased in presence of cycloheximide, which on its own markedly increased the β_2-adrenoceptors mRNA levels by about more than 5 fold within 1.5 hrs (Fig. 3-24, C)



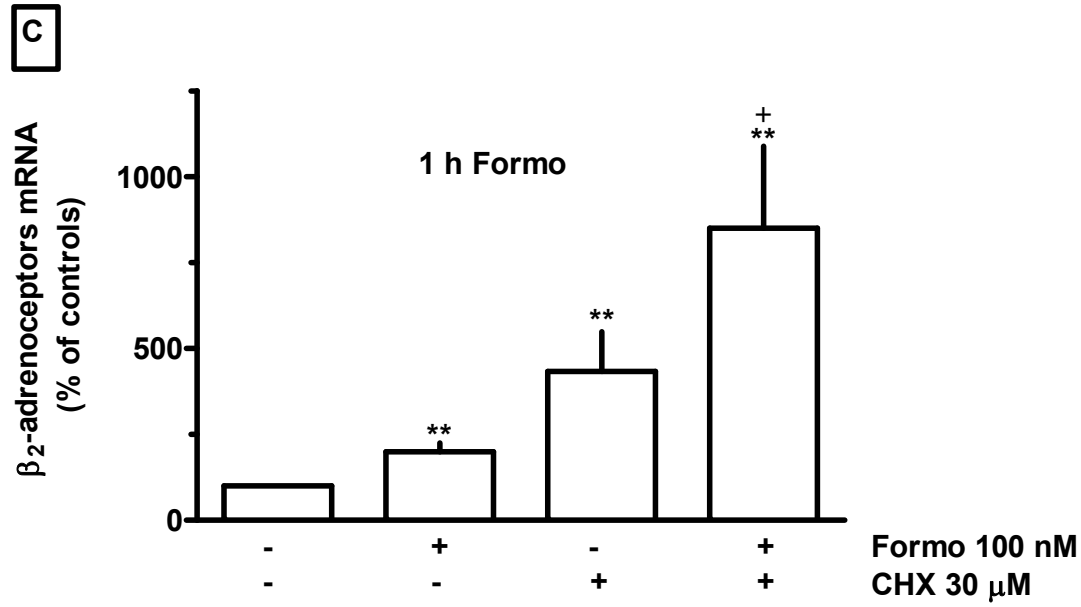


Figure 3-24: Effect of formoterol (Form 100 nM) and/or actinomycin D (30 μM) **(A and B)** or cycloheximide (CHX, 30 μM) **(C)** on β_2-adrenoceptors mRNA expression in MRC-5 human lung fibroblasts. $3*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS followed by 24 hrs FCS-free period. Subsequently, cells were additionally cultured in absence or presence of test substances as described. Actinomycin D being present 15 minutes before formoterol and cycloheximide 30 minutes before formoterol. Thereafter total RNA was extracted, DNase-treated and used for RT-qPCR.β_2-adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=4-6, significance of differences vs. control: *p<0.05, **p<0.01; ***p<0.001; vs. CHX: +P< 0.001

3.4 Effects of Selective cAMP Analogue on β_2-Adrenoceptors mRNA Expression Levels in MRC-5 Human Lung Fibroblast Cell Line

To elucidate whether cAMP-regulated effectors, protein kinase A (PKA) and exchange protein activated by cAMP (Epac) participate in β_2-adrenoceptors transduction pathway mediating β_2-adrenoceptors regulation in human lung fibroblast, the effect of cAMP analogue was studied. As described in figure 3-25, A, exposure to the selective PKA agonist (6-Bnz-cAMP 500 μM) or/and to the selective Epac agonist (8-CPT-2'-O-Me-cAMP 300 μM) for 1 and 4 hrs did not significantly affect the β_2-adrenoceptors mRNA levels, but it was reduced by about 40 % after exposure to their combination. However, in the presence of cycloheximide, 6-Bnz-cAMP8, but not

8-CPT-2'-O-Me-cAMP, it caused a significant further increase in β_2-adrenoceptors mRNA levels (Fig. 3-25 B).

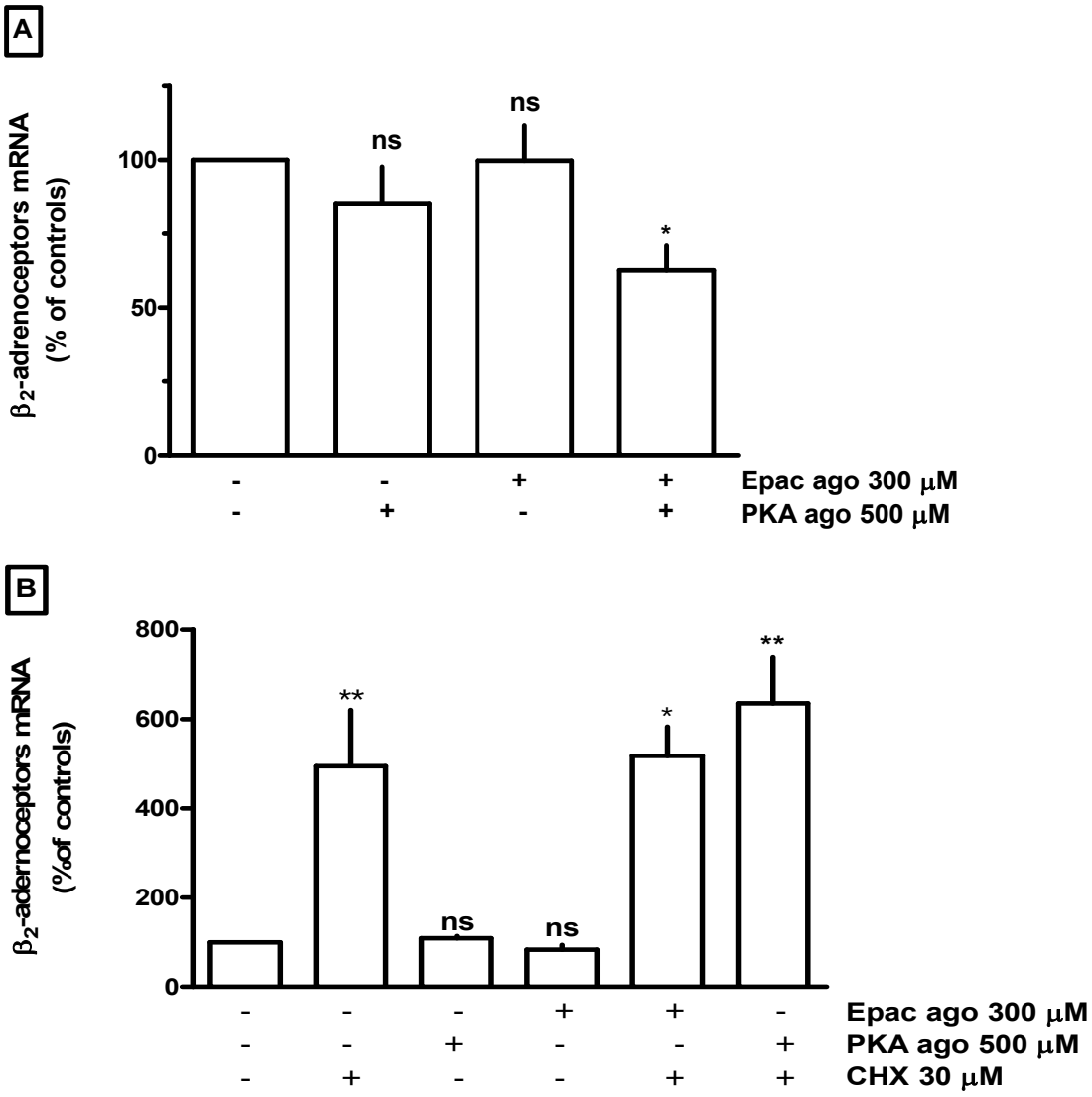


Figure 3-25: Effects of the selective PKA (6-Bnz-cAMP, 500 μM) and/or Epac (8-CPT-2'-O-Me-cAMP, 300 μM) agonist (ago) **(A)** or cycloheximide (CHX, 30 μM) alone and in combination with the Epac or PKA agonist **(B)** on β_2-adrenoceptors mRNA expression in MRC-5 human lung fibroblasts. $3.5*10^5$ cells were seeded in 6-well plates and cultured for 24 hrs in presence of 10% FCS. Followed by 24 hrs FCS-free period. Subsequently, the tested substances were added. Thereafter total RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-7. Significance of differences vs. control: *p<0.05, **p<0.01 ns = not significant

3.5 Effects of Corticosteroids and their Combinations on β_2-Adrenoceptors mRNA Expression Levels in MRC-5 Human Lung Fibroblast Cell Line

The effect of corticosteroids on β_2-adrenoceptors mRNA was also studied. As shown in figure 3-26, exposure of MRC-5 cells to dexamethasone (1 µM) and budesonide (100 nM) induced a sharp increase and clear up-regulation in the β_2-adrenoceptors mRNA level expression after 8, 24, 48, and 72 hrs.

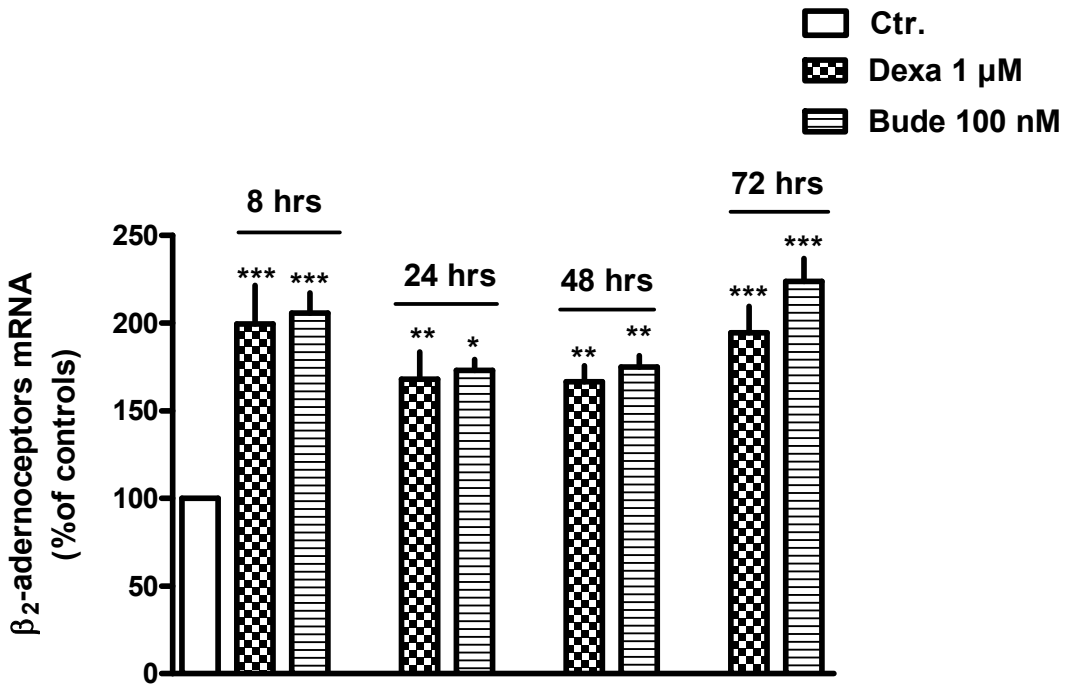


Figure 3-26: Time-dependent effects of dexamethasone (Dexa.1µM), budesonide (Bude 100 nM), on expression of β_2-adrenoceptors mRNA in MRC-5 cells. $3.5*10^5$ cells were seeded in 6-well dishes and cultured in presence of 10% FCS for 24 hrs (to nearly confluence) followed by 24 hrs FCS-free period. Cells were stimulated with corticosteroids for 8, 24, 48 and 72 hrs. Total RNA was extracted, treated with DNase, and used for real time PCR with primers specific for the human β_2-adrenoceptors or GAPDH. β_2-Adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are mean + SEM of n=4-6. Significance of differences: *P<0.05, **P<0.05, ***P<0.01 vs. controls (Ctr.).

mRNA expression levels of β_2-adrenoceptors were not significantly affected after exposure to 100 nM of non-selective β-adrenoceptors agonist isoprenaline for 8 or 24 hrs, but was up-regulated by about 64% after 48 hrs of exposure. The expression levels were reduced by 20%, 26% and 11% after exposure to 100 nM of selective β_2-adrenoceptors agonist formoterol for 8, 24, and 48 hrs respectively.

Furthermore, the dexamethasone-induced up-regulation of β_2-adrenoceptors was not affected in presence of 100 nM isoprenaline, whereas 100 nM formoterol resulted in a down-regulation of β_2-adrenoceptors also in the presence of 100 nM budesonide.
In other words dexamethasone-induced up-regulation of β_2-adrenoceptors was not changed either in presence or absence of non-selective β-adrenoceptors agonist isoprenaline, whereas the down-regulation caused by 100 nM formoterol was not frustrated by 100 nM budesonide after 8, or 24 hrs but it was partially counteracted after 48 hrs (data not shown).

Moreover, in order to ensure the stability and efficiency of test substances, and to determine optimal conditions for the effect of β-adrenoceptors agonists and corticosteroids on expression of β_2-adrenoceptors during the long incubation period protocols (48 hrs), FCS free medium and test substances were changed once at 24 hrs. These alterations showed again clear up-regulation in the β_2-adrenoceptors mRNA level expression after exposure to 1 μM dexamethasone or 100 nM budesonide. Furthermore, exposure to 100 nM of non-selective β-adrenoceptors agonist isopre-naline induced the β_2-adrenoceptors mRNA level expression as previously seen. But exposure to 100 nM of selective β_2-adrenoceptors agonist formoterol did not signify-cantly affect the receptor expression level. Additionally, under these culture conditions, incubation of a combination of corticosteroids and β-adrenoceptors agonists showed an additive up-regulation effect of β_2-adrenoceptor mRNA level expression by about 116% for dexamethasone-isoprenaline combination and 83% for budesonide-formoterol combination (data not shown).

Furthermore, the effect of actinomycin D on the stimulatory effect of dexamethasone was also tested. After addition of dexamethasone (1 μM) 15 minutes prior to actinomycin D, the short half-life of β_2-adrenoceptors mRNA was not affected (Fig. 3-27).

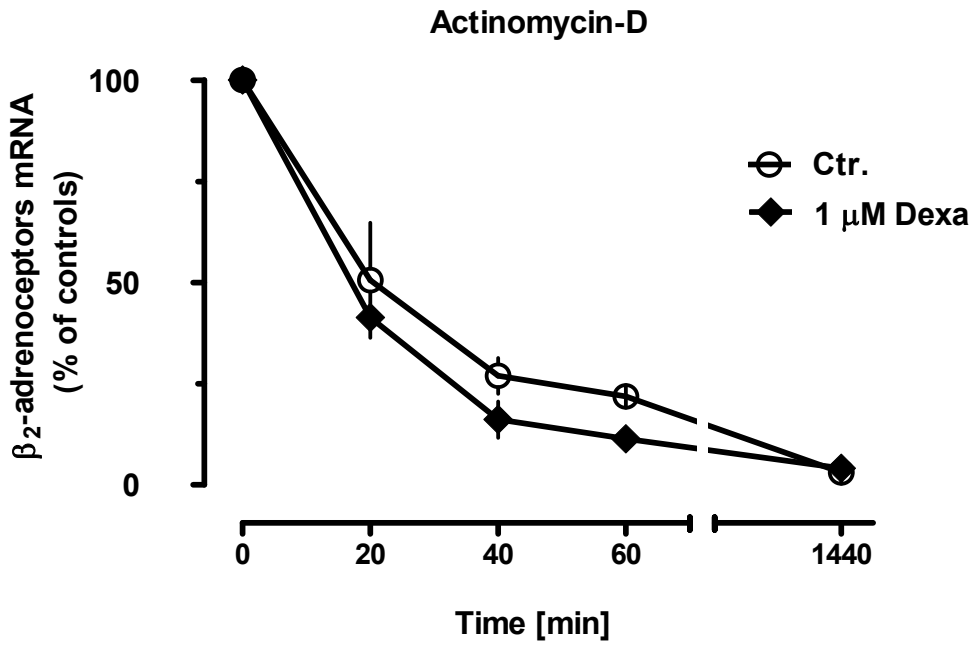


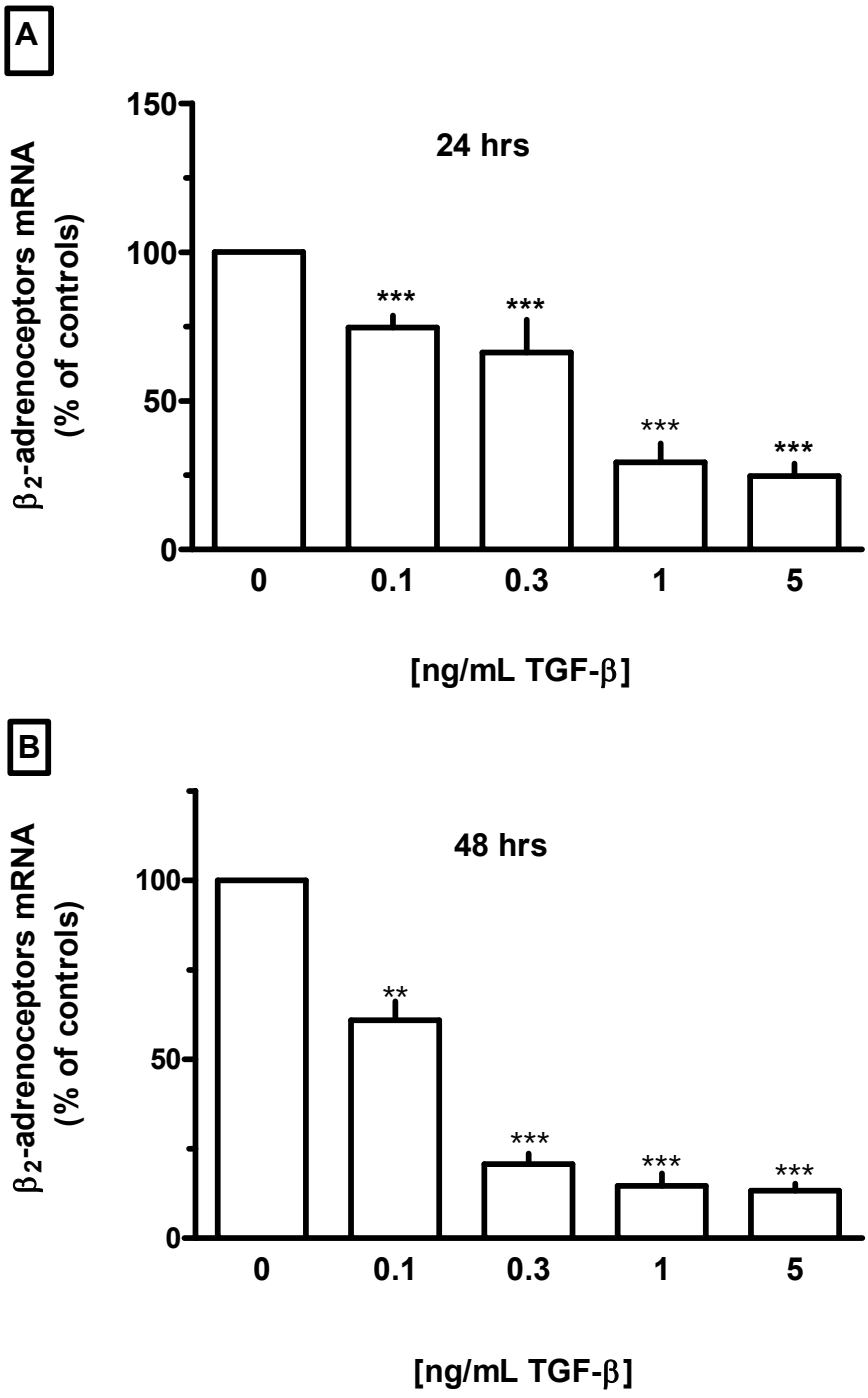
Figure 3-27: Effect of dexamethasone (1 μM) and/or actinomycin D (30 μM) on β_2-adrenoceptors mRNA expression in MRC-5 human lung fibroblasts. $3*10^5$ cells were seeded in 35 mm dishes and cultured for 24 hrs in presence of 10% FCS followed by 24 hrs FCS-free period, cells were cultured for additional 1 hr in absence or presence of test substances. Actinomycin D being present 15 minutes before dexamethasone. Thereafter total RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=4-6, significance of differences vs. control: *p<0.05, **p<0.01; ***p<0.001.

3.6 Effects of Cytokines on β_2-Adrenoceptors mRNA Expression Level

In light of the central role that TGF-β plays in mediating the pathogenesis of lung fibrosis, the effect of TGF-β on the expression of β_2-adrenoceptors mRNA was studied.

The expression levels of β_2-adrenoceptors mRNA after exposure to TGF-β (0.1, 0.3, 1, 5 ng/ml) were markedly reduced in a time and concentration-dependent manner. As shown in figure 3-28, A and B, the results of real time (qPCR) analysis demonstrated a progressive time and concentration reduction in the levels of β_2-adrenoceptors mRNA expression by TGF-β with a maximum of more than 86% inhibition after 48 hrs.

The maximum reduction effect was reached after exposure either to 1 or 5 ng/mL TGF-β for the same time. In other words the expression level of β_2-adrenoceptors mRNA was reduced nearly to the same level when 1 and 5 ng/mL were used. For this reason and for confirmation of these observations the effect of 1 ng/ml TGF-β for various periods was studied. The results again demonstrated TGF-β down-regulated β_2-adrenoceptors mRNA expression levels in a time-dependent manner; whereas, a similar effect was observed after an extension of time for 6 - 48 hrs (Fig. 3-28, C).



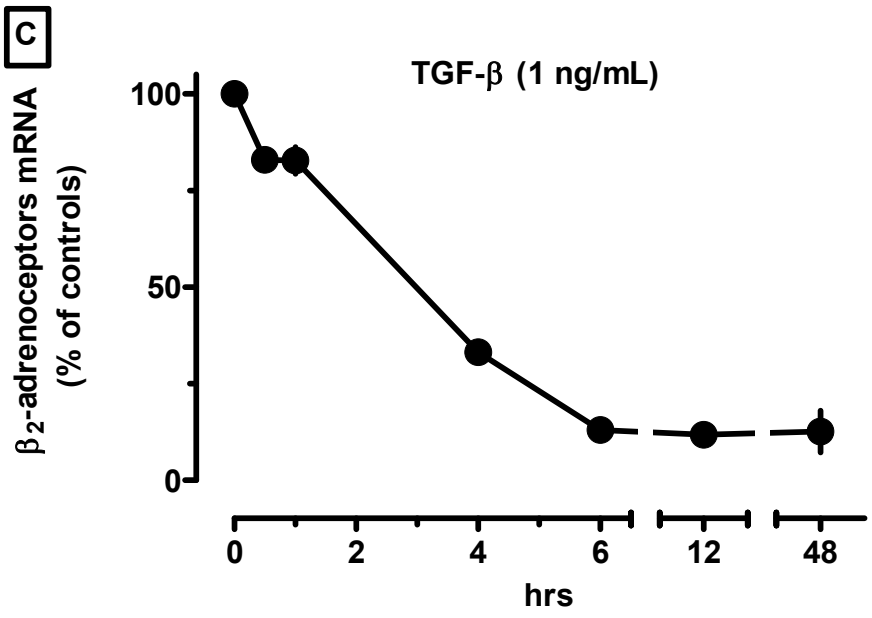
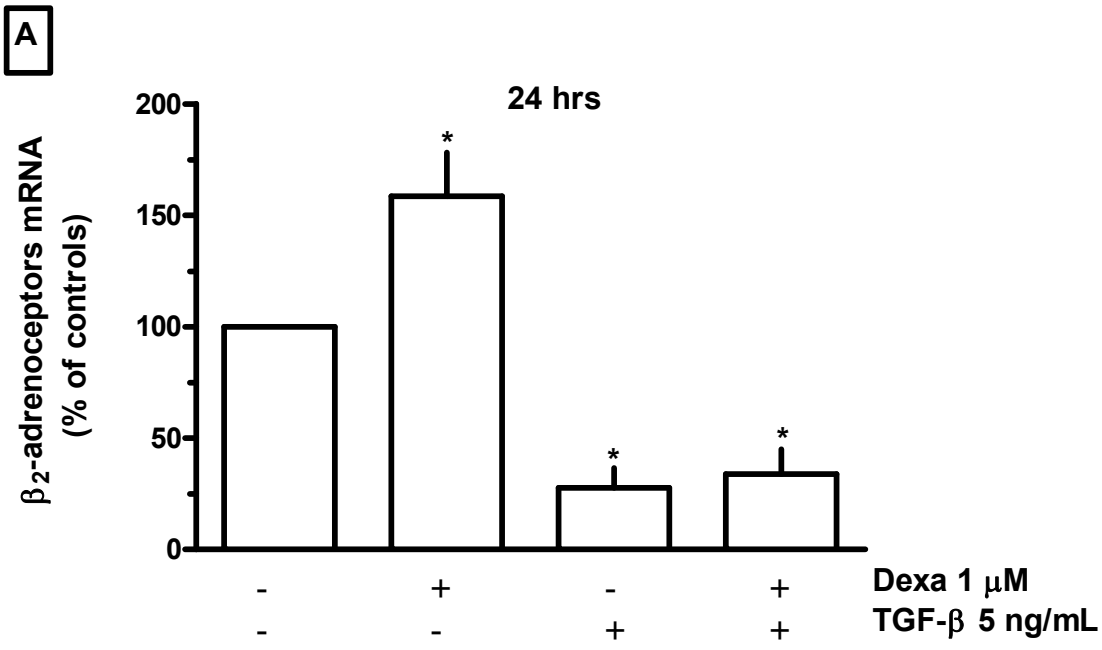
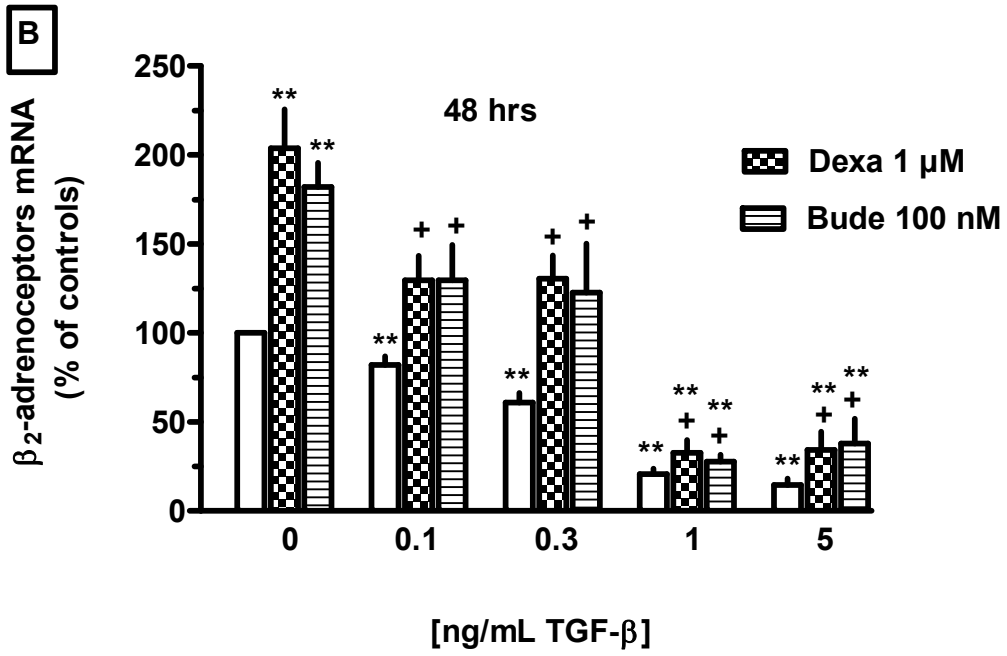


Figure 3-28: Effect of TGF-β on β_2-adrenoceptors mRNA expression in MRC-5 fibroblasts.$3.5*10^5$ cells were cultured and grown in the presence of 10% FCS for 24 hrs (to nearly confluence) followed by 24 hrs under serum-deprived conditions, and stimulated with different concentration of TGF-β for 24 hrs **(A)**, 48 hrs **(B)** or different time schedule **(C)**. Thereafter total RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-6, significance of differences vs. control: **p<0.001, ***p<0.0001

Previous experiments had elucidated the marked reduction in β_2-adrenoceptors mRNA expression levels caused after stimulation of MRC-5 human lung fibroblast cell line by TGF-β. The effect of corticosteroids in presence of TGF-β was also studied. As described in figure 3-29, A, dexamethasone partially opposes TGF-β induced down-regulation of β_2-adrenoceptors mRNA level. As shown in figure 3-29, B, the real time PCRs analysis elucidates again that both dexamethasone and budesonide significantly reversed partially TGF-β induced reduction of β_2-adrenoceptors mRNA levels.

Although corticosteroids were added 48 hrs prior to TGF-β addition, they were not able to protect their stimulatory effects on β_2-adrenoceptors mRNA but could still partially reverse the TGF-β mediated down-regulation of β_2-adrenoceptors mRNA (Fig.3-29, C).


A
24 hrs
β2-adrenoceptors mRNA
(% of controls)
200
150
100
50
0
*
*
*
- + - +
- - + +
Dexa 1 μM
TGF-β 5 ng/mL


B
48 hrs
β2-adrenoceptors mRNA
(% of controls)
250
200
150
100
50
0
Dexa 1 μM
Bude 100 nM
0
0.1
0.3
1
5
[ng/mL TGF-β]

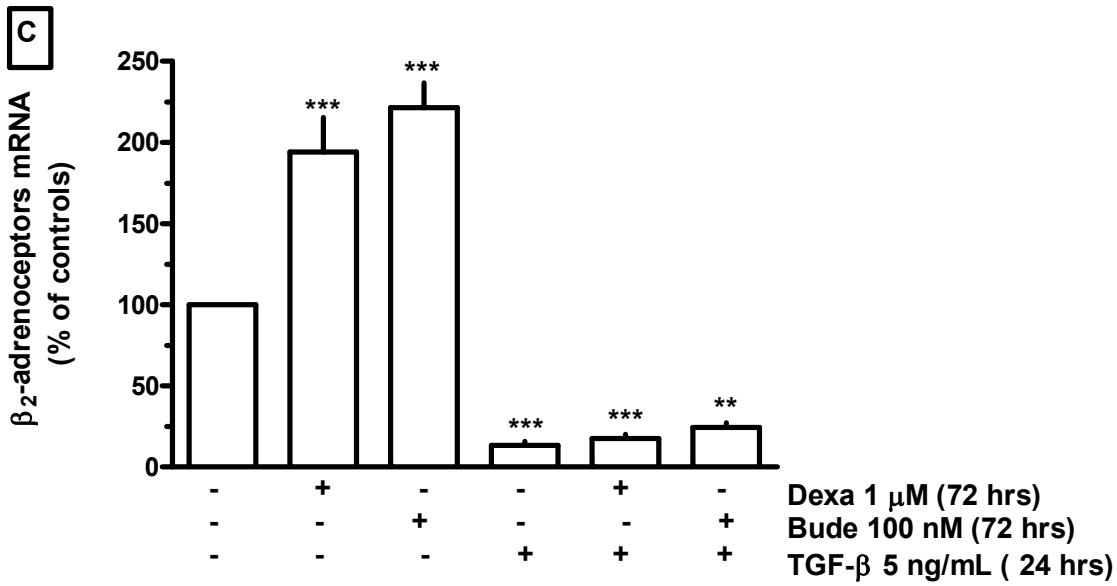


Figure 3-29: Time and concentration-dependent effect of TGF-β in absence and presence of dexamethasone (Dexa 1 μM) and budesonide (Bude 100 nM) on β_2-adrenoceptors mRNA expression in MRC-5 fibroblasts. $3.5*10^5$ cells were cultured and grown in presence of 10% FCS for 24 hrs (to nearly confluence) followed by 24 hrs under serum-deprived conditions, followed by **(A)**, addition of 1 μM dexamethasone and 5 ng/mL TGF-β at the same time for 24 hrs, **(B)**, addition of 1 μM dexamethasone, 100 nM budesonide and different concentration of TGF-β (0.1, 0.3, 1 and 5 ng/mL) for 48 hrs, **(C)**, 1 μM dexamethasone or 100 nM budesonide were present 48 hrs prior to 5 ng/mL TGF-β which was present in the last 24 hrs **(C)**. Cells were harvested, RNA was extracted, DNase-treated and relative expression analysed by real time RT-PCR. Given are means + SEM of n=3-6, significance of differences vs. control: **p<0.001, ***p<0.0001; vs. TGF-β: +P< 0.001

Many reports have shown that β_2-adrenoceptors agonists may have pharmacological properties useful for controlling inflammation. Exposure to β_2-adrenoceptors agonist formoterol (1 nM) induced a small down-regulation of β_2-adrenoceptors mRNA expression, whereas TGF-β induced strong down-regulation of β_2-adrenoceptors mRNA expression, which was not significantly affected and appeared to be slightly increased in presence of 1 nM formoterol (Fig. 3-30).

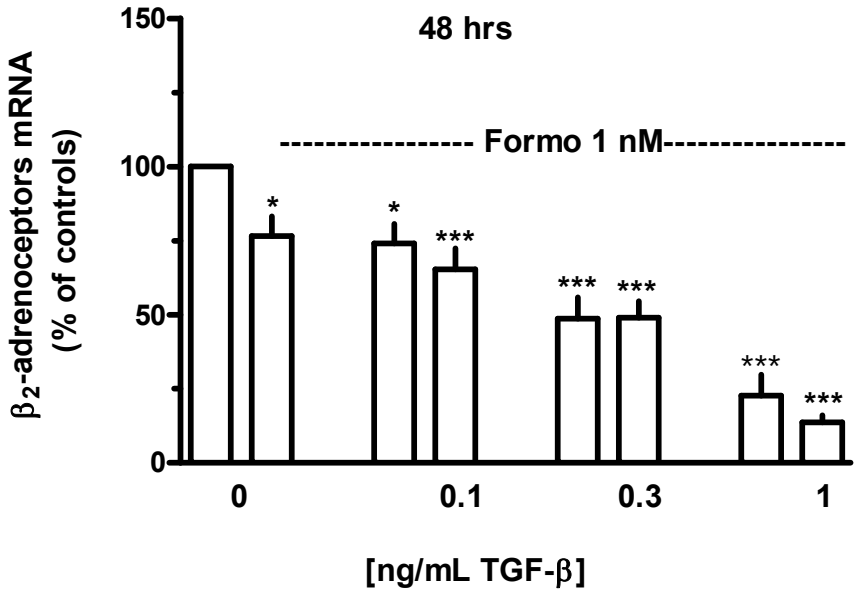


Figure 3-30: Concentration dependent effect of TGF-β in absence and presence of formoterol (Formo 1 nM) on β_2-adrenoceptors mRNA expression in MRC-5 fibroblasts. $3.5*10^5$ cells were cultured and grown in presence of 10% FCS for 24 hrs (to nearly confluence) followed by 24 hrs under serum-deprived conditions, followed by addition of formoterol (Formo 1 nM) and stimulated with different concentration of TGF-β in the same time for 48 hrs. Total RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=7, significance of differences: vs. controls, *P<0.05, ***p<0.0001

The sharp stimulatory effect of CHX resulted after 6.5 hrs was significantly counteracted when the cells were stimulated with 1 ng/ml TGF-β after 30 minutes of CHX addition and incubated for further 6 hrs (Fig. 3-31).

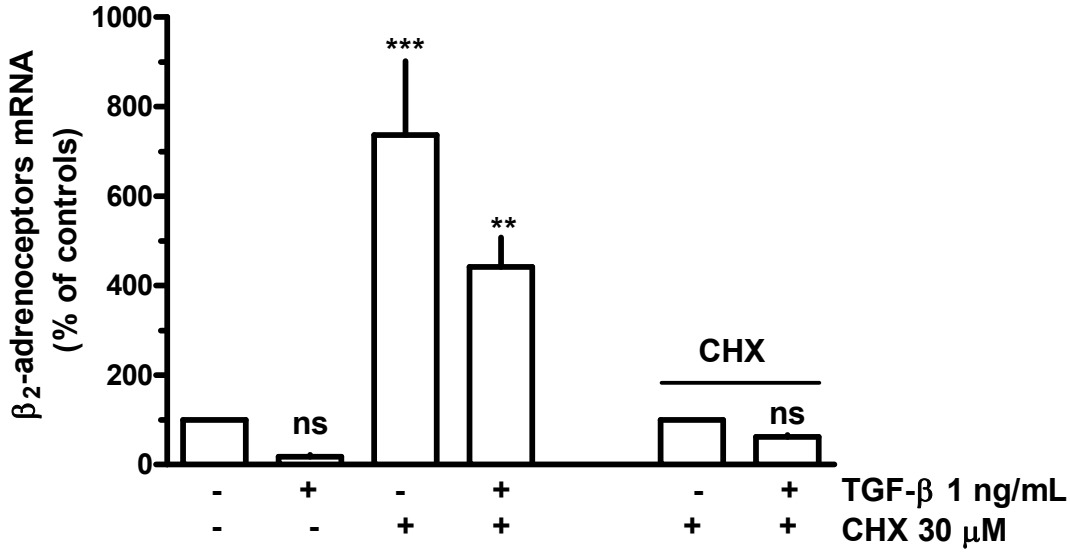


Figure 3-31: Effect of cycloheximide (CHX 30 μM) on β_2-adrenoceptors mRNA expression in MRC-5 human lung fibroblasts, in absence and presence of 1 ng/mL TGF-β. $3.5*10^5$ cells were seeded in 6-well plates and cultured for 24 hrs in presence of 10% FCS. Followed by 24 hrs FCS-free period, cells were cultured for additional 24 hrs in serum-deprived medium in absence or presence of test substances. Cycloheximide being present 30 minutes before TGF-β. Total RNA was extracted, DNase-treated and used for RT-qPCR. β_2-Adrenoceptors mRNA was normalized over GAPDH in each individual sample and then expressed as % of the controls of the individual cell preparation ($2^{-(\Delta\Delta CT)}*100$). Given are means + SEM of n=3-7, significance of differences: vs. controls, **p< 0.05, ***p<0.001

On the basis of hypothesis that the Th2 cytokines plays a pivotal role in the pathogenesis of lung diseases, the effect of IL-13 on the β_2-adrenoceptors mRNA expression was also tested. Exposure to IL-13 (10 ng/mL) did not affect the expression level of β_2-adrenoceptors mRNA (data not shown).

4. Discussion

4.1 β-Adrenoceptors Expression in Human Lung Fibroblasts

As outlined in the introduction, there is some support that the action of β-adrenoceptors agonists in treatment of asthma and/or COPD is not confined to bronchodilation. Thus, β-adrenoceptors agonists have been shown to exert various inhibitory effects on pro-fibrotic features in pulmonary fibroblasts. However, until now, the expression pattern of β-adrenoceptors subtypes in human lung fibroblasts has not been characterized and the pharmacological characterization of their functions was missing.

For this purpose semi-quantitative reverse transcriptase polymerase chain reactions (RT-PCR's) were used for determination of expression pattern of β-adrenoceptors in MRC-5, HEL-299 cell line as well as in primary human lung fibroblasts.

The current study clearly demonstrated stable and similar expression of mRNA encoding β_2-adrenoceptors in human lung fibroblasts, but no transcripts for β_1- or β_3-adrenoceptors were detected.

The expression profile of β_2-adrenoceptors was found to be constant under different culture conditions. Moreover, the expression of β_2-adrenoceptors was confirmed at protein level using Western blot analysis.

Various studies have reported that the β_2-adrenoceptors expression and its functional coupling to adenylyl cyclase (**AC**) was very high in the human airways cells (Barnes 1995; Johnson 1998; Nijkamp et al. 1992), suggesting that the cell line MRC-5 represents a suitable model to study the regulation and functional characteristics of the β_2-adreno-ceptors signaling in the respiratory system.

4.2 Functional Characteristics of β_2-Adrenoceptors Expression in Human Lung Fibroblasts

In agreement with the expression pattern of β-adrenoceptors, it is demonstrated in this study that the β_2-adrenoceptors mediates inhibitory effects on proliferation and collagen synthesis.

The observations in this study have showed the anti-proliferative and inhibition of collagen synthesis of long-acting selective β_2-adrenoceptors agonist formoterol and non-selective β-adrenoceptors agonist isoprenaline on MRC-5 cell line and primary human lung fibroblasts.

The magnitude of inhibition exerted by the long-acting selective β_2-adrenoceptors agonists formoterol and olodaterol (Bouyssou et al. 2010) was similar to that caused by the non-selective agonist isoprenaline.

Isoprenaline and formoterol inhibited [^{3}H]-thymidine incorporation in a concentration-dependent manner with an IC_{50} of about 2 and 0.06 nM, respectively. Furthermore, both isoprenaline and formoterol inhibited [^{3}H]-proline incorporation in a concentration-dependent manner with an IC_{50} of 2 and 0.1 nM, respectively.

In contrast, it has been demonstrated earlier that the short-acting β-adrenoceptors agonist salbutamol, as well as the long-acting β-adrenoceptors agonist formoterol, by themselves did not show any significant anti-proliferative action on different cultures of primary human lung fibroblasts proliferation (Descalzi et al. 2008).

The inhibitory effects of formoterol on human lung fibroblasts proliferation and collagen synthesis were mimicked by a direct activator of adenylyl cyclase forskolin and the prostanoid EP_2 receptor agonist butaprost, and were potently antagonized by the β_2-adrenoceptors selective antagonist ICI 118,551; whereas no effect was exerted by the β_1-adrenoceptors selective antagonists CGP 20712. These data suggest the competitive interaction between agonist and antagonist. The apparent pA_2 value of ICI 118,551 determined in the present experiments (9.6) agrees well with its affinity

values determined in binding studies on human β_2-adrenoceptors log k_D, -9.26, k_i, 1.8 nM, (Baker 2005; Smith and Teiler 1999). However ICI 118,551 has inverse agonist properties (Bond et al. 1995), but did not exert effects on its own. Therefore, significant constitutive activity of the β_2-adrenoceptors in human lung fibroblasts may be excluded. It has been reported in previous study in our laboratory that prostaglandins can exert strong inhibitory effects on pulmonary fibroblasts proliferation mediated by EP_2 receptors, which are like β-adrenoceptors-coupled to adenylyl cyclase (Haag et al. 2008b).

This study showed that indomethacin on its own had no effect on proliferation nor significantly affected the inhibitory effect of isoprenaline. Thus, at least under the standard culture conditions, any interference with endogenously released prostaglandins might be excluded. Although using different model and varying species, in contrast to several reports (Descalzi et al. 2008; Lindemann and Racke 2003; Spoelstra et al. 2002), the observations in this study showed that budesonide and dexamethasone did not affect human lung fibroblasts proliferation and collagen synthesis.

4.2.1 Cytokines Control of Proliferation and Collagen Synthesis in MRC-5

The cells were stimulated with TGF-β, an important pro-inflammatory cytokine, whose levels are increased during airways diseases (Chung and Barnes 1999) resulting in an increase in ECM deposition (Romberger et al. 1992). The present study showed that TGF-β alone exerted only a very weak stimulatory effect on proliferation which was not further enhanced by the additional presence of budesonide or/and indomethacin. TGF-β had been reported to induce the proliferation of many different cell types (Ghayor et al. 2005; Khalil et al. 2005; Martinez-Salgado et al. 2006). The explanations for this large species and used model differences have to be illuminated in future studies. In consonance with the marked down-regulation effect of TGF-β on the expression of β_2-adrenoceptors showed in this study, TGF-β resulted in a loss of the inhibitory effect of formoterol, an effect not counteracted in presence of budesonide. Furthermore, the strong inhibitory effect of the EP_2 receptor agonist butaprost was also markedly attenuated in presence of TGF-β alone or in combination with budesonide.

On the other hand, TGF-β markedly stimulated collagen synthesis which was augmented in presence of dexamethasone and budesonide. Furthermore, this strong stimulatory effect of TGF-β in combination with budesonide was not significantly affected by the additional presence of indomethacin, excluding significant interference with endogenously released prostanoids.

However, previous studies have documented that Th2 cytokines are potent inducers of collagen production in vivo in the lung (Lee et al. 2001), in vitro in skin and keloid fibroblasts (Oriente et al. 2000), stimulated fibroblast proliferation (Saito et al. 2003), and in contrast to previous observations from our laboratory in rat fibroblasts (Lindemann and Racke 2003). Unexpectedly, neither IL-4 nor IL-13 showed any stimulatory effects on proliferation or collagen synthesis of human lung fibroblasts. Furthermore, neither IL-4 nor IL-13 affected the inhibitory effect of formoterol on proliferation. Moreover, both IL-4 and IL-13 slightly reduced collagen synthesis in MRC-5 cells and the effect was even greater in presence of formoterol.

4.2.2 Regulation of α-Smooth Muscle Actin

Under standard culture conditions, MRC-5 human lung fibroblasts express significant levels of α-smooth muscle actin (α-SMA), the most important marker of fibroblast differentiation into significantly larger contractile myofibroblast, which may reflect some kind of fibroblast activation, probably caused by serum constituents of culture medium.

Formoterol, the EP_2 receptor agonist butaprost, and the direct activator of adenylyl cyclase forskolin, caused a reduction in the expression of α-SMA protein by about 50%-70% after 24 hrs of exposures. A similar reduction by formoterol was seen at mRNA level, suggesting that transcriptional effects might be responsible for the β_2-adreno-ceptors mediated down-regulation of α-SMA. Since these effects of formoterol persisted after 48 hrs, it suggests that the β_2-adrenoceptors in human lung fibroblasts do not undergo rapid desensitization during prolonged agonist exposure.

As observed previously (Baouz et al. 2005), the findings in this study confirmed that the β_2-adrenoceptors agonist formoterol inhibited α-SMA expression, suggesting that

it could have a role in inhibition of formation of myofibroblasts, the inducer of remodelling and consequently the sub-epithelial fibrosis.

Since myofibroblasts are key players in the airway remodelling process associated with chronic obstructive and inflammatory airways diseases (Davies et al. 2003; Westergren-Thorsson et al. 2010), a sustained suppressive action of long-acting β_2-adrenoceptors agonists on myofibroblast differentiation could be of particular therapeutic value.

In agreement with others, the present study demonstrates that prolonged exposure to TGF-β induces marked increase in α-SMA mRNA and protein expression in human lung fibroblasts. It is well known that exposure of fibroblasts to TGF-β stimulates the differentiation of fibroblasts into myofibroblasts with expression of α-SMA (Kuang et al. 2006; Phan 2002; Vaughan et al. 2000). It is also known that Smad signal transduction pathways plays a crucial role in mediating several TGF-β actions in fibroblasts, but other signaling pathways may potentially contribute in α-SMA expression (Gu et al. 2007). However, the inhibitory effect of formoterol and the strong inhibitory effect of butaprost and forskolin vanished in the presence of TGF-β, this indicates that the pathways mediating the stimulatory effect of TGF-β are not affected by these compounds.

Many studies have documented that glucocorticoids may affect diverse functions of airway smooth muscle (Hirst and Lee 1998; Panettieri 2004) among them the enhancement of β_2-adrenoreceptors number and function. In line with recently reported data, the present study reports that exposure to glucocorticoids caused significant reduction in α-SMA protein under basal culture conditions, but not when α-SMA protein was increased by exposure to TGF-β (Goldsmith, et al. 2007).

In contrast to other studies (Tsai et al. 2003), glucocorticoids induced significant increase in α-SMA mRNA in this study. These observations may confirm the same observations which were seen after treatment of airway smooth muscle cells with glucocorticoids, suggesting that this treatment inhibits protein expression at least in part by reducing the translation of mRNA (Goldsmith et al. 2007).
Generally, β-adrenoceptors by coupling to Gs mediate activation of adenylyl cyclase leading to an increase in cellular cAMP. As previously outlined, there is substantial

evidence that lung fibroblasts proliferation and collagen synthesis are inhibited after elevation of cAMP as a result of AC activation. This study shows that the activation of adenylyl cyclase by forskolin causes, like activation of β_2-adrenoceptors, down-regulation of α-SMA expression.

As outlined in the introduction, beside protein kinase A (PKA), the classic effector of cAMP (Skalhegg and Tasken 2000), alternative cAMP effectors have also been identified, among them Epac (exchange protein activated by cAMP) of which two variants [Epac1 and Epac2, new nomenclature RapGEF3 (Rap guanine nucleotide exchange factor 3) and RapGEF4 (Rap guanine nucleotide exchange factor 4), respectively] have been identified (de Rooij et al. 1998; Kawasaki et al. 1998). Recent studies, including our laboratory observations, documented that the inhibitory effects of cAMP on proliferation and collagen synthesis in human lung fibroblasts are mediated via different signaling pathways. PKA pathway mediates inhibition of collagen synthesis, whereas Epac signaling mediates inhibition of proliferation (Haag et al. 2008b; Huang et al. 2007). In the present study, both the selective PKA agonist 6-Bnz-cAMP as well as the selective Epac agonist 8-CPT-2'-O-Me-cAMP (Bos 2006; Holz et al. 2008) caused a reduction of α-SMA expression, both at protein and mRNA levels in MRC-5 human lung fibroblasts. In previous experiments, using the same cells and the same concentration of 6-Bnz-cAMP and lower concentration of 8-CPT-2'-O-Me-cAMP as used in the present study, it was observed that 6-Bnz-cAMP and 8-CPT-2'-O-Me-cAMP caused a marked and selective inhibition of either the collagen synthesis or proliferation, respectively (Haag et al. 2008b). These observations argue in favour of the selectivity of these tools and therefore support the conclusion that both PKA and Epac can mediate inhibitory effects on α-SMA expression.

The observation that the greatest reduction in α-SMA expression occurred when the cells were exposed to a combination of both agonists, suggests some degree of additivity of both cAMP signaling pathways.

In agreement with the effect of the up-stream acting β-adrenoceptors agonist, the selective PKA and Epac agonists caused comparable reduction of α-SMA mRNA and protein, indicating that both signal pathways may act via transcriptional effects. It is noteworthy that a dual cAMP signaling with PKA and Epac acting in the same dire-

ction has recently also been reported for the regulation of airway smooth muscle phenotype (Roscioni et al. 2011a,b).

It is worth attention here that the definite role of PKA and/or Epac in mediating the various inhibitory effects of β-adrenoceptors in human lung fibroblasts must be illuminated in future studies with selective knockdown of these signaling pathways.

4.3 Regulation of β_2-Adrenoceptors mRNA Expression in Human Lung Fibroblasts

As outlined in the introduction, due to their bronchodilatory action, β-adrenoceptors agonists are an essential element in the control of chronic obstructive airways diseases. Since β-adrenoceptors agonists and glucocorticoids represent the corner stone in long-term treatment of chronic obstructive airways disease, the changes in expression of β_2-adrenoceptors caused by exposition to various substances was investigated further.

Signal transduction is a basic process in cell communication within a single cell or tissue. In the airways β_2-adrenoceptors mediate physiological, pathological and pharmacological effects by activation of their intracellular second messenger system adenylyl cyclase. Interestingly, the expression of β_2-adrenoceptors in human lung fibroblasts appears to be highly regulated at mRNA level.

For this purpose RT-qPCR technique was performed to investigate the regulation of β_2-adrenoceptors mRNA expression. In previous reports, regulation of β_2-adrenoceptors has been investigated in human lymphocyte (Brodde et al. 1987) as well as in cell-line such as DDT1 MF-2 hamster vas deferens cells and H9c2 rat heart cell-line (Dangel et al. 1996; Hadcock and Malbon 1988a; Hadcock and Malbon 1988b; Mak et al. 1995b). In full agreement with previous reports the data in this study showed that the half-life of β_2-adrenoceptors mRNA is within 25 minutes relatively short. Considering that the inhibition of RNA synthesis may occur with a certain delay after addition of actinomycin D to the culture medium, the half-life of β-adrenoceptors mRNA may even be shorter. Therefore, β_2-adrenoceptors mRNA levels are expected to reflect immediate changes in receptor gene transcription.

Accordingly, significant changes of β_2-adrenoceptors mRNA levels were observed within 20 minutes after test substances exposure. It is interesting to consider that

stimulation of MRC-5 human lung fibroblasts by long acting β_2-adrenoceptors agonist formoterol induced dual mode of action: a marked up-regulation of β_2-adrenoceptors mRNA expression which was very rapid in onset but transient, and a time-dependent substantial down-regulation over time as described previously in other reports by the use of other techniques (Nishikawa et al. 1996) reversed again after prolongation of the agonist exposure time to 48 hrs.

Furthermore, several previous radioligand-binding studies on polymorphonuclear leukocytes and lymphocytes obtained from normal and asthmatic humans, have elucidated down-regulation of β_2-adrenoceptors after in vitro and in vivo treatment with β_2-adrenoceptors agonists (Aarons et al. 1983; Brodde et al. 1985; Galant et al. 1978; Hataoka et al 1993). In contrast, (Hauck et al. 1990), there was a failure to demonstrate the down-regulation of pulmonary β_2-adrenoceptors in lung obtained from patients treated with terbutaline before undergoing lobectomy for lung carcinoma.

Generally, the β_2-adrenoceptors is a prototypical G-protein-coupled receptors that interacts with the stimulatory G-protein of adenylyl cyclase, (Gs) to increase cellular cAMP (Gilman 1987; Kobilka 1992). Worth mentioning is that the stimulatory effect of β-adrenoceptors' agonists on β_2-adrenoceptors mRNA could be fully mimicked by forskolin as a direct adenylyl cyclase catalyst activator, indicating that an increase of cAMP is the crucial transduction signalling pathway in this effect. In agreement with our observations, transient β_2-adrenoceptors-cAMP-mediated up-regulation of β_2-adrenoceptors gene expression, which has also been previously described in DDT_1-MF-2 hamster smooth muscle cells (Collins et al. 1988), and evidence for a cAMP responsive element in the β_2-adrenoceptors gene was presented (Collins et al. 1990). Furthermore, a cAMP-mediated reduction in β_2-adrenoceptors mRNA was also observed in transfected Chinese hamster fibroblasts expressing human β_2-adrenoceptors (Bouvier et al. 1989).

The rapid formoterol-induced up-regulation of β_2-adrenoceptors mRNA was competitively blocked by the β_2-adrenoceptors antagonist ICI 118,551. Although, formoterol evoked dual mode of action, only the stimulatory effect appears to be the result of a direct cAMP-mediated regulation of β_2-adrenoceptors gene, as the

inhibitory effect was not only blocked by CHX, but also converted to marked stimulatory effect, as well as the formoterol stimulatory effect was blocked by actinomycin D. This suggests that this effect was caused by an increase in transcription rate and was independent of protein synthesis, as an up-regulation was also seen after exposure to protein synthesis inhibitor CHX.
Interestingly, CHX on its own caused a rapid and sharp increase in β_2-adrenoceptors mRNA expression level, suggesting that basal β_2-adrenoceptors gene expression in human lung fibroblasts may be under the inhibitory control of short-living suppressor proteins.

Furthermore, the up-regulation after short exposure as well as the down-regulation effect seen after 4 hrs of formoterol appears to be markedly increased after exposure of cells to a combination of formoterol and CHX. This effect of cycloheximide on β_2-adrenoceptors mRNA suggests that the initial direct stimulatory signal was still operating after 4 hrs, but mainly obstructed by newly synthesized inhibitory factors presumably induced following β_2-adrenoceptors activation by adenylyl cyclase-cAMP signaling pathway.

At present it is still unknown whether the β_2-adrenoceptors-cAMP signaling pathway system enhances the action of these suppressor proteins or induces biosynthesis of additional inhibitory regulators. Therefore, this hypothesis will be a challenge for future studies to elucidate and identify these regulators which could be potential targets for drugs aiming to improve and maintain β_2-adrenoceptors function during prolonged agonist asthma and COPD treatment.

As outlined in the introduction, cellular cAMP signaling transduction can be transmitted either by the classic effector (PKA) (Skalhegg and Tasken 2000) or alternative cAMP effector Epac of which two variants, Epac1 and Epac2, have been identified (de Rooij et al. 1998; Kawasaki et al. 1998). Our observations showed that the inhibitory effect of the β_2-adrenoceptors agonist formoterol could be effectively mimicked after exposure to selective PKA agonist 6-Bnz-cAMP within 1, 2 and 24 hrs, whereas the selective Epac agonist 8-CPT-2'-O-Me-cAMP (Bos 2006; Holz et al. 2008) failed to exert any effect on β_2-adrenoceptors mRNA, but the combination of

both appears to additively exert an inhibitory effect on β_2-adrenoceptors mRNA after the same exposure time.

On the other hand, the effects seen after PKA and Epac agonists exposure were not only prevented by cycloheximide, but also converted into sharp up-regulation.

Worth mentioning is that previous observations after using the same cell line and concentrations of PKA agonist 6-Bnz-cAMP and Epac agonist CPT-2'-O-Me-cAMP elucidated and confirmed that these agonists mediated a marked and selective inhibition of collagen synthesis and proliferation, respectively (Haag et al. 2008b). This suggests that both PKA and Epac may participate in the cellular transmission of β_2-adrenoceptors-cAMP-mediated regulation of β_2-adrenoceptors expression, but PKA appears to play the major part.

4.3.1 Glucocorticoids Control of β_2-Adrenoceptors Expression

It has been reported that glucocorticoids have the ability to increase the number of β_2-adrenoceptors in human lung measured by radioligand-binding study, and dexamethasone also was found to enhance the β_2-adrenoceptors transcription rate via a GRE in 5'-flanking region of the gene (Davies and Lefkowitz, 1984; Malbon and Hadcock, 1988), and a similar effect of dexamethasone on β_2-adrenoceptors expression in cultured cell lines has been confirmed in other reports (Hadcock and Malbon 1988b).

In the present study, the effect of treatment of MRC-5 cell line with glucocorticoids and β-adrenoceptors agonists alone or in combination on mRNA expression level of β_2-adrenoceptors by using of real-time PCR's technique was investigated. As observed previously, (Mak et al. 1995b; Mano et al. 1979) the observations in the present study also provide evidence that an increase in β_2-adrenoceptors mRNA expression levels after exposure to glucocorticoids such as dexamethasone and budesonide could be detected. This confirms earlier reports that glucocorticoids can induce up-regulation of β-adrenoceptors in different cell lines in culture (Collins et al. 1988; Feve et al. 1990; Hadcock and Malbon 1988b).

As outlined previously in this study, the β_2-adrenoceptors mRNA half-life and stability is relatively short, and in agreement with earlier reports (Mak et al. 1995a) the present study showed that dexamethasone has not been found to alter the half-life of β_2-adrenoceptors mRNA in MRC-5 human lung fibroblasts cell culture.

It is known that glucocorticoids enhance and modulate the efficiency of coupling between the β_2-adrenoceptors and Gs protein resulting in adenylyl cyclase activation and cAMP accumulation (Dooley et al. 1986). Down-regulation of β_2-adrenoceptors in the lung after chronic use of β_2-adrenoceptors agonist in vivo has been detected but was reversed after treatment with glucocorticoids (Davies and Lefkowitz 1984; Nishikawa et al. 1993). Such an effect may have a clinical role in preventing the development of tolerance to β_2-adrenoceptors agonists in asthmatic patients treated with these compounds.

5. Conclusion

In conclusion, human lung fibroblasts express β_2-adrenoceptors which mediate inhibitory effects on different pro-fibrotic features. The expression of β_2-adrenoceptors in human lung fibroblasts appear to be highly regulated at transcriptional level, suggesting that β_2-adrenoceptors expression may rapidly respond to physiological or pathological changes, as well as pharmacological interventions. The effect of cycloheximide suggests that the β_2-adrenoceptors gene is under strong inhibitory control from short-living but as yet unidentified suppressor proteins. The time-dependent regulation of β_2-adrenoceptors gene expression (up and down-regulation) following β_2-adrenoceptors activation appears to be mediated via activation of adenylyl cyclase-cAMP signaling. However, only stimulatory effects appear to be caused by direct stimulation of the β_2-adrenoceptors gene expression.

6. Summary

Background: Both asthma and COPD are chronic lung diseases characterized by airway remodelling in which fibrotic alterations represents major part. This study focuses on the role of classical anti-obstructive drugs in the modulation of long-term remodelling processes in asthma and COPD, in an attempt to explore β-adrenergic mechanisms in lung fibroblasts.

Methods: Cell proliferation and collagen synthesis were analyzed by measuring incorporation of [^{3}H]-thymidine and [^{3}H]-proline, respectively. Expression of β-adrenoceptors and α-smooth muscle actin (α-SMA) a marker of myofibroblast differentiation, was measured at the level of mRNA by semi-quantitative and real time RT-PCR and at the protein level by western blot analysis.

Results: In human lung fibroblasts (primary cells as well as MRC-5 cell line) stable expression of mRNA encoding β_2-adrenoceptors, but no transcripts for β_1- and β_3-adrenoceptors could be demonstrated.

β-Adrenoceptors agonists inhibited cell proliferation and collagen synthesis both in MRC-5 cells and PhLFb maximally by about 30-40 % and a detailed pharmacological characterization demonstrated that both effects were mediated via β_2-adrenoceptors.

MRC-5 cells showed basal expression of α-SMA which was inhibited both at mRNA and protein level by about 50% by the β_2-adrenoceptors selective agonist formoterol.

Both, PKA and Epac selective agonist mimicked the effect of β-adrenoceptors agonists on α-SMA mRNA and protein expression with some tendency for additive effects, whereas previous studies had shown that PKA and Epac agonists inhibited selectively either collagen synthesis or proliferation, respectively.

The effects of β-adrenoceptors agonists on proliferation, collagen synthesis and α-SMA expression were lost after exposure to transforming growth factor-β_1 (TGF-β) which alone had no effect on proliferation, but caused a marked increase in collagen synthesis and a marked up-regulation of α-SMA expression.

After inhibition of mRNA synthesis by actinomycin D (30 μM), β_2-adrenoceptors mRNA decreased with a half-life of 25 min, whereas inhibition of protein synthesis by cycloheximide (CHX), (30 μM) resulted in a rapid increase of β_2-adrenoceptors

mRNA (about 3- and 6- fold within 1.5 and 6.5 hrs, respectively). Indicating that β_2-adrenoceptors expression is highly regulated at mRNA level.

TGF-β induced a marked and long lasting down-regulation of β_2-adrenoceptors mRNA (about 90 % within 6 hrs). Also corticosteroids (dexamethasone and budesonide) alone caused a marked and long lasting up-regulation of β_2-adrenoceptors mRNA, but they were not able to prevent or oppose the TGF-β induced down-regulation.

Exposure to β-adrenoceptors agonists resulted in a rapid (maximum effect within 1 h), but transient up-regulation of β_2-adrenoceptors mRNA level. This effect was mimicked by forskolin, a direct activator of adenylyl cyclase. Furthermore, as β-adrenoceptors agonists-induced up-regulation of β_2-adrenoceptors mRNA also in presence of CHX, but not in presence of actinomycin D, it can be concluded that a cAMP direct stimulation of the expression of the β_2-adrenoceptors gene may be involved.

In conclusion: Human lung fibroblasts exclusively express β_2-adrenoceptors which mediate inhibition of various aspects of pro-fibrotic processes. The β_2-adrenoceptors are highly regulated at mRNA level, and the up-regulation after β-adrenoceptors agonists-induced up-regulation of β_2-adrenoceptors gene may be a mechanism to oppose agonist-induced receptor desensitization which may contribute to the maintenance of β_2-adrenoceptors function during long term agonist exposure. Under clinical conditions, anti-fibrotic effects may associate the therapeutic effect of β_2-adrenoceptors agonists treatment of asthma and COPD.

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8. List of Publications and Published Congress Abstracts

I. Published Paper

1. Juergens, U. R., Racké, K., Uen, S., Haag, S., Lamyel F., Stöber, M., Gillissen, A., Novak, N., & Vetter, H. Inflammatory responses after endothelin B (ET B) receptor activation in human monocytes: New evidence for beneficial anti-inflammatory potency of ETB-receptor antagonism. Pulmonary pharmacology and therapeutics 2008; 21 (3), 533-539.

2. Lamyel F, Warnken-Uhlich M, Seemann WK, Mohr K, Kostenis E, Ahmedat AS, Smit M, Gosens R, Meurs H, Miller-Larsson A, and Racké K. The β_2-subtype of adrenoceptors mediates inhibition of pro-fibrotic events in human lung fibroblasts. Naunyn Schmiedebergs Arch Pharmacol. 2011; 384:133-145.

3. N Kämpfer, F Lamyel, I Schütz, M Warnken, K Hoffmann, I von Kügelgen and Kurt Racké. Dual regulation of β_2-adrenoceptor messenger RNA expression in human lung fibroblasts by β_2–cAMP signaling; delayed up regulated inhibitors oppose a rapid in onset, direct stimulation of gene expression. Naunyn-Schmiedeberg's Arch Pharmacol 2014; 387:649–657

II. Published Conference Abstracts

1. Kurt Racké, Fathi B. Lamyel, Uwe R. Juergens, Mareille Warnken. Characterization of expression and functional role of β_2-adrenoceptors in human lung fibroblasts. Proceeding of British Pharmacological Society at http://www.pA2-online.org/abstracts/Vol7Issue2abst015P.pdf 2009.

2. K. Racké, F. B. Lamyel, M. Warnken and R.U.Juergens. Expression and Functional Role of β_2-Adrenoceptors in Human Lung Fibroblasts. . Am J Respir Crit Care Med 179; 2009:A5617.

3. K. Racké, F. B. Lamyel, M. Warnken, S. Haag, U. R. Juergens. Inhibitory Effects of cAMP Elevating Agents On α-Smooth Muscle Actin Expression in Expression in Human Lung Fibroblasts Are Mediated via Protein Kinase A (PKA) And Epac. Am J Respir Crit Care Med 181; 2010:A3532.

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5. Mareille Warnken Uhlich, Fathi B. Lamyel, Ina Schütz, Kurt Racké. Auto-receptor-mediated up-regulation of β_2-adrenoceptor mRNA expression in human lung fibroblasts. Naunyn Schmiedebergs Arch Pharmacol. 2011; 383:46.

6. Nora Kämpfer, Fathi Lamyel, Ina Schütz, Mareille Warnken, Michael Pieper, Kurt Racké. β_2-Adrenoceptor-cAMP signaling exerts dual effects on β_2-adrenoceptor expression in human lung fibroblasts, delayed up-regulated inhi-bitory factors oppose a rapid in onset, direct stimulation of gene expression. Proceeding of British Pharmacological Society at http://www.pA2online.org/abstracts/Vol9Issue3abst077P.pdf 2011

7. Kurt Racké, Fathi B. Lamyel, Mareille Warnken. Beta$_2$-adrenoceptor mRNA expression in human lung fibroblasts is highly up-regulated by β_2-adrenoceptor agonists and corticosteroids, but down-regulated by TGF-β. Am J Respir Crit Care Med 183; 2011:A2147.

8. Racké K.; Lamyel, F.; Kaempfer, N.; Schütz I., Warnken M. A β_2-adrenoceptor-cAMP mediated, immediate stimulation of β_2-adrenoceptor gene expression in human lung fibroblasts is opposed by a delayed up-regulation of inhibitory factors. Naunyn Schmiedebergs Arch Pharmacol. 2012; 385:S71-S72.

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